notes on methodology

A method for the rapid qualitative and quantitative analysis of 4,4-dimethyl sterols

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Summary A simple and relatively rapid technique has been developed for the separation of several 4,4-dimethyl steryl acetates, some of which contain sterically hindered nuclear double bonds. The method involves thin-layer chromatography on silver nitrate-impregnated silica gel and silver nitrate-impregnated alumina. The separated steryl acetates may then be analyzed quantitatively by gas–liquid chromatography.

Supplementary key words thin-layer chromatography · silica gel–silver nitrate · alumina–silver nitrate · gas–liquid chromatography

The number of potential intermediates in the biosynthetic pathway between lanosterol (Fig. 1, *1a*) and cholesterol is very large (1) and the order in which the methyl groups are removed, the nuclear double bond isomerized, and the side-chain double bond saturated remain in some doubt. Studies in this laboratory are concerned with differentiating between those compounds which constitute the major biosynthetic pathway and those which, although they are convertible to cholesterol, participate only to a minor or negligible extent. In particular, we are interested in the mechanism and sequence of events by which the 14α-methyl group of lanosterol is removed. Fig. 1 shows the structures of some of the compounds that have been variously proposed as intermediates in the early stages of cholesterol biosynthesis from lanosterol. It is important, therefore, that methods are available not only for the separation and identification of these types of compounds but for an accurate measurement of the small mass of these materials that may be present in biological samples during cholesterol biosynthesis from acyclic precursors.

In the past, techniques involving the use of silver nitrate-impregnated thin-layer plates of silica gel (2–6) or alumina (7) have been successful only in separating compounds differing in the number of double bonds (e.g., *1a* and *1b*, Fig. 1). Compounds containing a single double bond (e.g., *1b, 2b, 3, 5,* and *6b*, Fig. 1) are either inseparable or only partially separable from each other in these systems. Column chromatography involving the use of silver nitrate-impregnated stationary phases (8, 9) has provided good resolution, but the method is time consuming and unsuitable for routine analysis involving many samples.

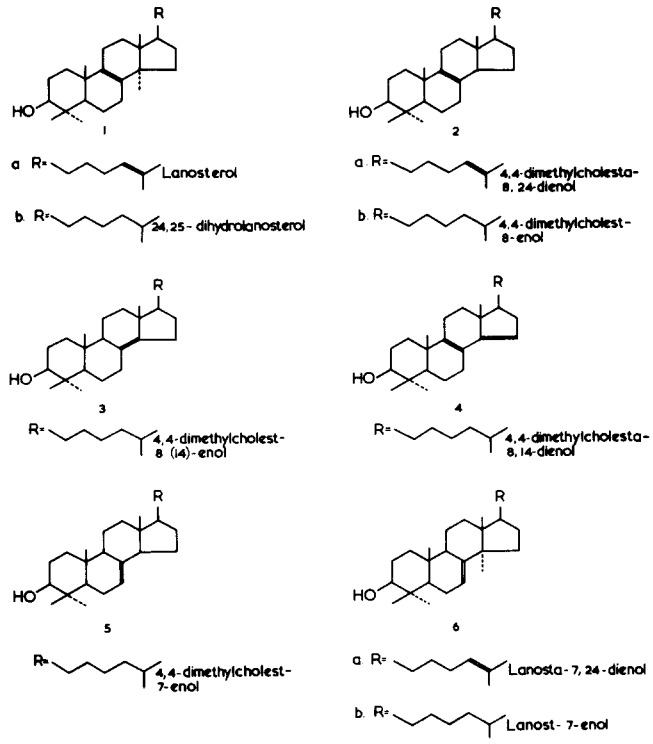

Fig. 1. Structures of some known and proposed 4,4-dimethyl sterol precursors of cholesterol.

The present report describes a method for the rapid and routine separation, by thin-layer argentation chromatography, of the acetates of a number of 4,4-dimethyl sterols that may be involved in cholesterol biosynthesis. The weights of the separated acetates may then be determined by GLC. The technique has been of practical importance in this laboratory in the separation, identification, and quantitative analysis of small amounts of these types of materials present in biological extracts.

