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101. Kyosuke Tsuda, Yoshihiro Sato,*1 Nobuo Ikekawa,*2 Sayoko Tanaka, Hideaki Higashikuze,*1 Soichi Suzuki, Hisao Ohkubo, and Yusaku Anazawa*3 : Studies on Bile Acids and Bile Alcohols. I.*4 Gas Liquid Chromatography of Human Bile Acids.

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The microanalysis of bile acids by gas liquid chromatography was first developed by VandenHeuvel, Sweely and Horning[1] in 1960. Since then many studies[2] on bile acid separation have been reported but the only application of this method to the analysis of human bile acids has been described by Blomstrand[3] in 1961.

Recently[4] we have investigated the gas liquid chromatographic behavior of many bile acids by using different liquid phases. This paper describes the sample preparation from human bile and the suitable column conditions for gas liquid chromatographic separation, as a preliminary experiment for application to clinical analysis.

Human bile samples for this study were collected from gallbladders at the operating table, and in one case hepatic bile was collected through the choledochostomy. The conjugate bile acids were saponified with alkali by a method similar to that reported by Blomstrand.[3] After removal of neutral lipids, the acid fraction of this hydrolysis product was used for gas liquid chromatographic samples without partitioning. In the work of Blomstrand, the partition method was applied to the separation of bile acids from neutral lipids and fatty acids using the solvent system of petroleum ether and

Table Ia. Partition of Palmitic Acid in Petroleum Ether in the Solvent System of Ethanol-Water-Petr. Ether (35:15:30 ml.)

Palmitic acid used (mg.)	Number of times of partition and quantity partitioned (mg.)			
	1	2	3	4
48.1	27.6	9.0	3.5	0.5
34.5	21.1	7.7	2.0	1.0
22.3	17.5	2.2	1.5	1.0
10.6	7.5	2.5	0.5	0.5

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*4 This paper constitutes Part XLVIII of a series entitled "Steroid Studies" by K. Tsuda; Part XLVII: This Bulletin, **12**, 473 (1964).

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3) R. Blomstrand : Pro. Soc. Exp. Biology and Med., **107**, 126 (1961).
4) Reported at the 83rd Annual Meeting of Pharmaceutical society of Japan, Nov., 2, 1963 (Tokyo, Japan). The results will be reported in a forthcoming paper.

Table Ib. Partition of Bile Acids in Petroleum Ether in the Solvent System of Ethanol-Water-Petr. Ether (35:15:30 ml.)

Bile acids used (mg.)	Number of times of partition and quantity partitioned (mg.)			
	1	2	3	4
10.1[a)]	0.4	1.0	1.0	1.2
199.0[b)]	3.1	7.8	10.2	10.8
99.8[b)]	2.2	3.1	3.5	4.6
51.9[b)]	0.8	1.2	1.6	1.7
10.1[b)]	0.6	0.4	0.5	0.7
200.2[c)]	2.2	4.3	3.2	3.4
99.9[c)]	0.4	0.7	1.7	2.6
49.5[c)]	0.0	0.0	0.0	0.0

a) Lithocholic acid *b*) Deoxycholic acid *c*) Cholic acid

Table II. Relative Retention Times of Acetates of Bile Acid Methyl Esters on 3/4% SE-52

Compd.	R.R.T.	Compd.	R.R.T.
cholestane	0.23	3α,12-oxo	1.26
3α	0.72	3α,7α,12α	1.44
3α,$\Delta^{7,9}$	0.66	3α,6α	1.55
3α,Δ^{7}	0.76	3α,7β	1.58
3α,12α	1.00[a)]	3α,7α,12-oxo	1.93
3α,7α	1.20	3α,7β,12α	1.98

Column (0.4×150 cm.) 3/4% SE-52 on Anakrom A (80~100 mesh) at 235°
Carrier gas, N_2 (90 ml./min.) (Inlet Press, 2.0 kg.)
a) 15.6 min.

