Separation of 24- and 25-dehydrocholesterols; and the impure state of commercial desmosterol preparations

JAMES A. SVOBODA and MALCOLM J. THOMPSON

Insect Physiology Laboratory, Entomology Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705

SUMMARY Chromatographic methods for the separation of 24- and 25-dehydrocholesterols are described. The purities of three commercial samples of 24-dehydrocholesterol examined by thin-layer and gas–liquid chromatography were only 42, 79, and 80%, respectively; a commercial sample of radioactive 24-dehydrocholesterol was shown to contain 40% 25-dehydrocholesterol.

KEY WORDS 24- and 25-dehydrocholesterols · separation · silver nitrate · thin-layer · column · chromatography · radiochemical purity

THE LEAST INVOLVED procedure for the preparation of 24-dehydrocholesterol (desmosterol) usually results in a mixture of 24- and 25-dehydrocholesterols. Since both compounds were found to be present in commercial preparations of desmosterol, even in a sample of desmosterol-26,27-^{14}C, and since they are inseparable by GLC, we have developed chromatographic techniques for their separation using the acetates.

Methods. All melting points were determined on a Kofler[1] block. Infrared spectra were taken in carbon disulfide solutions with a Perkin-Elmer model 221 prism-grating double beam spectrophotometer. The GLC analyses were made on model 10 and 15 Barber-Colman instruments with radium sulfate β-ionization detectors. The support was deactivated and coated according to the method of Horning, VandenHeuvel, and Creech (1). Radioactive samples were counted with a Packard Tri-Carb scintillation spectrometer. All solvents for chromatography were redistilled over sodium.

24- and 25-Dehydrocholesterol Acetates from 25-Hydroxycholesterol Acetate. The reaction of 26-norcholesten-25-on 3β-ol (generously provided by Schering Corp., Bloomfield N.J.) with methyl magnesium iodide according to the procedure of Dauben and Bradlow (2) gave 25-hydroxycholesterol in 90% yield. The 25-hydroxycholesterol acetate was prepared and then dehydrated with phosphorous oxychloride in benzene–pyridine 2:1 at reflux temperature for 1 hr to give a

Abbreviations: TLC, thin-layer chromatograpy; GLC, gas–liquid chromatography; RRT, relative retention time.

[1] Mention of a company name or a proprietary product does not necessarily imply endorsement by the U. S. Department of Agriculture.

mixture of 24- and 25-dehydrocholesterol acetates, as determined by infrared analysis.

24-Dehydrocholesterol Acetate. The mixture of 24- and 25-dehydrocholesterol acetates in 50-mg portions was applied in streaks (Radin-Pelick Streaker, Applied Science Laboratories Inc., State College, Pa.) to several preparative thin-layer plates (20 × 20 cm, 500 μ thickness) of Silica Gel H (Brinkmann Instruments Inc., Westbury, N.Y.) impregnated with 20% (w/w) silver nitrate (3) and developed with benzene (without wicks). After the solvent had moved 19 cm, all of each plate except the two sides was covered with another glass plate; the plates were sprayed with 50% sulfuric acid solution and heated until color developed. There were two bands with R_f values of 0.81 and 0.64, containing 24- and 25-dehydrocholesterol acetates respectively. The upper band was then marked off and removed from the plate, and the sterol acetate was eluted from the adsorbent with ether. Any residue or impurities derived from the thin-layer adsorbent could be readily removed by filtration of the eluate through a column of neutral Woelm alumina, activity grade II, with benzene. The results from several plates showed that about 56% of the original mixture consisted of 24-dehydrocholesterol acetate. Recrystallization from methanol–acetone gave plates, mp 96–97°C, α_D −43°, ν in CS_2 1739 cm^{-1} (acetate), 1666 cm^{-1}, doublet at 835 and 820 cm^{-1} (Δ^5- and Δ^{24}-double bonds); (reference 4: mp 99–100°C, α_D −39°).

