DETERMINATION OF SERUM BILE ACIDS IN HEPATOBILIARY DISEASES BY GAS LIQUID CHROMATOGRAPHY (FASTING SERUM BILE ACID LEVELS AND ENDOGENOUS BILE ACID TOLERANCE TEST)[*]

By

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ABSTRACT

The methods and results of a gas-liquid chromatographic analysis of bile acids in serum are presented. The analysis of bile acids in serum involves enzymatic hydrolysis (cholylglycine hydrolase), preparation of propionated methyl ester derivatives of bile acid and gas chromatographic procedure with 2.5% OV-1.

Adequate separation of the individual bile acids, (cholic, chenodeoxycholic and deoxycholic acid) was achieved with vitamine E caprylate as an internal standard. A detector response was linear and recovery of radioactive taurocholic acid and non-radioactive vitamine E caprylate added to the serum was 82.10 ± 6.86 and $80.96 \pm 1.62\%$ respectively. The serum fasting bile acid concentrations of normal controls were 3.17 ± 2.34 for total bile acids, 1.17 ± 1.25 for cholic acid, 1.34 ± 2.11 for chenodeoxycholic acid and 1.29 ± 0.95 μg/ml for deoxycholic acid. The differences in the serum total bile acid levels, magnitude of the increase in the serum concentration between cholic and chenodeoxycholic acid and serum concentration level of deoxycholic acid which were all characterized in various hepatobiliary diseases seemed to be useful for the diagnosis and differential diagnosis of hepatobiliary diseases. However, these serum bile acid concentrations were frequently observed overlapped in some of the individuals among hepatobiliary diseases. An endogenous bile acid tolerance test with 2 μg/kg caerulein injection demonstrated more distinction in serum bile acid levels between normal and chronic active hepatitis and between chronic active hepatitis and liver cirrhosis than indicated by a fasting total bile acid level alone.

Percent chenodeoxycholic acid increased more than any other individual bile acids during the endogenous bile acid tolerance test suggesting the most important role of chenodeoxycholic acid.

INTRODUCTION

Assay of fasting and postprandial serum bile acids is well known to provide an index useful in the diagnosis of the hepatobiliary diseases. La Russo and his coworkers reported that the bile acid tolerance test was more sensitive than other conventional liver function tests[1,2].

The recent studies on the bile acid chemistry developed three types of methodology for measurement of these components, the enzymatic fluorimetric method[3] (3α-hydroxysteroid dehydrogenase) to measure total bile acids, radio-immunoassay[4] to determine a partial component in the several bile acid conjugates, e. g. cholylglycine, and gas-chromatography[5] to determine the individual bile acids.

Sandberg et al.[5] were the first to describe the third method of analyzing serum bile acids using gas chromatography and their studies have stimulated many investigators to develop this procedure. In 1977, Ross et al.[6] established a sensitive and simple method for the analysis of serum bile acids using hydrolyzing enzyme (cholylglycine hydrolase) based on the method of Nair et al.[7] However, the bile acid derivatives by their method were found to stay stable for only several days and best analyzed immediately after trifluoroacetylation.

The method described in this paper provides bile acid derivatives of excellent stability which can be stored for two months at room temperature. It facilitates less labour-intensive assay while retaining the sensitivity and accuracy of the previous gas-chromatographic methods. In this study, using this simple method not only the determination of fasting serum bile acids but also the bile acid tolerance test were performed in patients with chronic liver diseases.

METHODS

I. Subjects:

Twelve healthy subjects and forty-eight patients were used in this investigation. The healthy controls were six men and women without hepatobiliary, metabolic and intestinal diseases chosen from the medical staffs, nurses and laboratory assistants of our medical department. All of them showed normal serum levels of S-GOT, S-GPT, γ-GPT, alkaline phosphatase, albumin and bilirubin by conventional liver function tests.

