Since compound (XII) was an unstable oil on heating and turned into a resin, crude (XII) was used to afford (XIV).

6-Isopropyl-1,4-dimethylazulene (XIV) (Entry No. 11 in Table I)——A mixture of XII (150 mg.) and thionyl chloride (0.1 ml.) in pyridine (1 ml.) was left for 2 hr. at room temperature and treated under the same manner as IX. The residue was chromatographed on Al_2O_3 to give a colorless oil (XIII, 124 mg.). A mixture of XIII (27.2 mg.) and 10% Pd-C (20 mg.) was heated at 320~322° for 1 min. in a nitrogen atmosphere and treated as described above to give an oily blue-violet azulene (XIV, 3.0 mg., 11.0% yield), UV λ_{max} mμ (log ε): 242.5 (4.27), 285 (4.69), 291 (4.69), 343 (3.51), 351 (3.59) and 368 (3.30), and Visible Spectrum λ_{max} mμ(ε): 539(216), 563 (257), 585 (286), 613 (245), 638 (219), 675 (107) and 706 (72), IR $\nu_{max}^{CS_2}$ cm^{-1}: 1330, 1287, 1198, 1040, 1020, 880, 865, 826, 775 and 713. 2,4,6-Trinitrobenzene adduct: Dark purple prisms, m.p. 149.5~150.5° (from EtOH). *Anal.* Calcd. for $C_{15}H_{18}\cdot C_6H_3O_6N_3$: C, 61.31; H, 5.15; N, 10.21. Found: C, 61.27; H, 5.21; N, 10.06. This azulene (XIV) was identical with 6-isopropyl-1,4-dimethylazulene synthesized by Plattner[7] by comparison of infrared and visible spectra.

Summary

A microtechnique for dehydrogenation of some perhydroazulene and -naphthalene derivatives was developed, and the optimum reaction conditions were discussed.

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96. Kyosuke Tsuda*1, Yoshihiro Sato,*1 Nobuo Ikekawa,*2 Sayoko Tanaka,*1 Hideaki Higashikuze,*1 and Ryuzaburo Ohsawa*3 : Studies on Bile Acids and Bile Alcohols. II.*4 Separation of Bile Acids by Gas Liquid Chromatography.

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Gas liquid chromatography has already been recognized to be a powerful tool for the qualitative and quantitative analysis of steroids. Horning and his co-workers[1] were the first to report the separation of bile acids by this technique and the application of such separation in the analysis of acetate, trifluoroacetate or trimethylsilyl ether of bile acids have since been demonstrated by several workers.[2]

In view of its potential use in the field of bile acids, a systematic program was undertaken in this laboratory to explore the conditions under which favorable separa-

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*4 This paper constituted Part XLIX of a series "Steriod Studies." Reported at the 83rd Annual Meeting of Pharm. Soc. of Japan, Nov., 2, 1963 (Tokyo).

1) W. J. A. VandenHeuvel, C. C. Sweeley, E. C. Horning: Biochem. Biophys. Res. Comm., **3**, 33 (1960).
2) *a*) J. Sjövall, C. R. Meloni, D. A. Turner: J. Lipid Res., **2**, 317 (1960). *b*) W. J. A. VandenHeuvel, J. Sjövall, E. C. Horning: Biochim. Biophys. Acta, **48**, 596 (1961). *c*) R. Blomstrand: Proc. Soc. Exp. Biol. Med., **107**, 126 (1961), *d*) W. L. Holmes, E. Stack: *Ibid.*, **54**, 163 (1962). *e*) R. I. Ellin, A. I. Mendeloff, D. A. Turner: Anal. Biochem., **4**, 198 (1962). *f*) D. A. Bloomfield: Anal. Chem., **34**, 737 (1962). *g*) J. Sjövall: Acta Chem. Scand., **16**, 1716 (1962). *h*) H. Danielson, P. Beneroth, K. Hellsrom, J. Sjövall: J. Biol. Chem., **237**, 3657 (1962). *i*) M. Makita, W. Wells: Anal. Biochem., **5**, 523 (1963). *j*) A. Kuksis, B. A. Gordon: Canadian J. Biochem. Physiol., **41**, 1355 (1963).

tion of variety of bile acid derivatives might be obtained using gas liquid chromatography. A part of this studies on the application to the separation of human bile acids was already reported in the previous paper.[3] This paper presents the results obtained in investigations into the separation of twenty-eight kinds of bile acids using selective and nonselective phases.[4]

Experimental

Apparatus——Gas chromatograph : A Barber-Colman Model 10 instrument with argon ionization detector and a Shimadzu Model GC-1B with hydrogen flame ionization detector (dual column and differential flame) were used for this study. Chromatographic column : The U-shape glass and stainless steel columns were used in a Barber-Colman Model 10 and a Shimadzu Model GC-1B, respectively. Packings, column-sizes and operating conditions were given in Table I.