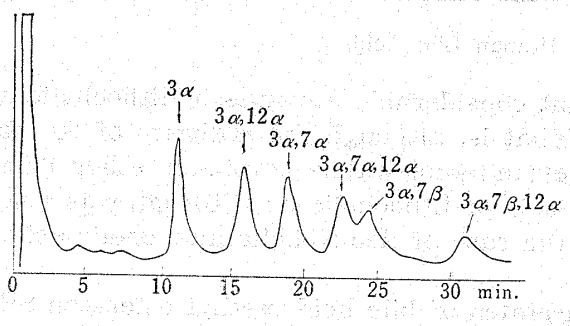

Fig. 1. Separation of Methyl Ester Acetates of Bile Acids

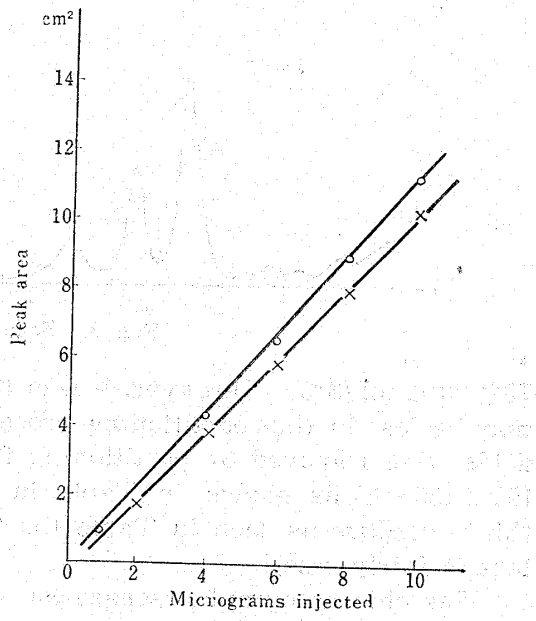

Fig. 2. Simple Calibration Curves of Acetates of Bile Acid Methyl Ester
—○—○— Lithocholic and deoxycholic acid
—×—×— Chenodeoxycholic, ursodeoxycholic, and cholic acid

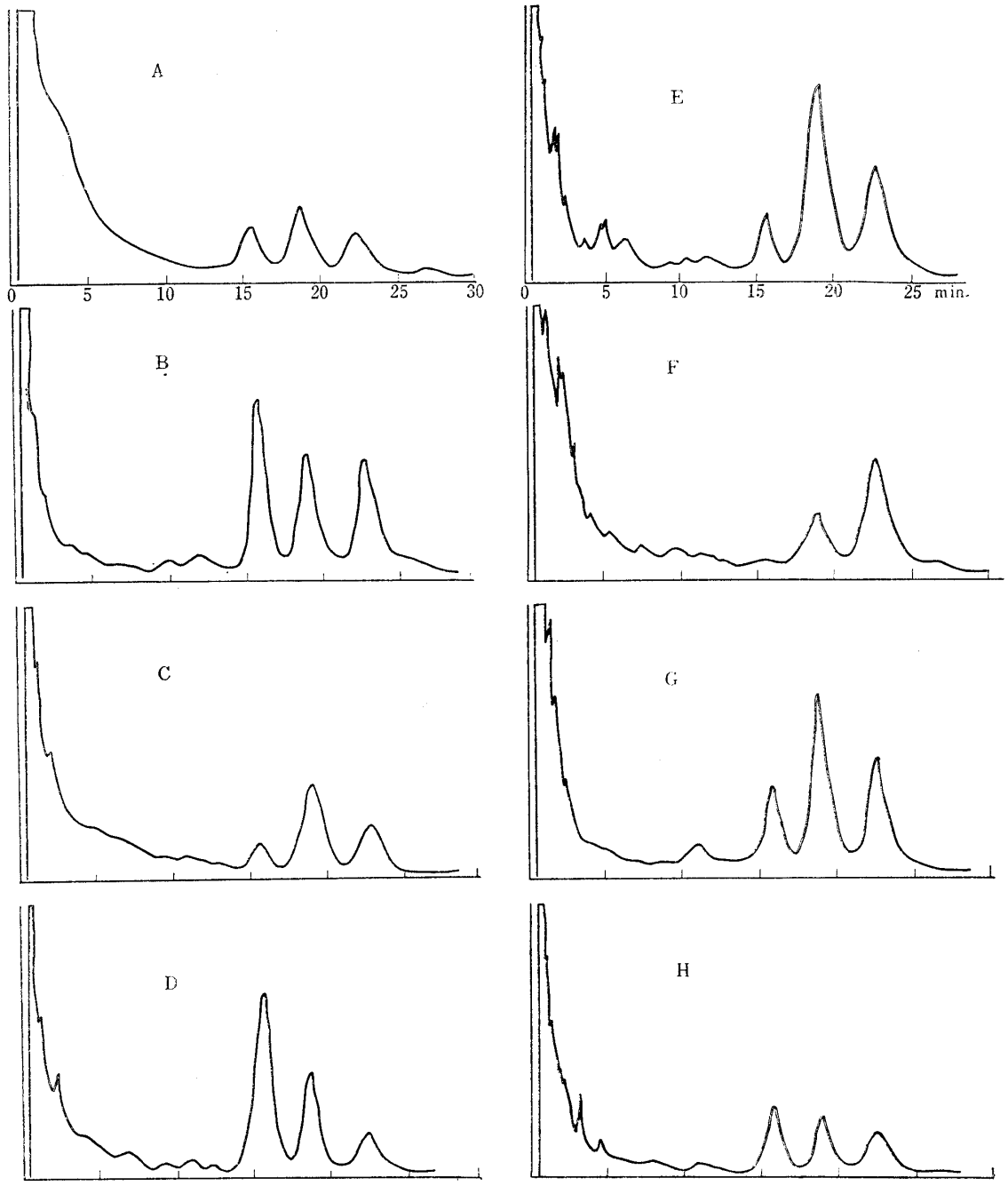

Fig. 3. Separation of Human Bile Acids

70% ethanol (3:5). However, it was found that considerable amounts of lithocholic acid may be lost in this partitioning procedure. That is, although the majority of the fatty acids were removed by partitioning four times; ethanol–water–petroleum ether (35 ml.: 15 ml.:30 ml.) as shown in Table Ia, about 40% of lithocholic acid (10 mg.) was lost by this procedure as seen in Table Ib. But in the case of deoxycholic and cholic acid the loss is fairly small.