24-Dehydrocholesterol. Saponification of the 24-dehydrocholesterol acetate with 2% methanolic KOH solution gave the free sterol as plates after recrystallization from methanol, mp 121–122°C, α_D −41°, ν in CS_2 3610 cm^{-1} (hydroxyl), 1665 cm^{-1}, doublet at 835 and 821 cm^{-1} (Δ^5- and Δ^{24}-double bonds), doublet at 958 and 950 cm^{-1} (typical of a $\Delta^{5,24}$-sterol); (reference 4: mp 117–118°C, α_D −38°). Only a single peak was seen on three GLC systems (Table 1).

TABLE 1 GLC Data from Synthesized Material

Compound	Relative Retention Times*		
	QF-1†	NGS‡	SE-30§
24-Dehydrocholesterol acetate	4.95	7.60	2.94
25-Dehydrocholesterol acetate	5.13	7.67	2.91
24-Dehydrocholesterol	3.06	8.51	2.03
25-Dehydrocholesterol	3.18	8.53	2.00

* Retention time relative to that of cholestane.

† Column 6 ft × 4 mm I.D., 3.0% QF-1 (methyl fluoroalkyl silicone) (10,000 centistokes) on 100–140 mesh Gas-Chrom P, 40 psi, 230°C, cholestane time 5.5 min.

‡ Column 6 ft × 4 mm I.D., 0.75% neopentyl glycol succinate polyester on 100–140 mesh Gas-Chrom P, 32 psi, 215°C, cholestane time 2.5 min.

§ Column 6 ft × 4 mm I.D., 0.75% SE-30 (methyl polysiloxy gum, General Electric) on 100–140 mesh Gas-Chrom P, 16 psi, 232°C, cholestane time 5.0 min.

25-Dehydrocholesterol Acetate. The lower band containing the 25-dehydrocholesterol acetate was removed, and the sterol acetate was eluted from the thin-layer adsorbent with ether. The 25-dehydrocholesterol acetate represented about 44% of the original mixture. Recrystallization from methanol–acetone gave plates mp 113–114°C, α_D −40°, ν in CS_2 1735 cm^{-1} (acetate), 1650 and 890 cm^{-1} [strong, $-C(CH_3)=CH_2$]; (reference 5: mp 112°C, α_D −44°).

25-Dehydrocholesterol. Saponification of the 25-dehydrocholesterol acetate with a solution of 2% methanolic KOH gave the free sterol as plates from methanol, mp 135–136°C, α_D −44°, ν in CS_2 3610 cm^{-1} (hydroxyl), 1649 and 885 cm^{-1} [strong, $-C(CH_3)=CH_2$]; (reference 5: mp 132.5°C, α_D −41°). One peak was found when the material was examined by GLC.

The R_f values of 24- and 25-dehydrocholesterol acetates on an analytical silver nitrate-impregnated Silica Gel H plate (250 μ thickness) were 0.79 and 0.56 respectively.

Purification of 24-Dehydrocholesterol Acetate by Column Chromatography. A 97 mg sample of the 24- and 25-dehydrocholesterol acetate mixture was chromatographed on a column, washed with benzene–hexane 30:70, of 12 g of 20% silver nitrate-impregnated Unisil (Clarkson Chemical Co., Williamsport, Pa.) (6). Fractions (10 ml) were collected as follows: 24 fractions of benzene–hexane 30:70, 20 fractions of benzene–hexane 40:60, and 20 fractions of benzene. These fractions, as monitored by TLC, showed that fractions 11–25 contained about 42% of the original mass as pure 24-dehydrocholesterol acetate. The remaining fractions still consisted of a mixture of 24- and 25-dehydrocholesterol acetates. Rechromatography permitted further recovery of uncontaminated 24-dehydrocholesterol acetate. Because of considerable tailing of 24-dehydrocholesterol acetate on this column, only a small percentage of pure 25-dehydrocholesterol acetate could be obtained by column chromatography.

