The separation of sterol intermediates in cholesterol biosynthesis by high pressure liquid chromatography

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Abstract A three-step procedure has been developed for the separation of complex mixtures of sterol intermediates in cholesterol biosynthesis. The method has been applied to the separation of sterol intermediates formed from [^{14}C]mevalonate by normal rat hepatocyte culture cells. In Step 1, a short gravity-flow silicic acid column (1.2 × 6.5 cm) separates the incubation products into four classes consisting of *A*) squalene + squalene oxide, *B*) methyl sterol precursors, *C*) C_{27} sterols, and *D*) polar compounds. In Step 2, the components of classes *B*) and *C*) are further resolved by reverse-phase high pressure liquid chromatography (HPLC) on a μBondapak-C_{18} column. In Step 3, (after acetylation) high pressure liquid chromatography on a μPorasil column of peaks obtained from Step 2 is conducted. This last step resolves mixtures which may be present in peaks resulting from Step 2. The relative retention times of unknown radioactive sterols are compared with authentic co-chromatographed reference sterols in both Steps 2 and 3. Relative retention time factors for several functional groups encountered in sterol intermediates in cholesterol biosynthesis have been determined for both reverse-phase and silicic acid HPLC systems. The use of these functional group factors allows one to calculate a predicted relative retention time for a variety of structural possibilities. The HPLC techniques described utilize single columns, isocratic solvent systems, and comparatively short (<30 min) elution times, and the three-step procedure is capable of resolving complex mixtures of sterol intermediates.—**Hansbury, E., and T. J. Scallen.** The separation of sterol intermediates in cholesterol biosynthesis by high pressure liquid chromatography. *J. Lipid Res.* 1980. **21:** 921–929.

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Studies from several laboratories have demonstrated the variety and complexity of sterol intermediates[1] which are formed when either radioactive acetate or mevalonate are incubated with rat liver homogenates (1), liver or brain slices (2), or with intact primary hepatocyte cells (3). Therefore, it has been necessary to devise chromatographic procedures capable of separating the various sterol intermediates from cholesterol and from each other.

The pioneer studies of Frantz (4) and Klein and Szczepanik (5) provided a major step forward in this regard; however, the chromatographic techniques used (gravity flow silicic acid columns of 100 cm in length) required 3 or 4 days to conduct, and only partial resolution of certain of the sterol intermediates was obtained. Gas–liquid chromatography has been employed, both alone (6) and in conjunction with silicic acid column chromatography (7) or TLC (8), to separate cholesterol and its sterol precursors. While gas–liquid chromatography is a useful technique, a major disadvantage is the difficulty of sample recovery. Furthermore, gas–liquid chromatographs are not easily adapted for the measurement of radioactive sterols. Several thin-layer chromatographic procedures have been utilized (9); however, recovery of the purified sterol and the relatively low resolution of conventional TLC procedures (10) are significant problems.

High pressure liquid chromatography (HPLC), on the other hand, has some important advantages over the other separation techniques mentioned above: i) the time required for chromatographic separation is usually measured in minutes; ii) the sample is not

Abbreviations: HPLC, high pressure liquid chromatography; TLC, thin-layer chromatography; RRT, relative retention time; RI, refractive index; Ac, acetate.

[1] Cholesterol, Δ^5cholesternol; cholestanol, 5α-cholestan-3β-ol; desmosterol, $\Delta^{5,24}$-cholestadienol; 7-dehydrocholesterol, $\Delta^{5,7}$-cholestadienol; dihydrolanosterol, 4,4,14α-trimethyl-Δ^8-cholestenol; lanosterol, 4,4,14α-trimethyl-$\Delta^{8,24}$-cholestadienol; lathosterol, Δ^7-cholestenol; zymostenol, Δ^8-cholestenol; zymosterol, $\Delta^{8,24}$-cholestadienol.

TABLE 1. Reverse-phase HPLC of sterol intermediates in cholesterol biosynthesis

Sterol	Relative Retention Time	
	Observed[a]	Calc.[d]
4,4,14α-Trimethyl-Δ^8-cholestenol (dihydrolanosterol)	1.00	0.98
4,4-Dimethyl-Δ^8-cholestenol	0.96	0.95
Cholestanol	0.93	0.93
4-Methyl-Δ^8-cholestenol[b]	0.82	0.81
14α-Methyl-Δ^8-cholestenol[c]	0.74	0.74
Δ^5-Cholestenol (cholesterol)	0.72	0.72
Δ^7-Cholestenol (lathosterol)	0.72	0.72
Δ^8-Cholestenol (zymostenol)	0.72	0.72
$\Delta^{8(14)}$-Cholestenol	0.69	0.69
4,4,14α-Trimethyl-$\Delta^{8,24}$-cholestadienol (lanosterol)	0.67	0.68
4,4-Dimethyl-$\Delta^{8,24}$-cholestadienol	0.66	0.66
4,14-Dimethyl-$\Delta^{8,24}$-cholestadienol	0.57	0.58
4-Methyl-$\Delta^{8,24}$-cholestadienol[b]	0.56	0.56
$\Delta^{5,7}$-Cholestadienol (7-dehydrocholesterol)	0.54	0.54
14α-Methyl-$\Delta^{8,24}$-cholestadienol	0.51	0.51
$\Delta^{8,24}$-Cholestadienol (zymosterol)	0.50	0.49
$\Delta^{5,24}$-Cholestadienol (desmosterol)	0.49	0.49
$\Delta^{5,7,24}$-Cholestatrienol	0.38	0.37

[a] The relative retention times were based upon the retention time of 4,4,14α-trimethyl-Δ^8-cholestenol (1.00), whose average retention time was 22.8 min (nine determinations).

[b] The presumed 4-methyl-Δ^8-cholestenol and 4-methyl-$\Delta^{8,24}$-cholestadienol were peaks obtained from incubations of normal rat primary hepatocyte cells with [^{14}C]mevalonate. Havel, C., E. Hansbury, T. J. Scallen, and J. A. Watson. Unpublished data. Their retention times were consistent with those calculated from functional group factors, but for which authentic standards were unavailable.

[c] The relative retention time of 14α-methyl-Δ^8-cholestenol was calculated from the retention time for authentic 14α-methyl-$\Delta^{8,24}$-cholestadienol by dividing the retention time of this sterol by 0.69, the retention factor for the Δ^{24}-bond.

