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Thin-layer chromatography of bile acids*

PETER ENEROTH

Department of Chemistry,
Karolinska Institutet,
Stockholm, Sweden

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SUMMARY

Solvent systems suitable for thin-layer chromatographic separation of 40 different bile acids are described. The influence of substituents and chain length on the separation factors in different solvent systems has been examined.

Preparative separation of bile acids has been achieved by adsorption and partition chromatography, and microscale separations have been made by various paper-chromatographic methods (1). Using these methods, good separations and acceptable sensitivity can be achieved. Methods are lacking, however, that permit rapid qualitative analysis of a large number of samples; e.g., for the monitoring of effluent from column chromatograms. The only method meeting the requirements of extreme rapidity and sensitivity has been published by Hamilton and Dieckert (2), who used glass fiber papers impregnated with silicic acid or monopotassium phosphate. Recently, the gas-liquid chromatographic separation of bile acids has been described (3, 4).

Since the publication of a standardized procedure for thin-layer chromatography (TLC) (5), some papers concerning the separation of steroids with this technique have appeared (6). It was thought that this simple and rapid procedure could be adapted to the systematic analysis of compounds in the bile acid series, and solvent systems have been worked out for most of the unconjugated bile acids of biochemical interest. During the course of this investigation, the separation of a few common bile acids and their conjugates has been described (7, 8, 9).

EXPERIMENTAL METHODS

Thin-layer chromatographic equipment from Firma Desaga, Heidelberg, was used. The general procedure was that previously described by Stahl except that the layers were prepared from a suspension of 58 ml of distilled water and 30 g of Kieselgel G (from Firma Desaga, Heidelberg). The plates had the dimensions 5 x 20 or 20 x 20 cm and were used in jars measuring 550 cm^3 and 4,300 cm^3, respectively. All solvents were redistilled before use and were measured exactly by pipetting since in some instances even small changes in the composition of the solvent system interfered with reproducibility.

The chromatoplates were activated in an oven at 110–120° for 1–3 hr before use. The compounds to be analyzed were dissolved in a suitable solvent (e.g., acetone or methanol) and applied to the film through a sharpened micropipette (5–10 μg in 3-4 μl). During this procedure, the chromatoplates were warmed on an electrical hot plate. The glass plates were allowed to cool to room temperature and were then placed in the jars and developed with the ascending technique. The jars were sealed with aluminium foil and a heavy glass plate. All runs were performed at room temperature (18–20°) without using the so-called "Kammerübersättigung" technique (10). When the solvent front was 17–18 cm from the starting line, the plates were taken out of the jars and dried in an oven at 150°. The plates were then sprayed with concentrated sulfuric acid (reagent grade) and heated in an oven at 240°. The spots thus obtained had a maximum diameter of 1.5 cm. The time required for a run varied with the solvent system used but never exceeded 3 hr.

RESULTS AND DISCUSSION

With the method used, the solvent fronts become concave, and the so-called "Kammerübersättigung" technique was used in an attempt to avoid this. How-

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TABLE 1. SOLVENT SYSTEMS FOR TLC OF BILE ACIDS

System No.	Components	Ratio
N 1	Diethyl oxalate–dioxane	40:10
N 2	Diethyl oxalate–isopropyl alcohol	48:8
S 1	Benzene–dioxane–acetic acid	75:20:2.0
S 2	" " " "	20:10:2.0
S 3	" " " "	15:5:2.0
S 4	" " " "	55:40:2.0
S 5	Cyclohexane–ethyl acetate–acetic acid	10:15:4.0
S 6	" " " " "	7:23:3.0
S 7	Benzene–isopropyl alcohol–acetic acid	30:10:1.0
S 8	Cyclohexane–isopropyl alcohol–acetic acid	30:10:1.0
S 9	Trimethylpentane–isopropyl alcohol–acetic acid	30:10:1.0
S 10	" " " "	60:20:0.5
S 11	Trimethylpentane–ethyl acetate–acetic acid	10:10:2.0
S 12	" " " "	5:25:0.2
S 13	" " " "	50:50:0.7
S 14	" " " "	10:10:0:25
S 15	"	10:10:0.1