24-Dehydrocholesterol-26,27-^{14}C Acetate. A commercial sample of 24-dehydrocholesterol-26,27-^{14}C was acetylated, and the radiochemical purity of the compound was checked by TLC. The spot that corresponded to 24-dehydrocholesterol acetate contained 57.6% of the recovered radioactivity; the spot corresponding to 25-dehydrocholesterol acetate contained 39.8% of the recovered activity.

Commercial Desmosterol Preparations. Three commercial sources of unlabeled desmosterol, referred to here as source 1, source 2, and source 3, claimed purity of their desmosterol to be 99, 95, and 98%, respectively. The samples, received as the free sterol in crystalline form, were immediately stored in benzene to prevent further

deterioration[2] (7). Each sample was examined by GLC as the free sterol (by the systems specified under Table 1) and then acetylated. Initial GLC analyses showed that desmosterol from sources 1 and 2 contained a component that exhibited a relative retention time (RRT) less than that of desmosterol on all three GLC systems. The GLC analyses of desmosterol from source 3 indicated the presence of at least eight compounds, and part of the sample was insoluble in benzene. Preparative TLC of acetylated commercial desmosterol showed the presence of 25-dehydrocholesterol acetate in every sample. Analyses by GLC of the 24-dehydrocholesterol acetate recovered from sources 1 and 2 showed a component other than 24-dehydrocholesterol acetate whereas the corresponding 25-dehydrocholesterol acetate from both sources yielded only one peak. Neither 24- nor 25-dehydrocholesterol acetate could be obtained in a pure state from source 3 by preparative TLC. The quantitative GLC analyses (SE-30 system) of the acetates recovered from preparative TLC showed that the original purity of desmosterol from sources 1, 2 ,and 3 was about 79, 80, and 42%, respectively. The purified 24- and 25-dehydrocholesterols and their acetates obtained from sources 1 and 2 were further identified by direct comparison of their physical properties and infrared spectra.

Table 1 summarizes the RRT's of our synthetic 24- and 25-dehydrocholesterols and their acetates. The purified 24- and 25-dehydro compounds obtained from the commercial samples also gave these values. Although the RRT values of the two sterols differ slightly, these differences do not permit the detection of one in the presence of the other by GLC. Thus, a GLC analysis of desmosterol should not be used as the sole criterion to establish purity.

Discussion. The TLC method presented was able to separate a newly synthesized mixture of 24- and 25-dehydrocholesterol acetates in which no deterioration had occurred, and to provide these compounds in a state of purity previously not available. However, certain impurities, perhaps decomposition products of 24-dehydrocholesterol (7), could not be completely removed from commercial samples of desmosterol by this method. We have used successfully the column chromatography system on $AgNO_3$-impregnated Unisil for the preparation of large quantities of 24-dehydrocholesterol.

We have examined three different commercial samples of desmosterol with an assigned purity of 95% or higher, and one commercial sample of labeled desmosterol. The GLC analyses, after preparative TLC, showed that the original purity of the unlabeled desmosterol preparations was from 42 to 80%. From 7 to 10% 25-dehydrocholesterol was found in these samples, which suggests that they were synthesized via the dehydration of 25-hydroxycholesterol. In the 26,27-^{14}C-labeled compound, the proportion of 58% labeled desmosterol and 40% labeled 25-dehydrocholesterol closely agrees with the ratio of 24- and 25-dehydrocholesterol acetates (56:44) we obtained from the dehydration of 25-hydroxycholesterol acetate with phosphorous oxychloride.

The above results indicate the necessity for thorough examination of preparations of desmosterol before they are used.

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[2] In our laboratory, 24-dehydrocholesterol has been shown to be very stable over long periods of time, even at room temperature, when it is stored in benzene; the crystalline material undergoes rapid deterioration at room temperature.

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