Samples[*5]——Bile acid methyl esters were analyzed as a trifluoroacetate or an acetate prepared by the following method. Trifluoroacetates[2b,2g] were prepared by heating 0.3~1 mg. of the methyl ester in 0.2 ml. of trifluoroacetic anhydride in a small glass stoppered test tube at 30° for 15 min. The reagent was then evaporated under a stream of nitrogen and the acetone solution of the residue was injected into the chromatograph. Acetates were prepared by refluxing with an excess of redistilled acetic anhydride for 4 hr. The reaction mixture was then evaporated to dryness and the acetone solution of the residue was injected.

Results and Discussion

In order to examine the gas liquid chromatographic behavior of various kinds of bile acids and also to compare the relative retention times on several liquid phases, trifluoroacetates of methyl esters were analyzed both on SE-30 (methyl silicone, G. E.) as a nonselective phase and on QF-1-0065 (trifluoropropyl methyl silicone, D. C.), NGS (neopentyl glycol succinate), and CNSi (cyanoethyl methyl silicone, G. E.), as selective phases. Free hydroxy bile acid methyl esters were analyzed on CNSi and on SE-52 (methyl phenyl silicone, G. E.) which is also one of selective phases. On the latter phase, acetates of methyl esters were also separated giving a good results on the application of human bile acids analyses, which has already been published in the previous paper of this series.[3]

Relative retention times of twenty-eight bile acids on these phases were listed in Table I. In the analysis on 0.4% CNSi and on 0.75% SE-52 phases of hydroxy free bile acids, the separation was not found to be so desirable for an analysis of naturally occuring bile acids. Separation of a mixture of trifluoroacetate methyl esters of lithocholic, deoxycholic, chenodeoxycholic, hyodeoxycholic, and cholic acids on QF-1 is shown in Fig. 1. A detector response of the nine standard bile acids was shown in Fig. 2. It was observed to be linear over a range of 1~40 γ in the analysis by an argon ionization detector (Barber-Colman Model 10). The response tested here decreased with an increasing hydroxy substitution in a molecule and, in other words, with an increasing retention time.

As pointed out by Sjövall, an equatorial hydroxy group gives a longer retention time than an axial one at the same carbon atom on QF-1. In the case of trifluoroacetates, the results obtained on QF-1 in this studies also indicated that the equatorial compounds are generally retained much longer than the corresponding axial isomers. For examples, in the four groups of $3\beta<3\alpha$; $3\alpha,7\alpha<3\alpha,7\beta$; $3\beta,6\beta<3\beta,6\alpha<3\alpha,6\alpha$, and

*5 Bile acids described in this report are all cholanic acid derivatives (5β), unless otherwise noted.

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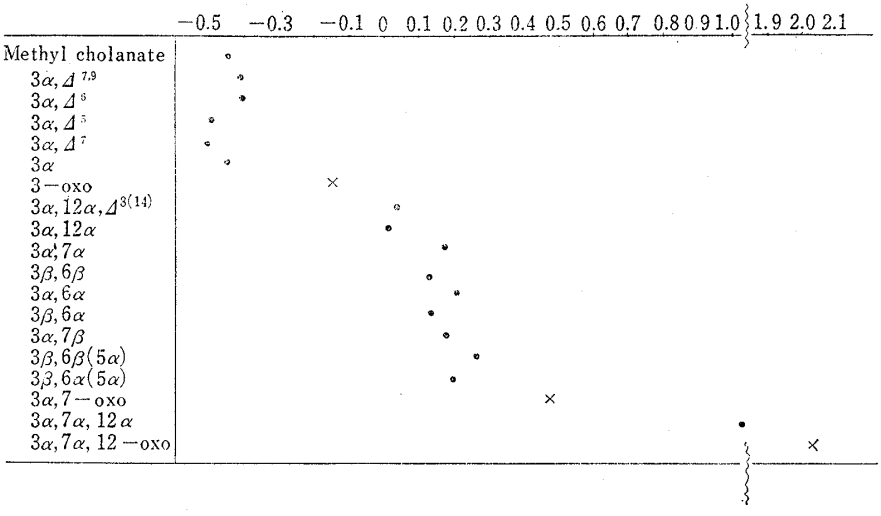
Table I. Relative Retention Times of Bile Acid Derivatives

