Esterified and total 7α-hydroxycholesterol in human serum as an indicator for hepatic bile acid synthesis

Hitoshi Oda, Hiroyuki Yamashita, Keigo Kosahara, Syoji Kuroki, and Fumio Nakayama

Department of Surgery I, Kyushu University Faculty of Medicine, 3-1-1 Maidashi, Fukuoka 812, Japan

Abstract Serum levels of 7α-hydroxycholesterol and activities of hepatic microsomal cholesterol 7α-hydroxylase in surgical patients were analyzed by capillary gas–liquid chromatography–selected ion monitoring technique using a new internal standard, 5α-cholestane-3β,7β-diol. We found that concentrations of 7α-hydroxycholesterol obtained after alkaline hydrolysis were higher than those without alkaline hydrolysis, indicating that a considerable amount of 7α-hydroxycholesterol in human serum is present in esterified form. Esterified 7α-hydroxycholesterol could also be quantitatively hydrolyzed with cholesterol esterase, suggesting that fatty acid is bound at the 3β-position of the cholestenediol. The serum levels of esterified and free 7α-hydroxycholesterol in patients with cholelithiasis were 198.0 ± 90.3 and 48.3 ± 19.8 pmol/ml (mean ± SD), respectively, and were similar to those in patients without hepatobiliary diseases. After treatment with chenodeoxycholic acid (300 mg per day) for 7 to 10 days, esterified and free 7α-hydroxycholesterol levels decreased to 64.9 ± 33.6 and 20.5 ± 11.1 pmol/ml, respectively. Activity of cholesterol 7α-hydroxylase was also inhibited. Treatment with ursodeoxycholic acid (600 mg per day) for 7 to 10 days had no inhibitory effect on serum 7α-hydroxycholesterol levels and the enzyme activity. In all groups, high correlations were found between the activity of cholesterol 7α-hydroxylase and serum levels of 7α-hydroxycholesterol: free (r = 0.71, n = 38, $P<0.001$); esterified (r = 0.87, n = 38, $P<0.001$); total (r = 0.87, n = 38, $P<0.001$). Esterified and total 7α-hydroxycholesterol was more highly correlated with the enzyme activity than the free form. We conclude that a significant amount of 3β-acyl esters of 7α-hydroxycholesterol is present in human serum and that serum levels of esterified and/or total 7α-hydroxycholesterol are likely to reflect the activity of hepatic cholesterol 7α-hydroxylase and thus the amount of primary bile acids synthesized in the liver. —**Oda, H., H. Yamashita, K. Kosahara, S. Kuroki, and F. Nakayama.** Esterified and total 7α-hydroxycholesterol in human serum as an indicator for hepatic bile acid synthesis. *J. Lipid Res.* 1990. **31:** 2209–2218.

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The first and rate-limiting step in the major pathway for conversion of cholesterol to primary bile acids, i.e., cholic acid (CA) and chenodeoxycholic acid (CDCA), is catalyzed by the hepatic microsomal cholesterol 7α-hydroxylase (1). In humans, as well as in experimental animals, it has been shown that interruption of the enterohepatic circulation of bile acids by external biliary drainage or by treatment with cholestyramine increases enzyme activity and bile acid synthesis, while administration of CA and CDCA decreases both enzyme activity and bile acid biosynthesis (2–5). It is thus assumed that the enzyme activity is regulated by the concentration of bile acids returning to the liver via a negative feedback mechanism and that the activity of hepatic cholesterol 7α-hydroxylase should reflect the amount of bile acids synthesized in the liver. However, measurement of the activity of cholesterol 7α-hydroxylase has not been widely used because it necessitates surgical liver biopsy. Einarsson et al. (6) and Björkhem et al. (7) investigated serum free 7α-hydroxycholesterol concentration and microsomal cholesterol 7α-hydroxylase activity in patients with cholelithiasis using isotope dilution–mass spectrometry. They found that serum 7α-hydroxycholesterol level correlated with cholesterol 7α-hydroxylase activity only when the enzyme activity was increased by prior administration of cholestyramine (7).