Forty-eight patients comprised 13 with acute hepatitis (7 in acute stage and 6 in convalescence), 12 with chronic active hepatitis, 12 with liver cirrhosis, 8 with obstructive jaundice (obstruction were all from pancreatic cancer or metastatic cancer from stomach) and 3 with primary biliary cirrhosis. The diagnosis of hepatic diseases was based on clinical symptoms, liver function tests, liver scintigraphy, biopsy and laparotomy in most of the cases. The diagnosis of biliary diseases was made mainly on the basis of various clinical symptoms, biliary enzyme levels, ultrasonography, computed tomography and percutaneous transhepatic cholangiography.

II. Blood samples:

Blood was obtained by venopuncture at fast. Blood from normal controls, chronic active hepatitis and liver cirrhosis was withdrawn at fast, 30, 60 and 120 minutes after administration of 2 μg/kg caerulein (endogenous bile acid tolerance test). Serum was separated by centrifugation at 2,500 r. p. m. for 10 minutes and stored at $-20°C$ until analysis. Concurrently oral cholecystography was undertaken to ascertain the gallbladder contraction.

III. Analysis of serum bile acids:

The analytical procedure of serum bile acids

Table 1. Analytical procedure of serum bile acids by gas liquid chromatography

0. 5-2 ml Serum + 50 μg vitamine E caprylate as an internal standard

$\downarrow$

Deproteination and extraction with appropriate ethanol

$\downarrow$

Hydrolysis with 10 unit cholylglycine hydrolase for 18 hours at 37°C in 2 ml 0. 1 M acetate buffer (pH 5. 6)

$\downarrow$

Extraction with ethyl ether after acidification with 0. 2 ml 3 N-HCl

$\downarrow$

Methylation with excess diazomethane

$\downarrow$

Propionation with 0. 1 ml propionic acid and 0. 1 ml pyridine for 2 hours at 110-120°C

$\downarrow$

Determination by gas-liquid chromatography (Shimadzu GC-6A) equipped with chromatopac-E1A (2. 5% OV-1 glass column)

are summarized in Table 1.

A. Enzyme preparation:

The hydrolyzing enzyme (cholylglycine hydrolase) was purchased from Sigma Co., U.S.A. The acetone powder of the enzyme was dissolved in 30 ml 50% glycerine solution and was kept in a freezer. This procedure was based on the method reported by Ross[6] and Nair et al.[7]

B. Bile acid extraction:

Fifty micrograms of vitamine E caprylate (an internal standard) in an acetone solution was added to 0.5-2 ml of serum. Protein was removed by adding 3 ml of ethanol and centrifuging at 2,500 r.p.m. for 10 minutes. The supernate was kept overnight at 4°C and centrifuged again to remove peptides. The supernate was evaporated to dryness under nitrogen stream.

The dry extract was added with 1 ml methanol : water (1 : 1), mixed with 2 ml 0.1 M acetate buffer (pH 5.6) and 0.2 ml enzyme preparation (10 units) and incubated in a stoppered tube at 37°C for one hour. The medium was acidified with 3N–HCl and hydrolyzed bile acids were extracted three times with ethyl ether.

C. Formation of bile acid derivatives:

The combined extracts were taken to dryness under nitrogen stream and methylated in a diazomethan-ether solution (as revealed by persistent yellow colour).

The solvent was evaporated under nitrogen stream, and methylated bile acids were propionated[8] in propionic anhydride and dehydrated in a pyridine solution at 110–120°C in an oil bath for 2 hours. After an excess solution was evaporated under nitrogen stream, the sample was stored at room temperature or −20°C until analysis by gas chromatography.

D. Gas liquid chromatography:

Analysis of bile acid derivatives was performed on a Shimadzu GC-6A gas chromatograph equipped with dual FID using 2 m × 4 mm glass column packed with 2.5% OV–1 on Shimalite 100-120 mesh.

Peak analyses and calculations were automatically performed by data analyzer. A Shimadzu chromatopac EIA was connected to the amplifier of the gas chromatograph. The temperature of injection port and detector was 300°C and column temperature was 260°C. Vitamine E caprylate was kindly donated by Eisai Pharmaceutical Co., Tokyo.