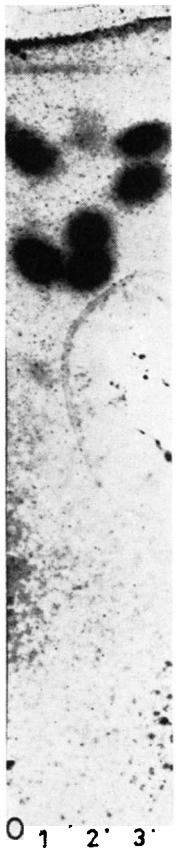

FIG. 1. Separation of 3,7- and 3,12-diketocholanic acids and their methyl (Me) esters with system S10. Compounds are enumerated in their respective order from the starting line. 0 = origin *Position 1:* 3,7-diketo; Me-3,12-diketo. *2:* 3,-7-diketo; 3,12-diketo. *3:* Me-3,7-diketo; Me-3,12-diketo.

ever, this technique necessitated changing the solvent systems, which in some cases resulted in reduced separation factors; it therefore did not offer any advantages. The mobility of the acids is given in relation to one of three "standard" acids run close to the sample. The ratio between the absolute mobility of a compound and the absolute mobility of the "standard" cholic, desoxycholic, and lithocholic acids has been called R_C, R_D, and R_L, respectively.

In this connection, it is pointed out that with the method used (i.e., the layers not prewashed), the neutral systems listed in Table 1 give rise to two fronts after spraying and heating with sulfuric acid. The second front appears at about half the distance between the starting line and the true front. This phenomenon is probably due to preferential adsorption of one solvent component and neither disturbs the separations nor influences the detection of the compounds.

During the course of the investigation, it was soon found that acid solvent systems were superior to basic and neutral ones. In analyzing a completely unknown mixture of bile acids, however, the neutral systems had the advantage of permitting a rough screening of the bile acids present. After this preliminary chromatography, the appropriate acid system could be chosen for more accurate analysis.

Although some overlapping occurred, it was convenient to classify the bile acids tested into three classes according to the number of hydroxyl and/or keto groups. Thus, if each hydroxyl group is given a value of 2 and each keto group a value of 1, this rough classification would be as follows: I, bile acids having a value of at least 5; II, bile acids having a value of at least 3; III, bile acids having a value of less than 3.

Most of the members within each class were separated; i.e., mixtures of 5–10 μg of each compound yielded distinct individual spots. Some exceptions were noted; only an incomplete separation of 3α, 7α-dihydroxy-12-ketocholanic acid from 3α,12α-dihydroxy-7-ketocholanic acid and of 7α-hydroxycholanic acid from 12α-hydroxycholanic acid could be obtained. It was not possible to find any system for the separation of 7-ketocholanic and 12-ketocholanic acids. 3,7-Diketocholanic and 3,12-diketocholanic acids were separated but very small changes in the composition of the solvent system could cause incomplete separation, it is therefore recommended that these substances be run both as free acids and as methyl esters. No other method is available for the separation of this biologically important pair of bile acids. A chromatogram is shown in Fig. 1.

