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BILE ACIDS

XXVIII. GAS CHROMATOGRAPHY OF NEW BILE ACIDS AND THEIR DERIVATIVES

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SUMMARY

The new phases OV-1 and OV-17 are compared with QF-1 in the gas chromatographic analysis of 80 methyl 5β- and 5α-cholanoates and their complete trimethylsilyl (TMSi) ethers. The 5α-cholanoates were slower than their 5β-isomers in elution from the columns by factors of 1.22, 1.11, and 1.20 for QF-1, OV-1, and OV-17, respectively. Methyl α-muricholate can be effectively separated on OV-17 from methyl cholate; the complete TMSi ethers of deoxycholate, cholate, α-, β-, and ω-muricholates can be separated on OV-17 from the TMSi ether of hyocholate. OV-17 resembles PhSi-35 in its polarity and selectivity.

INTRODUCTION

The coupling of gas chromatography with mass spectrometry[1] has provided a new tool for the determination of structure of the components from the effluent of the gas chromatograph. With this new tool has arisen a need for stationary phases with exceptional thermal stability and low bleed rate. A new series of silicones containing 0–65 % phenyl groups in place of methyl groups in dimethylpolysiloxane polymers has provided phases of particular interest to the mass spectrometrist. HORNING *et al.*[2,3] have reported on the use of OV-1 and OV-17 for the separation of urinary acids as the methyl ester trimethylsilyl (TMSi) derivatives and the TMSi derivatives of a number of steroids; SUPINA *et al.*[4] have reported on the evaluation of six of these silicones for the separation of lipids. KUKSIS[5] has studied the chromatography of seven bile acids as their methyl esters on 1 % OV-17, and concluded that this phase is similar in retention characteristics to PhSi-35. The studies reported here present the results of gas chromatographic analysis of a series of 80 bile acids as their methyl esters and their TMSi derivatives on the silicone phases OV-1 and OV-17 in comparison with the fluorosilicone QF-1. Mass spectra were determined to ascertain the structures of the materials studied.

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MATERIALS AND METHODS

Gas chromatography

An F and M Model 402 gas chromatograph fitted with a hydrogen flame detector was used isothermally in these studies. The columns were silanized glass U-shaped tubes (6 ft. × 4 mm, I.D.) packed individually with 3 % OV-1, OV-17, or QF-1 on 100–120 mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, Pa.). Since the silicones* OV-1 and OV-17 are reported to be stable up to 350° and 375°, respectively, the following conditions were used: column, 260°; flash heater, 275°; detector, 275°; helium, 40 p.s.i. at a flow rate of 40 cc/min. With the fluorosilicone QF-1 the conditions were: column, 230°; flash heater, 245°; detector, 245°; helium, 40 p.s.i. at a flow rate of 40 cc/min. Methyl deoxycholate was used as internal standard. Relative retention time, R_t, relates to methyl deoxycholate (1.00) or its TMSi derivative (1.00). Absolute retention times of methyl deoxycholate were: on QF-1, 29.0 min; on OV-1, 38.4 min; on OV-17, 44.0 min. For the TMSi derivative of methyl deoxycholate the retentions were: QF-1, 10.0 min; OV-1, 27.3 min; OV-17, 14.2 min.

Mass spectrometry

An LKB Model 9000 Gas Chromatograph Mass Spectrometer fitted with molecule separators of the Becker Ryhage type was used as reported previously[6]. A silanized coiled glass column (6 ft. or 8 ft. × 0.25 in., O.D.) packed with 3 % OV-1, OV-17, or QF-1 on Gas-Chrom Q was used for gas chromatography under the following conditions: ion source, 310°; ionizing energy, 70 eV; ionizing current, 60 μA; for QF-1: flash heater, 240°; column, 215°; molecule separator, 255°; for OV-1 or OV-17: flash heater, 285°; column, 260°; molecule separator, 290°.

Bile acids and derivatives. Cholic, deoxycholic and hyodeoxycholic acids were gifts of the Wilson Laboratories, Chicago, Ill. 3β-Hydroxy-Δ^5-cholenoic acid was obtained from Mann Research Laboratories. All other 5β-acids reported in Table I were available in this laboratory from current or previous studies. Methyl 3β,6β-dihydroxy-5α-cholanoate was a generous gift of Dr. PETER ZIEGLER, Canadian Packers Ltd., Toronto, Canada. All other 5α-acids and their methyl esters were prepared in these laboratories[6–9]. In general, methyl esters of bile acids were obtained from the free acid by treatment with diazomethane. Samples of the methyl esters were prepared for gas chromatography in acetonitrile. TMSi ethers were prepared from the methyl esters with a mixture of trimethylsilyl chloride, hexamethyldisilazane, and dry pyridine as reported by MAKITA AND WELLS[10] and discussed by WELLS *et al.*[11]. The solutions were made up to a concentration of 1–2 μg of methyl ester per μl. After the mixture had stood for a minimum of 10 min on the warm top of the oven, a sample of 1–2 μl of solution was injected into the gas chromatograph. Samples were discarded after 24 h, although decreased amounts of TMSi ethers could be detected as long as four days after preparation.

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* The stationary phases OV-1 and OV-17 are commercially available polymers of dimethylpolysiloxane and phenylmethylpolysiloxane (50 % phenyl), respectively. QF-1 is a commercially available fluorosilicone. Thus, OV-1 is a non-polar phase comparable to SE-30, and OV-17 has polarity intermediate between OV-1 and QF-1.