Experimental

Materials. Silica gel H was obtained from E. Merck, Darmstadt, Germany. Alumina G (containing 10% gypsum by weight) was obtained from M. Woelm, Eschwage, Germany. Analar grade silver nitrate and rhodamine 6G were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Analar benzene and reagent grade hexane were obtained from Hopkin & Williams Ltd., Chadwell Heath, Essex, U.K., and both were distilled prior to use. Cholesterol was purified from a commercial preparation (BDH Biochemicals). Lanosteryl acetate was prepared from a

Abbreviations: GLC, gas–liquid chromatography, TLC, thin-layer chromatography.

commercial sample of lanosterol (Aldrich Chemical Co., Inc., Milwaukee, Wis.) by the method of Bloch and Urech (10). 24,25-Dihydrolanosteryl acetate was prepared from this material by catalytic hydrogenation using PtO_2. Lanosta-7,24-dienyl acetate[1] and lanost-7-enyl acetate were prepared by the method of Marker, Wittle, and Mixon (11). 4,4-Dimethylcholesta-8,14-dienyl acetate, 4,4-dimethylcholest-8(14)-enyl acetate, and 4,4-dimethylcholest-8-enyl acetate were prepared by previously published methods (12). 4,4-Dimethylcholest-7-enyl acetate was the kind gift of Dr. J. L. Gaylor, Cornell University, Ithaca, N.Y., and was also prepared by hydrogenation of 4,4-dimethylcholesta-5,7-dienol using Raney nickel catalyst (13) followed by acetylation.

Thin-layer chromatography. A solution of 4 g of silver nitrate in 80 ml of water was mixed with silica gel H (40 g). The resultant slurry was sufficient for the preparation of five plates, each 0.3 mm thick. Silver nitrate-impregnated plates of alumina were prepared by adding a solution of silver nitrate (14 g) in water (57 ml) to alumina G (50 g). The slurry was used to prepare five plates (20 × 20 cm), each 0.3 mm thick. All plates were air-dried at room temperature and then heated to 110°C for 2 hr. Each steryl acetate (40 μg, contained in 10 μl of a mixture of chloroform and methanol 2:1, v/v) was applied as a strip 4 cm wide and 2 cm from the bottom of the plate. For chromatography on silica gel–silver nitrate, a mixture of benzene and hexane 50:50 (v/v) was used as the developing solvent. Alumina–silver nitrate plates were placed in chromatographic tanks containing 200 ml of a mixture of benzene–hexane (either 15:85, v/v, or 25:75, v/v). The tank containing the solvent was allowed to equilibrate at 5°C for 2 hr prior to chromatography, and the plates were developed at the same temperature. The solvent was allowed to rise 16.5 cm from the origin, and the time required for complete development was about 2.0 hr. In all cases, the tanks were lined with chromatography paper prior to addition of the solvent, and after development the sterol bands were visualized by spraying the plates with a solution of rhodamine 6G in acetone (0.1%, w/v). The fractions containing the separated components of the mixture were scraped from the plate, and the steryl acetates were obtained by elution with ether (60 ml) through a filter funnel containing silica gel H to remove contaminating rhodamine 6G.

Gas–liquid chromatography. GLC was carried out on a Varian Associates gas chromatograph series 2740 equipped with dual flame ionization detectors and 6 ft × ⅛ inch ID glass columns. The columns were packed with stationary phases of either 1.5% SE-30 or 1.5% QF-1 supported on Varoport 30 (80–100 mesh). The operating conditions were: column temperature, 220°C; injector temperature, QF-1, 272°C, SE-30, 266°C; detector temperature, 262°C. Nitrogen gas flow rates were 30 ml/min (QF-1) and 50 ml/min (SE-30). Cholesterol was used as internal standard in all determinations.

[1]Lanosteryl acetate was converted to the 24,25-dibromide prior to isomerization. The reaction product was debrominated with sodium iodide.

TABLE 1. Chromatographic properties of 4,4-dimethyl steryl acetates on silica gel–silver nitrate and alumina–silver nitrate

Compound	Silica Gel–Silver Nitrate	Alumina–Silver Nitrate	
	R_F	R_F	$R_S \times 100$
4,4-Dimethylcholest-7-enyl acetate	0.30	0.20	235
4,4-Dimethylcholest-8-enyl acetate	0.30	0.21	247
4,4-Dimethylcholest-8(14)-enyl acetate	0.30	0.27	318
24,25-Dihydrolanosteryl acetate	0.30	0.34	400
Lanost-7-enyl acetate	0.30	0.29	341
Lanosteryl acetate	0.23	0.08 (0.25)	94 (92)
Lanosta-7,24-dienyl acetate	0.23	0.06 (0.20)	70 (74)
4,4-Dimethylcholesta-8,14-dienyl acetate	0.14		

With silica gel–silver nitrate plates, benzene–hexane 50:50 (v/v) was used. Benzene–hexane 15:85 (v/v) was used as solvent for the alumina–silver nitrate plates, which were developed at 5°C. Figures in parentheses refer to the R_F and R_S values of the respective compounds in a solvent system consisting of benzene–hexane 25:75 (v/v). R_S values were measured using cholesteryl acetate as standard.