TABLE 2. RELATIVE MOBILITIES OF BILE ACIDS IN DIFFERENT SOLVENT SYSTEMS

Solvent systems*	N1	N2	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15
Rel. mobility†	R_D	R_L	R_D	R_C	R_C	R_C	R_C	R_C	R_C	R_C	R_D	R_L	R_D	R_D	R_L	R_L	R_L
Mobility of standard (cm)	5.0	9.7	5.4	4.7	8.5	4.0	5.2	5.1	9.0	4.7	9.8	12.0	9.1	5.7	9.1	8.9	7.5
Compounds‡																	
3α,7α,12α,23ξ		0.13	0.04	0.17	0.12	0.07	0.13	0.28	0.06	0.08							
3α,7α,23ξ			0.15	0.62	0.43	0.25	0.85	0.43	0.26	0.28							
3α,7α,16α			0.67	2.08	1.41	2.50	2.14	2.54	1.62	1.79	0.88		0.72				
3α,7α,12α-C_{27}			0.13	1.52	1.28	1.61	1.96	1.88	1.38	1.89	0.85						
3α,7α,12α	0.34	0.34	0.17	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.76	0.50	0.22	0.16			
3β,7α,12α				1.27	1.14	1.44	1.00	1.00	0.87	1.00							
3α,7β,12α			0.30	1.46	1.17	1.47	1.59	1.75	1.22	1.40							
3α,6α,7α			0.30	1.35	1.05	1.30	1.47	1.49	1.12	1.36							
3α,6β,7α				1.15	0.98	1.17	1.17	1.14	0.82	0.83							
3α,6β,7β				1.25	0.89	1.11	1.12	0.86	0.92	0.87							
7α,12α,3-keto			1.31	2.82	1.55	2.94	2.28	3.02	1.66	2.04	0.87	0.81	0.63	1.26			
3α,12α,7-keto			0.45	1.95	1.15	1.92	1.83	2.32	1.37	1.63	0.66	0.62	0.44	0.60			
3α,7α,12-keto		0.66	0.45	1.95	1.15	1.94	1.85	2.32	1.37	1.63	0.66	0.62	0.48				
3α,7,12-diketo	1.16	0.85	1.07	2.56	1.30	2.58	1.94	2.65	1.52	1.39	0.54	0.53	0.52				
3α,7α	1.00		1.00								0.91	0.82	0.88	1.00			
3β,7α			1.26								0.85	0.73	0.95	1.33			
3α,7β			1.14								0.77	0.77	0.78	1.00			
3α,12α	1.00	0.86	1.00	2.62	1.28	2.59	3.48	3.68	1.67	2.33	1.00	0.83	1.00	1.00	0.21		
3β,12α			1.36								0.96	0.81	1.06	1.40			
3α,12β			1.58								1.00	0.87	1.09	1.53	0.34		
3α,6α		0.46	0.63	1.86	1.12	1.80	2.02	2.24	1.24	1.70	0.67	0.71	0.50	0.56	0.10		
7α,12α			2.51									0.98	1.62	2.23	0.80	0.84	
3,7,12-triketo	1.58	1.00	2.13								0.55	0.46	0.91	1.78	0.46	0.41	0.41
3α,7-keto	1.38	0.90	1.63								0.88	0.70	0.95	1.44	0.45	0.42	
3α,12-keto	1.38		1.78								1.03	0.80	1.17		0.55	0.54	
7α,3-keto		0.99	2.13									0.93	1.35	2.11	0.74		
12α,3-keto			1.85								1.22	0.87	1.45		0.54		
3,7-diketo	1.56	1.08	2.65								1.10	0.85			0.93	0.91	0.93
3,12-diketo	1.56	1.08	2.65								1.17	0.91			0.93	0.91	0.95
3α	1.56	1.00	2.54								1.28	1.00	1.60	2.27	1.00	1.00	1.00
3β												1.04			1.13	1.08	1.15
7α		1.12	3.20									1.08			1.35	1.24	1.40
7β												1.01			1.19	1.10	1.21
12α												1.12	1.71		1.37		1.40
12β												1.12			1.28		1.32
3-keto	1.62	1.13	3.10									1.12			1.50	1.21	1.35
7-keto												1.16			1.62	1.27	1.50
12-keto												1.16			1.62	1.27	1.50
unsubst.															1.73	1.58	1.89

* See Table 1.

† See text.

‡ Hydroxyl groups have been indicated by Greek letters. The notation -C_{27} means a coprostanic acid.

