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DETERMINATION OF SERUM BILE ACIDS AND ITS USE AS A HEPATIC FUNCTION TEST

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Abstract

Serum bile acid of 26 normal subjects and 61 patients with hepatobiliary disorders was determined by Murphy's enzymatic-fluorimetric method which was improved by the authors. The fasting serum bile acid concentration was below 1 μg/ml in normal subjects. It was elevated in about 90% of patients.

Observation of serum bile acid in a patient with acute hepatitis throughout the whole course of illness suggested that the determination of serum bile acid is a valuable measure for pursuing the clinical course of this disease.

Variations of the serum bile acid level after taking meal (including two boiled eggs) were examined on ten normal subiects and 14 patients with hepatic parenchymal damages. The postprandial concentration rose abnormally high in all of the patients, whereas no rise was seen in normal subjects. The degree of postprandial rise was parallel to the abnormality of ICG test, (with a correlation coefficient $\gamma = +0{,}90$). It is worth while to special mention that the postprandial serum bile acid concentration was abnormally high even in some of the patients who showed normal ICG tests. It is therefore said that the serum bile acid will be a sensitive indicator of hepatobiliary disorders.

INTRODUCTION

It was recently reported by Kaplowitz and his associates[1] that determination of bile acids in blood was a sensitive test for diagnosis of hepatobiliary diseases, being particularly useful for observation of their clinical course. However, the procedures hitherto introduced for the determination of bile acids employ extraction with organic solvents and chromatographies (including gas, thin layer and paper) in a complicated combination which demands specialized skill. Moreover, they require a

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large amount of blood (not less than 10 ml) for the starting specimen. These difficulties are the obstacle to the use of the blood bile acid estimation as a routine hepatic examination in laboratories of ordinary level. Four years ago, Murphy et al.[2] developed a n enzymatic-fluorimetric method for determination of bile acids which employs 3α-hydroxysteroid dehydrogenase extracted from *Pseudomonas testosteroni*. This enabled us bile acid estimation with a relatively small amount of blood by a simpler manipulation. Lately we have had an offer of the samples of 3α-hydroxysteroid dehydrogenase from Nyegaard (Oslo, Norway), and have successfully modified Murphy's procedure with this enzyme specimen so that the necessary blood may be further diminished in volume. We determined bile acids in the blood samples obtained from the patients with various hepatobiliary diseases in order to compare the fasting and the postprandial levels. Routine hepatic tests were run simultaneously. Scrutiny of the results indicated that the postprandial rise of serum bile acid level was one of the most sensitive indicator of hepatobiliary disturbance. This paper aims to present the results obtained in our investigation.

MATERIALS AND METHODS

Materials: The materials consisted of 26 normal subjects (namely, 10 males and 16 females), 16 patients with acute hepatitis, 26 with chronic hepatitis, 9 with liver cirrhosis, and 10 with biliary tract disorders.

Collection of blood samples: Blood samples (2-3 ml) were collected from all the patients after they had been fasted for 12 hours. Some of them (10 normal subjects and 14 patients with hepatic parenchymal damages) were asked to eat two boiled eggs at breakfast, and their blood samples were again collected two hours later. A patient with acute hepatitis (48 year old woman who gave positive test to Australia antigen), was pursued by repeated blood bile acid estimations in a sequence of several day intervals from the time of her hospitalization.

Bile acid determination: 1.0 ml of serum separated from each blood sample was assayed for bile acids by the enzymatic-fluorimetric method using 3α-hydroxysteroid dehydrogenase which is described below.

A. *Reagents*

(1) Enzyme solution: With 1 unit of freeze-dried 3α-hydroxysteroid dehydrogenase (1 unit is equivalent to the activity which oxidizes 1 μM of 3α-hydroxysteroid) are mixed 0.5 M of NAD, 10^{-3} M of EDTA and 10^{-3} μM of cleland and kept in a vial. The content of the vial is dissolved in 2.9 ml of aqueous hydrazine hydrate (0.1M) solution. This is called the enzyme solution.

(2) 0.1M hydrazine hydrate (aqueous) solution: Ensure that the solution has a pH of 9.5.