Results and discussion

Table 1 shows the R_F values, on silica gel–silver nitrate, of the acetates of some established and proposed 4,4-dimethyl sterol precursors of cholesterol. Although there is a useful separation of compounds containing different numbers of double bonds and of those compounds containing conjugated and nonconjugated double bond systems, there is no separation of those compounds containing only one double bond. Variations in the amount of silver nitrate incorporated into the silica gel (10% w/w, 20% w/w, or 30% w/w), the type of solvent system used (benzene–petroleum ether 50:50 [v/v], chloroform–petroleum ether 30:70 [v/v], chloroform–petroleum ether 15:85 [v/v], and ethyl acetate–petroleum ether 3:97 [v/v]), or a combination of these were also unsuccessful in separating these types of compounds. The acetates of these 4,4-dimethyl sterols were then chromatographed on silver nitrate-impregnated plates of alumina. Table 1 shows the R_F and R_S values in this system. Mixtures containing all of these compounds showed separation of all the components except 4,4-dimethylcholest-8-enyl acetate and 4,4-dimethylcholest-7-enyl acetate (Fig. 2). These results demonstrate useful separations of compounds containing hindered double bonds in the Δ^8, $\Delta^{8(14)}$, and Δ^7 positions, and the rapidity of the method allows the routine analysis of many samples. The very large separation between lanosteryl acetate and dihydrolanosteryl acetate illustrates the remarkable sensitivity of this system to small changes

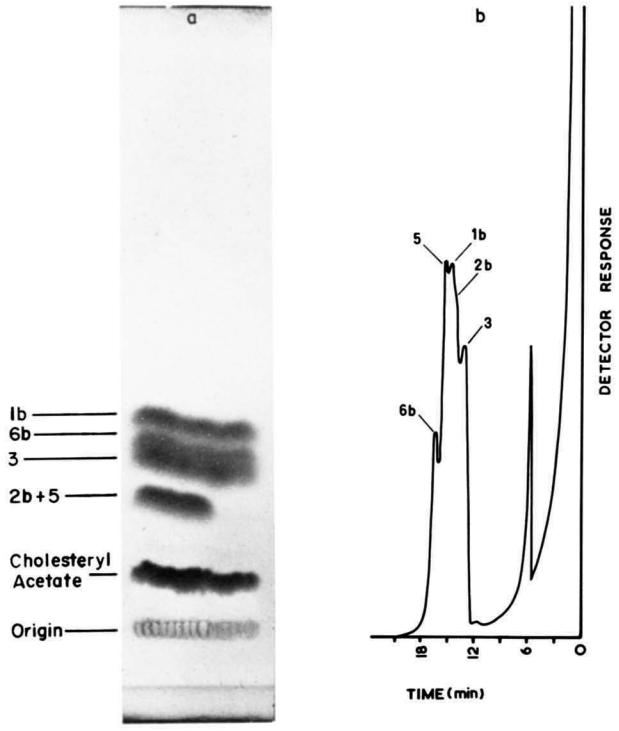

Fig. 2. (*a*) TLC separation of the acetates of some 4,4-dimethyl sterols. A mixture of the acetates of *1b* (40 μg), *2b* (20 μg), *3* (40 μg), *5* (20 μg), and *6b* (40 μg) (Fig. 1) was chromatographed on an alumina–silver nitrate plate as described in the text. The solution containing *2b* and *5* was spotted out along a distance of 2 cm at the origin, the remainder along a distance of 4 cm. In this case (for visual representation), the developed plate was sprayed with 50% sulfuric acid and charred at 110°C for 10 min. (*b*) GLC of a 4,4-dimethyl steryl acetate mixture. A mixture of the acetates of *1b* (1 μg), *2b* (1 μg), *3* (0.5 μg), *5* (1 μg), and *6b* (0.5 μg) (Fig. 1) and cholesterol (0.3 μg) in a total of 3.0 μl of solvent was chromatographed by GLC. The operating conditions are given in the text.