IV. *Recovery experiment:*

Recovery experiments were undertaken using 24–[14]C–taurocholic acid and non-radioactive vitamine E caprylate added to the human serum samples. Radioactivity of ^{14}C–taurocholic acid and its derivative was determined with an Aloca L.S.C. liquid scintillation spectrometer. Five micrograms of non-radioactive vitamine E caprylate in acetone was directly injected into a gas chromatograph equipped with chromatopac-EIA. The same amount of this compound was also added to the normal serum and after regular preparative procedure for bile acids determination as mentioned above, injected into a gas chromatograph and counted by chromatopac-EIA. The counts obtained after the preparative procedure (found) were divided by the counts after direct injection (added) and the values thus obtained were considered as recovery (expressed by percentage) of vitamine E caprylate[9].

RESULTS

The analysis of three bile acids and vitamine E caprylate showed a linear detector response over the range examined and under the conditions described above.
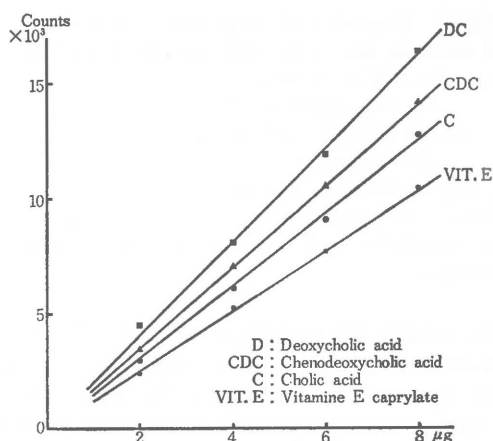

Fig. 1. Detector response of bile acid delivatives and internal standard. Relationship between counts detected by chromatopac–EIA and amounts of bile acids analyzed as propionated methyl esters and vitamine E caprylate on a 2.5% OV–1 column.

The retention times, relative retention times and separation factors were listed in Table 2.

The typical charts of standards and samples

Table 2. Retention data and resolution of authentic bile acids in gas chromatographic analysis

Bile acid	Rt (min)	RRt	Resolution $2_{\triangle}t/(Wb_1+Wb_2)$
Deoxycholic acid	12.00	0.500	
			1.26
Chenodeoxycholic acid	13.54	0.563	
			2.62
Cholic acid	17.24	0.718	
			2.31
Vitamin E Caprylate	24.00	1.000	

Rt   : Retention time
RRt : Relative retention time
$_{\triangle}$t   : Distance between peak maxima
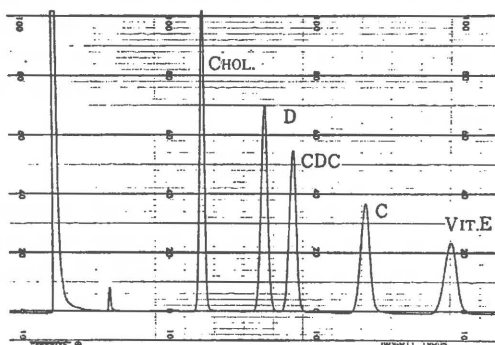Wb_1 and Wb_2 : Base width of peak 1 and 2 respectively

Fig. 2. Separation of propionated methyl esters of authentic bile acids and vitamine E caprylate on 2.5% OV-1 column.
D : Deoxycholic acid
CDC : Chenodeoxycholic acid
C : Cholic acid
Vit. E : Vitamine E caprylate
(Chol. : Free cholesterol, propionated methyl ester of lithocholic acid has the identical retention volume)

from serum are presented in Figs. 2 and 3. Adequate distances between adjacent two peaks were obtained by employing the combination of methyl ester propionate as bile acid derivatives and OV-1 as stationary phase.