[d] The calculated retention times were obtained by utilizing the functional group factors in Table 5. For example, $\Delta^{5,24}$-cholestadienol is (0.93) (0.77) (0.69) = 0.49.

Reverse-phase HPLC was conducted on a μBondapak-C_{18} (3.9 mm × 30 cm, Waters Associates) column eluted with acetonitrile–water 88:12. Prior to chromatography, the samples were filtered through a 0.5 μm fluoropore filter and organic prefilter contained in a Swinny filtration apparatus. The filtrates were evaporated under nitrogen and then they were redissolved in an appropriate volume of filtered toluene for injection onto the column. Detection of authentic samples was by change in refractive index, utilizing a Waters Model ALC/GPC liquid chromatograph. Other chromatographic conditions were as described in Fig. 3.

destroyed, and purified sterols can be isolated (via a fraction collector) and utilized for further experiments; and iii) the resolution attained is generally better than that achieved with other techniques. These advantages are important because of the extreme complexity possible with mixtures of sterol intermediates in cholesterol biosynthesis. For example, in the methyl sterol fraction, sterols can be found with one or two methyl groups at C-4, a methyl group at C-14, carbon-carbon double bonds at Δ^8 or Δ^7 (and possibly other locations, e.g., $\Delta^{8,(14)}$, $\Delta^{(8,14}$, and $\Delta^{7,14}$). In the C_{27} sterol fraction carbon-carbon double bonds may be encountered at Δ^5, Δ^7, $\Delta^{5,7}$, and Δ^8 (and also possibly at $\Delta^{8(14)}$, $\Delta^{8,14}$, and $\Delta^{7,14}$). All of these possibilities can exist either with or without the Δ^{24}-bond in the side-chain.

In an earlier report from our laboratory (11), it was demonstrated that reverse-phase HPLC was a rapid and effective technique for the separation of Δ^{24}-sterols from their side-chain saturated counterparts. For example, desmosterol ($\Delta^{5,24}$-cholestadienol) was separated from cholesterol (Δ^5-cholestenol) in 10 min.

However, a number of sterol intermediates which were isomeric (contained the same number of carbon-carbon double bonds) were not separated by reverse-phase HPLC. The present article describes a three-step procedure which is capable of achieving separations not possible with the earlier procedure.

MATERIALS AND METHODS

Materials

Cholesterol was purified through the dibromide (12) and recrystallized from acetone. [^{14}C]4,4-Dimethyl-Δ^8-cholestenol was prepared as previously described (13). 4,4,14α-Trimethyl-$\Delta^{8,24}$-cholestadienol (lanosterol) was from Sigma, St. Louis, MO. 4,4,14α-Trimethyl-Δ^8-cholestenol (dihydrolanosterol) was from Schwarz-Mann, Orangeburg, NY, and was recrystallized from chloroform–methanol. Cholestanol, cholestanyl acetate and $\Delta^{5,7}$-cholestadienyl acetate were from Steraloids, Inc., Wilton, NH. $\Delta^{5,24}$-Cholestadienol (desmosterol) was from Organon, West Orange, NJ. $\Delta^{8(14)}$-Cholestenol and Δ^7-cholestenol were from Research Plus Steroid Laboratories, Inc., Denville, NJ. The preparation of $\Delta^{5,7,24}$-cholestatrienol was reported in an earlier paper (14). Δ^8-Cholestenol and Δ^8-cholestenyl acetate were generous gifts from Dr. G. J. Schroepfer, Jr., Rice University. $\Delta^{8,24}$-Cholestadienol (zymosterol), 14α-methyl-$\Delta^{8,24}$-cholestadienol, 4,14α-dimethyl-$\Delta^{8,24}$-cholestadienol, and 4,4-dimethyl-$\Delta^{8,24}$-cholestadienol were generous gifts from Dr. A. C. Oehlschlager, Simon Fraser University. Acetonitrile, cyclohexane, *i*-octane, and toluene were either HPLC or Spectro grade and were purchased from Burdick and Jackson Laboratories, Muskegon, MI, Fisher Scientific Co., Fair Lawn, NJ, or MCB, Cincinnati, OH. Those solvents which had not been filtered during manufacture were filtered through a Millipore (fluoropore) filter (0.5 μm), and toluene was first dried over molecular sieve (Linde, type 3A). Acetylations of sterols not previously noted were performed using an acetylation kit (acetic anhydride–pyridine) purchased from Applied Science, State College, PA. Unisil silicic acid (200–325 mesh) was from Clarkson Chemical Co., Williamsport PA.

Chromatography

High pressure liquid chromatography was carried out on a Waters liquid chromatograph Model ALC/GPC that was equipped with a refractive index detector, a Houston recorder, and a Buchler fraction collector. Sample preparation, measurement of radioactivity, and collection of fractions were conducted as previously described (11). Columns utilized and other details of chromatographic separations are described in the Table and Figure legends.

RESULTS

Reverse-phase HPLC of sterol intermediates in cholesterol biosynthesis

Table 1 summarizes (in reverse order of elution) the relative retention times of a number of sterols, using a single 30-cm reverse-phase HPLC column (μBondapak-C_{18}). **Table 2** compares these relative retention times according to the number or position of specific functional groups. As can be seen, a major advantage of reverse-phase HPLC is the separation of sterols which differ in the number of carbon-carbon double bonds, e.g., Δ^5-cholestenol from $\Delta^{5,24}$-cholestadienol. Another advantage is the ability to separate sterols according to the number of C-4 methyl groups; e.g., Δ^8-cholestenol from 4-methyl-Δ^8-cholestenol and from 4,4-dimethyl-Δ^8-cholestenol.

However, it is apparent from Tables 1 and 2 that reverse-phase HPLC failed to separate sterols which differed only in the presence or absence of the C-14 methyl group; e.g., 4,4,14α-trimethyl-$\Delta^{8,24}$-cholestadienol from 4,4-dimethyl-$\Delta^{8,24}$-cholestadienol.

Reverse-phase HPLC is also ineffective for the separation of isomeric C_{27} sterols, as for example Δ^5-cholestenol from Δ^7-cholestenol and Δ^8-cholestenol, or the corresponding sterols having a Δ^{24}-bond in the side chain, e.g., $\Delta^{5,24}$-cholestadienol from $\Delta^{8,24}$-cholestadienol.