in the polarity of molecules. Because of the large difference in polarity between Δ^{24} and 24,25-dihydro compounds, the separation of individual compounds containing a Δ^{24} bond was not as large as that observed in the 24,25-dihydro series. In biosynthetic studies, therefore, we have found it convenient to separate the former compounds from the latter by TLC on silica gel–silver nitrate, followed by chromatography of each fraction independently on alumina–silver nitrate. The solvent systems used were benzene–hexane 25:75 (v/v) for Δ^{24} bond-containing steryl acetates and benzene–hexane 15:85 (v/v) for 24,25-dihydro steryl acetates. The R_F and R_S values for the Δ^{24} bond-containing compounds in the more polar solvent system are presented in Table 1 and show a better separation of the Δ^7 and Δ^8 compounds. In addition to the better separation of the Δ^{24} bond-containing compounds, this procedure eliminates any confusion that may arise as a result of the possible simultaneous occurrence of a large number of 4,4-dimethyl-Δ^{24} and 4,4-dimethyl-24,25-dihydro sterols in biosynthetic studies using acyclic

TABLE 2. GLC characteristics of 4,4-dimethyl sterols and 4,4-dimethyl steryl acetates

Compound	RRT[a] (SE-30)	RRT (QF-1)
4,4-Dimethylcholest-8-enol	1.640	1.416
4,4-Dimethylcholest-8(14)-enol	1.590	1.399
4,4-Dimethylcholest-7-enol	1.822	1.612
4,4-Dimethylcholesta-8,14-dienol	1.627	1.405
24,25-Dihydrolanosterol	1.591	1.482
Lanosterol	1.740	1.592
4,4-Dimethylcholest-8-enyl acetate	2.275	2.391
4,4-Dimethylcholest-8(14)-enyl acetate	2.183	2.314
4,4-Dimethylcholest-7-enyl acetate	2.533	2.639
4,4-Dimethylcholesta-8,14-dienyl acetate	2.258	2.334
24,25-Dihydrolanosteryl acetate	2.227	2.543
Lanosteryl acetate	2.437	2.748
Lanost-7-enyl acetate	2.529	2.826
Lanosta-7,24-dienyl acetate	2.813	3.089

Because relative retention times differed slightly from day to day, all samples were chromatographed on the same day.

[a] Relative retention time (cholesterol = 1).

precursors of cholesterol. With all compounds studied, application of quantities greater than 200 μg to a band width of 4 cm resulted in considerable "tailing," with concomitant decrease in resolution.

Quantitative estimation of the weight of steryl acetate present in each fraction eluted from the plate was carried out by GLC. To determine the efficiency of recovery of the steryl acetates from the TLC plate, 40 μg of each of the acetates of *1b*, *2b*, *3*, and *6b* (see Fig. 1) was chromatographed independently and in quadruplicate. The recoveries were 87 ± 9% (SEM), 88 ± 3%, 98 ± 13%, and 101 ± 9%, respectively, as measured by GLC. The differences in relative retention times (RRT, cholesterol = 1, Table 2) of the separated monounsaturated steryl acetates are large enough to provide confirmation of identity. Thus, GLC may also be used for confirmation of structure as well as for quantitative estimation of the separated steryl acetates. However, although there is a significant difference in RRT between these compounds when each is chromatographed independently, this difference is not sufficient for complete separation by GLC when they are chromatographed as a mixture (Fig. 2).

We have used these techniques to identify, and estimate both radioactivity and mass of, 4,4-dimethyl sterols formed from [2-^{14}C]mevalonic acid in vitro under different biosynthetic conditions (14). In practice, the ^{14}C-labeled 4,4-dimethyl sterol fraction was separated from 4-demethyl and 4α-methyl sterols by TLC on silica gel. Each radioactive sterol fraction was located by radioautography. To ensure the complete elimination of any contaminating 4α-methyl sterols, the 4,4-dimethyl sterol fraction was rechromatographed in the same system. The complete absence of 4α-methyl sterols in the recovered fraction was demonstrated by radioautography. The 4,4-dimethyl sterol fraction was acetylated and the Δ^{24} bond-containing steryl acetates were separated from 24,25-dihydro compounds by TLC on silver nitrate-impregnated sili-

ca gel prior to chromatography of each fraction on alumina–silver nitrate and subsequent assay of each separated component by GLC. Virtually complete recovery of radioactivity from the alumina–silver nitrate plates was obtained. In this way, we have been able to demonstrate the presence of *1a, 1b, 2a,* and *2b* (Fig. 1) by their chromatographic properties and mass spectra. The actual weights of these compounds present at the end of the incubations, and their specific radioactivities, have been measured. This type of information has allowed us to calculate the steady-state levels of 4,4-dimethyl sterols and has proved of value in elucidating the major metabolic pathway in the early stages of cholesterol biosynthesis from lanosterol.

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