

Column Chromatography of Steryl Acetates on a Silver Nitrate-Impregnated Silicic Acid

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Abstract

This investigation deals with the separation of complex steryl acetate mixtures by an improved procedure for column chromatography on a silver nitrate-impregnated silicic acid. Separations were effected on the basis of the number and position of double bonds. Also, some relationship could be observed between the chemical structure of steryl acetates and the mobility. The application of this technique for the sterol mixture isolated from a short-necked clam, *Tapes philippinarum*, gave the complete separation of several components; brassicasterol, 22-dehydrocholesterol, 22-trans-24-norcholesta-5, 22-dien-3 β -ol, 28-isofucosterol, desmosterol, 24-methylencholesterol, and an unknown sterol.

Some classes of marine invertebrates have been pointed out by gas-liquid chromatography (GLC) to contain very complex sterol mixtures in their tissues¹⁾. Since the separation of sterols with closely related structures is generally difficult, the conclusive elucidation of sterol components, especially minor ones, has been achieved about relatively a few animals. Up to the present, several workers have proposed the methods for separation of sterols by thin-layer chromatography (TLC) using a silver nitrate-impregnated silicic acid²⁻¹¹⁾ and using a reversed-phase adsorbent¹²⁻¹⁶⁾. Recently, IDLER and SAFE have reported the improved procedure for preparative separation of steryl acetates on a silver nitrate-impregnated thin-layer plate¹¹⁾.

Generally, a relatively large amount of material is required for conclusive elucidation of chemical structure of unknown sterols by infrared absorption (IR) spectroscopy, nuclear magnetic resonance (NMR) spectrometry, and X-ray diffraction analysis. However, the above mentioned TLC techniques are conceived not to be so excellent as a method for isolation of minor components from very complex sterol mixtures. Our studies on marine sterols often made it necessary for us to isolate a large amount of pure minor components occurring in mollusks for elucidation of chemical structures or for use in feeding experiments. In a previous paper¹⁷⁾, the authors have succeeded in isolating several sterols from a short-necked clam, *Tapes philippinarum*, by column chromatography on a silver nitrate-impregnated silicic acid. In a present study, the attempt to isolate more completely each component from complex sterol mixtures was carried out. The present paper deals with the improved procedure for separation of steryl acetate mixtures by column chromatography on a mixture of silicic acid and silver nitrate.

Materials and Methods

Chemicals. All solvents used were reagent grade and redistilled before use. Silicic acid (100 mesh, analytical reagent for chromatography) was purchased from Mallinckrodt Works,

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U.S.A. Cholesterol, desmosterol, campesterol, stigmasterol, and β -sitosterol were obtained from Nakarai Chemicals Co. and/or Gas-Chrokogyo Co., Japan. 22-Dehydrocholesterol^{17,18)}, 22-*trans*-24-norcholesta-5, 22-dien-3 β -ol¹⁹⁾, brassicasterol²⁰⁾, 24-methylenecholesterol¹⁷⁾, and 7-cholestenol²¹⁾, were isolated from the several sources such as mollusks and algae in this laboratory. Fucosterol was kindly supplied by Dr. A. SAITO, Fisheries Research Board of Canada, or isolated from a brown alga, *Sargassum piluliferum*, in this laboratory. The purity of these sterols was extensively checked by GLC using at least two or more columns²²⁾, IR spectroscopy, and mass spectrometry.

Column chromatography. In this study, column chromatography was carried out by slight modification of the procedures reported by VROMAN and COHEN⁹⁾. The two types of adsorbents were prepared for separation of sterol acetates. The adsorbent-1, silicic acid-silver nitrate (4 : 1, w/w), was prepared as follows : To 165 ml of silver nitrate solution (silver nitrate 30 g/165 ml distilled water), 120 g of silicic acid was added and mixed thoroughly. The mixture was poured into several 1 L-Erlenmeyer flasks and then dried in an oven at 100–110°C for 24 hours. The adsorbent-2, silicic acid-silver nitrate (5 : 1, w/w), was prepared by the essentially same manner as the adsorbent-1. After activation, the adsorbent was stored in a plastic desiccator covered with a black paper or aluminum foil until use. For preparation of a column 27 cm long and 3.0 cm in diameter, about 250 ml of n-hexane was added to 150 g of the adsorbent-1, and then packed in a column equipped with glass filter (No. 3) at the bottom without addition of pressure. A sterol acetate mixture dissolved in a small volume of n-hexane was then placed on the column. The samples of sterol acetate mixtures examined were as follows: (1) Mixture A; mixture of β -sitosterol, campesterol, cholesterol, stigmasterol, brassicasterol, 22-dehydrocholesterol, 22-*trans*-24-norcholesta-5, 22-dien-3 β -ol, desmosterol, fucosterol, and 24-methylenecholesterol, (2) mixture B; mixture of cholesterol, and 7-cholestenol, (3) mixture C; the sterol mixture isolated from the short-necked clam, *T. philippinarum*, in June, 1972. These sterol mixtures were acetylated with acetic anhydride-dry pyridine (1 : 1, v/v) and then subjected to column chromatography. In this study, some investigation on chromatographic conditions was carried out. Finally, the separation of sterol mixtures A, B, and C was accomplished by using the columns and solvent systems as described in Table 1 and Figs. 1, 2, and 3. The solvent usually flows at a rate of about 15–20 ml/hr. The eluate from the column was collected in 50 ml fractions. The sterol acetates in fractions were monitored by GLC on 1.5% OV-17.