Silicic acid HPLC of sterol intermediates in cholesterol biosynthesis

Table 3 summarizes (in order of elution) the relative retention times of a number of sterol acetates using a single 30-cm silicic acid (μPorasil) HPLC column. **Table 4** compares these retention times according to the number or position of specific functional groups. In contrast to reverse-phase HPLC, which did not separate isomeric sterols containing one carbon-carbon double bond or the corresponding sterols with a Δ^{24}-bond in the side chain, silicic acid HPLC of the sterol acetates successfully resolved these compounds; e.g., Δ^5- from $\Delta^{8,(14)}$-, from Δ^8-, and from Δ^7-cholestenyl acetates. The sterol acetates with a side chain Δ^{24}-bond were also well separated; e.g., $\Delta^{5,24}$-cholestadienyl acetate from $\Delta^{8,24}$-cholestadienyl acetate.

Whereas reverse-phase HPLC did not separate sterols which differed in the presence or absence of a methyl group at C-14, silicic acid HPLC of the sterol acetates readily resolved these compounds, e.g., 4,4,14α-

TABLE 2. Separation of sterols by reverse-phase high pressure liquid chromatography according to number or position of specific functional groups

Sterols with One Double Bond	Rel. Ret. Time[a]	Sterols with Two Double Bonds	Rel. Ret. Time[a]	Sterols with Three Double Bonds	Rel. Ret. Time[a]
4,4,14α-Trimethyl-Δ^8-cholestenol	1.00	4,4,14α-Trimethyl-$\Delta^{8,24}$-cholestadienol	0.67		
4,4-Dimethyl-Δ^8-cholestenol	0.96	4,4-Dimethyl-$\Delta^{8,24}$-cholestadienol	0.66		
4-Methyl-Δ^8-cholestenol[b]	0.82	4-Methyl-$\Delta^{8,24}$-cholestadienol[b]	0.56		
14α-Methyl-Δ^8-cholestenol[c]	0.74	14α-Methyl-$\Delta^{8,24}$-cholestadienol	0.51		
Δ^8-Cholestenol	0.72	$\Delta^{8,24}$-Cholestadienol	0.50		
Δ^5-Cholestenol	0.72	$\Delta^{5,24}$-Cholestadienol	0.49		
Δ^7-Cholestenol	0.72				
$\Delta^{8(14)}$-Cholestenol	0.69				
		$\Delta^{5,7}$-Cholestadienol	0.54	$\Delta^{5,7,24}$-Cholestatrienol	0.38
Sterols with No C-4 Methyl Groups		Sterols with One C-4 Methyl Group		Sterols with Two C-4 Methyl Groups	
Δ^8-Cholestenol	0.72	4-Methyl-Δ^8-cholestenol[b]	0.82	4,4-Dimethyl-Δ^8-cholestenol	0.96
$\Delta^{8,24}$-Cholestadienol	0.50	4-Methyl-$\Delta^{8,24}$-cholestadienol[b]	0.56	4,4-Dimethyl-$\Delta^{8,24}$-cholestadienol	0.66
14α-Methyl-$\Delta^{8,24}$-cholestadienol	0.51	4,14-Dimethyl-$\Delta^{8,24}$-cholestadienol	0.57	4,4-Dimethyl-$\Delta^{8,24}$-cholestadienol	0.66
Sterols with No C-14 Methyl Groups		Sterols with One C-14 Methyl Group			
4,4-Dimethyl-Δ^8-cholestenol	0.96	4,4,14α-Trimethyl-Δ^8-cholestenol	1.00		
Δ^8-Cholestenol	0.72	14α-Methyl-Δ^8-cholestenol	0.74		
4,4-Dimethyl-$\Delta^{8,24}$-cholestadienol	0.66	4,4,14α-Trimethyl-$\Delta^{8,24}$-cholestadienol	0.67		
4-Methyl-$\Delta^{8,24}$-cholestadienol[b]	0.56	4,14-Dimethyl-$\Delta^{8,24}$-cholestadienol	0.57		
$\Delta^{8,24}$-Cholestadienol	0.50	14α-Methyl-$\Delta^{8,24}$-cholestadienol[c]	0.51		

Legend same as for Table 1.

TABLE 3. Silicic acid HPLC of sterol acetate intermediates in cholesterol biosynthesis

Sterol	Relative Retention Time	
	Observed[a]	Calc.[c]
4,4,14α-Trimethyl-Δ^8-cholestenyl-Ac[b] (dihydrolanosteryl-Ac)	1.00	0.97
4,4,14α-Trimethyl-$\Delta^{8,24}$-cholestadienyl-Ac (lanosteryl-Ac)	1.12	1.15
4,4-Dimethyl-Δ^8-cholestenyl-Ac	1.13	1.17
14α-Methyl-Δ^8-cholestenyl-Ac[c]	1.19	1.21
Δ^5-Cholestenyl-Ac (cholesteryl-Ac)	1.22	1.23
Cholestanyl-Ac	1.25	1.25
4,14-Dimethyl-$\Delta^{8,24}$-cholestadienyl-Ac	1.33	1.33
4-Methyl-Δ^8-cholestenyl-Ac[d]	1.34	1.36
4,4-Dimethyl-$\Delta^{8,24}$-cholestadienyl-Ac	1.36	1.38
$\Delta^{8(14)}$-Cholestenyl-Ac	1.36	1.36
14α-Methyl-$\Delta^{8,24}$-cholestadienyl-Ac	1.41	1.43
$\Delta^{5,24}$-Cholestadienyl-Ac (desmosteryl-Ac)	1.42	1.45
Δ^8-Cholestenyl-Ac (zymostenyl-Ac)	1.46	1.46
Δ^7-Cholestenyl-Ac (lathosteryl-Ac)	1.55	1.55
$\Delta^{5,7}$-Cholestadienyl-Ac (7-dehydrocholesteryl-Ac)	1.56	1.56
4-Methyl-$\Delta^{8,24}$-cholestadienyl-Ac[d]	1.62	1.60
$\Delta^{8,24}$-Cholestadienyl-Ac (zymosteryl-Ac)	1.73	1.73
$\Delta^{5,7,24}$-Cholestatrienyl-Ac	1.88	1.84

[a] The relative retention times were based upon the retention time of 4,4,14α-trimethyl-Δ^8-cholestenyl acetate (1.00), whose average retention time was 9.75 min (14 determinations).