(3) 50% ethanol: Ethanol (guaranteed grade) is diluted with distilled water in 1:1 (volume), and the pH of the diluent adjusted to 9.5-10.5 by addition of a minute amount of 0.1N NaOH.

(4) Ether: n-heptane (1:1) mixture.

(5) Pure ethanol (guaranteed grade).

(6) Standard solution: 100 mg of the sodium salt of glycocholic acid is dissolved in a sufficient volume of 50% ethanol solution and then made to 100 ml. This solution is used as a stock solution (1000 μg/ml). Every time on use, bile acid solutions of 100 μg/ml, 50 μg/ml, 10 μg/ml and 1 μg/ml are prepared by diluting the stock solution with 50% ethanol.

B. *Procedure*

(1) Extraction of bile acids: (a) Transfer 5 ml of pure ethanol into a centrifuge tube, and boil in a boiling water bath. Add 1.0 ml of serum to the ethanol, and continue to heat for five minutes with constant stirring, centrifuge to separate supernatant fluid from precipitate, and collect the supernate.

(b) Pour 5 ml of boiling ethanol over the precipitate, heat in the boiling water bath for five minutes, centrifuge, and combine the supernate with the supernate obtained in (a).

(c) In the same way as in (b), add 5 ml of boiling ethanol again over the precipitate, stirr up and centrifuge. Combine the supernate with the supernate got in (a) and (b).

(2) Concentration and dissolution of the supernate: Evaporate the whole amount of the supernates obtained in (1) under reduced pressure to dryness in a rotary evaporator (this procedure can be completed in about ten minutes), and dissolve the residue in 5 ml of 50% ethanol.

(3) Isolation and purification of bile acids: Add 15 ml of the ether: heptane (1:1) mixture to the solution prepared in (2), shake in a shaker for 15 minutes, centrifuge (at 2000 r.p.m. for five minutes), and remove the supernate with an aspirator.

(4) Preparation of a solution of bile acid extract: Evaporate the bottom layer obtained in (3) in vacuum to dryness, and dissolve the residue in 0.5 ml of 50% ethanol.

(5) Enzymatic reaction: Transfer 0.1 ml of the solution of bile acid extract gained in (4), into each one of two Test Tubes S and B. Add 2.9 ml of the enzyme solution to Test Tube S (serum sample), and 2.9 ml of 0.1M hydrazine hydrate solution to Test Tube B (serum blank). Transfer 0.1 ml of 50% ethanol to Test Tube C_0 (reagent blank), and 0.1

ml of the 50 μg/ml solution of bile acids (standard solution) to the test Tube C_1, and then add 2.9 ml of the enzyme solution to each one of these two. Allow all of these four test tubes to stand at room temperature (18 to 22°C) for 30 minutes.

(6) Fluorimetry: In a fluorimeter (the Hitachi model MPF 2A), adjusting emission light to 460 nm, and fluorescence light to 348 nm, read the intensity of fluorescence of the solution of the Test Tubes B, S, C_0 and C_1, namely b, s, c_0 and c_1 in a cuvette (optical path 0.5 cm).

(7) Calculation: The concentration of bile acids in serum is calculated by the following equation.

$$\text{Concentration of bile acid in serum } (\mu g/ml) = \frac{S-(C_0+b)}{C_1-C_0} \times \frac{50}{2}$$

C. *Routine hepatic tests*

In parallel with the determination of bile acids in blood, biochemical hepatic tests such as serum alkaline phosphatase (Alk P), serum leucine aminopeptidase (LAP), serum transaminases (S-GOT and S-GPT), serum cholinesterase (ChE), icteric index (serum bilirubin), A/G ratio (as well as cellulose acetate membrane electrophoresis of serum proteins), and indocyanine green excretion test (ICG) were performed in the conventional way.

