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A study of the separation of substituted cholanic acids by gas-liquid chromatography*

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SUMMARY

Suitable conditions for the gas-liquid chromatography of a number of substituted methyl cholanates are described. The effects of various functional groups on the retention times have been studied, using as liquid phase two types of silicone gum rubbers differing in their content of phenyl groups.

The methods most commonly used for the chromatographic separation of bile acids involve reversed phase partition chromatography (1, 2, 3), partition chromatography with aqueous acetic acid as stationary phase (2, 4), and paper chromatography with acidic solvents (5, 6). The last-mentioned type of solvents was originally developed by Ahrens and Craig (7) for the countercurrent distribution of bile acids. Silicic-acid chromatography of methyl esters of bile acids has been used in some cases (8) and adsorption chromatography on impregnated glass paper (9) has proved to be very valuable for separation of extremely small amounts of bile acids. By combining several of the methods mentioned, most of the known biologically occurring bile acids can be separated. In analyses of very complicated mixtures, however, the procedures become tedious and time-consuming. Recently Vanden Heuvel *et al.* (10) showed that bile acids could be separated by gas-liquid chromatography. In connection with studies of human fecal bile acids, we have therefore further investigated the gas-liquid chromatographic behavior of a number of substituted cholanic acids. This paper describes suitable conditions for the separation of the methyl esters of some hydroxy-, acetoxy- and ketocholanic acids.

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METHODS

Gas-Chrom P, 100 to 120 mesh (Applied Science Laboratories), was used to support the stationary phase. The washing and coating of the support was carried out by the technique used by Horning *et al.* (11). Forty grams of the support was washed repeatedly with concentrated hydrochloric acid, then with distilled water, until the supernatant was neutral (fine particles were removed by decanting), and finally with acetone. After drying in an oven at 80° for 1 to 2 hours, the support was siliconized for 15 minutes with a 5% solution of dimethyldichlorosilane (General Electric Company) in toluene, under intermittent water pump vacuum to remove air from the particles. After washing with methanol until neutral, the support was dried overnight at 80°.

The stationary phases described in this work were two types of silicone rubber gum, kindly made for us by Dr. A. C. Martellock, of the Silicone Products Department, General Electric Company. They were modifications of "SE-52" and consisted of the $-(Si(CH_3)_2O)-$ and the $-(Si(C_6H_5)_2O)-$ groupings. One polymer (GE No. 287-149-243) had 20 moles per cent, and the other (GE No. 287-149-244) 35 moles per cent, of the latter groupings. One-half gram of the material was dissolved in 100 ml of toluene, 20 g of the support was added, and intermittent water pump vacuum was applied. After about 15 minutes the excess of solution was filtered off on a suction funnel and the wet support dried in an oven at 80° for 1 to 2 hours. Although the exact amount of stationary phase on the support cannot be stated, this procedure was reproducible and appeared to cause the least damage to the

support. The coated support was packed into 183 cm × 6 mm columns by tapping against the floor; packing by vibration seemed to result in tailing peaks. A Barber-Coleman Model 10 apparatus with an argon ionization detector was used. Two milligrams of the bile acids was dissolved in 0.25 ml of methanol and freshly distilled diazomethane in ether was added in excess until the solution was slightly colored. The solvent was removed by blowing with nitrogen and 0.5% to 1% solutions of the methyl esters were prepared in acetone. Two-tenths to 2 μl of these solutions were injected into the column with a Hamilton syringe. Suitable conditions for columns prepared as described were: Column temperature 215° to 220°, flash heater temperature 260° to 290°, detector temperature 235° (radium foil), argon pressure 10 to 15 psi, flow rate 40 to 70 ml/minute.

RESULTS AND DISCUSSION

Table 1 gives the retention times, relative to methyl deoxycholate, of the substituted methyl cholanates tested on two types of column. One type had the silicone rubber with 20 moles per cent phenyl groups as stationary phase, the other one had the polymer with 35 moles per cent phenyl groups. Figures 1 and 2 show the differences in the separating abilities of the two phases.

Methyl deoxycholate was chosen as the reference compound since it has a retention time which is in the middle of the range of retention times found for the various compounds. The conditions were chosen so as to retain methyl deoxycholate for about 20 minutes. Except in the case of the methyl esters of 3α-hydroxy-6-ketocholanic and 3α-hydroxy-6-keto-allocholanic acids (kindly given to us by Dr. E. A. Doisy, Jr., St. Louis University, St. Louis, Mo.), no signs of destruction were noted. These two compounds, however, were apparently partly degraded in the flash heater. The peak shape of keto and acetoxy compounds was symmetrical; di- and trihydroxy compounds showed a slight tailing, which can be prevented by using more polar stationary phases. As seen in Table 1, addition of a hydroxyl group to the cholanic acid nucleus approximately doubles the retention times (see also Table 2).