[b] Ac, acetate.

[c] The relative retention time of 14α-methyl-Δ^8-cholestenyl acetate was calculated from the retention time for authentic 14α-methyl-$\Delta^{8,24}$-cholestadienyl acetate by dividing the retention time of this sterol acetate by 1.18, the retention factor for the Δ^{24}-bond.

[d] The presumed 4-methyl-Δ^8-cholestenyl acetate and 4-methyl-$\Delta^{8,24}$-cholestadienyl acetate were peaks obtained from incubations of normal rat primary hepatocyte cells with [^{14}C]mevalonate. Havel, C., E. Hansbury, T. J. Scallen, and J. A. Watson. Unpublished data. Their retention times were consistent with those calculated from functional group factors, but for which authentic standards were unavailable.

[e] The calculated retention times were obtained by utilizing the functional group factors in Table 5. For example, $\Delta^{5,24}$-cholestadienol-Ac is (1.25) (0.98) (1.18) = 1.45.

Silicic acid HPLC was conducted on a μPorasil (3.9 mm × 30 cm, Waters Associates) column eluted with *i*-octane–cyclohexane–toluene 50:30:20. Prior to chromatography the sterols were acetylated overnight at 37°C in pyridine–acetic anhydride 2:1. The samples were evaporated to dryness under a stream of nitrogen in a warm water bath, brought back in 6 ml of ethanol–water 50:50, and then extraction was performed three times with 6-ml portions of petroleum ether. The combined petroleum ether fractions were then filtered as described in Table 1 and as previously described (11).

trimethyl-$\Delta^{8,24}$-cholestadienyl acetate from 4,4-dimethyl-$\Delta^{8,24}$-cholestadienyl acetate.

Like reverse-phase HPLC, silicic acid HPLC is capable of resolving sterols which differ in the presence or absence of the Δ^{24}-bond; e.g., $\Delta^{5,7}$-cholestadienyl acetate from $\Delta^{5,7,24}$-cholestatrienyl acetate.

Sterols which differed only in the number of C-4 methyl groups were also separated; e.g., Δ^8-cholestenyl acetate from 4-methyl-Δ^8-cholestenyl acetate and from 4,4-dimethyl-Δ^8-cholestenyl acetate.

However, using silicic acid HPLC, 4,4-dimethyl sterols were not well separated from 4,14-dimethyl sterols; e.g., 4,4-dimethyl-$\Delta^{8,24}$-cholestadienyl acetate from 4,14-dimethyl-$\Delta^{8,24}$-cholestadienyl acetate; separation of these two compounds (as the unesterified sterols) was achieved by reverse-phase HPLC (Tables 1 and 2). In addition, sterols which differed by the addition of both a methyl group at C-4 and a Δ^{24}-bond were not resolved; e.g., 4,4,14α-trimethyl-$\Delta^{8,24}$-cholestadienyl acetate and 4,4-dimethyl-Δ^8-cholestenyl acetate. These two sterols were, however, easily resolved as the unesterified sterols by reverse-phase HPLC (Tables 1 and 2).

A three-step chromatographic procedure for the resolution of sterol intermediates in cholesterol biosynthesis

An examination of Tables 1 and 3 show that neither reverse-phase HPLC nor silicic acid HPLC alone was capable of resolving all of the compounds tested. However, the three-step chromatographic procedure shown in **Fig. 1** is capable of this task. The first step is a short silicic acid column (1.2 × 6.5 cm) (15), which separates compounds into four general classes: *A*, squalene and squalene-2,3-oxide; *B*, methyl sterol precursors (sterols with 30, 29, or 28 carbon atoms); *C*, C_{27} sterols; and *D*, polar sterols (two or more hydroxyl groups).

Squalene and squalene-2,3-oxide (Step 1, Class A, Fig. 1) can easily be separated by reverse-phase HPLC, using 100% acetonitrile as the eluting solvent (11). The retention time for squalene under these conditions was 5.16 min, and for squalene-2,3-oxide, it was 3.80 min.

The methyl sterol fraction (Class B from Step 1, Fig. 1) was then subjected to the second step, which was reverse-phase HPLC (μBondapak-C_{18}) with acetonitrile–water 88:12 as the eluting solvent. Fig. 1 shows the resolution achieved by this technique. In two instances separation was not attained. For example, 4,4,14α-trimethyl-$\Delta^{8,24}$-cholestadienol (lanosterol) and 4,4-dimethyl-$\Delta^{8,24}$-cholestadienol were not resolved by reverse-phase HPLC; however, when these fractions were pooled and acetylated, then chromatographed by HPLC on μPorasil silicic acid, resolution was achieved.

The application of this procedure to the separation of radioactive sterols formed from [^{14}C]mevalonate by normal primary rat liver hepatocyte cells[2] is shown in **Fig. 2.** In Fig. 2A, the reverse-phase HPLC separa-

[2] Havel, C., E. Hansbury, T. J. Scallen, and J. A. Watson. Unpublished data.

TABLE 4. Separation of sterol acetates by silicic acid high pressure liquid chromatography according to number or position of specific functional groups

