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Note

Separation of sterols using digitonin-impregnated thin-layer chromatography plates

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The sterols isolated from marine invertebrates are often very complex mixtures. Their similarities in conventional thin-layer chromatography (TLC) lead to difficulties in the isolation and identification of minor sterol components. In such instances, preliminary fractionation of the sterol mixture is often essential.

It has been shown[1] that slow addition of a solution of digitonin to a sterol mixture results in fractional precipitation of sterols with a long side-chain, a Δ^{22}-double bond, and a short side-chain. When used for preliminary fractionation, this method has led to the isolation and identification of a large number of minor sterol constituents of marine organisms. However, it required a large amount of material (at least 50 mg of sterol mixture) and fatty alcohols interfered.

The aim of this work was to avoid these disadvantages by use of TLC or paper chromatography. TLC plates of silica gel, impregnated with an excess of digitonin, were previously used by Taylor[2] and Bojesen *et al.*[3] for separating digitonide-forming sterols (*i.e.*, 3β-hydroxy sterols) from those which either do not or only weakly bind digitonin. We have studied the possibility of fractionating 3β-hydroxy sterol mixtures by TLC on adsorbents impregnated with limited amounts of digitonin.

EXPERIMENTAL

Abbreviations

C_{19}, Δ^5 = androst-5-en-3β-ol; C_{21}, Δ^5 = pregn-5-en-3β-ol; C_{26}, $\Delta^{5,22}$ = (22*E*)-24-norcholesta-5,22-dien-3β-ol; C_{27}, Δ^5 = cholest-5-en-3β-ol; C_{27}, $\Delta^{5,22}$ = cholesta-5, 22-dien-3β-ol; C_{28}, Δ^5 = 24-methylcholest-5-en-3β-ol; C_{28}, $\Delta^{5,22}$ = 24-methylcholesta-5,22-dien-3β-ol; C_{28}, $\Delta^{5,24}$ = 24-methylenecholest-5-en-3β-ol; C_{29}, Δ^5 = (24*R*)-24-ethylcholest-5-en-3β-ol; and C_{29}, $\Delta^{5,22}$ = (24*S*)-24-ethylcholesta-5, 22-dien-3β-ol.

TLC separation

The TLC (20 × 5 cm) were coated with a slurry of 2 g of silica gel G

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(Merck, Darmstadt, G.F.R.) in 6 ml of an aqueous solution of digitonin, the ratio of digitonin to silica gel being varied. After the plates had been dried, the sterol mixture was applied on a line 4 cm from the bottom edge of the plate. The developing solvent was *tert.*-butanol–water–ethyl acetate (5:1:5). Development was stopped when the solvent front reached 1 cm below the top edge of the plate. The amounts of digitonin used and of sterol mixture applied are given in Tables I–III. Zones of equal length were scraped off and the adsorbent was suspended in a minimum amount of pyridine (about 3 ml).

The digitonides decomposed on heating for 5 min in a boiling water-bath. The digitonin released was precipitated with about 10 ml of diethyl ether. Silica gel and digitonin were removed by centrifugation and the solution was washed with 20 ml of hydrochloric acid and then with 20 ml of saturated sodium hydrogen carbonate solution. After evaporation of the solvent, the free sterols were converted into acetates and analysed by gas–liquid chromatography (GLC) (3% OV-17, 280 °C) and gas chromatography–mass spectrometry (GC–MS) (3% OV-17, electron-impact mode).

The sterol mixture from *Rhizostoma pulmo*, weighing 32 mg, was fractionated by this method on two 20 × 20 cm plates, each prepared by coating with 8 g of silica gel G in 24 ml of an aqueous solution containing 0.140 g of digitonin.

Separation by paper chromatography

Sheets of Whatman No. 1 paper (13 × 35 cm) were first extracted with diethyl ether and the developing solvent. The paper was then impregnated by dipping it into a 1.4% solution of digitonin in ethanol in such way that dry strips, 7.5 cm long, were left at both ends. The sterol mixture (8 mg) was applied to one of the unimpregnated zones 5,5 cm from its end. After development for 20 h in the solvent system *tert.*-butanol–water–ethyl acetate (5:1:5), the digitonin-impregnated strip was cut into two halves of equal length. Further work proceeded as with silica gel/digitonin.

RESULTS AND DISCUSSION

Standard mixtures, containing 3β-hydroxy sterols with side-chains of different lengths, with or without a C-22 double bond, were used. The amounts of digitonin

TABLE I

SEPARATION OF STEROLS DIFFERING IN THE SIDE-CHAIN LENGTH

A: 2 mg of sterol mixture, 0.035 g of digitonin, 2 g of silica gel. B: 2.5 mg of sterol mixture, 0.020 g of digitonin, 2 g of silica gel. C: 4 mg of sterol mixture, 0.035 g of digitonin, 2 g of silica gel.

Composition of the standard mixture		*Composition of the zones (%)*											
		A				*B*				*C*			
Sterol	*%*	*1**	*2*	*3*	*4*	*1**	*2*	*3*	*4*	*1**	*2*	*3*	*4*
C_{19}, Δ^5	19	8	22	33	44	10	16	37	60	4	25	32	76
C_{21}, Δ^5	16	12	20	30	32	5	26	31	26	8	27	31	19
C_{27}, Δ^5	34	35	32	23	8	35	35	22	10	38	28	22	5
C_{29}, Δ^5	30	45	25	14	—	48	22	9	—	50	19	13	—

* In all tables the zone numbers increase with distance from the start.

and sterol mixture, the composition of the solvent system and the standard mixture were varied. The sterols formed more or less oblong spots, their length depending on the conditions used. The spot was segmented into several zones of equal length, each was extracted with pyridine and the digitonin was precipitated with diethyl ether. The composition of the isolated fractions was determined by GLC. The results are given in Tables I–IV. The best fractionation was achieved when silica gel impregnated with 1–1.7% of digitonin was used. The solvent system used was satisfactory; water is necessary for the formation of digitonides.