Recently, we have independently developed a highly sensitive, accurate, and reproducible method of determining the activity of hepatic microsomal cholesterol 7α-hydroxylase (8). This method uses 5α-cholestane-3β,7β-diol as a new internal standard, Bond-Elut silica cartridge columns for more convenient and efficient clean-up procedure, and capillary gas–liquid chromatography–selected ion monitoring (GLC–SIM) (8). The method is also applicable to the analysis of serum 7α-hydroxycholesterol levels in both free and esterified forms. In a preliminary study, we found not only free 7α-hydroxycholesterol but also a considerable amount of esterified 7α-hydroxycholesterol was present in human serum, although Björkhem et al. (7) was only concerned with free 7α-hydroxycholesterol.

Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid; GLC–SIM, gas–liquid chromatography–selected ion monitoring; TLC, thin-layer chromatography; TMS, trimethylsilyl; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

In the present study, we confirm the presence of esterified 7α-hydroxycholesterol in human serum and report the correlation between the activity of hepatic microsomal cholesterol 7α-hydroxylase and serum concentrations of free, esterified, and total 7α-hydroxycholesterol in surgical patients. The effect of oral administration of either CDCA or ursodeoxycholic acid (UDCA) on the enzyme activity and serum 7α-hydroxycholesterol levels was also studied.

MATERIALS AND METHODS

Chemicals

7α-Hydroxycholesterol, 7β-hydroxycholesterol, and 5α-cholestane-3β,7β-diol were synthesized as described previously (8). TMSI-H (hexamethyldisilazane–trimethylchlorosilane–pyridine 2:1:10) was purchased from Gasukuro Kogyo Inc., Tokyo, Japan. Nicotinamide adenine dinucleotide phosphate (reduced form, NADPH) was purchased from Kojin Co., Ltd., Tokyo, Japan. Cholesterol, 7-ketocholesterol, and ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), and cholesterol esterase (Sigma No. C-3766, from bovine pancreas) were purchased from Sigma Chemical Co., St. Louis, MO. Coprostanol was obtained from Steraloids Inc., Wilton, NH. Silica gel G was obtained from Merck, Darmstadt, West Germany. Bond-Elut silica cartridge columns (part number, 601303; Bonded Phase, SI; size, 3 cc) were obtained from Analytichem International, Harbor City, CA. Sterols used in this study were checked for purity by thin-layer chromatography (TLC) using ether and benzene–ethyl acetate 3:2 (v/v) as solvent systems and by gas–liquid chromatography (GLC) as their trimethylsilyl (TMS) ether derivatives. Purities of the sterols were better than 99%.

Patients

Altogether, 38 patients were included in this study. Of these, 30 were patients with cholelithiasis who underwent elective cholecystectomy (19 women and 11 men, aged 37 to 72 years, mean age 53.9 years). Some were administered either CDCA (400 mg/day, 6 women and 3 men) or UDCA (600 mg/day, 8 women and 1 man) for 7 to 10 days before the operation. Eight patients with gastric cancer were subjected to elective gastrectomy (4 women and 4 men, aged 34 to 72 years, mean age 56.6 years). They had no evidence of abnormal lipid metabolism or liver dysfunction and served as controls.

Experimental procedure

The patients were admitted to the Kyushu University Hospital at least 10 days before the operation and were given a regular hospital diet. After a 12-h fast, blood samples were obtained in the morning (between 7 and 8 AM) on the day of operation. Serum was collected and stored at −20°C until analyzed. In the preliminary study, it was confirmed that serum 7α-hydroxycholesterol levels did not change significantly during the storage at −20°C for at least 2 months. Care was taken to avoid unnecessary thawing and re-freezing. The patients were operated on between 9 and 10 AM. A surgical liver biopsy specimen (0.5–1.5 g) was obtained and a small piece of the liver specimen was fixed in 10% formalin and sent for histologic examination. The rest of the biopsy sample was immediately placed in ice-cold homogenizing medium and transported to the laboratory within 10 min for determination of microsomal cholesterol 7α-hydroxylase activity as described below.

Preparation of liver microsomes

The liver homogenate (10% w/v) was prepared in 50 mM Tris-HCl buffer, pH 7.4, containing 0.3 M sucrose, 10 mM EDTA, and 10 mM DTT. The microsomal fraction was prepared according to the procedure described by Einarsson et al. (6). A small aliquot was used for protein determination by the method of Lowry et al. (9).