Results

Column chromatography of authentic sterol acetate mixtures. The mixture A (1.5 g) was chromatographed on the adsorbent-1 (150 g, 27 cm \times 3.0 cm) with n-hexane-benzene. As shown in Table 1 and Fig. 1, a considerable well separation of individual sterol acetates was achieved. Also some relationship could be observed between the chemical structure of sterol acetates and mobility; that is, under the chromatographic conditions adopted, sterol acetates were eluted ahead in the order of Δ^5 -C₂₉-, Δ^5 -C₂₈-, Δ^5 -C₂₇-, $\Delta^{5,22}$ -C₂₉-, $\Delta^{5,22}$ -C₂₈-, $\Delta^{5,22}$ -C₂₇-, $\Delta^{5,22}$ -C₂₆-, $\Delta^{5,24(28)}$ -C₂₉-, $\Delta^{5,24}$ -C₂₇-, and $\Delta^{5,24(28)}$ -C₂₈-sterol acetates. The above-mentioned regularity may give some information for elucidation of chemical structures of unknown sterols. 24-Methylenecholesterol was completely separated from the other sterol acetates. Also,

Table 1. Chromatography of the mixture A on the adsorbent-1.

Fraction	Solvent system	ml	% Composition of steryl acetates detected in each fraction
1—9	n-Hexane	450	nil
10—18	n-Hexane-benzene (90:10)	450	nil
19—30	n-Hexane-benzene (85:15)	600	nil
31—34	n-Hexane-benzene (80:20)	200	Cholesterol (50—60), campesterol (13—20), β -sitosterol (20—35)
35—37	ditto	150	Cholesterol (64—67), campesterol (18—28), β -sitosterol (5—15)
38—40	ditto	150	Cholesterol (70—82), campesterol (11—25), β -sitosterol (5—7)
41—43	n-Hexane-benzene (75:25)	150	Cholesterol (10—50), campesterol (0—5), stigmasterol (45—90)
44—47	ditto	200	Stigmasterol (2—97), brassicasterol (3—98)
48—50	ditto	150	Brassicasterol (100)
51—53	n-Hexane-benzene (73:27)	150	Brassicasterol (0—85), 22-dehydrocholesterol (15—100)
54—56	ditto	150	22-Dehydrocholesterol (100)
57—60	ditto	200	22-Dehydrocholesterol (0—50), C_{26} -sterol* (50—100), fucosterol (0—2)
61—64	n-Hexane-benzene (71:29)	200	C_{26} -Sterol (0—80), fucosterol (0—80)
65—72	ditto	400	Desmosterol (0—100)
73—75	n-Hexane-benzene (68:32)	150	Desmosterol (0—5), 24-methylenecholesterol (0—100)
76—83	ditto	400	24-Methylenecholesterol (100)
84—90	ditto	350	24-Methylenecholesterol (100)
91—100	ditto	500	24-Methylenecholesterol (0—50)**

* 22-*trans*-24-Norcholesta-5, 22-dien-3 β -ol. ** A small amount of unknown sterol was detected. Each fraction (50ml) was collected and the percentage composition of each steryl acetate was determined by GLC on 1.5% OV-17.