Hydrolysis of glycine and taurine-conjugates by cholylglycine hydrolase reached completion under the condition described above, and analysis by thin layer chromatography[6] (developed in 2, 2, 4-trimethylpentane : ethyl acetate : glacial acetic acid; 10 : 10 : 2) proved that there was no conjugated material remaining after hydrolysis of 10 mM standard glyco- and tauro-
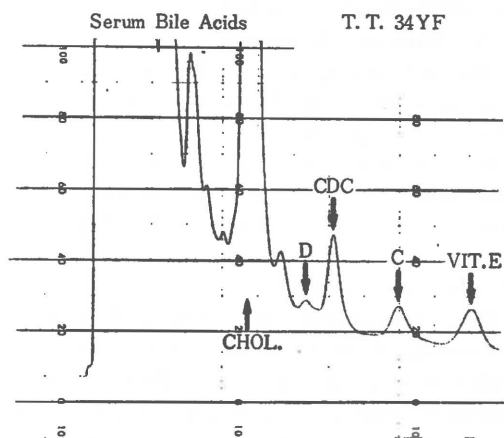
Fig. 3. An example of gas chromatogram showing peaks of deoxycholic (D) chenodeoxycholic (CDC) cholic (C) and vitamine E caprylate (VIT. E)
CHOL: Peak of cholesterol

conjugated cholic and chenodeoxycholic acids. $24-^{14}C$ labelled taurocholic acids were employed for the recovery experiment. The percentage recovery in each step was $90.82\pm2.12\%$ after deproteination, $82.10\pm6.78\%$ after extraction with ether and final percentage recovery of taurocholic acids was $82.10\pm4.08\%$ which was almost identical to that of internal standard, that was, $80.96\pm1.62\%$ as shown in Table 3.

Repeated analysis of bile acid methyl ester propionates kept at room temperature revealed the identical height of peaks after 2 months suggesting that no decomposition of compounds occurred during this period (See Fig. 4)

Figure 5 shows total serum bile acid and Table 4 shows both the fasting total and individual serum bile acid concentrations in health and hepatobiliary diseases. The mean values$\pm$ standard deviation of total bile, cholic, chenodeoxycholic and deoxycholic acids in normal controls were 3.17$\pm$2.43, 1.17$\pm$1.25, 1.34$\pm$2.11 and 1.29$\pm$0.95 μg/ml respectively.

All hepatobiliary diseases examined in this study had elevated fasting serum total bile acid levels. Acute hepatitis showed marked elevation of cholic and chenodeoxycholic acids and moderate elevation of deoxycholic acid in acute stage but in convalescence elevated cholic and chenodeoxycholic acids apparently returned near the normal range with the lowered deoxycholic acid level below normal. High elevation of chenodeoxycholic acid was followed by moder-

Table 3. Results of recovery experiments of ^{14}C taurocholate and non-radioactive vitamine E caprylate added to the human serum samples

Delivatives examined	Stage of procedure	added C. P. M.	found C. P. M.	% recovery	Mean±S. D.
[14]C-taurocholate	After deproteination	103051	96084	93.2%	93.30±1.95%
			96506	93.6%	
			97544	94.7%	
			92785	90.0%	
			97703	94.8%	
	After extraction with ether	〃	83358	80.9%	83.00±3.11%
			85341	82.8%	
			81676	79.3%	
			89530	86.9%	
			87860	85.3%	
	After propionation	〃	85106	82.6%	82.10±4.08%
			78701	76.4%	
			82178	79.7%	
			87808	85.2%	
			88997	86.4%	
Vitamine E caprylate		216867*	172842*	79.1%	80.96±1.62%
		209280*	171818*	82.1%	
		202256*	165243*	81.7%	

* Counts detected by chromatopac E1A connected to gas chromatograph

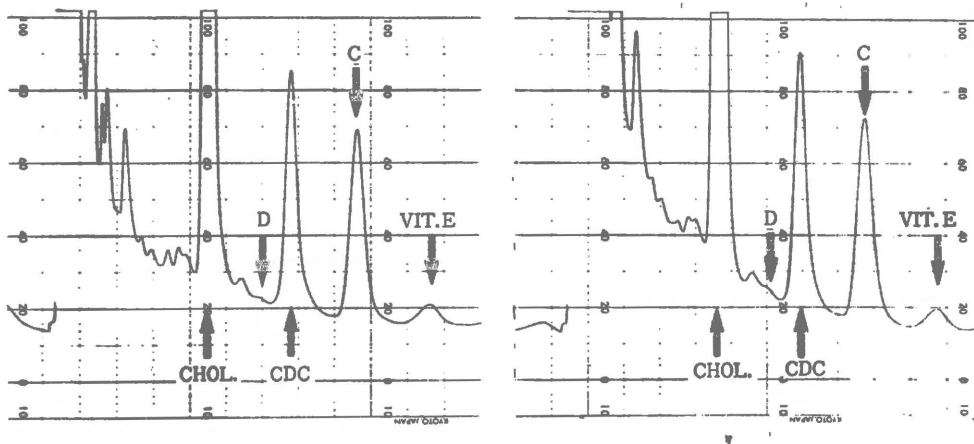## Immediately after preparation   2 months after preparation