RESULTS

Table I contains the relative retention times of the 5β- and 5α-cholanoates. SJÖVALL[12], MAKITA AND WELLS[10], KUKSIS[13], and OKISHIO AND NAIR[14] have previously presented data for esters of some bile acids on QF-1; the data of Table I present for the first time a rather complete comparison of retention times for the 5β-derivatives and their new 5α-analogs. Where comparisons can be made, agreement is good between the values of Table I and the published values for the fluorosilicone QF-1, although the data were obtained from columns at different temperatures and different concentrations of the phase on the support. The data in Table I show smaller relative retention times for the non-polar OV-1 than for the other phases. The poorer selectivity of OV-1 for ketones is especially reflected in the grouping of the monoketo monohydroxy derivatives with the diols and the monoketo-diols or diketo monohydroxy derivatives with the triols.

From an analysis of 45 methyl 5β-cholanoates most of the generalizations discussed by KUKSIS[5] are confirmed. Thus, on the more selective fluorosilicone, QF-1, the order of elution of monohydroxy derivatives (in increasing R_t) is C_{12}, C_7, C_6 and C_3 with elution of the axial derivative prior to the equatorial alcohol. With the less selective silicone OV-1 all derivatives are grouped rather closely with little distinction between the axial and equatorial hydroxyl groups at C_{12}, C_7, C_6 or C_3. Results from the column of OV-17 tend to approach that of QF-1, but also show less ability to differentiate between the conformational or configurational isomers.

Among the 5β-dihydroxy derivatives eluted from QF-1, four diaxial diols (7α,12α; 6β,12α; 3β,12α; 3β,7α) are eluted before the equatorial-axial 3α,12α-diol, methyl deoxycholate. The axial 12α- and 7α-hydroxyl groups are shielded by the side chain and the C_{18} angular methyl groups, and the A/B *cis* configuration of the ring structure, respectively, and hence are unlikely to interact appreciably with the liquid phase. The 6β-hydroxyl group encounters 1,3-diaxial interference especially with the C_{19} angular methyl group. This difference is not found in the 5α-series and will be discussed below.

Two pairs each of equatorial/axial:diaxial (3α,12α/3β,12α; 3α,7α/3β,7α) and of diequatorial:equatorial/axial (3α,7β/3α,7α; 3α,6α/3α,6β) diols are eluted from QF-1 as predicted, *i.e.*, the equatorial/axial diol follows the diaxial diol, and is followed by the diequatorial diol. Although, the phases OV-1 and OV-17 are unable to distinguish well between the axial and equatorial 3-hydroxyl groups in the 3,12- and 3,7-diols, the 3α,6-diols are distinguished on QF-1, OV-1 or OV-17.

The four monoketo esters are eluted from QF-1, OV-1 and from OV-17 as predicted in the order of increasing R_t: C_{12}, C_7, C_6, and C_3. On OV-17 the 3,7-dione is eluted prior to the 3,12-dione, whereas the reverse is the case on QF-1. The monohydroxy monoketo derivatives are eluted within a narrower range on OV-17 than on QF-1, and are eluted with or slightly later than the diketones from each phase. The Rohrschneider constants (Table II) for the three phases[4] suggest that a better separation of ketones from alcohols may not be expected for OV-17 over QF-1. Figs. 1 and 2 show the separation of methyl lithocholate, 3-dehydrolithocholate, deoxycholate, ursodeoxycholate, chenodeoxycholate, and hyodeoxycholate on OV-1 and OV-17 respectively. The peaks are well defined and separated on OV-1; the 3α,7-diols are not separated on OV-17. KUKSIS[5] was unable to separate urso- and hyodeoxycholic acids

TABLE I

RELATIVE RETENTION TIMES OF METHYL CHOLANOATES

All relative retention times are referred to methyl deoxycholate = 1.00. Absolute times of elution of methyl deoxycholate are: QF-1, 29.0 min; OV-1, 38.4 min; OV-17, 44.0 min. Methyl deoxycholate was injected simultaneously with each of the esters.