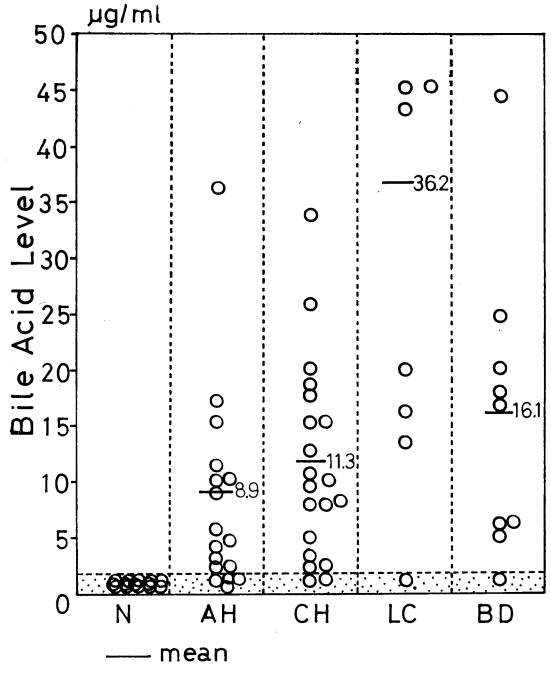

Fig. 1. Fasting serum bile acid level in normal subjects and patients with various hepatobiliary disorders. N: Normal subjects, AH: Acute hepatitis, CH: Chronic hepatitis, LC: Liver cirrhosis, BD: Biliary obstruction.

RESULTS

The fasting serum bile acid concentration was below 1.0 μg/ml in both males and females of the 26 normal subjects. The fasting serum bile acid levels of the patients with acute hepatitis, chronic hepatitis, liver cirrhosis and biliary obstruction are presented in Fig. 1. It is apparent from this figure that the mean fasting serum bile acid concentration ascended higher from the normal range to the levels of acute hepatitis, chronic hepatitis, biliary tract disease and liver cirrhosis in the order listed. However, there was a large overlapping between the disease groups, so that it was difficult to diagnose the diseases of individual cases in terms of the bile acid concentration. Abnormal serum bile

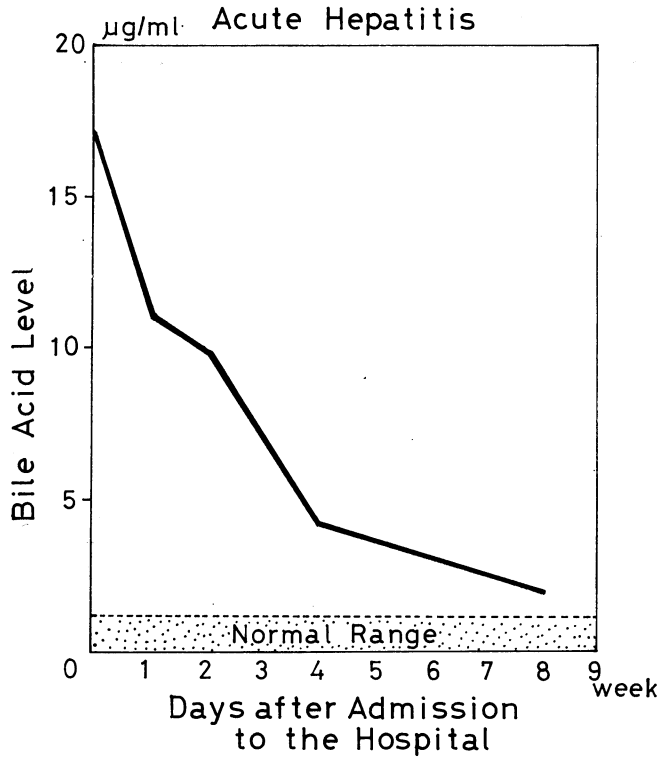

Fig. 2. Viscitude of the serum bile acid level with lapse of time in a patient with acute hepatitis who took a beneficial course. On the first day of her admission to the hospital her hepatic tests were (serum protein 7.5 g/dl, A/G 0.79, LAP 67 u/dl, alk. phosphatase 27 u/dl, serum bilirubin 6.5 mg/dl, S-GOT 41 Mizobe u., S-GOT 63 Mizobe u., cholinesterase 0.55⊿pH, γ-Glb 24.6% and positive Au-antigen), while on the 6th hospitalization week they became (SP 8 g/dl, A/G 0.82, LAP 55 u/dl, alk. phos. 14 u/dl, bilirubin 1.2 mg/dl, S-GOT 10u., GPT 7u., ChE 0.64 ⊿pH and γ-Glb 26.6%).

acid values were noted in 90% of all patients with hepatobiliary disorders. The bile acid concentration in blood serum was followed up on a female patient (aged 48) of acute hepatitis (Au-antigen positive) who cured uneventfully after the onset of the disease. As illustrated in Fig. 2, the bile acid level descended straight in accordance with the amelioration of symptoms and signs, although it still remained in a slightly supernormal range even after all of the other hepatic function tests had been restored normal.