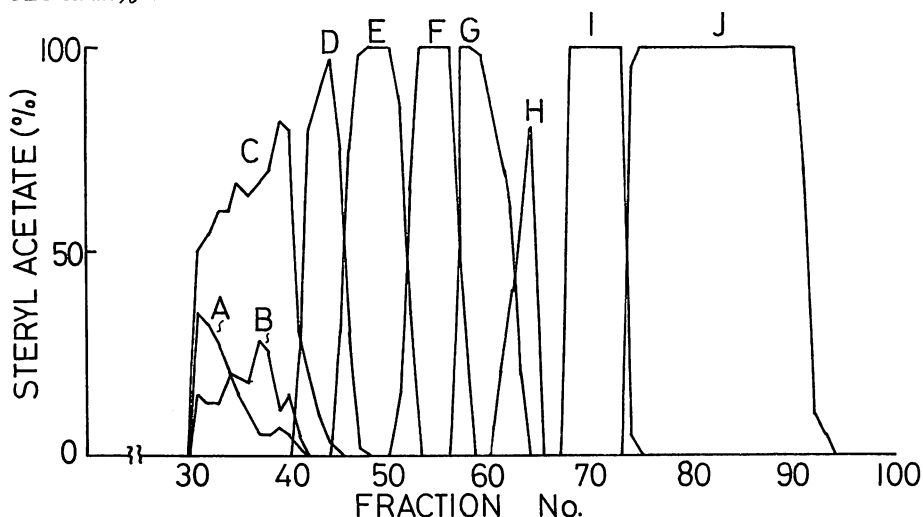


Fig. 1. Chromatographic separation of the mixture A.

Conditions : column 27.0cm long \times 3.0cm i. d., adsorbent-1 150g, sample 1.0g. A, β -sitosteryl acetate ; B, campesteryl acetate ; C, cholesteryl acetate ; D, stigmasteryl acetate ; E, brassicasteryl acetate ; F, 22-dehydrocholesteryl acetate ; G, 22-*trans*-24-norcholesta-5, 22-dien-3 β -ol acetate ; H, fucosteryl acetate ; I, desmosteryl acetate ; J, 24-methylenecholesteryl acetate

Δ^5 -steryl acetates such as cholesteryl, campesteryl, and β -sitosteryl acetates were widely separated from the diene steryl acetates examined. However, the separation of C_{27} -, C_{28} -, and C_{29} - Δ^5 -steryl acetates which differ from each other only in the length of side chain at C_{24} was insufficient. These results were similar to that of separation of steryl acetates by TLC reported by IDLER and SAFE¹¹⁾. As compared with the Δ^5 -steryl acetates, the $\Delta^{5,22}$ -steryl acetates could be considerably well separated into the individual C_{26} -, C_{27} -, C_{28} -, and C_{29} -steryl acetates. In this chromatography, some fractions were shown to give pure (single peak in GLC) or highly enriched $\Delta^{5,22}$ -steryl acetates. Moreover, when "enriched $\Delta^{5,22}$ -steryl acetate" was rechromatographed two or more times by this chromatographic technique using the adsorbent-1 or adsorbent-2, every $\Delta^{5,22}$ -steryl acetate examined could be isolated as pure steryl acetates.

The results of the chromatography of mixture B are given in Fig. 2. Cholesterol was completely separated from both cholesterol and 7-cholestenol. However, the separation of cholesterol and 7-cholestenol was insufficient under the chromatographic conditions adopted in this study.

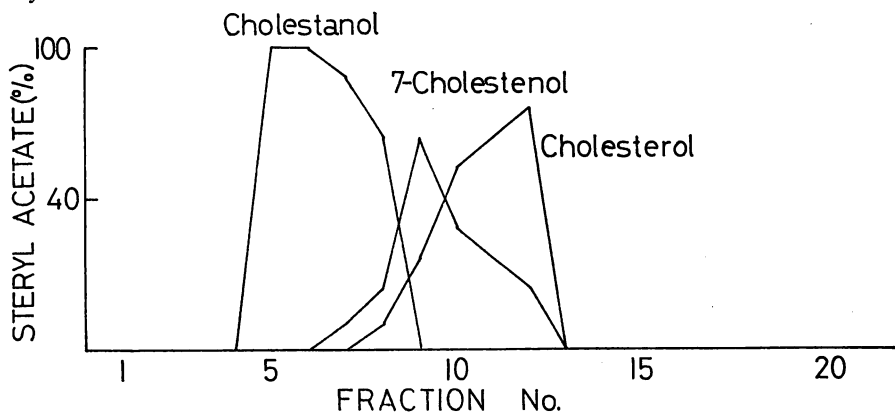


Fig. 2. Chromatographic separation of the mixture B.

Conditions: column 21.0cm long \times 1.5cm i. d., adsorbent-2 22g, sample 30mg. The elution was carried out with 100ml each of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50% benzene in n-hexane. Fraction (50ml) was collected and monitored by GLC.