Assay of microsomal cholesterol 7α-hydroxylase activity

The standard assay system consisted of 0.5 ml of the microsomal preparation corresponding to 0.25–1.0 mg of protein, 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA and 1 mM NADPH in a total volume of 1.0 ml. The enzyme assay was carried out in duplicate for 15 min at 37°C in room air. Care was taken to avoid unnecessary exposure to light. The reaction was terminated by addition of 10 ml of chloroform–methanol 2:1 (v/v). Two hundred pmol of 5α-cholestane-3β,7β-diol, dissolved in 50 μl of ethanol, was added as the internal standard. The organic phase was evaporated to dryness under a stream of nitrogen. Sample purification by a Bond-Elut silica cartridge column and analysis of actual mass of 7α-hydroxycholesterol by GLC–SIM were performed as described previously (8). Activity of cholesterol 7α-hydroxylase was calculated by subtracting the amount present in the zero-time assay from that at the end of the incubation period and expressed as pmol/min per mg protein.

Determination of serum 7α-hydroxycholesterol

Two hundred pmol of 5α-cholestane-3β,7β-diol dissolved in 50 μl of ethanol was added to 0.5 ml of serum. After addition of 0.7 ml of 0.9% NaCl solution and 1.8 ml of absolute ethanol in order to achieve 60% ethanol concentration, sterols were extracted three times, each with 3 ml of n-hexane; in our preliminary study, 7α-hydroxycholesterol and 5α-cholestane-3β,7β-diol were extracted quantitatively with hexane from 60% ethanol solution (data not shown). The n-hexane layer was divided into two portions and the solvent was evaporated to dryness

under a stream of nitrogen. One sample (for free 7α-hydroxycholesterol) was dissolved in 200 μl of benzene–ethyl acetate 9:1 (v/v) and applied onto a Bond-Elut silica cartridge column for purification as described below. Another sample (for total 7α-hydroxycholesterol) was hydrolyzed in 2.0 ml of 2.5% KOH in 90% ethanol at 55°C for 45 min (10). After addition of 1.2 ml of distilled water, the sterols were extracted three times each with 3-ml portions of n-hexane. The organic layer was washed twice with 1 ml of distilled water–ethanol 1:1 (v/v) and evaporated under nitrogen. The residue was dissolved in 200 μl of benzene–ethyl acetate 9:1 (v/v) and applied onto a Bond-Elut silica cartridge column. The column was eluted with 6 ml of benzene–ethyl acetate 9:1 (v/v) to remove most of the cholesterol and then eluted with 6 ml of benzene–ethyl acetate 2:3 (v/v) to recover 7α- and 7β-hydroxycholesterols, 7-ketocholesterol, and 5α-cholestane-3β,7β-diol (8). The solvent of the latter fraction was evaporated under nitrogen and the residue was treated with 100 μl of TMSI-H at 45°C for 60 min. After evaporation of the reagent, the residue was dissolved in 1% pyridine–n-hexane solution and analyzed by GLC–SIM. In the SIM mode, the ion at *m/z* 456 (M–90) was scanned for TMS ether derivatives of 7α- and 7β-hydroxycholesterols, *m/z* 458 (M–90) for the internal standard, and *m/z* 472 (M) for 7-ketocholesterol. The equipment and operating conditions for GLC–SIM were described in detail in a recent publication (8). The concentration of esterified 7α-hydroxycholesterol was calculated by subtracting the amount of free form (without hydrolysis) from that of total 7α-hydroxycholesterol (with alkaline hydrolysis).

Enzymatic hydrolysis with cholesterol esterase

In some experiments, enzymatic hydrolysis was performed instead of alkaline hydrolysis to study whether esterified 7α-hydroxycholesterol could also be hydrolyzed enzymatically. The incubation system consisted of 0.1 M phosphate buffer, pH 7.0, containing 1 unit of cholesterol esterase and 300 μmol of sodium cholate in a total volume of 2 ml. Incubations were conducted for 60 min at 37°C (11). After extraction with n-hexane, purification, and derivatization, 7α-hydroxycholesterol was analyzed by GLC–SIM.

Purification of serum 7α-hydroxycholesterol by TLC

In another experiment, sample preparation was carried out by TLC according to Björkhem et al. (7), instead of a silica cartridge column, to compare two different purification procedures. In brief, the sterol extract with or without hydrolysis was applied to a precoated silica gel G plate (0.5 mm thick, Merck) which was then developed in benzene–ethyl acetate 1:1 (v/v). The average R_f values were: 5α-cholestane-3β,7β-diol, 0.16; 7α-hydroxycholesterol, 0.16; 7β-hydroxycholesterol, 0.21; 7-ketocholesterol, 0.28; and cholesterol, 0.51. On TLC using the above systems, 5α-cholestane-3β,7β-diol and 7α-hydroxycholesterol had similar R_f values. The band corresponding to 7α-hydroxycholesterol and 5α-cholestane-3β,7β-diol was scraped off and the sterols were eluted with methanol. Further analysis was carried out as described above.