Fig. 4. Stability of propionated methyl esters of bile acids and vitamine E caprylate: gas chromatographic charts immediately and two months after preparation of the sample.

CDC : Chenodeoxycholic acid

C : Cholic acid

VIT. E : Vitamine E caprylate

Chol. : Cholesterol

Table 4. Fasting total and individual bile acids in normal and hepatobiliary diseases   (μg/ml)

	No. of cases	Total bile acids	Cholic acid	Chenodeoxy- cholic acid	Deoxycholic acid
Normal controls	12	3.17± 2.43	1.17± 1.25	1.34± 2.11	1.29±0.95
Acute hepatitis acute stage	6	170.92±129.89	87.29±68.29	78.68±61.07	4.95±4.96
Acute hepatitis convalescent stage	7	7.83± 4.78	3.56± 2.43	3.60± 5.18	0.65±0.52
Chronic active hepatitis	12	14.30± 12.65	3.03± 3.00	8.43± 7.73	2.83±3.57
Liver cirrhosis	12	43.59± 28.73	11.97± 8.41	28.70±21.56	3.22 3.92
Obstructive jaundice	8	110.67± 77.81	46.36±43.11	63.90±48.37	0.39±0.85
Primary biliary cirrhosis	3	94.13± 18.23	52.71±12.19	37.85± 6.07	2.68±2.37

(mean±standard deviation)

ate elevation of cholic acid in chronic active hepatitis and liver cirrhosis. Elevation of chenodeoxycholic acid was also higher than that of cholic acid in obstructive jaundice, but as compared with chronic diffuse parenchymal liver damages, obstructive jaundice tended to have a higher cholic acid ratio. Deoxycholic acid was strongly diminished in obstructive jaundice. This bile acid (=deoxycholic acid) did not decrease in primary biliary cirrhosis and a more increased cholic acid ratio was found in primary biliary cirrhosis than in obstructive jaundice.

gallbladder was evaluated by oral cholecystography during the test. Elevation of total bile acids after gallbladder contraction was observed both in health and diseases. Cirrhosis of the liver showed the largest elevation of serum total bile acids which remained high after two hours in many cases.

Fig. 6. Endogenous bile acid tolerance test. Serum bile acid levels at fast and 30, 60, 120 minutes after 2 μg/kg caerulein injection in normal controls, chronic active hepatitis and liver cirrhosis.
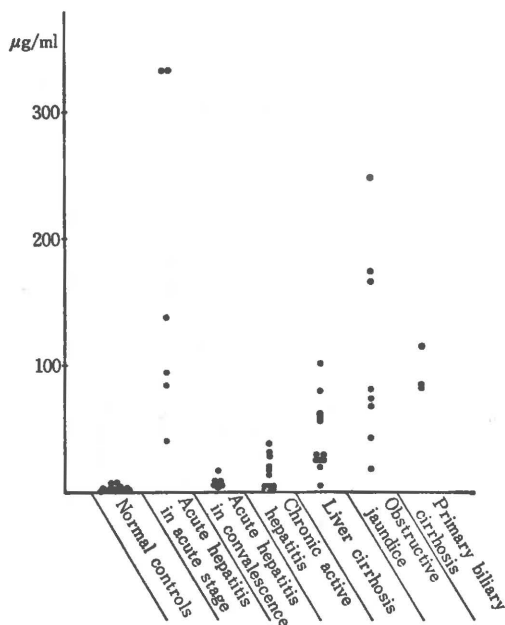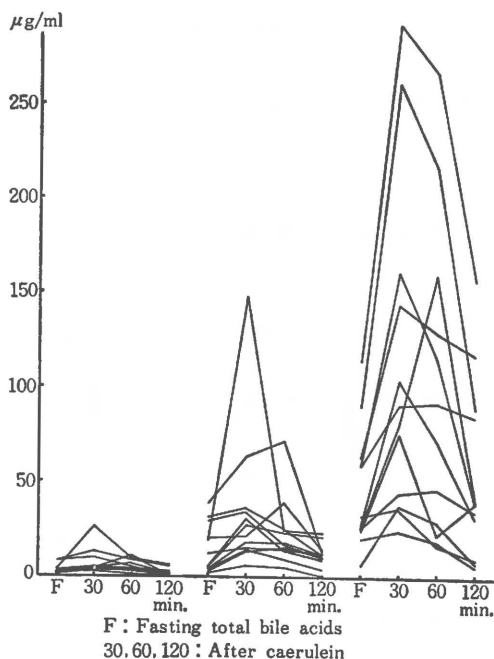