No.	Compound	QF-1	NGS	CNSi		SE-30	SE-52	
		TFA[1]	TFA[2]	TFA[3]	OH-Free[4]	TFA[5]	OH-Free[6]	Acetate[7]
1	Methyl Cholanate	0.31	0.44	0.35	0.31	0.71	—	—
2	3α,$\Delta^{7,9}$	0.57	0.91	0.67	—	0.98	—	0.66
3	3α,Δ^{6}	0.62	0.90	0.73	0.37	1.01	0.63	—
4	3α,Δ^{5}	0.65	0.94	0.77	0.33	1.09	—	—
5	3α,Δ^{7}	0.67	1.09	0.78	—	1.14	—	0.76
6	3β	0.68	0.89	0.76	0.34	1.08	—	—
7	3α	0.69	1.06	0.81	0.37	1.13	0.32	0.72
8	3α,12α,$\Delta^{8(14)}$	0.86	0.90	0.82	1.00	0.86	1.04	—
9	3α,12α	1.00[a]	1.00[b]	1.00[c]	1.00[d]	1.00[e]	1.00[f]	1.00[g]
10	3α,7α	1.26	1.50	1.53	1.13	1.17	1.12	1.20
11	3-oxo,Δ^{6}	1.30	3.34	2.13	0.40	1.69	—	—
12	3-oxo,$\Delta^{7,9}$	1.33	2.96	1.98	—	1.68	—	—
13	3β,6β	1.32	—	1.50	1.26	1.18	—	—
14	3β,6α	1.43	—	1.59	1.35	1.30	—	—
15	3α,6α	1.45	1.93	1.62	1.42	1.28	1.24	1.55
16	3α,7β	1.48	2.12	1.77	1.21	1.40	1.22	1.58
17	3-oxo	1.54	3.39	2.22	0.45	1.69	—	—
18	3β,6β(5α)	1.63	—	—	1.64	1.39	—	—
19	3β,6α(5α)	1.76	—	—	—	1.55	—	—
20	3α,7α,12α	1.93	1.94	2.27	3.50	1.09	1.86	1.44
21	3α,7β,12α	—	—	—	—	—	—	1.98
22	3-oxo,12α	—	—	—	1.44	—	—	—
23	3α,7-oxo	2.48	—	3.80	—	2.00	—	—
24	3α,12-oxo	—	—	—	1.22	—	—	1.20
25	3α,7α,12-oxo	4.00	—	6.50	—	1.97	—	1.93
26	3,7-dioxo	—	—	—	1.50	—	—	—
27	3,12-dioxo	—	—	—	1.51	—	—	—
28	3,7,12-trioxo	—	—	—	4.36	—	—	—
29	Cholestane	0.18	0.17	0.18	—	0.72	—	0.23

1) 1% QF-1, 0.6×200 cm., at 220°, inlet pressure 1.6 kg./cm², *a*) 8.1 min.
2) 0.4% NGS 0.4×145 cm., at 210°, 2.0 kg./cm², *b*) 10.9 min.
3) 0.6% CNSi, 0.4×150 cm., at 216°, 2.4 kg./cm², *c*) 13.0 min.
4) 0.4% CNSi, 0.4×75 cm., at 230°, 2.4 kg./cm², *d*) 7.1 min.
5) 1% SE-30, 0.6×200 cm., at 220°, 1.6 kg./cm², *e*) 12.7 min.
6) 0.75% SE-52, 0.4×150 cm., at 260°, 2.0 kg./cm², *f*) 5.4 min.
7) 0.75% SE-52, 0.4×150 cm., at 235°, 2.0 kg./cm², *g*) 15.6 min.