Gas chromatographic separation of the acetates of bile acid methyl esters on SE-52 on Anakrom A used in the former study[4] was successful in separating the major gallbladder bile acids. In Table II relative retention times of substituted methyl cholanyl acetates employed as standards in this study are listed. Fig. 1 shows the chromatographic separation of standard mixture of six kinds of methyl ester acetates of bile

acids : lithocholic, deoxycholic, chenodeoxycholic, ursodeoxycholic, cholic, and 3α,7β,12α-trihydroxycholanic acid, which are known as human bile acids. Typical calibration curves of five bile acids are given in Fig. 2. This shows that detector response was linear over a range of the usual injection amounts, and the molar response was variable, requiring calculation of individual compounds. But the same simple calibration curves were given by the two groups of lithocholic and deoxycholic acid and of chenodeoxycholic, ursodeoxycholic and, cholic acid respectively.

In Fig. 3, gas chromatograms from eight patients are shown. A, B, and C are normal patients who were diagnosed as having a duodenal ulcer, case D, E, and F patients with cholelithiasis, and G a patient with common duct stone with acute cholecystitis. In patient F hepatic bile was analyzed and in this analysis a trace amount of deoxycholic acid and a larger ratio of cholic acid were detected. The data summarized in Table Ⅲ shows the relative ratios of lithocholic (L), deoxycholic (D), and cholic (C) to chenodeoxycholic acid (CD), and the total amount of bile acids. Because of limited case studied, it is not possible to make any definite conclusions for clinical diagnosis from this data.

Table Ⅲ. Bile Acid Ratios and their Quantities in Gallbladder Bile of Patients

Patient	Sex	Age	Diagnosis	L	D	CD	C	TA[b] (mg./ml.)
A	♀	19	duodenal ulcer	0.00	0.63	1.00	0.96	48
B	♂	25	〃	0.06	0.81	1.00	0.81	138
C	♀	20	〃	0.04	0.21	1.00	0.81	45
D	♀	55	cholelithiasis	0.03	1.62	1.00	0.37	105
E	♂	38	〃	0.05	0.21	1.00	0.63	23
F	♂	45	cholelithiasis; choledochostomy performed[a]	0.02	0.03	1.00	2.10	—
G	♂	65	common duct stone with subsiding jaundice	0.05	0.34	1.00	0.79	44
H	♀	40	acute cholecystitis	0.03	1.10	1.00	0.76	2

a) The drainage bile was analyzed.
b) TA : total acids L : lithocholic acid D : deoxycholic acid C : cholic acid
CD : chenodeoxycholic acid

Experimental

1) Preparation of Gas Chromatography Samples——Two ml. of the bile collected from gallbladder was refluxed 30 min. with 40 ml. of EtOH on water bath, filtered through filterpaper, evaporated to dryness *in vacuo*, and hydrolyzed in 4 ml. of H_2O, 2 ml. of ethylene glycol and 2 ml. of 4*N* NaOH for 20 hr. at 140~145° in an oil bath. After dilution of hydrolysate with H_2O, neutral lipids were removed with Et_2O, and from the aqueous layer acidic compounds were extracted with Et_2O after acidification with 10% HCl. The Et_2O extract was washed with H_2O, dried over anhyd. Na_2SO_4, evaporated to dryness, and methylated with CH_2N_2 in MeOH. Then the methyl esters were refluxed 4 hr. with Ac_2O giving the corresponding acetates. After removal of Ac_2O *in vacuo*, this ester acetate mixture was used for gas liquid chromatography as a Me_2CO solution.

2) Apparatus——A Shimadzu Seisakusho Gas Chromatograph Model GC-1B instrument with hydrogen flame detector (dual column and differential flame) was used. The two U-shape stainless steel column (75 cm.×4 mm., i.d.) connected in series were used. The column packing was 3/4% SE-52 on Anakrom A (80~100 mesh), acid washed and siliconized, and prepared by the filtration technique. After packing the column was conditioned for 36 hr. at 250° with N_2 flow at the rate of 25 ml./min. The same packing was used for the reference and for the sample column.

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Summary

The microanalysis of human bile acids by gas liquid chromatography using 3/4% SE-52 packing is discussed. Bile acid ratios of lithocholic, deoxycholic, chenodeoxycholic and cholic acid in gallbladder bile were estimated.

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