Substituent	*5β*			*5α*			*Ratio 5α/5β*		
	QF-1	*OV-1*	*OV-17*	*QF-1*	*OV-1*	*OV-17*	*QF-1*	*OV-1*	*OV-17*
None	0.15	0.35	0.20	0.18	0.40	0.24	1.20	1.14	1.20
12α	0.27	0.55	0.39	0.37	0.60	0.49	1.37	1.09	1.21
12β	0.31	0.56	0.41	—	—	—	—	—	—
7α	0.34	0.60	0.44	0.40	0.64	0.51	1.18	1.07	1.16
7β	0.36	0.59	0.46	0.43	0.67	0.52	1.19	1.14	1.13
6β	0.38	0.62	0.47	—	—	—	—	—	—
6α	0.45	0.65	0.52	—	—	—	—	—	—
3β	0.44	0.64	0.51	0.55	0.72	0.60	1.25	1.13	1.18
3α	0.49	0.62	0.52	0.50	0.72	0.58	1.02	1.12	1.12
12-Keto	0.49	0.57	0.43	0.60	0.63	0.51	1.23	1.11	1.19
7-Keto	0.57	0.58	0.46	0.72	0.66	0.57	1.26	1.14	1.24
6-Keto	0.78	0.64	0.58	—	—	—	—	—	—
3-Keto	1.00	0.71	0.61	1.06	0.76	0.71	1.06	1.07	1.16
7α,12α	0.70	0.91	0.84	0.90	0.99	0.99	1.29	1.09	1.18
6β,12α	0.77	0.94	0.91	—	—	—	—	—	—
3β,12α	0.86	1.00	1.00	1.16	1.16	1.20	1.35	1.16	1.20
3β,7α	0.94	1.06	1.10	1.29	1.18	1.34	1.37	1.11	1.22
3α,12α	1.00	1.00	1.00	1.07	1.12	1.19	1.07	1.12	1.19
3α,7α	1.18	1.08	1.14	1.22	1.18	1.27	1.04	1.09	1.11
3α,6β	1.20	1.11	1.21	—	—	—	—	—	—
3α,7β	1.27	1.06	1.13	—	—	—	—	—	—
3α,6α	1.50	1.20	1.32	—	—	—	—	—	—
3β,6β	—	—	—	1.27	1.20	1.29	—	—	—
7,12-Diketo	1.45	0.91	0.85	1.97	0.96	1.06	1.36	1.06	1.25
3,12-Diketo	2.86	1.05	1.29	3.52	1.15	1.51	1.23	1.10	1.17
3,7-Diketo	3.05	1.04	1.21	3.96	1.17	1.52	1.30	1.17	1.26
12-Keto-7α	—	—	—	1.40	1.03	1.12	—	—	—
7-Keto-12α	1.10	1.13	0.88	1.56	1.09	1.13	1.42	0.96	1.28
12-Keto-6β	1.19	0.99	1.11	—	—	—	—	—	—
12-Keto-3α	1.61	1.00	1.12	—	—	—	—	—	—
7-Keto-3α	1.80	1.03	1.20	1.97	1.23	1.42	1.09	1.19	1.18
7-Keto-3β	—	—	—	2.12	1.26	1.41	—	—	—
3-Keto-12α	1.82	1.07	1.17	2.23	1.19	1.41	1.23	1.11	1.21
3-Keto-7α	2.40	1.23	1.43	2.32	1.27	1.60	0.97	1.03	1.12
3-Keto-6α	2.93	1.32	1.59	—	—	—	—	—	—
3β,7α,12α	1.90	1.63	2.08	2.78	1.88	2.71	1.46	1.15	1.30
3α,7β,12α	2.20	1.52	2.07	—	—	—	—	—	—
3α,7α,12α	2.33	1.66	2.26	2.68	1.77	2.73	1.15	1.07	1.21
3α,6β,7β	2.33	1.68	2.30	2.10	1.78	2.34	0.90	1.06	1.02
3α,6β,7α	2.42	1.84	2.65	—	—	—	—	—	—
3α,6α,7β	2.67	1.69	2.25	—	—	—	—	—	—
3α,6α,7α	3.14	1.85	2.56	—	—	—	—	—	—
7-Keto-3α,6α	1.78	1.48	1.59	—	—	—	—	—	—
7-Keto-3α,12α	3.51	1.59	2.27	4.62	1.95	2.89	1.32	1.23	1.27
12-Keto-3α,7α	3.85	1.73	2.57	—	—	—	—	—	—
7,12-Diketo-3α	4.55	1.50	2.18	—	—	—	—	—	—
3-Keto-7α,12α	4.79	1.88	2.79	6.10	1.96	3.12	1.27	1.04	1.12
6-Keto-3α,7β	—	—	—	2.06	1.52	1.88	—	—	—
7-Keto-3β,12α	—	—	—	4.50	1.85	2.87	—	—	—
3,7-Diketo-12α	5.38[a]	1.54	2.31	7.14[b]	1.84	3.04	1.32	1.20	1.32
3,12-Diketo-7α	—[b]	1.91	3.30	—[b]	—[b]	—[b]	—	—	—
3,7,12-Triketo	6.40	1.39	2.25	9.24	1.64	2.98	1.44	1.18	1.32

[a] The peak was not well formed, so this value is tentative.
[b] This material is retained too long on the column to provide meaningful data.

TABLE II

ROHRSCHNEIDER CONSTANTS OF THREE SILICONE PHASES

For a more detailed discussion on these constants and the method of acquisition, see ref. 15.

Phase	*X*	*Y*	*Z*	*U*	*S*
OV-1	0.16	0.20	0.50	0.55	0.48
OV-17	1.30	1.66	1.79	2.53	2.47
QF-1	1.09	1.86	3.00	3.94	2.41

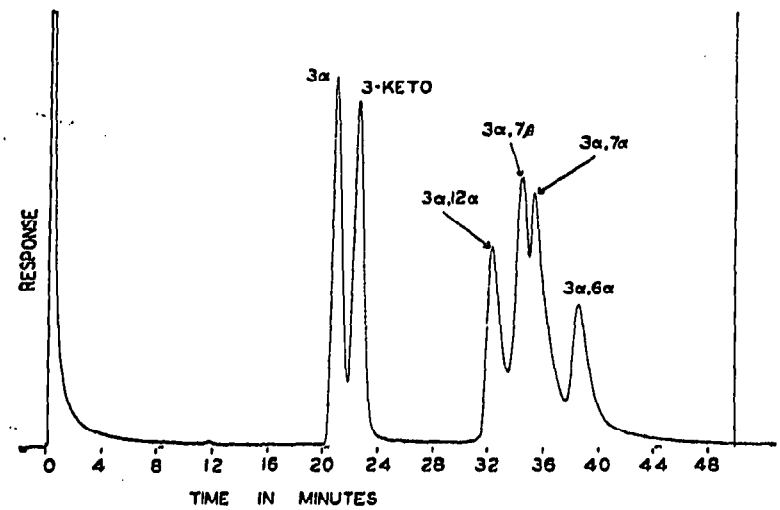

Fig. 1. Chromatographic separation of methyl 5β-cholanoates on 3% OV-1. A mixture of methyl lithocholate (3α), 3-keto-5β-cholanoate (3-keto), deoxycholate (3α,12α), ursodeoxycholate (3α, 7β), chenodeoxycholate (3α,7α) and hyodeoxycholate (3α,6α) was injected into a column of 3% OV-1 on Gas-Chrom Q at 260°. The time of elution of methyl deoxycholate differs from that stated in Table I since a different column was used in this separation.