Separation of steryl acetate mixture isolated from the short-necked clam. The sterols isolated from the short-necked clam were found by GLC on 1.5% OV-17 to contain a number of sterols as given in Table 2. The sterols were acetylated and then the steryl acetates (1.0g) were chromatographed on the adsorbent-1 (150g, 27cm \times 3.0cm). As shown in Fig. 3, the steryl acetate mixture was considerably separated into several components. Also, the large scale column (sample 12g, adsorbent-1 750g, 64cm \times 5cm) was found to give the essentially similar separation of steryl acetate to that in the above column. By repeated uses of this chromatography on the adsorbents 1 or 2, the various sterol components were isolated as pure steryl acetates. The results showed that this method is effective for separation of individual sterols from very complex sterol mixture containing monoene- and diene- Δ^5 -sterols but not Δ^7 -sterols. The authors have previously succeeded in isolating 22-dehydrocholesterol, desmosterol, 24-methylenecholesterol, and 22-*trans*-24-norcholesta-5, 22-dien-3 β -ol from another sample of the short-necked clam^{17, 19)}. In addition to the above four sterols, three sterols (S-1, S-2, and S-3) were isolated from the short-necked clam as pure steryl acetates in the present study. The sterols S-1 and S-2 were identified as brassicasterol and 28-isofucosterol, respectively, on the basis of the data mentioned below. S-1 acetate: m. p. 157°C; IR spectrum, $\nu_{\text{max}}^{\text{CHCl}_3}$ 3

Table 2. GLC on 1.5% OV-17 of the sterols isolated from the short-necked clam, *T. philippinarum*.

Peak	RRT*	% Composition	Identified as
1	0.66	2.5	22- <i>trans</i> -24-Norcholesta-5, 22-dien-3 β -ol
2	0.88	0.3	22- <i>cis</i> -Cholesta-5, 22-dien-3 β -ol
3	0.92	5.0	22- <i>trans</i> -Cholesta-5, 22-dien-3 β -ol
4	1.00	32.6	Cholesterol
5	1.05	0.1	Unknown sterol
6	1.13	12.5	Brassicasterol
7	1.18	0.4	Desmosterol
8	1.30	8.4	Campesterol
9	1.33	20.8	24-Methylencholesterol
10	1.41	3.6	Stigmasterol
11	1.60	12.0	β -Sitosterol
12	1.70	0.1	Fucosterol
13	1.78	1.8	28-Isocosterol

* Relative retention time to cholesterol (retention time, 23.0 min.)

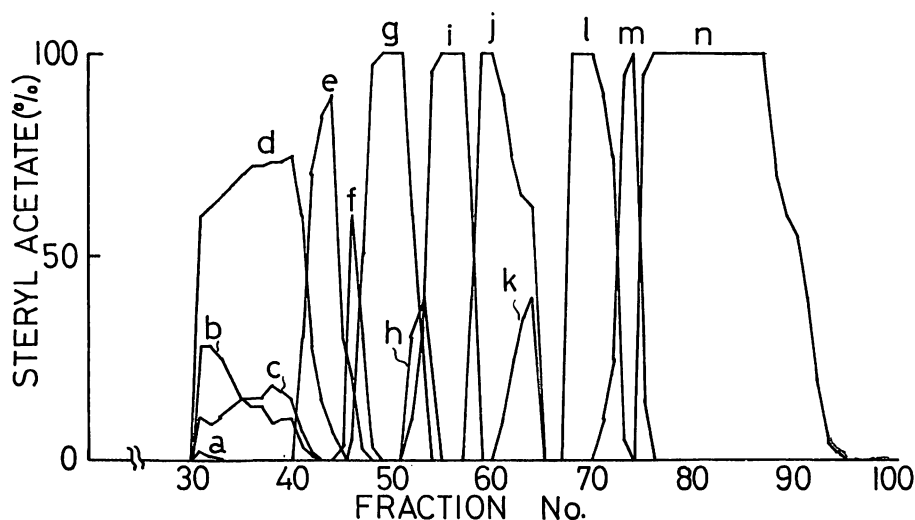


Fig. 3. Chromatographic separation of the mixture C.