From Table 2 and Fig. 2, it is seen that, as expected, the unsubstituted cholanic acid is least retarded followed by the monoketones 7-keto-, 12-keto-, and 3-ketocholanic acids. It is also evident that, among the monohydroxy acids, 3α-hydroxy- and 3β-hydroxycholanic acids are the most strongly adsorbed. It can be concluded that, in the systems used, bile acids that carry an oxygen function at C3 are more retarded than those having it at C7 or C12. Furthermore 3α-hydroxy-, 7β-hydroxy-, and 12β-hydroxycholanic acids, having equatorial substituents, are more retarded than the corresponding axially substituted acids (3β-hydroxy-, 7α-hydroxy-, and 12α-hydroxycholanic acids). Since the mobility of 7α-hydroxycholanic acid is slightly less than that of 12α-hydroxycholanic acid and since the same is true for 7β-hydroxy-

cholanic acid when compared to 12β-hydroxycholanic acid, it might be concluded that a hydroxyl group at C7 causes a stronger adsorption than one at C12. Thus, the following order of increasing mobility has been found:

$$3\alpha < 3\beta < 7\beta < 12\beta < 7\alpha \leqq 12\alpha < \text{3-keto} < \text{7-keto} \leqq \text{12-keto}$$

For more polar acids, the situation is far more complicated (Figs. 3–5), especially since the composition of the solvent system may profoundly alter the influence of certain substituents (see below). The powerful effect, however, of a substituent in the 3α-position as compared to positions 7 and 12 is quite evident from Table 2. For instance, 7α,12α-dihydroxycholanic acid is much less retarded than any dihydroxy acid containing the 3α-hydroxyl group. Furthermore, 7α,12α-dihydroxy-3-ketocholanic acid is less adsorbed than both 3α,12α-dihydroxy-7-keto- and 3α,7α-dihydroxy-12-ketocholanic acids (Figs. 3, 4). From the data obtained with the dihydroxy acids, it appears that a hydroxyl group in the C6-position causes a stronger adsorption than one at C7 or C12 (Fig. 4).

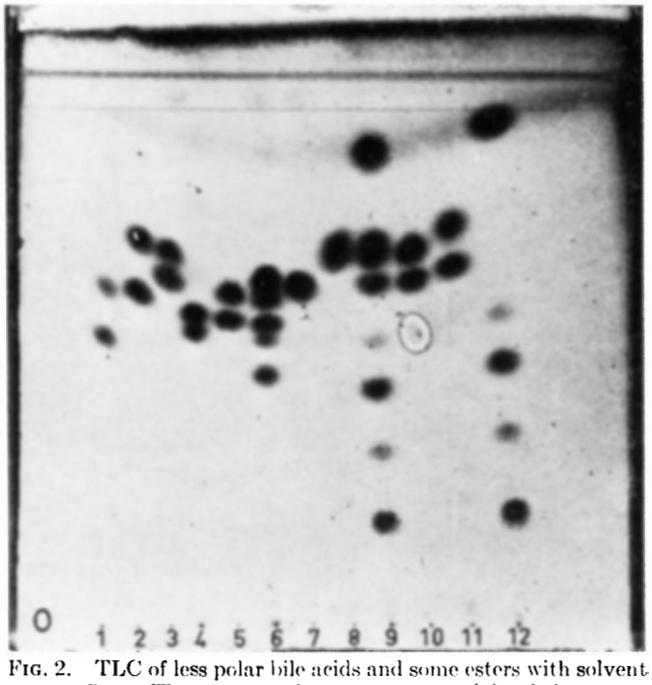

FIG. 2. TLC of less polar bile acids and some esters with solvent system S15. The compounds are enumerated in their respective order from the starting line. Me = methyl ester. 0 = origin *Position 1:* 3α; 3β. *2:* 7β; 7α. *3:* 12β; 12α. *4:* 3β; 7β. *5:* 7β; 12β. *6:* 3α; 3β; 7β; 12β; 7α; 12α. *7:* 7α; 12α. *8:* 7-keto; 12-keto. *9:* 3,7,12-triketo; Me-3,7,12-triketo; 3,7-diketo; Me-3,7-diketo; 3-keto; 7 keto; 12 keto; cholanic. *10:* 3-keto; 7-keto. *11:* 3-keto; 12-keto. *12:* 3,7,12-triketo; Me-3,7,12-triketo; 3,7-diketo; Me-3,7-diketo; cholanic.