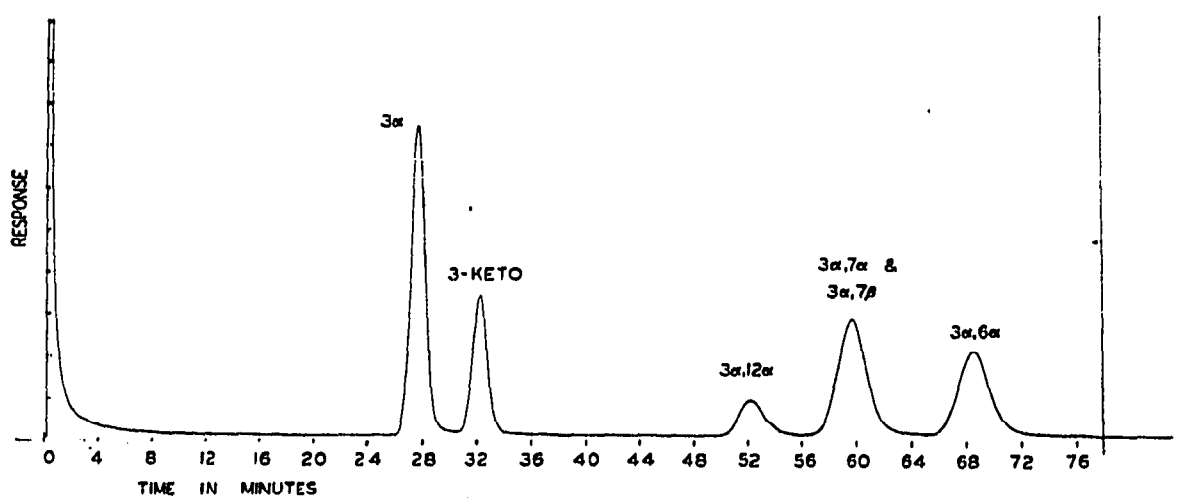

Fig. 2. Separation of methyl 5β-cholanoates on 3% OV-17. The same mixture of esters used for Fig. 1 was injected into a column of 3% OV-17 on Gas-Chrom Q at 260°. Chenodeoxycholate (3α,7α) and ursodeoxycholate (3α,7β) are not separated on this phase.

as their methyl esters on a 4-ft. column of 1 % OV-17 at 235°, or by programmed elution from 230–280°.

Among the trihydroxy derivatives the triaxial triol (3β,7α,12α) is eluted before the equatorial-diaxial triol (3α,7α,12α) from each of the phases, but the non-specific OV-1 shows little distinction between them; QF-1 offers the best separation. However, the diequatorial axial triol (3α,7β,12α) is eluted before cholate on each phase. The order of elution of the muricholates (3α,6,7-triols) varies. From QF-1 the order (6β,7β; 6β,7α; 6α,7β; 6α,7α) differs from that on OV-1 (6β,7β; 6α,7β; 6β,7α; 6α,7α) or that on OV-17 (6α,7β; 6β,7β; 6α,7α; 6β,7α). Thus, of the trihydroxy acids present in rat bile OV-17 offers a separation of α-muricholate (3α,6β,7α-triol, R_t 2.65) from cholate (R_t 2.26), a separation not attained on QF-1 because of the relatively large amount of cholate.

In the 5α-series Rings A and B are *trans* and the 3α- and 3β- substituents are axial and equatorial, respectively, the reverse of the 5β-series. This difference is immediately evident in their order of elution from QF-1 and OV-17, but not from the non-selective OV-1. Three examples appear as contradictions to the generalization that selective phases retain the 5α-cholanoates longer than the corresponding 5β-cholanoates: the ester of the 7-keto-12α-ol on OV-1, and the esters of the 3-keto-7α-ol and the 3α,6β,7β-triol on QF-1. From the ratios of the relative retention times (5α/5β) the longer average retention times of the 5α-derivatives on each of the three phases has been calculated to be: for QF-1, 1.22 (range 0.90–1.49); for OV-1, 1.11 (range 0.96–1.23); and for OV-17, 1.20 (range 1.02–1.32); *i.e.* on the average a 5α-cholanoate would be expected to have a retention time 1.22 times that of the 5β-derivative on QF-1, 1.11 on OV-1 and 1.20 on OV-17*.

Table III contains the relative retention times of the TMSi ethers of the methyl 5β- and 5α-cholanoates. Formation of the TMSi derivatives of the hydroxy esters results in greater volatility as the etherified nucleus tends to approach the hydrocarbon nature of methyl cholanoate; *i.e.* on QF-1 the slowest TMSi derivative of the triols (3α,6α,7β, R_t 1.88) was eluted as the tris-TMSi derivative in 18.8 min. Despite this shorter elution time the retention times of the TMSi ethers respond dramatically to the conformation of the substituents. In general those TMSi derivatives of the 5α-series are retained longer than the corresponding TMSi derivative of the 5β-series in which an axial substituent of the 5β-series appears as an equatorial substituent in the 5α-series. This is particularly true of the 3β(e),5α-derivatives; *i.e.* 3β; 3β,7α; 3β,12α; and 3β,7α,12α-hydroxy-5α-cholanoates are each retained longer than the corresponding derivatives of the 3β(a)-5β-cholanoates. On the other hand the TMSi derivatives of the 3α-ol, 3α,7α-diol, 3α,7α,12α- and 3α,6β,7β-triols of the 5α-cholanoates are eluted from each of the phases before their respective 5β-cholanoates. HOSHITA *et al.*[16] have reported that the TMSi derivatives of methyl *allo*-cholate, *allo*-chenodeoxycholate, and *allo*-deoxycholate each exhibit retention times on 1 % SE-30 of 0.96 relative to that of the TMSi ethers of the corresponding 5β-cholanoates. HOFMANN AND MOSBACH[17] have reported retention times of 0.74 on 0.5 % Hi-Eff 8B and 0.91 on 1 % QF-1 for the TMSi derivative of methyl *allo*-deoxycholate relative to the TMSi ether of methyl deoxycholate. The present studies show that the TMSi ether of methyl

* These ratios also indicate a lower precision in measurement of R_t for those substances eluted substantially later than the standard; *i.e.*, the ratio of R_t for 5α/5β derivatives is generally larger than the average value for substituted ketones.