The serum bile acid concentrations in fasting state and two hours after breakfast with two boiled eggs were compared in 10 normal subjects. They were within the range of 0 to 1.0 μg/ml before breakfast and 0.5 to 1.4 μg/ml after taking two boiled eggs. In all of the 14 patients with hepatic parenchymal damages, the postprandial concentration rose by at least 3.5 μg/ml over the fasting level (Fig. 3). It deserves special

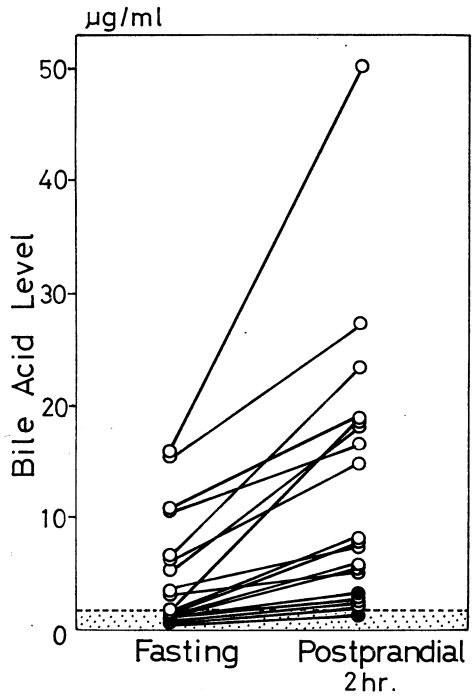

Fig. 3. Comparison of postprandial serum bile acid levels with fasting serum bile acid concentrations in 10 normal subjects (●) and 14 patients with hepatobiliary disorders (○).

mention that the postprandial bile acid levels ascended distinctly high from the fasting levels which had been entirely normal in three of the

patients of acute hepatitis in convalescent stage. Their hepatic function tests except the postprandial serum bile acid were normal. Table 1 shows the postprandial rise of serum bile acid level in comparison with the results of various hepatic tests carried out on these patients simultane-

Table I. Laboratory data

Name	diagnosis	bile acid (μg/ml)		Alk.p. (u)	LAP (u)	LDH_5 (isozyme)	GPT (u)	GOT* (u)	ΔpH Cholin est.	A/G	γ-globulin (%)	ICG (%)
		preprandial	postprandial									
H.S.	AH	<0.5	5.0	13	40	-	27	15	0.74	1.28	18.9	1.0
I.T.	AH	6.0	13.5	13	41	+	17	17	0.75	0.91	25.3	25.2
M.H.	AH	<0.5	4.5	8	50	-	14	11	1.03	1.35	10.3	11.5
D.H.	AH	1.0	6.0	6	35	-	7	6	0.45	1.46	17.0	2.5
Y.H.	AH	10.0	17.0	19	52	+	71	65	0.46	0.92	17.8	25.5
Y.H.	AH	10.0	15.5	17	72	+	77	87	0.69	1.05	17.6	25.5
B.S.	AH	2.3	3.5	8	35	+	30	16	0.75	1.28	14.0	7.0
O.H.	AH	3.0	4.7	5	31	+	6	8	0.53	1.30	24.3	13.0
T.T.	CH	6.0	22.5	20	38	+	7	20	0.28	0.64	20.5	32.6
W.K.	CH	<0.5	17.0	10	34	+	8	10	0.53	0.96	21.8	29.2
D.S.	CH	4.2	15.0	10	50	+	33	41	0.73	0.87	23.0	28.5
S.Y.	CH	15.0	50.0	13	35	+	4	7	0.34	1.14	17.7	52.0
I.N.	CH	0.5	6.5	20	46	-	8	10	0.32	1.02	20.8	14.0
T.O.	LC	13.5	24.0	20	48	+	7	13	0.32	0.63	31.6	57.0
Detection rate of abnormality (%)		71	100	35.6	35.6	71.7	50	50	72	50	36	79
Correlation coefficient (to fasting bile acid level)				0.69	0.24		0.17					0.83
Correlation coefficient (to post-prandial bile acid level)				0.38	0.13		0.16					0.90