Conditions: column 27.0cm long \times 3.0cm i. d., adsorbent-1 150g, sample 1.5g. The elution was carried out with the same solvents as those in the mixture A. Fraction (50ml) was collected and monitored by GLC. The steryl acetates detected are as follows: a, unknown 1; b, β -sitosteryl acetate; c, campesteryl acetate; d, cholesteryl acetate; e, stigmasteryl acetate; f, unknown 2 (C_{28} -sterol); g, brassicasteryl acetate; h, unknown 3 (22-*cis*-cholesta-5, 22-dien-3 β -ol acetate?); i, 22-dehydrocholesteryl acetate; j, 22-*trans*-24-norcholesta-5, 22-dien-3 β -ol acetate; k, fucosteryl acetate; l, 28-isofucosteryl acetate; m, desmosteryl acetate; n, 24-methylencholesteryl acetate

968, 971 cm^{-1} ($\Delta^{5,22}$); mass spectrum, m/e at 380 ($M^+ - \text{CH}_3\text{COOH}$, $M^+ =$ molecular ion), 365 [$M^+ - (\text{CH}_3\text{COOH} + \text{CH}_3)$], 337 [$M^+ - (\text{CH}_3\text{COOH} + 43$ ($C_{25} - C_{27}$))], 255 [$M^+ - (\text{CH}_3\text{COOH} + R)$, $R =$ alkyl side chain], 253 [$M^+ - (\text{CH}_3\text{COOH} + R + 2H)$], 228 [$M^+ - (\text{CH}_3\text{COOH} + R + 27)$], 213 [$M^+ - (\text{CH}_3\text{COOH} + R + 42)$], and 211 [$M^+ - (\text{CH}_3\text{COOH} + R + 42 + 2H)$]; NMR

spectrum, τ 4.70 (doublet, proton at C-6), τ 4.80–4.88 (2H, olefinic protons at C-22 and C-23), τ 9.11 (6H, isopropyl group at C-25 to C-27). S-2 acetate: m. p. 129–130°C; IR spectrum, $\nu_{\text{max}}^{\text{CHCl}_3}$ 811 cm^{-1} (ethylidene group at C-24); mass spectrum, m/e at 394 ($M^+ - \text{CH}_3\text{COOH}$, M^+ = molecular ion), 379 [$M^+ - (\text{CH}_3\text{COOH} + \text{CH}_3)$], 296 [$M^+ - (\text{C}_{23} - \text{C}_{27} + \text{CH}_3\text{COOH} + 1\text{H})$], 281 [$M^+ - (\text{C}_{23} - \text{C}_{27} + \text{CH}_3\text{COOH} + 1\text{H} + \text{CH}_3)$], 255 [$M^+ - (\text{R} + \text{CH}_3\text{COOH})$], 253 [$M^+ - (\text{R} + \text{CH}_3\text{COOH} + 2\text{H})$], 228, and 213 [$M^+ - (\text{R} + \text{CH}_3\text{COOH} + 42)$]; NMR spectrum, τ 8.46 (doublet, protons at C-29), τ 4.95 (quartet, proton at C-28), τ 7.2 (multiplet, proton at C-25).

On the other hand, the steryl acetate S-3 revealed a close retention time to that of brassicasteryl acetate in GLC on 1.5% OV-17. The molecular weight of sterol S-3 determined by mass spectrometry was 398. However, the melting point and IR spectrum were not identical with those of authentic brassicasterol. These results suggest that the sterol S-3 is probably an analogue closely related to brassicasterol. Elucidation of the chemical structure of this sterol will be performed in this laboratory.

Discussion

In earlier studies, several TLC techniques have been proposed for separation of free sterols or steryl acetates²⁻¹⁶. Especially, the argentative TLC method reported by IDLER and SAFE¹¹ has been excellent for preparative separation of complex sterol mixtures containing a number of sterols with different carbon numbers and/or substitutes at side chain. In conjunction with GLC or combined GLC-mass spectrometry, application of their method will facilitate the understanding of components of complex sterol mixtures occurring in natural sources. On the other hand, only a few methods have been reported for separation of sterol mixtures by column chromatography^{9, 23-25}. By column chromatography on a silver nitrate-impregnated silicic acid, VROMAN and COHEN⁹ have succeeded in isolating each component from the acetates of binary sterols; 24,25-dihydrolanosterol-lanosterol, cholesterol-desmosterol, cholestanol-cholesterol, and 7-dehydrocholesterol-cholesterol. Recently, YASUDA²⁶ and the authors^{17, 19} have independently performed the isolation of several sterols from the short-necked clam by application of the method of VROMAN and COHEN⁹.

In the present study, it was found that some modification of solvent systems in the chromatography devised by VROMAN and COHEN⁹ resulted in a considerable wide separation of individual sterols from complex sterol mixtures. Also, the chromatographic technique used in the present study was available for a large scale column. Accordingly, by the combined use of this column chromatography and GLC, the presence of unknown sterols which are not detected by using only GLC due to small quantities may be demonstrated.

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