Sterols with One Double Bond	Rel. Ret. Time[a]	Sterols with Two Double Bonds	Rel. Ret. Time[a]	Sterols with Three Double Bonds	Rel. Ret. Time[a]
4,4,14α-Trimethyl-Δ^{8}-cholestenyl-Ac[b]	1.00	4,4,14α-Trimethyl-$\Delta^{8,24}$-cholestadienyl-Ac	1.12		
4,4-Dimethyl-Δ^{8}-cholestenyl-Ac	1.13	4,4-Dimethyl-$\Delta^{8,24}$-cholestadienyl-Ac	1.36		
14α-Methyl-Δ^{8}-cholestenyl-Ac[d]	1.19	14α-Methyl-$\Delta^{8,24}$-cholestadienyl-Ac	1.41		
Δ^{5}-Cholestenyl-Ac	1.22	$\Delta^{5,24}$-Cholestadienyl-Ac	1.42		
4-Methyl-Δ^{8}-cholestenyl-Ac[c]	1.34	4-Methyl-$\Delta^{8,24}$-cholestadienyl-Ac[d]	1.62		
$\Delta^{8(14)}$-Cholestenyl-Ac	1.36				
Δ^{8}-Cholestenyl-Ac	1.46	$\Delta^{8,24}$-Cholestadienyl-Ac	1.73		
Δ^{7}-Cholestenyl-Ac	1.55				
		$\Delta^{5,7}$-Cholestadienyl-Ac	1.56	$\Delta^{5,7,24}$-Cholestatrienyl-Ac	1.88
Sterols with No C-4 Methyl Groups		Sterols with One C-4 Methyl Group		Sterols with Two C-4 Methyl Groups	
14α-Methyl-$\Delta^{8,24}$-cholestadienyl-Ac	1.41	4,14-Dimethyl-$\Delta^{8,24}$-cholestadienyl-Ac	1.33	4,4-Dimethyl-$\Delta^{8,24}$-cholestadienyl-Ac	1.36
Δ^{8}-Cholestenyl-Ac	1.46	4-Methyl-Δ^{8}-cholestenyl-Ac[d]	1.34	4,4-Dimethyl-Δ^{8}-cholestenyl-Ac	1.13
$\Delta^{8,24}$-Cholestadienyl-Ac	1.73	4-Methyl-$\Delta^{8,24}$-cholestadienyl-Ac[d]	1.62	4,4-Dimethyl-$\Delta^{8,24}$-cholestadienyl-Ac	1.36
Sterols with No C-14 Methyl Groups		Sterols with One C-14 Methyl Group			
4,4-Dimethyl-$\Delta^{8,24}$-cholestadienyl-Ac	1.36	4,4,14α-Trimethyl-$\Delta^{8,24}$-cholestadienyl-Ac	1.12		
4,4-Dimethyl-$\Delta^{8,24}$-cholestadienyl-Ac	1.36	4,14-Dimethyl-$\Delta^{8,24}$-cholestadienyl-Ac	1.33		
Δ^{8}-Cholestenyl-Ac	1.46	14α-Methyl-Δ^{8}-cholestenyl-Ac[c]	1.19		
4-Methyl-$\Delta^{8,24}$-cholestadienyl-Ac[d]	1.62	4,14-Dimethyl-$\Delta^{8,24}$-cholestadienyl-Ac	1.33		
$\Delta^{8,24}$-Cholestadienyl-Ac	1.75	14α-Methyl-$\Delta^{8,24}$-cholestadienyl-Ac	1.41		

Legend same as for Table 3.

tion of the methyl sterol fraction (Class B from the first step) is shown. Five peaks (B-I thru B-V) are observed. Peak B-III is a mixture of 4,4,14α-trimethyl-$\Delta^{8,24}$-cholestadienol (lanosterol) and 4,4-dimethyl-$\Delta^{8,24}$-cholestadienol. The fractions from B-III (Fig. 2A) were pooled, acetylated, and then silicic acid HPLC (Step 3) was carried out (Fig. 2B). Two radioactive sterols were resolved (as the acetates): lanosteryl acetate (B-IIIa) and 4,4-dimethyl-$\Delta^{8,24}$-cholestadienyl acetate (B-IIIb).

Fig. 1 shows the approach for the analysis of C_{27} sterols. First, the C_{27} sterols are isolated as a class using the first step (1.2 × 6.5 cm silicic acid column). Next, the C_{27} sterols (Class C)[3] are subjected to reverse-phase HPLC (Step 2). As can be seen in Fig. 1, this yields three sterol peaks, mono-, di-, and tri-unsaturated sterols. Each of these is acetylated and chromatographed by means of silicic acid HPLC (Step 3). This procedure produces resolution of the isomeric sterols contained in each peak.

An application of this three-step procedure to the separation of C_{27} sterols formed from [^{14}C]mevalonate by normal primary rat liver hepatocyte cells[2] is shown in **Fig. 3.** Class C (C_{27} sterol fraction from Step 1) was subjected to reverse-phase HPLC (Step 2) (Fig. 3A). Two major peaks were observed: C-I corresponds to a mixture of sterols containing two carbon-carbon double bonds (di-unsaturated), while peak C-III corresponds to a mixture of sterols containing one carbon-carbon double bond (mono-unsaturated). Peak C-III was acetylated and silicic acid HPLC (Step 3) was carried out (Fig. 3B). Two radioactive sterol acetates were resolved. As expected, the major peak (C-IIIa) was cholesteryl acetate and the smaller second peak (C-IIIb) corresponded with Δ^{7}-cholestenyl acetate.

Table 5 shows the calculated functional group factors for commonly encountered functional groups in sterol intermediates formed during cholesterol biosynthesis. This procedure allows one to compare an observed relative retention time for an unidentified peak with one calculated for a given structural possibility. The reference compounds used for these calculations were cholestanol (RRT = 0.93 on reverse-phase HPLC) and cholestanol acetate (RRT = 1.25 on normal-phase silicic acid HPLC). For example, the calculated relative retention time (relative to dihydrolanosterol) for 4,4,14α-trimethyl-$\Delta^{8,24}$-cholestadienol (lanosterol) by reverse-phase HPLC = (RRT cholestanol) (Δ^{8} factor) (Δ^{24} factor) (14α-methyl factor)

[3] It is possible that monomethyl sterols containing the methyl group at C-14, such as 14α-methyl-$\Delta^{8,24}$-cholestadienol, might be present in Class C. While sterols of this type are known to occur in yeast cells, no evidence exists that sterols of this type are found in mammalian cells, such as the liver system utilized here.