AH : acute hepatitis, CH : chronic hepatitis, L : liver cirrhosis. Normal values for GOT and GPT are 1-15 and 1-10 Mizobe units. One Mizobe unit is equal to 1/2.23 international unit for GOT and to 1/2.54 international unit for GPT.

ously. It will be seen from this table that there is a close parellelism between the serum bile acid level and the abnormality of indocyanine green test (coefficients of correlation were $\gamma = +0.83$ and $\gamma = +0.90$ for the fasting bile acid-ICG and the postprandial-ICG, respectively).

DISCUSSION

Because bile acids are primarily the ingredients of bile, investigations were made at first on the assay of bile acid in bile. The bile acid level in blood is incomparably lower than that in bile, so that it was impossible to apply the method invented for the determination of bile acids in bile to estimation of the blood bile acids. On account of the technical difficulty to create a method of high sensitivity, the assay of blood bile acids has been left unsolved for a long time. It was only ten or several years ago that extration of serum with organic solvents was combined with the use of countercurrent distribution and paper chromatography, to isolate serum bile acids, and sensitivity of estimation was significantly raised by addition of sulfuric acid to the purified extract for fluorescence photometry.[3] This method enabled the investigators to surmise the bile acid level in blood for the first time. However, this method was not so suitable for routine laboratory use.

Frosch et al.[4] in 1967 employed thin layer chromatograpy, and obtained the result that the determination of bile acids would be of value for observation of acute hepatitis. They were successful in estimating blood bile acids to the level as low as 0.5 μg/ml. At length, in 1973, gas chromatography was introduced to bile acid determination by Kaplowitz and his associates.[1] By this technique they discovered diurnal variation of bile acid level in blood for the first time, and compared the fasting and the postprandial bile acid concentrations in blood. In their experiment the fasting serum bile acid concentration remained in normal range in only 25% of patients with liver disease, and in none of them the postprandial concentration was normal.[5] Unfortunately Kaplowitz's procedure is not so simple as useful for routine tests in the laboratories of ordinary level.

In 1964, Iwata and Yamasaki[6] invented a new method in which the extract of serum with organic solvents was treated with 3α-hydroxysteroid and NAD, and the increase in ultraviolet absorbance (at 340 nm) of the NADH produced by this treatment was measured spectrophotometrically. The sensitivity of this method was markedly elevated six years later (1970) when Murphy[2] employed fluorimetry for the quantita-

tion of NADH instead of spectrophotometry. However, relatively large amount of blood (10 ml) or blood serum (5 to 3 ml) was still required as starting specimen for the analysis. This made a hindrance to the adoption of Murphy's method as a routine test. Our method is an improvement of Murphy's which finishes total procedure of bile acid estimation within 1.5 hours with a specimen of only 1 ml of serum.

The principle of our method is as follows:—To boiling ethanol in a centrifuge tube is added the serum sample to coagulate proteins. They are precipitated by centrifugation to separate the supernate into which all the bile acids contained in the serum are passed (Frosch 1967[4]). The supernatant is evaporated to dryness; the residue is dissolved in a suitable volume of 50% ethanol. Ether: heptane mixture is added to the solution, shaken, and then centrifuged to separate into two layers so as to allow the bile acids move into the bottom layer; the upper layer is discarded and evaporated to dryness; the residue is dissolved in 50% ethanol, to prepare the solution of bile acids extracted from serum.

To this solution of serum bile acids is added a reagent containing 3α-hydroxysteroid dehydrogenase, NAD and hydrazine hydrate. 3α-hydroxysteroid dehydrogenase converts the bile acids into the steroid ketone bodies in the presence of NAD through the following reaction: steroid alcohol (bile acids) + NAD$\rightleftarrows$steroid ketone bodies + NADH + H^+, and at the same time, NAD is reduced to NADH, which emits intense fluorescence. The steroid ketone bodies so produced are caught by the hydrazine hydrate and the reaction progresses toward the rightward direction, thus producing NADH which is equivalent in amount to bile acids.