Only three bile acids having hydroxyl groups in positions other than C3, C6, C7, and C12 have been tested. 3α,7α,16α-Trihydroxycholanic acid (pythocholic acid) was run in system S7 and found to have a R_C value of 0.86. This indicates a greater retarding effect of a 16α-hydroxyl group than of a 12α-hydroxy group. Since pythocholic acid is usually isolated in the lactone form, the values in Table 2 refer to this form (see also Fig. 3).

3α,7α,12α,23ξ-Tetrahydroxy and 3α,7α,23ξ-trihydroxycholanic acids move at a much slower rate than any other bile acid tested, showing the pronounced influence of a hydroxyl group in α-position to the carboxyl group. These acids showed tailing (Fig. 3) when run in the system listed in Table 2, but this could be overcome by increasing the amount of acetic acid in the solvent.

In order to evaluate the effect of the length of the side chain, 3α,7α,12α-trihydroxycoprostanic acid was run in several systems and was found to be less retarded

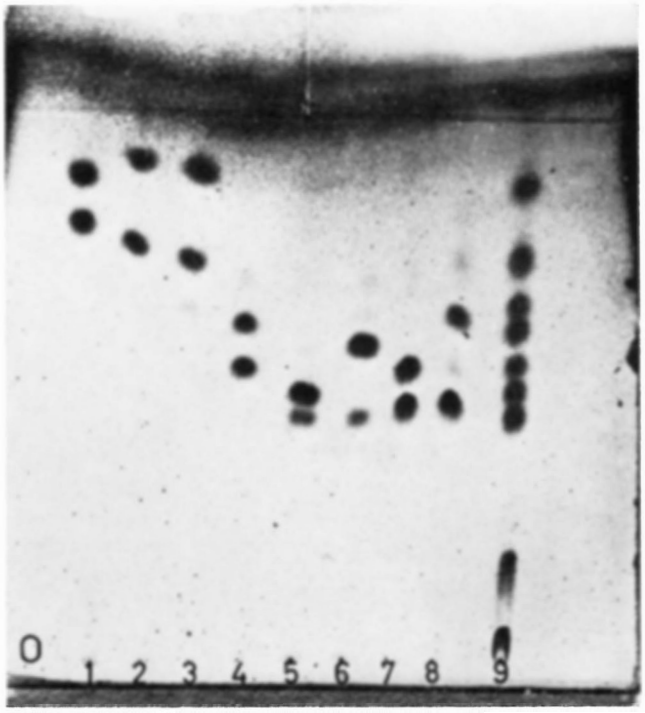

FIG. 3. TLC of trihydroxy bile acids with solvent system S7. The compounds are enumerated in their order from the starting line. Weak spots in some mixtures are due to impurities in the bile acid samples. 0 = origin. *Position 1:* 3α,12α,7-keto; 3α,7,12-diketo. *2:* 3α,7α,12-keto; 3α,12α. *3:* 3α,7α,12-keto; 7α,12α,3-keto. *4:* 3α,7α,12α; 3α,7β,12α. *5:* 3α,6β,7α; 3α,-6β7β. *6:* 3α,6β,7α; 3α,6α,7α. *7:* 3β,7α,12α; 3α,7α12α. *8:* 3β,7α,12α; 3α,7β,12α. *9:* 3α,7α,12α,23ξ; 3α,7α,23ξ; 3α,6β,7α; 3α,6β,7β; 3α,7α,12α; 3α,6α,7α; 3α,7β,12α; 3α,-7α,12α-C_{27}; 3α,7α,16α (as the lactone).

than 3α,7α,12α-trihydroxycholanic acid (Fig. 3). The same was true for 3α,7α-dihydroxycoprostanic acid when compared with the corresponding cholanic acid.