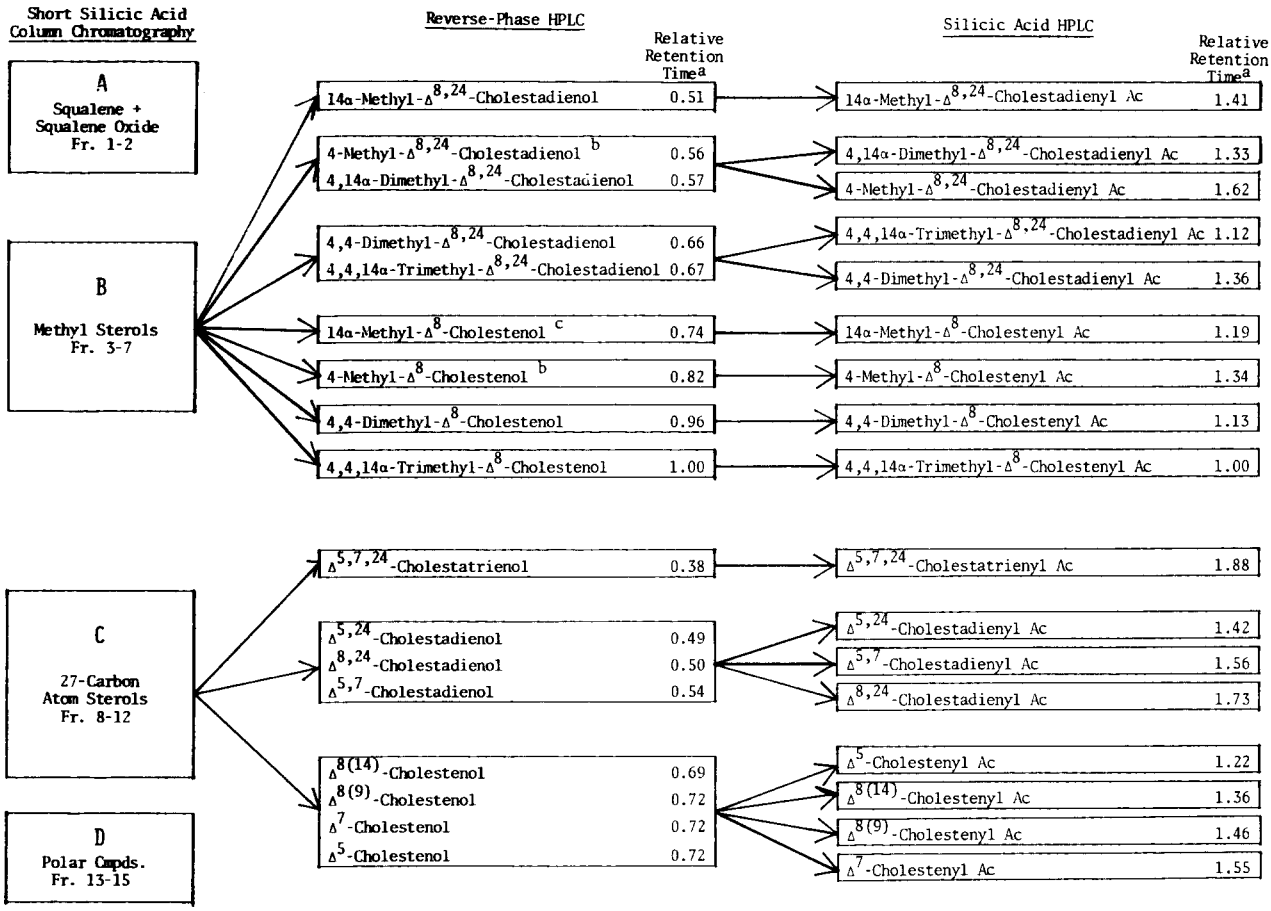

Fig. 1. Diagram of a three-step chromatographic procedure for the separation of sterol intermediates in cholesterol biosynthesis. The first step was a silicic acid column (Unisil 200–325 mesh (Clarkson Chemical Co.), 1.2 × 6.5 cm), conducted as previously described (Type II procedure) (15). The first twelve fractions of 8 ml each were eluted with toluene, which had been dried over molecular sieve, followed by elution with toluene–ethyl acetate 1:1 for the last three fractions. Aliquots were removed for the measurement of radioactivity, then the fractions within each class, e.g., methyl sterols, were combined, filtered, and subjected to reverse-phase HPLC (Step 2) as described in Table 1, followed by silicic acid HPLC (Step 3) as described in Table 3. Footnotes are the same as for Table 1.

(4,4-dimethyl factor) = (0.93) (0.77) (0.69) (1.03) (1.33) = 0.68. This compares favorably with an observed value (Table 1) of 0.67.

For 4,4,14α-trimethyl-$\Delta^{8,24}$-cholestadienyl acetate (lanosteryl acetate) on normal phase silicic acid HPLC using the same technique: (RRT cholestanyl acetate) (Δ^8 factor) (Δ^{24} factor) (14α-methyl factor) (4,4,-dimethyl factor) = (1.25) (1.17) (1.18) (0.83) (0.80) = 1.13. This agrees well with the observed value (Table 3) of 1.12.

DISCUSSION

Thowsen and Schroepfer (16) have used silicic acid HPLC (μPorasil) of sterol acetates as a means of separating various C_{27} sterols. Four μPorasil columns were connected in series (total length, 120 cm) and the elution time required was approximately 3½ hr. The method was applied to C_{27} sterols with a saturated side-chain. C_{27} sterols with double bonds in the indicated nuclear locations were eluted in the following sequence: Δ^5, $\Delta^{8(14)}$, Δ^8, Δ^7, $\Delta^{5,7}$, $\Delta^{8,14}$, and $\Delta^{7,14}$, using hexane–benzene 9:1 as the eluting solvent.

A medium pressure liquid chromatographic (MPLC) procedure has been devised by Pascal, Farris, and Schroepfer (17). An alumina column impregnated with silver nitrate (0.9 × 100 cm) was utilized. Side-chain saturated C_{27} sterol acetates were eluted in the following order: $\Delta^{8(14)}$, Δ^8, Δ^7, Δ^5, $\Delta^{7,14}$, $\Delta^{8,14}$, and $\Delta^{5,7}$. In some instances, e.g., Δ^8 and Δ^7, as well as, $\Delta^{7,14}$ and $\Delta^{8,14}$, only partial resolution was achieved. Approximately 17 hr were required for this particular separation. The principal advantage of this procedure is that the capacity of this system allows as much as 15 mg of sterol acetates to be separated. This can be an advantage if large quantities of tissues are analyzed, in which case significant quantities of cholesterol must