TABLE III

RELATIVE RETENTION TIMES OF TRIMETHYLSILYL ETHERS OF METHYL CHOLANOATES

All relative retention times are referred to the bis(trimethylsilyl) ether of methyl deoxycholate = 1.00. Absolute times of elution of this derivative are: QF-1, 10.0 min; OV-1, 27.3 min; OV-17, 14.2 min. This derivative was injected simultaneously with each of the TMSi derivatives listed.

Substituent	*5β*			*5α*			*Ratio 5α/5β*		
	QF-1	*OV-1*	*OV-17*	*QF-1*	*OV-1*	*OV-17*	*QF-1*	*OV-1*	*OV-17*
12α	0.59	0.58	0.62	0.66	0.60	0.64	1.12	1.03	1.03
12β	0.55	0.54	0.56	—	—	—	—	—	—
7α	0.65	0.61	0.63	0.62	0.57	0.57	0.97	0.94	0.91
7β	0.71	0.71	0.75	0.81	0.78	0.83	1.14	1.10	1.11
6β	0.63	0.62	0.63	—	—	—	—	—	—
6α	0.67	0.65	0.72	—	—	—	—	—	—
3β	0.83	0.81	0.86	1.20	0.82	1.20	1.45	1.01	1.40
3α	0.91	0.85	0.93	0.89	0.72	0.92	0.98	0.85	0.95
7α,12α	0.72	0.69	0.60	0.63	0.63	0.53	0.88	0.91	0.88
6β,12α	0.79	0.80	0.69	—	—	—	—	—	—
3β,12α	1.02	1.00	0.90	1.35	1.23	1.19	1.32	1.23	1.32
3β,7α	0.96	0.96	0.87	1.14	1.08	1.00	1.19	1.13	1.15
3α,12α	1.00	1.00	1.00	0.93	1.18	1.22	0.93	1.18	1.22
3α,7α	1.09	1.04	1.00	1.00	0.84	0.92	0.92	0.81	0.92
3α,6β	1.08	1.10	0.98	—	—	—	—	—	—
3α,7β	1.24	1.21	1.14	—	—	—	—	—	—
3α,6α	1.20	1.12	1.08	—	—	—	—	—	—
3β,6β	—	—	—	1.40	1.33	1.31	—	—	—
3β,7α,12α	0.96	0.96	0.79	1.18	1.01	0.79	1.23	1.05	1.00
3α,7α,12α	1.05	1.09	0.90	1.00	1.05	0.87	0.95	0.96	0.97
3α,6β,7α	1.05	1.12	0.84	—	—	—	—	—	—
3α,6α,7α	1.38	1.15	1.11	—	—	—	—	—	—
3α,6β,7β	1.44	1.45	1.18	1.41	1.44	1.17	0.98	0.99	0.99
3α,6α,7β	1.88	1.92	1.71	—	—	—	—	—	—
12-Keto-7α	—	—	—	2.43	0.90	1.18	—	—	—
7-Keto-12α	2.74	0.93	1.35	3.42	0.97	1.54	1.20	1.04	1.14
12-Keto-6β	2.13	0.95	1.27	—	—	—	—	—	—
7-Keto-3α	3.65	1.34	1.98	4.16	1.41	2.30	1.14	1.05	1.16
3-Keto-12α	4.10	1.09	1.67	3.04	1.17	1.85	0.74	0.93	1.11
3-Keto-7α	3.81	1.17	1.84	4.12	1.08	1.60	1.08	0.92	0.87
12-Keto-3α	3.19	1.33	1.91	—	—	—	—	—	—
3-Keto-6α	5.04	1.32	1.99	—	—	—	—	—	—
12-Keto-3α,7α	4.37	1.72	1.97	—	—	—	—	—	—
7-Keto-3α,12α	4.95	1.59	2.03	5.45	1.62	1.95	1.10	1.02	0.96
3-Keto-7α,12α	5.84	1.35	1.68	5.07	1.09	1.45	0.87	0.81	0.92
3,7-Diketo-12α	12.5	1.56	3.10	12.7	1.78	3.76	1.02	1.14	1.21
7,12-Diketo-3α	4.55	1.88	3.38	—	—	—	—	—	—
7-Keto-3β,12α	—	—	—	7.00	1.96	2.46	—	—	—

allo-deoxycholate is eluted before the comparable derivative of methyl deoxycholate on 3% QF-1, but not on OV-1 or OV-17. The data in Table III also show shorter relative retention times of the TMSi ethers of the 5α-cholanoates of the 7α-ol, 7α,12α-diol, and 3-keto-7α,12α-diol on each of the three phases, the 3-keto-12α-ol on QF-1 and OV-1, the 3-keto-7α-ol on OV-1, and the 7-keto-3α,12α-diol on OV-17. The mass spectra of the TMSi ethers of methyl 3-keto-7α,12α-dihydroxy-5α- and 5β-cholanoates

have been analyzed in support of the structure of the former derivative and are clearly bis TMSi ethers[6]. In contrast the TMSi ether of the 3α-hydroxy-7-keto-5α-cholanoate is not eluted more rapidly than its 5β-analog on either of the three phases. The TMSi derivative of the 3α,7β-diol is retained a little longer than that of the 3α,6α-diol on each of the three phases, whereas the 3α,6α-diol is retained longer than the 3α,7β-diol on each phase. The derivatives with the longest relative retention times are those of the keto compounds; by mass spectrometry[6] no evidence was found for the formation of enolic TMSi ethers.