For the purpose of identification, some useful effects were brought about mainly by changing from solvent systems containing ethylacetate–acetic acid to those having isopropyl alcohol–acetic acid or dioxane–acetic acid as the polar solvent component. For instance, 3α,6β,7β-trihydroxycholanic acid is more retarded

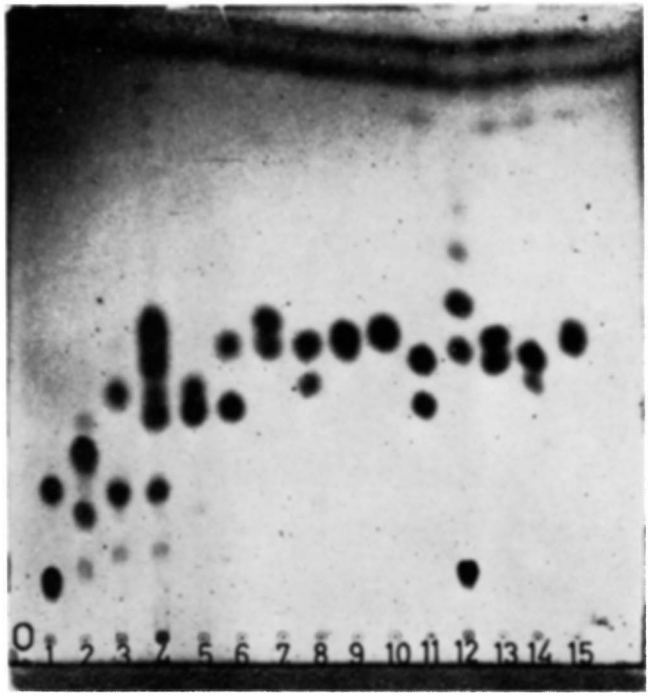

FIG. 4. TLC of dihydroxy bile acids with solvent system S11. The compounds are enumerated in their respective order from the starting line. The weak spots appearing in some mixtures are due to impurities in some of the samples. 0 = origin. *Position 1:* 3α,7α,12α; 3α,7,12-diketo. *2:* 3α,12α,7-keto; 7α,12α,3-keto. *3:* 3α,6α; 3α,7α. *4:* 3α,6α; 3α,7β; 3α,-7α; 3β,7α; 3α,12α; 3β,12α; 3α,12β; all appearing as two concurrent spots. *5:* 3α,7β; 3α,7α. *6:* 3α,7β; 3α,12α. *7:* 3α,12α; 3α,12β. *8:* 3α,7α; 3α,12α. *9:* 3α,12α; 3β,12α. *10:* 3β,12α; 3α,12β. *11:* 3α,7β; 3β,7α. *12:* 3α,7α,12α; 3α,12α; 7α,3-keto. *13:* 3β,7α; 3β,12α. *14:* 3α,7α; 3β,7α. *15:* 3β,7α; 3α12α.

than 3α,6β,7α-trihydroxycholanic acid in system S6 (see Table 2), while the reverse is true in system S7. Some other pairs of bile acids behaving in a similar way are shown in Fig. 6.

Since it was sometimes difficult to separate pairs of bile acids of biochemical interest, different derivatives of some of the acids listed in Table 2 were made and subjected to TLC. It was found that methyl esters were usually separated more easily than the free acids. Examples are shown in Table 3 and Fig. 1.