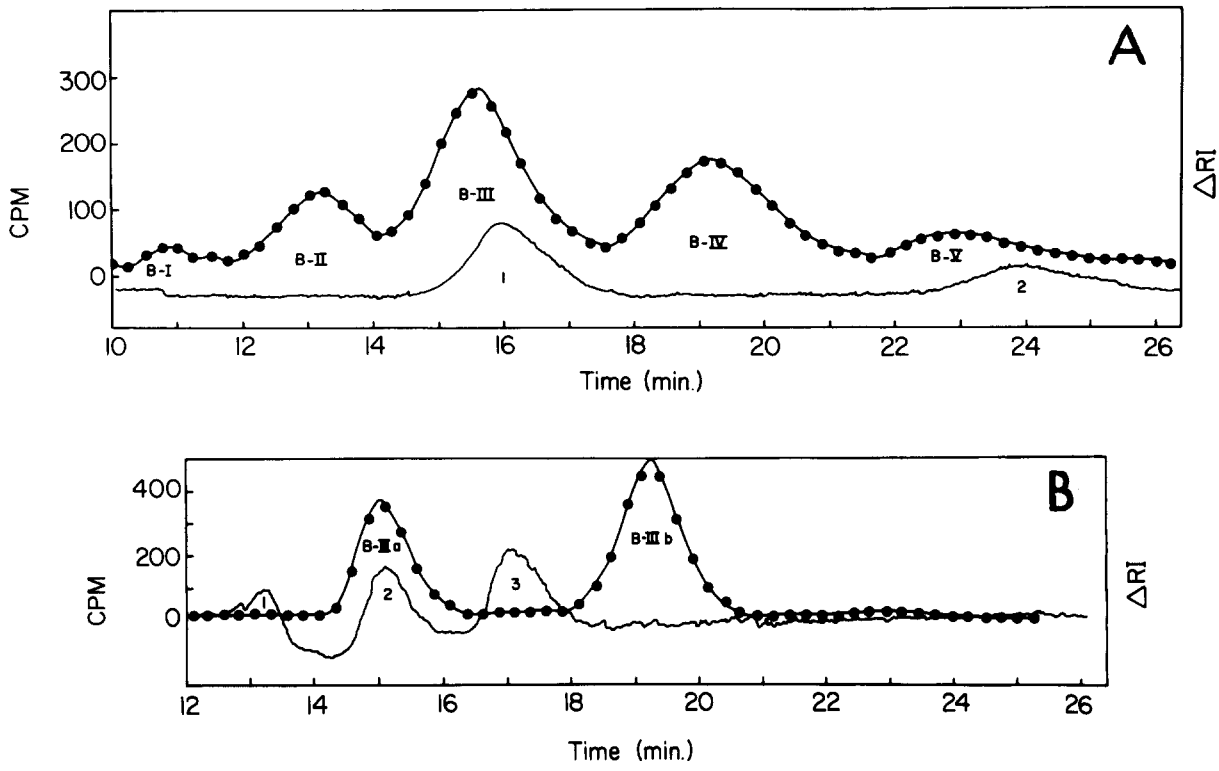

Fig. 2. A. Reverse-phase HPLC chromatogram of the methyl sterol region (Class B from Step 1, the short silicic acid column) on a μBondapak-C_{18} column (3.9 mm × 30 cm, see Table 1) eluted with acetonitrile–water 88:12. The sample was from an incubation of normal primary rat hepatocyte culture cells incubated with [^{14}C]mevalonate.[2] Aliquots of each fraction were assayed for radioactivity (scintillation counter), then the peaks were combined and acetylated for silicic acid HPLC as described in Fig. 2B; ● —— ●, radioactivity (CPM); ∿∿, ΔRI; solvent flow, 2.5 ml/min, attenuator 4X, chart speed 2.5 cm/min (Houston recorder), fraction collector (Buchler) set for 15 sec fractions of 0.63 ml each. Authentic RI standards of lanosterol, 1, and dihydrolanosterol, 2, were co-chromatographed with the sample. The chromatogram is corrected for an 0.8 min lag between the refractive index (RI) detector and the fraction collector. Tentative assignments for the radioactive peaks are: 4-methyl-$\Delta^{8,24}$-cholestadienol, B-II; 4,4-dimethyl-$\Delta^{8,24}$-cholestadienol and 4,4,14α-trimethyl-$\Delta^{8,24}$-cholestadienol, B-III; 4-methyl-Δ^{8}-cholestenol, B-IV; 4,4-dimethyl-Δ^{8}-cholestenol, B-V. B. The fractions comprising peak B-III in the reverse-phase HPLC chromatogram in Fig. 2A were combined, acetylated, and subjected to silicic acid HPLC on a μPorasil column (3.9 mm × 30 cm, see Table 3) eluted with *i*-octane–cyclohexane–toluene 50:30:20. The radioactive sample was co-chromatographed with the following authentic RI standards: dihydrolanosteryl acetate, 1, lanosteryl acetate, 2, cholesteryl acetate, 3: ● —— ●, radioactivity (CPM); ∿∿, RI. Other conditions, e.g., flow rate, recorder speed, fraction size, fraction time interval, were the same as in Fig. 2A. Small apparent discrepancies between some of the relative retention times shown in the Tables and those which can be calculated from the chromatographic tracings can be caused by small changes in solvent composition and/or the retentivity of a particular column. This emphasizes the importance of including authentic standards either within the run or immediately preceeding or following the run. For example, in Fig. 2B the relative retention time of the cholesteryl acetate standard, 3, varied from that given in Tables 3 and 4 by 7%. If the Peak B-IIIb relative retention time is also corrected by 7%, then this value is consistent with the retention time given for 4,4-dimethyl-$\Delta^{8,24}$-cholestadienyl acetate. Tentative assignments for the radioactive peaks are: 4,4,14α-trimethyl-$\Delta^{8,24}$-cholestadienyl acetate, B-IIIa; 4,4-dimethyl-$\Delta^{8,24}$-cholestadienyl acetate, B-IIIb.

be separated from trace quantities of radioactive sterols. HPLC columns with similar or greater capacities are available; however, no studies have as yet been published on their use in this application. Normally, the columns utilized in Tables 1 and 3 and Figs. 1–3, which can tolerate sterol loads of up to 1 mg, are quite sufficient for most biological studies with cultured cells, liver homogenates, or liver tissue studies (up to approximately 1 g of liver).

Trocha, Jasne, and Sprinson (18) have used reverse-phase HPLC in their studies of sterols from yeast cell mutants. The eluting solvents were: tetrahydrofuran–acetonitrile–water 5:5:2 and acetonitrile–water 10:1, using two 3.9 mm × 30 cm columns of μBondapak-C_{18}, connected in series.