Accordingly, if the fluorescence of the enzyme-treated solution is subjected to fluorimetry, the bile acid level in serum is estimated by comparing the intensity of fluorescence with that of the calibration curve constructed with the bile acid solutions of known concentrations.

The precautions which should be taken for the successful performance of our procedure are listed in the following.

1. *Purity of 3α-hydroxysteroid dehydrogenase:*[7,8]—This enzyme is extracted from *Pseudomonas testosteroni*, However, the extract usually contains 3α, and 17α-hydroxysteroid dehydrogenases as well as 12α-hydroxysteroid dehydrogenases as contaminant. Therefore, if enzyme preparation of insufficient purity is used, NADH is produced through the dehydrogenation of 17α-hydroxyl group and 12α-hydroxyl groug of bile acids by these contaminant enzymes intermixed with the one pro-

duced by 3α-hydroxysteroid dehydrogenase proper, thus resulting in unreasonably higher reading of bile acids.

The enzyme supplied by Nyegaard & Co. (Oslo, Norway) proved to be of the best quality without any contamination.

2. *Optimal temperature and time for enzymatic reaction*:—As shown in Fig. 4, the maximum intensity of fluorescence of NADH was attained in

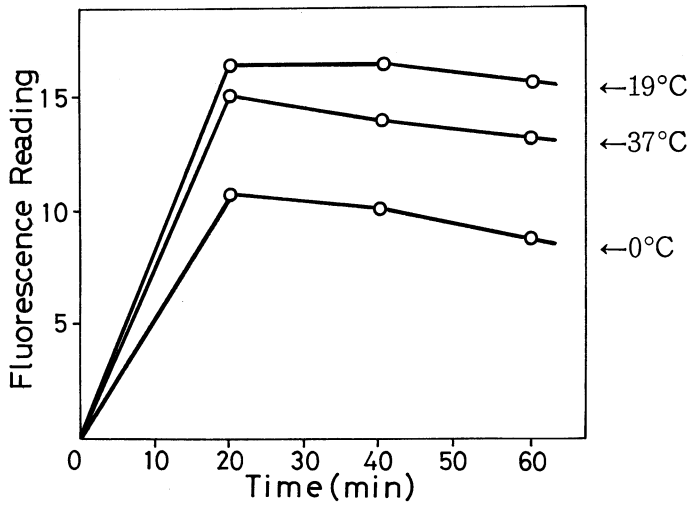

Fig. 4. Optimal temperature and time for enzymatic (3α-hydroxysteroid dehydrogenase) reaction as assessed by the intensity of fluorescence emitted by NADH produced (20 μg/ml glycocholic acid solution).

15 minutes when the enzyme was allowed to react at room temperature (19°C), and the intensity remained stable for at least 60 minutes. It was described by Iwata[6], Murphy[2] and Schwarz[9] that the optimal temperature for the enzymatic reaction was 37°C, and the maximum intensity of fluorescence was attained in 40 minutes, but it appears that the enzymes used by them were different from the one used by us, and this difference accounts for such dyscrepancy between them and us.

3. *Recovery rate*:—The rate of recovery of bile acids in the assay of serum bile acids was usually unsatisfactory. The rate was about 75% by Sandberg's gas chromatography,[10] and below 90% by Panveliwalla's thin layer-chromatography-sulfuric acid fluorimetry.[11] The rate of recovery was 82% by Iwata's enzymatic-spectrometric determination,[6] and about 85% by Murphy's enzymatic-fluorimetric determination.[2] In our

method a perfect recovery ranging from 98 to 101% was obtained, despite we employed the same extraction process as Murphy's.[2] This is attributable to the excellent quality of Nyegaard's 3α-hydroxysteroid dehydrogenase which is free from 12α-hydroxy-and 17α-hydroxysteroid dehydrogenases. The intensity of fluorescence (calibration curve) increased linearly in parellel with the bile acid concentration over the range between 1 μg/ml and 100 μg/ml (Fig. 5), but it remained the same as that in the 0 μg/ml so long as the bile acid concentration was below 1.0 μg/ml.