TABLE 3. COMPARISON BETWEEN THE SEPARATION OF BILE ACIDS BEFORE AND AFTER METHYLATION

	Cyclopentane–Tetrahydrofuran–Acetic Acid			
Compound	I 20:8.5: 0.25	II 20:8.5: 0.50	III 20:12.5: 0.30	IV 20:12.5: 0.50
3α,7α acid	0.86 } Not	0.81 } Not	0.87 } Almost	0.88 } Sep.
3α,12α acid	1.00 } sep.	1.00 } sep.	1.00 } sep.	1.00 }
3α,12α Me-ester	1.46 } Sep.	1.22 } Sep.	1.32 } Sep.	0.99 } Sep.
3α,12α Me-ester	1.77 }	1.59 }	1.67 }	1.08 }

In order to see whether a bile acid methyl ester could be separated from the ethyl ester, mixtures of methyl 3α,12α-dihydroxy-7-ketocholanate and ethyl 3α,12α-dihydroxy-7-ketocholanate were run in different systems. They could be just separated in solvent system S9 with R_D values of 0.77 and 0.84, respectively (Fig. 7). However, the systems used were primarily developed for the separation of free acids and it might be easier to separate these esters in other solvents. The systems reported here have also been found useful in the preparative TLC as described by Dahn and Fuchs (11).

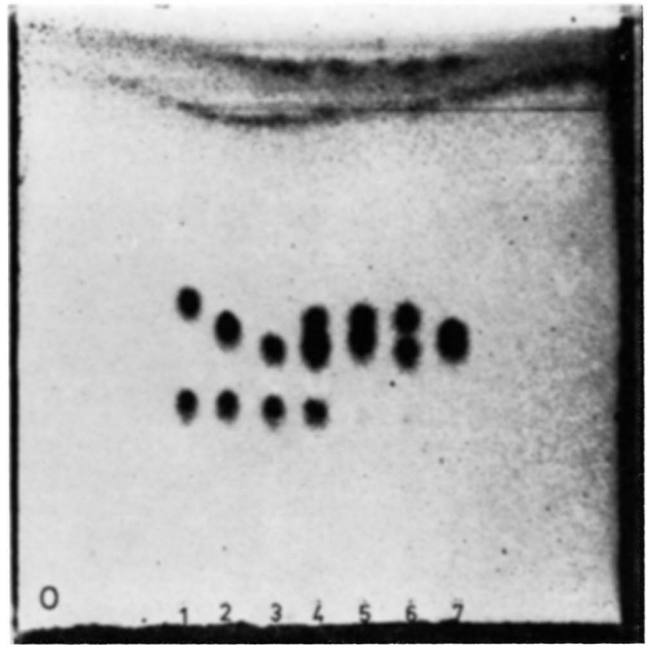

FIG. 5. TLC of dihydroxy bile acids with solvent system S12. Compounds are enumerated in their respective order from the starting line. 0 = origin. *Position 1:* 3α,12α; 3α,12β. *2:* 3α,12α; 3β,12α. *3:* 3α,12α; 3β,7α. *4:* 3α,12α; 3β,7α; 3β,12α; 3α,12β. *5:* 3β,12α; 3α,12β. *6:* 3β,7α; 3α,12β. *7:* 3β,7α; 3β,12α.