The relative retention times of the TMSi ethers of the trihydroxy 5β-cholanoates indicate that α- and β-muricholates (3α,6β,7α- and 3α,6β,7β-triols) are separable on QF-1, OV-1, or OV-17. Although the trimethylsilyl ethers (TMSi) of these triols are not separable from cholate on QF-1 or OV-1, they are separated on OV-17 (Fig. 3). Fig. 3 also shows the separation of the TMSi ethers of β-muricholate from cholate and α-muricholate in a mixture of these materials in the concentration reported[18,19] in rat bile (0.76:10:0.99). The area under the peaks of elution was measured with an Infotronics Model CRS-108 integrator and the results supported the ratio of injected material.

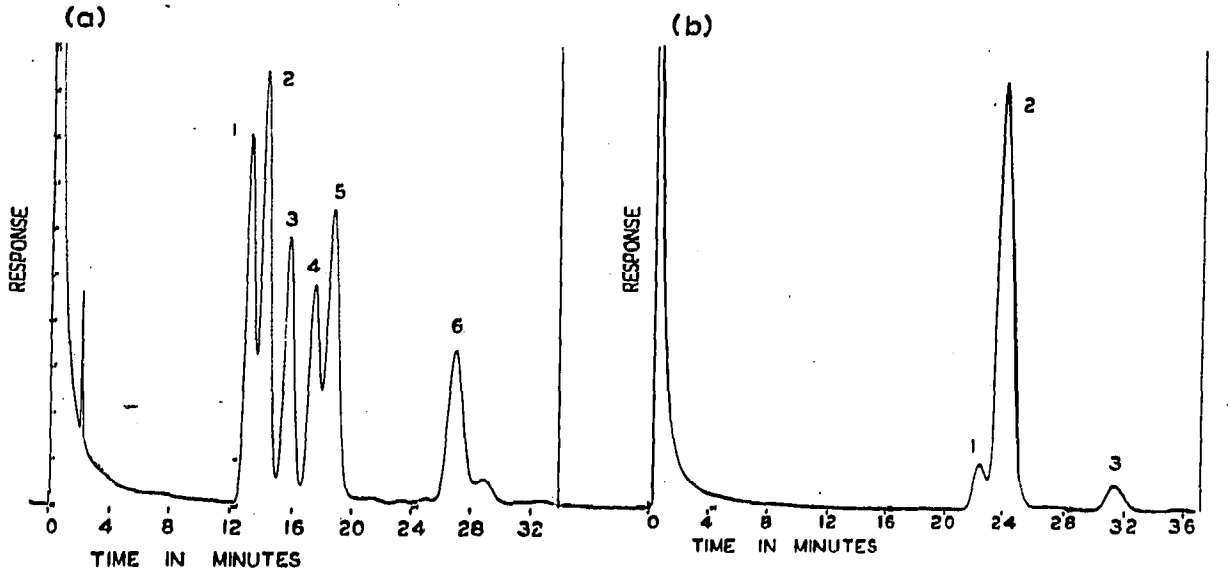

Fig. 3. Separation of TMSi ethers of methyl trihydroxy 5β-cholanoates on OV-17. (a) A mixture of methyl α-muricholate (1), cholate (2), deoxycholate (3), hyocholate (4), β-muricholate (5), and ω-muricholate (6) was converted to the completely silylated ethers and separated on 3% OV-17 on Gas-Chrom Q at 260°. Methyl deoxycholate was used as an internal standard. (b) A mixture of methyl α-muricholate (1) (3α,6β,7α), cholate (2) (3α,7α,12α), and β-muricholate (3) (3α,6β,7β) prepared in a ratio of 0.99:10.0:0.76 as reported for rat bile was completely silylated and separated on 3% OV-17 on Gas-Chrom Q at 260°. A different column of OV-17 was used in this separation.

In 1962 CLAYTON[20–23] proposed that the retention times of a polysubstituted steroid with noninteracting substituent groups was a product of the retention time of the unsubstituted nucleus and a series of constant factors characteristic of each substituent and its position in the molecule. SJÖVALL[12] has shown the concept can be extended to the bile acid series. Data in Table IV extend SJÖVALL's observations on