The isomeric mono- and dihydroxy compounds tested could all be separated (compounds 2, 4, 9, 14, 20), whereas separations of isomeric trihydroxy compounds were unsatisfactory (compounds 22, 23, 33, 34). Also, the stationary phases used did not differentiate an axial and an equatorial hydroxyl at C-7 (compounds 14, 15; and 22, 23), the only position tested in this respect. This is surprising since in reversed phase chromatography such isomeric compounds can be easily separated because of their different polarity. The monoketo compounds (Nos. 3, 6) are retained longer than the corresponding hydroxy compounds. This is also the case with the 3,12-disubstituted compounds (Nos. 9, 11, 13), whereas no change occurs on

TABLE 1. RETENTION TIMES RELATIVE TO METHYL DEOXYCHOLATE OF SUBSTITUTED METHYL CHOLANATES

No.	Compound	Rel. Retention Time 20*	35*	No.	Compound	Rel. Retention Time 20*	35*
1	Methyl cholanate	0.23	0.20	18	3α-acetoxy-7-keto	1.49	1.48
2	12α-hydroxy	0.42	0.38	19	3,7-diketo	1.16	1.21
3	12-keto	0.45	0.42	20	3α,6α-dihydroxy	1.34	1.30
4	3α-hydroxy	0.54	0.49	21	3α,6α-diacetoxy	1.92	1.72
5	3α-acetoxy	0.79	0.67	22	3α,7α,12α-trihydroxy	2.20	2.32
6	3-keto	0.62	0.58	23	3α,7β,12α-trihydroxy	2.30	2.36
7	3α-hydroxy-11-ene	0.53	0.48	24	3α,7α-diacetoxy-12-hydroxy	2.44	2.44
8	3β-hydroxy-5-ene	0.64	0.58	25	3α,7α,12α-triacetoxy	1.88	1.90
9	3α,12α-dihydroxy	1.00	1.00	26	3α,12α-dihydroxy-7-keto	2.20	2.36
10	3α,12α-diacetoxy	1.15	1.14	27	3α,12α-diacetoxy-7-keto	2.24	2.27
11	3α-hydroxy-12-keto	1.15	1.12	28	3α,7α-dihydroxy-12-keto	2.62	2.73
12	3α-acetoxy-12-keto	1.46	1.43	29	3α,7α-diacetoxy-12-keto	2.57	2.59
13	3,12-diketo	1.27	1.25	30	3α-hydroxy-7,12-diketo	2.18	2.34
14	3α,7α-dihydroxy	1.16	1.18	31	3α-acetoxy-7,12-diketo	2.52	2.72
15	3α,7β-dihydroxy	1.12	1.16	32	3,7,12-triketo	2.03	2.30
16	3α,7α-diacetoxy	1.35	1.29	33	3α,6α,7α-trihydroxy	2.41	2.62
17	3α-hydroxy-7-keto	1.14	1.21	34	3α,6β,7β-trihydroxy	2.20	2.24

* Mole per cent phenyl groups in the stationary phase.

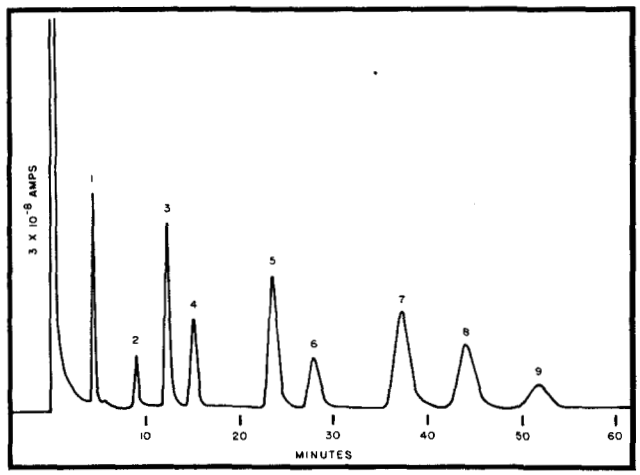

FIG. 1. Five-tenths per cent SE-52 silicone gum rubber, 20 moles per cent phenyl groups, on Gas-Chrom P, 100 to 120 mesh. Peak *1:* cholanic; peak *2:* 12-keto; peak *3:* 3-keto; peak *4:* 3α-acetoxy; peak *5:* 3α-12α-diacetoxy; peak *6:* 3α,7α-diacetoxy; peak *7:* 3α,7α,12α-triacetoxy and 3α,6α-diacetoxy; peak *8:* 3α,12α-diacetoxy-7-keto; peak *9:* 3α-acetoxy-7,12-diketo.

oxidation of the 3,7-dihydroxy compound to the ketones (compounds 14, 17, 19). Similarly, oxidation of the 7α-hydroxyl group in methyl cholate (No. 22) does not change the retention time (No. 26), whereas the corresponding change from 12α-hydroxy to 12-keto (No. 28) increases the retention time.