Fig. 5. Fasting Serum Total Bile Acids in Normal Controls and Hepatobiliary Diseases

Figure 6 shows the bile acid tolerance changes in health and patients with chronic active hepatitis and liver cirrhosis. Contraction of

The highest level of total bile acids was observed 30 minutes after the tolerance test in all three groups of health and diseases. Table 5

and Figure 7 summarize the percent bile acid compositions at fast and 30 minutes after the tolerance test in three groups. Percent deoxycholic acid decreased and percent chenodeoxycholic acid increased in all three groups.

Table 5. Percent composition of individual bile acid at fast and 30 minutes after endogenous bile acid tolerance test by 2 μg/kg caerulein injection

(%)

		Normal Control	Chronic Active Hepatitis	Liver Cirrhosis
At fast	D	37.30±31.24	20.60±17.79	10.29±12.07
	C D C	28.41±34.39	58.86±24.06	61.12±20.83
	C	34.39±29.12	20.55±15.33	28.59±13.50
30 min. after	D	30.86±19.44	10.04± 9.11	5.31± 7.43
	C D C	39.01±34.94	67.37± 5.71	64.95±16.65
	C	30.14±26.72	22.55±10.33	29.73±11.62

D : Deoxycholic acid
C D C : Chenodeoxycholic acid
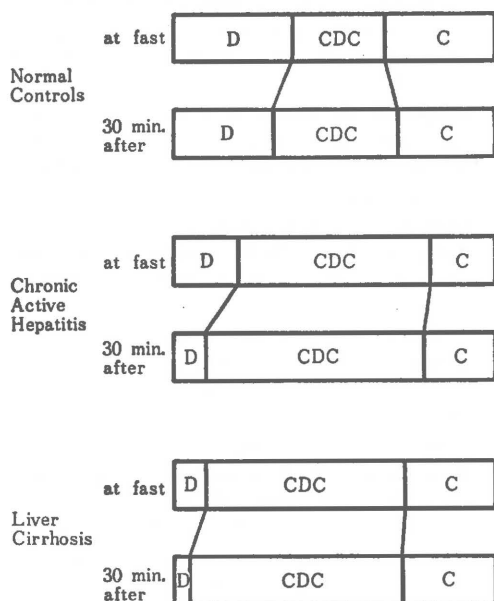C : Cholic acid
(mean±standard deviation)

Fig. 7. Percent bile acid composition at fast and 30 minutes after tolerance test in normal controls, chronic active hepatitis and liver cirrhosis.

DISCUSSION

The present procedure permits relatively rapid and simple assay of individual serum bile acids.

The separation of peaks was excellent as shown in Table 2 and Fig. 2.

7-Ketolithocholic acid[6], 7-Ketodeoxycholic acid[10] and 23-nor-deoxycholic acid[9] have been frequently used as an internal standard. Vitamine E caprylate is not bile acid but is very stable and extracted with organic solvents used in this procedure as well as bile acids and their derivatives. When this internal standard was added to plasma, the percentage recovery was 80.96±1.62%. Losses could be caused by incomplete extraction, remaining in the glass ware and unavoidable scattering during evaporation. This compound also has an appropriate retention time as shown in Table 2 and Fig. 2.