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TABLE IV

RELATIONSHIPS BETWEEN NATURE OF SUBSTITUENTS AND RELATIVE RETENTION TIMES OF SUBSTITUTED METHYL CHOLANOATES

Substituent	*QF-1*				*OV-1*				*OV-17*			
	5α		5β		5α		5β		5α		5β	
	Found	*Calcd.*	*Found*	*Calcd.*	*Found*	*Calcd.*	*Found*	*Calcd.*	*Found*	*Calcd.*	*Found*	*Calcd.*
None	—	1.00	—	1.00	—	1.00	—	1.00	—	1.00	—	1.00
12α	2.06	—	2.07	—	1.50	—	1.57	—	2.04	—	1.95	—
12β	—	—	2.07	—	—	—	1.60	—	—	—	2.05	—
7α	2.22	—	2.27	—	1.60	—	1.71	—	2.13	—	2.20	—
7β	2.39	—	2.40	—	1.68	—	1.69	—	2.17	—	2.30	—
6β	—	—	2.53	—	—	—	1.77	—	—	—	2.35	—
6α	—	—	3.00	—	—	—	1.86	—	—	—	2.60	
3β	3.05	—	2.93	—	1.80	—	1.83	—	2.50	—	2.55	—
3α	2.78	—	3.27	—	1.80	—	1.77	—	2.42	—	2.60	
12-Keto	3.33	—	3.27	—	1.58	—	1.63	—	2.13	—	2.15	—
7-Keto	4.00	—	3.80	—	1.65	—	1.66	—	2.38	—	2.30	—
6-Keto	—	—	5.20	—	—	—	1.83	—	—	—	2.90	—
3-Keto	6.11	—	6.67	—	1.90	—	2.03	—	2.96	—	3.05	—
7α,12α	5.00	4.57	4.67	4.70	2.48	2.40	2.60	2.68	4.13	4.34	4.20	4.29
6β,12α	—	—	5.13	5.24	—	—	2.69	2.78	—	—	4.55	4.58
3β,12α	6.44	6.28	5.73	6.06	2.90	2.70	2.86	2.87	5.00	5.10	5.00	4.97
3β,7α	7.17	6.77	6.27	6.65	2.95	2.88	3.03	3.13	5.58	5.32	5.50	5.61
3α,12α	5.94	5.73	6.67	6.77	2.80	2.70	2.86	2.78	4.96	4.93	5.00	5.07
3α,7α	6.78	6.17	7.87	7.42	2.95	2.88	3.08	3.03	5.29	5.15	5.70	5.72
3α,6β	—	—	8.00	8.27	—	—	3.17	3.13	—	—	6.05	6.11
3α,7β	—	—	8.47	7.85	—	—	3.03	2.99	—	—	5.65	5.98
3α,6α	—	—	10.00	9.81	—	—	3.43	3.29	—	—	6.60	6.76
7,12-Diketo	10.9	13.3	9.7	12.4	2.40	2.61	2.60	2.71	4.42	5.07	4.25	4.95
3,12-Diketo	19.6	19.6	19.1	21.8	2.88	3.00	3.00	3.31	6.29	6.30	6.45	[illegible]
3,7-Diketo	22.0	25.6	20.3	25.3	2.93	3.31	2.97	3.37	6.33	7.04	6.05	7.02

12-Keto-7α	7.78	7.39	—	—	2.58	2.53	—	—	4.67	4.54	—	—
7-Keto-12α	8.78	8.24	7.34	7.87	2.73	2.48	3.23	2.61	4.71	4.86	4.40	4.49
12-Keto-6β	—	—	7.94	7.85	—	—	2.83	2.89	—	—	5.55	5.05
12-Keto-3α	—	—	10.7	10.7	—	—	2.86	2.88	—	—	5.60	5.59
7-Keto-3α	10.9	11.1	12.0	12.4	3.08	2.97	2.94	2.94	5.92	5.76	6.00	5.98
7-Keto-3β	11.8	12.2	—	—	3.15	2.97	—	—	5.88	5.95	—	—
3-Keto-12α	12.4	12.1	12.1	13.8	2.98	2.85	3.06	3.19	5.88	6.04	5.85	5.95
3-Keto-7α	12.9	13.6	16.0	15.1	3.18	3.04	3.51	3.47	6.67	6.30	7.15	6.71
3-Keto-6α	—	—	19.5	20.0	—	—	3.77	3.78	—	—	7.95	7.43
3β,7α,12α	15.4	13.9	12.7	13.8	4.70	4.32	4.66	4.91	11.3	10.9	10.4	10.9
3α,7β,12α	—	—	14.7	16.2	—	—	4.34	4.69	—	—	10.4	11.7
3α,7α,12α	14.9	12.7	15.5	15.4	4.43	4.32	4.74	4.76	11.4	10.5	11.3	11.2
3α,6β,7β	11.7	—	15.5	19.8	4.45	—	4.80	5.29	10.2	—	11.5	14.1
3α,6β,7α	—	—	16.1	18.8	—	—	5.26	5.35	—	—	13.3	13.4
3α,6α,7β	—	—	17.8	23.5	—	—	4.83	5.56	—	—	11.3	15.5
3α,6α,7α	—	—	20.9	22.3	—	—	5.29	5.63	—	—	12.8	14.9
7-Keto-3α,12α	25.7	22.9	23.4	25.7	4.88	4.46	4.54	4.61	12.0	11.7	11.4	11.7
12-Keto-3α,7α	—	—	25.7	24.3	—	—	4.94	4.94	—	—	12.9	12.3
7,12-Diketo-3α	—	—	30.3	40.7	—	—	4.29	4.80	—	—	10.9	12.9
3-Keto-7α,12α	33.9	27.0	31.9	31.3	4.90	4.56	5.37	5.44	13.0	12.9	14.0	13.1
7-Keto-3β,12α	25.0	25.1	—	—	4.63	4.46	—	—	12.0	12.1	—	—
3,7-Diketo-12α	39.7	45.3	—	—	—	—	4.40	5.29	—	—	11.6	13.7
3,12-Diketo-7α	—	—	—	—	—	—	5.46	5.66	—	—	16.5	14.4
3,7,12-Triketo	51.3	81.2	42.7	82.9	4.10	4.94	3.97	5.49	12.4	13.5	11.3	15.1

QF-1 and include results on the newer phases, OV-1 and OV-17. With the exception of polyfunctional derivatives with long retention times (*e.g.* di- and triketonic esters) agreement between calculated and found values is generally better for the 5β- than the 5α-derivatives. Small errors in calculation in reference to methyl cholanoates are multiplied several times to magnify apparent differences between the calculated and found values. SJÖVALL[12] has commented on the potential interactions between the glycolic hydroxyl groups of the muricholic acids (3α,6,7-triols), and has suggested that such interactions may account for the discrepancies observed with these materials. Similar calculations have been made for the TMSi derivatives in Table III; in general, agreement between calculated and found values is reasonable for the phases OV-1 and OV-17. The data calculated from QF-1 indicate the trend, but the variance between calculated and found values probably reflects a combination of multiple calculations and variation in oven temperature from day to day.