TABLE II

SEPARATION OF CHOLESTEROL FROM STIGMASTEROL

4 mg of sterol mixture, 0.035 g of digitonin, 2 g of silica gel.

Composition of the standard mixture		*Composition of the zones (%)*		
Sterol	*%*	*1*	*2*	*3*
C_{27}, Δ^5	55	53	53	52
C_{29}, $\Delta^{5,22}$	47	47	47	48

TABLE III

SEPARATION OF SITOSTEROL FROM STIGMASTEROL

4 mg of sterol mixture, 0.035 g of digitonin, 2 g of silica gel.

Composition of the standard mixture		*Composition of the zones (%)*			
Sterol	*%*	*1*	*2*	*3*	*4*
C_{29}, Δ^5	40	49	40	35	27
C_{29}, $\Delta^{5,22}$	57	50	59	64	72

The results show that the sterols are fractionated in accordance with the length of their side-chain. Sterols with longer side-chains complex faster with digitonin and move more slowly on the TLC plates. For example, cholesterol (C_{27}, Δ^5) separated from sitosterol (C_{29}, Δ^5), having a side-chain which is two carbon atoms longer. Stigmasterol (C_{29}, $\Delta^{5,22}$) not only has two additional carbon atoms in the side-chain but also a C-22 double bond. Cholesterol and stigmasterol did not separate under the conditions used. Thus, the C-22 double bond compensate for the influence of the two extra carbon atoms in the side-chain. This is illustrated by the separation of a mixture of stigmasterol (C_{29}, $\Delta^{5,22}$) and sitosterol (C_{29}, Δ^5) (with the same carbon skeleton), which showed the enrichment of stigmasterol in the fast-moving fractions (Table III). The greater chromatographic mobility of 24-methyl-cholesta-5,22-dien-3β-ol in comparison with cholesterol is in accord with this statement (Table IV).

TABLE IV

SEPARATION OF CHOLESTEROL FROM C_{28}, $\Delta^{5,22}$

4 mg of sterol mixture, 0.035 g of digitonin, 2 g of silica gel.

Composition of the standard mixture		*Composition of the zones (%)*		
Sterol	*%*	*1*	*2*	*3*
C_{27}, Δ^5	54	59	53	48
C_{28}, $\Delta^{5,22}$	27	26	27	33

To explain the role of the adsorbent, we replaced the silica gel with paper. Chromatography of the same sterol mixture on digitonin-impregnated paper also resulted in the formation of a long spot in which the distribution of sterols, established by GLC, was the same as described above (Table V). In this instance, starting the zone of the chromatogram must not be impregnated with digitonin, because the solvent will not penetrate it. However, on view of the low capacity of paper and its impurities, which were always extracted by hot pyridine, this method is not suitable for practical purposes.

TABLE V

SEPARATION OF STEROLS ON DIGITONIN-IMPREGNATED PAPER

Composition of the standard mixture		*Composition of the zones (%)*	
Sterol	*%*	*1*	*2*
C_{19}, Δ^5	14	9	73
C_{27}, Δ^5	54	53	27
C_{29}, Δ^5	32	38	traces

Column chromatography on silica gel impregnated with digitonin failed to give a satisfactory separation.

The method reported may find application in the investigation of complex sterol mixtures. Fractions enriched in some sterols may be obtained. This is of special importance for the isolation of the recently discovered sterols with short side-chains[5] the separation of which is difficult because of their low concentrations in the sterol mixtures.

We have applied this method to the fractionation of the sterol mixture isolated from the Black Sea jellyfish, *Rhizostoma pulmo* (Coelenterate)[4]. GLC data indicated the presence of sterols with a short side-chain, but the concentration was less than 1% of the total sterols and they could not be identified by GC–MS. The results of TLC of this mixture on digitonin-impregnated silica gel are shown in Table VI. In this instance, the sterols with a short side-chain had a greater chromatographic mobility as well as the Δ^{22}-sterols and one sterol with a retention time corresponding to dinosterol than the sterols with a long side chain. The content

TABLE VI

SEPARATION OF STEROLS FROM RHIZOSTOMA PULMO

Main components of the sterol mixture from R. pulmo		*Composition of the isolated zones (%)*					
Sterol	*%*	*1*	*2*	*3*	*4*	*5*	*6*
$C_{26} + C_{25}$	<1	—	—	—	2	3	35
C_{26}, $\Delta^{5,22}$	1	traces	1	2	2	3	4
C_{27}, $\Delta^{5} + C_{27}$, $\Delta^{5,22}$ *	70	84	69	68	64	62	20
C_{28}, $\Delta^{5,22}$	14	8	14	19	23	28	traces
C_{28}, $\Delta^{5} + C_{28}$, $\Delta^{5,24} + C_{29}$, $\Delta^{5,22}$	8	5	10	7	8	6	traces

* The shoulder for the C_{27}, $\Delta^{5,22}$ sterol in GLC increased in the zones, remote from the start.

of the sterols with a short side-chain was higher in the upper zone of the chromatogram than in the crude sterol mixture. The GC–MS investigation revealed the presence of at least three sterols with short side-chains, having molecular weights of 342, 344 and 356. Their acetates lacked a molecular ion in the mass spectra, which is an indication of a C-5 double bond. On this basis, one of the compounds was identified as the C_{24} Δ^{5}-sterol, found recently in some *Gorgonia* and *Porifera* species[5], and the other two were identified as C_{24} and C_{25} sterol dienes ,as yet unknown, in which the location of one of the double bonds remains to be established.

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