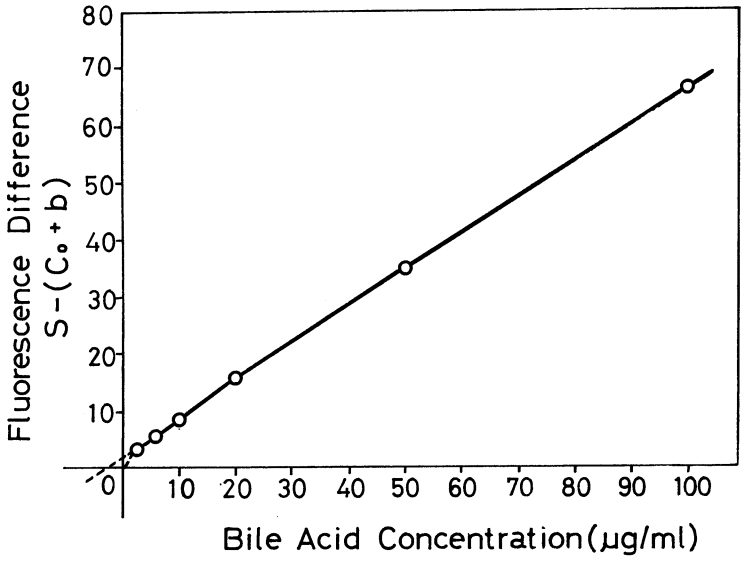

Fig. 5. The calibration curve of the present method.

This indicates that the maximum sensitivity of the method corresponds to 1 μg/ml of serum bile acids. The rate of increase in the fluorescence slowed down in the range over 200 μg/ml. The sensitivity of our method is, therefore, of the same order as that of Murphy's method; namely, 0.5 μg/ml (1.0 μM/l).

It may, however, be said that our method is more convenient to routine laboratory use than Murphy's method,[2] because only 1/3 as small amount of sample as that of Murphy's method is sufficient for our method.

4. *Precision*:—The coefficient of variation for Murphy's method[2] is 4.2%. In our method, it is within the range of 3.6 to 5.8%, being of the same precision as in Murphy's.

The normal values of fasting serum bile acid concentration estimated by our method was in a very narrow range between 0 and 1.0 μg/ml, being consistent with the normal values determined by gas chromatography by Kaplowitz and his associates.[1] This was, however, abnormal in 90% of patients with overt hepatobilliary disorders. This abnormality was, therefore, equally excellent in detection rate to the most sensitive liver function tests which are in use at present. However, serum bile acid is occasionally normal in convalescence of acute hepatitis. So, the true rate of detection may be somewhat lower, if convalescent patients are taken into consideration. Figure 2 illustrates how serum bile acid descended from a high level to the normal level in a straight way in a case of acute hepatitis who took a beneficial course, recovering health 8 weeks after onset of the disease. Frosch et al.[4] who followed up the serum bile acid level in patients with acute hepatitis by thin layer chromatography reported a similar result. It may, therefore, be said that the determination of the bile acid concentration in blood is a useful measure for observing the course of this disease. Comparison of the fasting serum bile acid concentration with the postprandial ones in 15 patients with hepatic parenchymal damages which is illustrated in Fig. 3, indicates that the postprandial bile acid levels are abnormal or increased significantly in all the patients. It is worthy of particular mentioning that the serum bile acid concentration revealed their abnormality in postprandial state even in the cases who had had normal fasting serum bile acid level. None of the hepatic function tests belonging to blood biochemical analysis were in good correlation with the serum bile acid concentration.

Osborn et al.[5] showed serum bile acid concentration to be closely correlated with serum bilirubin. Since in our study only a few jaundiced patients were available, we are not qualified to discuss about it. However, it is evident that serum bile acid concentration varies in a close parallelism to the ICG retention test ($\gamma = +0.90$). If it is granted that the ICG retention test is regared to be one of the most sensitive hepatic test currently in use, it may be said that the determination of serum bile acid concentration is equally sensitive. Furthermore, it is seen from Table 1 that the serum postprandial bile acid is increased even in some of the patients with normal ICG test. This may connote serum bile acid to be even more sensitive than the ICG test.

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