A number of other investigators have used HPLC to separate various sterols in the course of their studies. Smith and Hogle (19) separated the α-isomers from the β-isomers, when sterol benzoates were chromatographed using Corasil II with petroleum ether–methylene chloride as the eluting solvent. They were also able to separate sterol benzoates according to the number of carbon-carbon double bonds with Corasil II impregnated with 5% silver nitrate, using gradient elution techniques.

Rees, Donnahey, and Goodwin (20) separated C_{27},

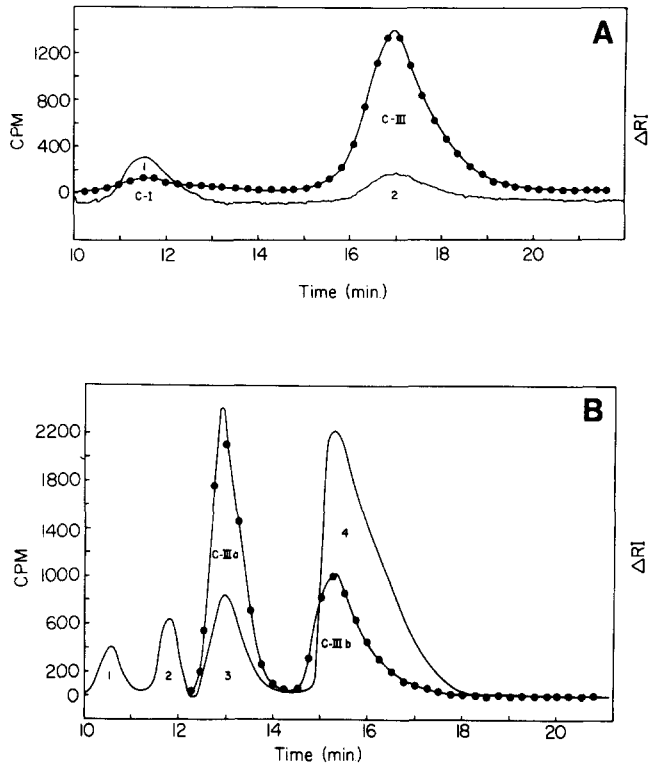

Fig. 3. A. Reverse-phase HPLC chromatogram of the C_{27} sterol region (Class C from Step 1) on a μBondapak-C_{18} column (3.9 mm × 30 cm, see Table 1), eluted with acetonitrile–water 88:12. The sample is from an incubation of normal primary rat hepatocyte cultured cells incubated with [^{14}C]mevalonate.[2] Authentic RI standards were: desmosterol, 1, cholesterol, 2. Other chromatographic conditions were as described in Fig. 2A. Tentative assignments for the radioactive peaks are: C_{27} sterols with 2 double bonds, C-I; C_{27} sterols with 1 double bond, C-III. B. The fractions comprising Peak C-III from the reversephase HPLC chromatogram in Fig. 3A were combined, acetylated, and subjected to silicic acid HPLC on a μPorasil column (3.9 mm × 30 cm, see Tables 3 and 4), eluted with *i*-octane–cyclohexane–toluene 50:30:20. The radioactive sample, ● —— ●, was co-chromatographed with the following RI standards, ∿∿∿: dihydrolanosteryl acetate, 1, lanosteryl acetate, 2, cholesteryl acetate, 3, and Δ^7-cholestenyl acetate, 4. Neither the Δ^7-cholestenyl acetate standard nor its radioactive counterpart correlate well with the relative retention times given in Table 3 because of overloading. Tentative assignments for the radioactive peaks are: cholesteryl acetate, C-IIIa; Δ^7-cholestenyl acetate, C-IIIb.

C_{28}, and C_{29} sterol acetates and certain sterol benzoates by reverse-phase HPLC. They also reported separations of sterols differing in the number of carbon-carbon double bonds.

Oxygenated sterols, both naturally occurring and auto-oxidation products, have been studied in recent papers by Ansari and Smith (21) and by Francisco et al. (22). Both laboratories employed multi-step chromatographic techniques, which included two HPLC columns in tandem.

HPLC procedures have also been utilized in the identification of sterols occurring in marine invertebrates as described by Popov and colleagues (23).

TABLE 5. Functional group retention factors encountered in sterol intermediates in cholesterol biosynthesis

Functional Group	Reverse-Phase HPLC	Silicic Acid HPLC
Δ^5	0.77	0.98
Δ^7	0.77	1.24
Δ^8	0.77	1.17
$\Delta^{8(14)}$	0.74	1.09
$\Delta^{5,7}$	0.58	1.25
Δ^{24}	0.69	1.18
14α-Methyl	1.03	0.83
4-Methyl	1.13	0.93
4,4-Dimethyl	1.33	0.80

The functional group retention factors were calculated relative to cholestanol for reverse-phase HPLC and relative to cholestanyl acetate for normal phase (silicic acid) HPLC. On reverse-phase HPLC, cholestanol had a relative retention time (relative to dihydrolanosterol) of 0.93; on silicic acid HPLC, cholestanyl acetate had a relative retention time of 1.25. For example, the relative retention time for cholesterol (Table 1) on reverse-phase HPLC is 0.72; functional group factor = 0.72/0.93 = 0.77 for Δ^5 factor.

The great complexity of sterol intermediates synthesized from radioactive acetate or mevalonate in various biological systems, e.g., primary hepatocyte cells and liver homogenates, mandates the use of sophisticated chromatographic techniques such as HPLC. The present article describes a high resolution three-step chromatographic procedure which separates all of the available sterol intermediates tested. The three steps are: Step 1, short silicic acid column for class separation; Step 2, reverse-phase HPLC of the unesterified sterols in each class; and Step 3, silicic acid HPLC of sterol acetates from the peaks obtained in Step 2. The application of this technique to the separation of radioactive sterols synthesized from [^{14}C]mevalonate by normal rat primary hepatocyte cultured cells was accomplished.

The results presented here support the conclusion that HPLC is an extremely valuable method for the separation of sterol intermediates in cholesterol biosynthesis, and it may be expected that HPLC techniques will be utilized to an even greater extent in the future study of cholesterol biosynthesis and regulation.

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