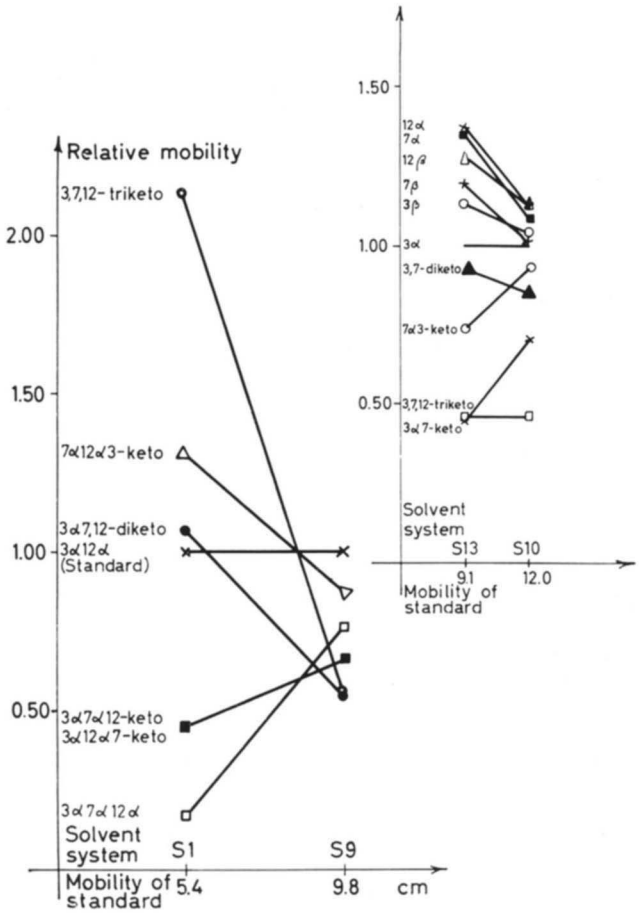

FIG. 6. Influence of solvent composition on separation factors.

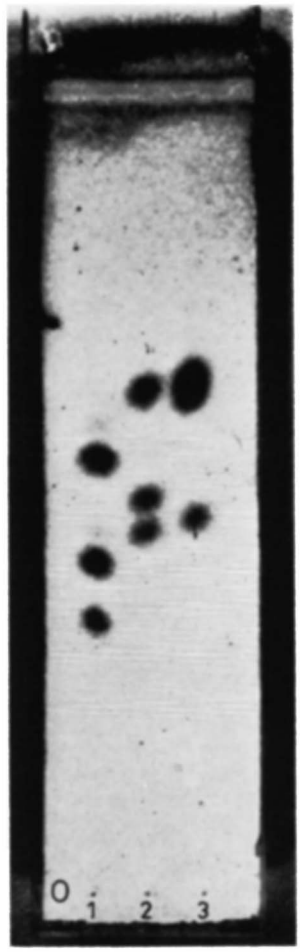

FIG. 7. TLC of keto bile acids with solvent system S9. Compounds are enumerated in their order from the starting line. Me = methyl ester; Et = ethyl ester. 0 = origin. *Position 1:* 3α,7,12-diketo; 3α,7α,12-keto; 3α,12α-7-keto; 7α,12α,3-keto. *2:* Me-3α,12α-7-keto; Et-3α,12α,7-keto; 3α,12β. *3:* Me-3α,12α,7-keto; 3α,12α; 3β12α.

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REFERENCES

1. Bush, I. E. *The Chromatography of Steroids.* Oxford, Pergamon Press, 1961, p. 45.
2. Hamilton, J. G., and J. W. Dieckert. *Arch. Biochim. Biophys.* **82**: 203, 1959.
3. VandenHeuvel, W. J. A., C. C. Sweely, and E. C. Horning. *Biochem. Biophys. Research Communs.* **3**: 33, 1960.
4. Sjövall, J., C. R. Meloni, and D. A. Turner. *J. Lipid Research* **2**: 317, 1961.
5. Stahl, E. *Pharmazie* **11**: 633, 1956.
6. Mangold, H. K. *J. Am. Oil Chemists' Soc.* **38**: 708, 1961.
7. Gänshirt, H., F. W. Koss, and K. Morianz. *Arzneimittel-Forsch.* **10**: 943, 1960.
8. Hofmann, A. F. *Anal. Biochem.* **3**: 145, 1962.
9. Hofmann, A. F. *J. Lipid Research* **3**: 127, 1962.
10. Stahl, E. *Arch. Pharm.* **292**: 411, 1959.
11. Dahn, H., and H. Fuchs. *Helv. Chim. Acta* **45**: 261, 1952.