Table II. Relationship of Relative Retention Times of Trifluoroacetates of Methyl Cholanates between on QF-1 and SE-30



$3\beta,6\beta(5\alpha)<3\beta,6\alpha(5\alpha)$ the fact was true as expected. The results of the following two groups, $3\beta,6\beta(5\beta)<3\beta,6\beta(5\alpha)$ and $3\beta,6\alpha(5\beta)<3\beta,6\alpha(5\alpha)$, showed that A/B *trans* compounds have longer retention times than the corresponding A/B *cis* ones. These relations on the retention times could be also observed in separation on the phases of NGS, CNSi, SE-30, and SE-52. It is interesting to point out that the trifluoroacetate of $3\alpha,7\alpha,12\alpha$-trihydroxy compound shows an exceptionally shorter retention time than that of some dihydroxy derivatives on the NGS and SE-30 phases.

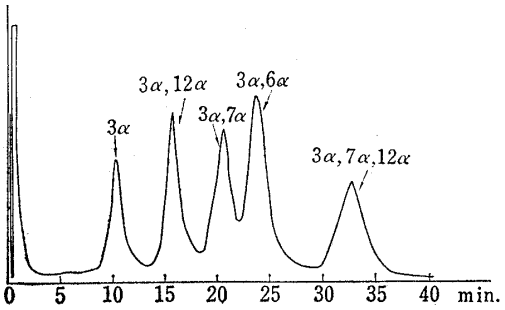


Fig. 1. Separation of a Mixture of Bile Acid Methyl Ester Trifluoroacetates on QF-1 Packing (For conditions see Table I)

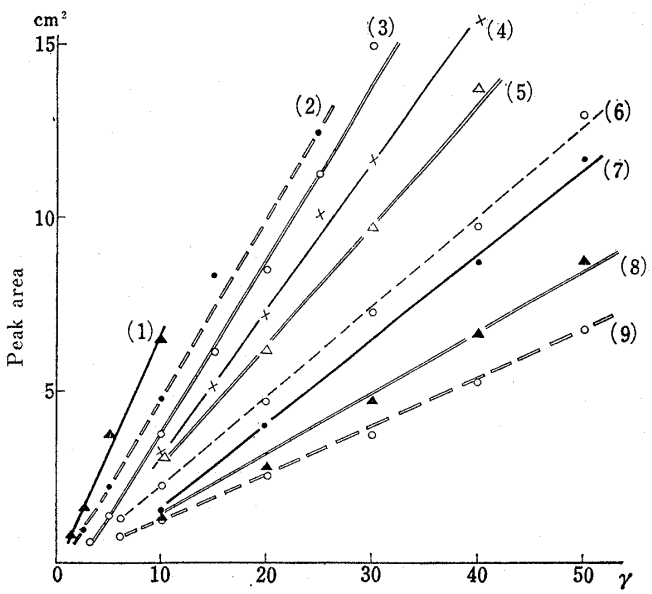


Fig. 2. Relationship of Various Amounts of Trifluoroacetates of the Methyl Esters of
1) Cholanic
2) 3α-Hydroxychol-6-enic
3) 3α-Hydroxychol-7-enic
4) 3α-Hydroxycholanic
5) 3α,12α-Dihydroxychol-8(14)-enic
6) 3α,12α-Dihydroxycholanic
7) 3α,6α-Dihydroxycholanic
8) 3α,7α-Dihydroxycholanic
9) 3α,7α,12α-Trihydroxycholanic acid (QF-1)

In the case of monotrifuoroacetoxy derivatives, the relative retention time on a selective phase QF-1 were much lower than that on a nonselective phase SE-30, on the other hand in the case of ditrifluoroacetoxy compounds, the both relative retention times were found to be about the same as shown in Table I. An interesting rule which was found in the relation of the relative retention times between on QF-1 and on SE-30 phases is shown in Table II. Sideways on Table II the differences of relative retention times on QF-1 and on SE-30 are plotted. As seen in the Table II, monohydroxy derivatives (3α-hydroxy unsaturated compounds in this case) dropped in a region of −0.5 to−0.4, dihydroxy in 0.0 to 0.3, and trihydroxy in around 1.0. On the other hand, monooxo derivatives such as 3-oxo, 3α,7-oxo, or 3α,7α,12-oxo showed more larger values than the corresponding mono-, di- or trihydroxy derivattves. This result will give a great value for an analysis and a structural elucidation of bile acids in a chemical reaction as well as in nature.

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Summary

The separation of twenty-eight kinds of standard bile acids by gas liquid chromatography using QF-1, NGS, CNSi, SE-30 or SE-52 packing is discussed.

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