Overall recovery of 24-^{14}C labelled bile acid was similar to those reported by other workers[5] and 82.10±4.08% at the stage after propionation. This recovery was almost equal to that of vitamine E caprylate, which convinced the authors to decide employing this compound as an internal standard. The average serum bile acid concentration from 12 normal subjects were 3.17±2.43 for total bile acids, 1.17±1.25 for cholic acid, 1.34±2.11 for chenodeoxycholic acid and 1.29±0.95 μg/ml for deoxycholic acid. These mean total and individual bile acid concentrations seem to be rather higher as compared with those assayed by gas liquid chromatography reported earlier[5,6,9] and to correspond to those obtained by enzyme method reported by Schwarz et al.[12]

Numerous bile acid derivatives and stationary phases have been used[11], in which the combination of trifluoroacetate and OV-210 was common in the recent studies. But trifluoroacetate derivatives were reported to be able to stay stable only for several days when stored without moisture at −20°C[6] and acetates were found to have a non-linear detector response of these derivatives[9].

In the present experiment the combination of propionated derivatives and 2.5% OV-1 was employed. These derivatives from serum samples proved stable at least for two months at room temperature. Repeated analyses of propionates of several pure bile acids showed identical peaks after 6 months when stored at −20°C.

In addition the propionated methyl esters had a satisfactory linear detector response as shown in Fig. 1. The increase in bile acids was dra-

matic in hepatobiliary diseases. Therefore, very small amount of serum was sufficient for the analysis by gas liquid chromatography. But the amount of serum usually used by the authors was 0.5 ml at least, although bile acids can be detected from 0.05 ml serum in some of hepatobiliary diseases with severe bilirubinemia.

These increased bile acids came from increases of cholic and chenodeoxycholic acids. But serum deoxycholic acid level often remained normal in these diseases.

Deoxycholic acid was not detected in most cases of obstructive jaundice, although other two bile acids increased predominantly. The disappearance of serum deoxycholic acid is probably caused by the interrupted enterohepatic circulation of bile acids and disturbed exposure of bile acids to intestinal bacteria. Thus, the changes not only in primary bile acids, i.e., cholic and chenodeoxycholic acids, but also in one of secondary bile acids, deoxycholic acid, seem to contribute to the diagnosis or differential diagnosis of the hepatobiliary diseases.

As shown in Table 4, the increase in serum total bile acids was moderate in chronic active hepatitis and predominant in liver cirrhosis on the average. However, in half of the patients with chronic hepatitis the total bile acid levels were within the normal range and more than half of liver cirrhosis had the same total bile acid levels as chronic active hepatitis did.

The increase in serum endogenous bile acids after gallbladder contraction has been applied to the bile acid tolerance test[13-15]. The endogenous bile acid tolerance test in the present study demonstrated more significant differences between normal controls and chronic active hepatitis and between chronic hepatitis and liver cirrhosis as compared with fasting total bile acid levels alone as shown in Fig. 6.

The combination of caerulein (2 μg/ml) and oral cholecystography was advantageous for the rapid contraction of gallbladder and its visual confirmation.

Figure 7 shows the percent bile acid compositions at fast and 30 minutes after tolerance test in normal controls, chonic active hepatitis and liver cirrhosis. Percent chenodeoxycholic acid increased after 30 minutes in all the 3 groups, indicating that chenodeoxycholic acid played the most important role in elevation of serum total bile acids during the tolerance test and that

the determination of this bile acid is most useful in the tolerance test for chronic parenchymal liver damages when one of the individual bile acids is chosen for the method due to radio- or enzyme immunoassay.

The present method did not permit determination of lithocholic and ursodeoxycholic acids.

Lithocholic acid can be detected in this procedure when cholesterol is removed using an appropriate solvent prior to the derivative formation. Ursodeoxycholic acid could not be separated from cholic acid on the 2.5% OV-1 column. However, these bile acids were detected in a very small amount and, therefore, negligible in human serum unless exogenous administration is performed.

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