From data at hand it is possible now to demonstrate the effects of change in a given phase by increasing the content of the "polar" group (phenyl, in this case). Thus, data in Table V compare relative retention times from OV-1 and OV-17 with those of SE-30 from VANDENHEUVEL AND BRALY[25], and phases of polysiloxanes prepared for and studied by SJÖVALL, *et al.*[24] with 20 and 35 mole percent of phenyl in place of methyl groups. The relative retention times from OV-17 and PhSi-35 are remarkably similar for all derivatives except the monoketodihydroxy- and diketo-monohydroxy-cholanoates. The superior selectivity of ketones by QF-1 is evident

TABLE V

COMPARISON OF RELATIVE RETENTION TIMES OF METHYL 5β-CHOLANOATES ON FIVE DIFFERENT PHASES

Substituent	*OV-1, 3 %* *260°*[a]	*PhSi-20* *215°*[b]	*PhSi-35* *215°*[b]	*OV-17, 3 %* *260°*[a]	*QF-1, 3 %* *230°*[a]	*SE-30, 0.5%* *208°*[c]
None	0.35	0.23	0.20	0.20	0.15	—
12α-	0.55	0.42	0.38	0.39	0.31	0.52
12-Keto	0.57	0.45	0.42	0.43	0.49	—
3α-	0.62	0.54	0.49	0.52	0.49	0.63
3-Keto	0.71	0.62	0.58	0.61	1.00	—
3α,12α-	1.00	1.00	1.00	1.00	1.00	1.00
3,12-Diketo	1.05	1.27	1.25	1.29	2.86	—
3α,7α-	1.08	1.16	1.18	1.14	1.18	1.10
3α,7β-	1.06	1.12	1.16	1.13	1.27	1.09
3α,7-Keto	1.03	1.14	1.21	1.20	1.80	1.04
3,7-Diketo	1.04	1.16	1.21	1.29	3.05	—
3α,6α-	1.20	1.34	1.30	1.32	1.50	1.22
3α,7α,12α-	1.66	2.20	2.32	2.26	2.33	1.72
3α,12α-7-Keto	1.59	2.20	2.36	2.27	3.51	—
3α,7α-12-Keto	1.73	2.62	2.73	2.57	3.85	—
3α-7,12-Diketo	1.50	2.18	2.34	2.18	4.55	—
3,7,12-Triketo	1.39	2.03	2.30	2.25	6.40	—
3α,6α,7α-	1.85	2.41	2.62	2.56	3.14	—
3α,6β,7β-	1.68	2.20	2.24	2.30	2.33	—

[a] This paper.
[b] Ref. 24.
[c] Calculated from data from. ref. 25.

from the data of Table V; however, the failure to separate the 3-keto-cholanoate from deoxycholate on QF-1 can be resolved with OV-1, OV-17, PhSi-20 or PhSi-35. As the phenyl content of these methylpolysiloxanes is increased, the separation of isomeric alcohols is improved[5] and tends to approach the fluorosilicone QF-1.

Relative retention times of eleven TMSi derivatives on five different phases are compared in Table VI. The TMSi ether of cholate is eluted earlier from OV-17 than from any of the other phases. The TMSi ethers of chenodeoxycholate and deoxycholate are not separated on OV-17 or the triphasic column of OKISHIO *et al.*[14,26]. The triequatorial ω-muricholate (3α,6α,7β-triol) is retained the longest on each column. Clearly, the choice of phases must be based upon the desired objectives. These data confirm the desirability of a preliminary separation of bile acids according to the number of substituent groups before chromatography as their TMSi ethers[5].

TABLE VI

COMPARISON OF RELATIVE RETENTION TIMES OF TRIMETHYLSILYL ETHERS OF METHYL 5β-CHOLANOATES ON VARIOUS PHASES

Substituent	*OV-1* 260°[a]	*OV-17* 260°[a]	*QF-1* 230°[a]	*QF-1* 210°[b]	*QF-1* *SE-30*; *NGS*[b]	*SE-30* 238°[c]
12α-	0.58	0.62	0.59	—	—	0.63
3α-	0.85	0.93	0.91	0.94	0.91	0.95
3α,12α-	1.00	1.00	1.00	1.00	1.00	1.00
3α,7α-	1.04	1.00	1.09	1.09	1.00	1.17
3α,7β-	1.21	1.14	1.24	1.06	1.27	1.35
3α,6α-	1.12	1.08	1.20	1.13	1.18	1.25
3α,7α,12α-	1.09	0.90	1.05	1.12	1.04	1.20
3α,6α,7α	1.15	1.11	1.38	1.41	1.41	—
3α,6α,7β-	1.92	1.71	1.88	2.09	2.32	—
3α,6β,7α-	1.12	0.84	1.05	—	—	—
3α,6β,7β-	1.45	1.18	1.44	—	—	—

[a] This paper.
[b] Calculated from data from ref. 26.
[c] Calculated from data from ref. 25.

In these studies* the conditions of MAKITA AND WELLS[10] have been used to form the completely silylated ethers of these esters, as opposed to the procedures of SJÖVALL[12], ENEROTH *et al.*[27,28] and BRIGGS AND LIPSKY[29] for the formation of partial derivatives. GRUNDY *et al.*[30] have identified fecal bile acids as complete silyl ethers on SE-30 or DC-560, but have not published retention times of these derivatives. VANDENHEUVEL AND BRALY[25] have reported on the gas chromatography of methane sulfonates and mixed silyl ethers of bile acids. OKISHIO *et al.*[14,26] have reported favorable results from a column of mixed phases (QF-1, SE-30 and NGS). EVRARD AND JANSSEN[31] have utilized a column of 1 % JXR to study fecal bile acids after oxidation of their methyl esters to the respective keto derivatives.

* Mass spectrometry has been carried out on most of the derivatives in Tables I and III; a manuscript on this subject is in preparation.

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