Several compounds with identical retention times can be separated after acetylation. Generally acetylation increases the retention time of mono- and disubstituted compounds, whereas for trisubstituted ones the effect is small and in some cases the retention time is decreased (compounds 22, 25; and 28, 29). Introduction of a 3α-carbethoxy group in deoxycholic acid more than doubled the retention time.

The number of CH_2-groups in the side chain has a pronounced effect on the retention time, as shown in Table 3. Comparison between compounds 37 and 41 shows that conversion of the C-27 methyl group in trihydroxy coprostane into the methyl ester group of methyl trihydroxy coprostanate increases the retention time about threefold.

The percentage of phenyl groups in the stationary phase influences the retention times of the compounds such that on the phase with the higher percentage of phenyl groups the hydroxyl and keto groups on the compounds cause a longer retention time (Table 1). Acetylation of hydroxyl groups results in a smaller relative retention time on the 35% column than on the 20% column.

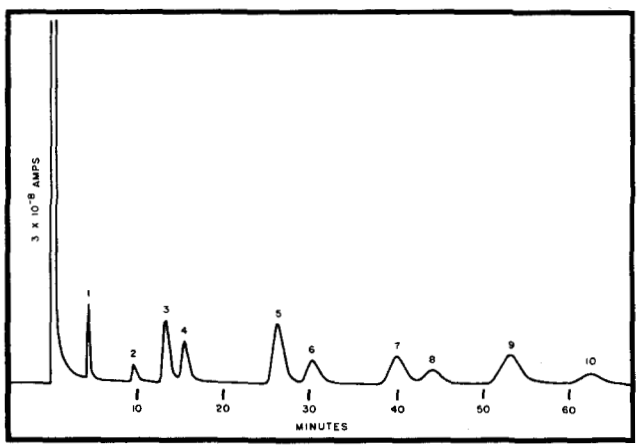

FIG. 2. Five-tenths per cent SE-52 silicone gum rubber, 35 moles per cent phenyl groups, on Gas-Chrom P, 100 to 120 mesh. Peak *1:* cholanic; peak *2:* 12-keto; peak *3:* 3-keto; peak *4:* 3α-acetoxy; peak *5:* 3α,12α-diacetoxy; peak *6:* 3α,7α-diacetoxy; peak *7:* 3α,6α-diacetoxy; peak *8:* 3α,7α,12α-triacetoxy; peak *9:* 3α,12α-diacetoxy-7-keto; peak *10:* 3α-acetoxy-7,12-diketo.

TABLE 2. RETENTION TIMES RELATIVE TO METHYL DEOXYCHOLATE OF MONO-, DI-, AND TRIHYDROXY COPROSTANES RELATED TO BILE ACIDS

No.	Compound	Rel. Retention Time 20*	35*
35	3α-hydroxy coprostane	0.36	0.27
36	3α,12α-dihydroxy coprostane	0.67	0.56
37	3α,7α,12α-trihydroxy coprostane	1.42	1.29

* Mole per cent phenyl groups in the stationary phase.

TABLE 3. EFFECT OF SIDE CHAIN LENGTH ON RELATIVE RETENTION TIME

No.	Compound	Rel. Retention Time 20*	35*
38	Methyl 3α,12α-diacetoxy bisnorcholanate	0.55	0.51
39	Methyl 3α,12α-diacetoxy norcholanate	0.86	0.82
10	Methyl 3α,12α-diacetoxy cholanate	1.15	1.14
40	Methyl 3α,7α,12α-trihydroxy bisnorcholanate	1.03	1.17
22	Methyl 3α,7α,12α-trihydroxy cholanate	2.20	2.32
41	Methyl 3α,7α,12α-trihydroxy coprostanate	4.37	4.23
37	3α,7α,12α-trihydroxy coprostane	1.42	1.29

* Mole per cent phenyl groups in the stationary phase.

Although it is not possible with the phases described in this paper to separate all possible fecal bile acids, the methods described should be of value for the tentative identification or quantitative determination of bile acids, particularly if the sample is analyzed before and after acetylation or oxidation.

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