Determination of serum cholesterol

Serum cholesterol concentration was determined by gas–liquid chromatography (as the TMS ether derivative) as described previously (12) using coprostanol as an internal standard.

Reproducibility, recovery, and statistical analysis

Reproducibility was investigated by analyzing five samples in triplicate by GLC–SIM. The result was analyzed according to analysis of variance (13). For the recovery study, 7α-hydroxycholesterol of known amounts was added to four sets of duplicate samples and the actual mass of 7α-hydroxycholesterol was determined. The recovery was calculated by dividing the amount recovered by that of 7α-hydroxycholesterol added. The estimated values were calculated by linear regression analysis (14). Data are expressed as mean ± SD. The statistical differences were analyzed by Student's unpaired or paired *t*-test and analysis of variance when necessary. Correlation between the enzyme activity and serum 7α-hydroxycholesterol concentration was tested by linear regression analysis (14).

RESULTS

Optimal assay conditions of microsomal cholesterol 7α-hydroxylase activity in human liver

Our assay system for measuring the activity of hepatic microsomal cholesterol 7α-hydroxylase was initially developed for the rat (8) and had to be adapted for analysis of the activity in human liver. Formation of 7α-hydroxycholesterol from endogenous cholesterol was linear with time up to 30 min and with microsomal protein concentration from 0.2 mg up to 2.5 mg in each incubation system. The reaction rate reached a plateau above 0.3 mM NADPH. We therefore chose 15 min of incubation time, 0.25–1.0 mg microsomal protein, and 1 mM NADPH as the optimal assay conditions.

Hydrolysis of serum esterified 7α-hydroxycholesterol

Typical mass fragmentograms obtained in the analysis of serum 7α-hydroxycholesterol with or without alkaline hydrolysis are shown in **Fig. 1**. The peak corresponding to 7α-hydroxycholesterol after alkaline hydrolysis was significantly greater than that without hydrolysis, suggesting the presence of esterified 7α-hydroxycholesterol in human serum. In the preliminary study, we confirmed that the structures of 7α-hydroxycholesterol and 5α-cholestane-

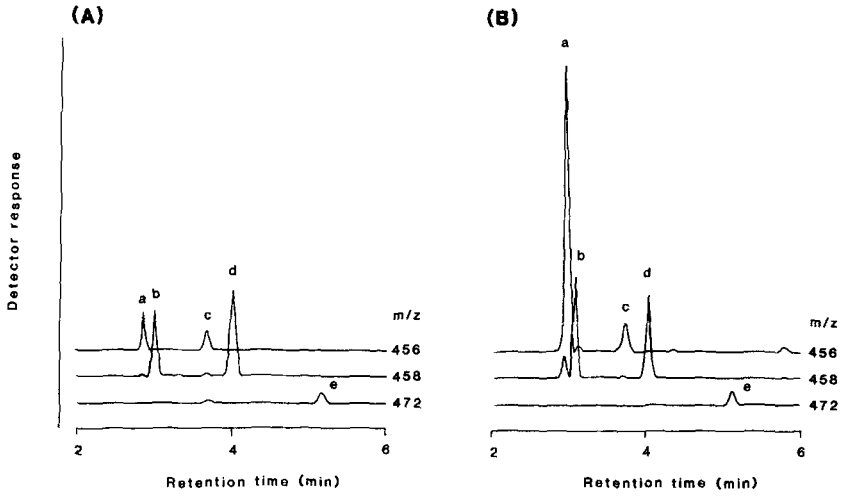


Fig. 1. Selected monitoring of the ions at *m/z* 456, *m/z* 458, and *m/z* 472 of TMS ether derivatives of serum lipid extract without hydrolysis (A), and with alkaline hydrolysis (B): (a) 7α-hydroxycholesterol, (b) cholesterol, (c) 7β-hydroxycholesterol, (d) 5α-cholestane-3β,7β-diol (internal standard), (e) 7-ketocholesterol.

3β,7β-diol (internal standard) were stable during alkaline hydrolysis, at least under the conditions used. The stability of natural 7α-hydroxycholesterol under alkaline conditions was also verified by a time-course experiment. After addition of potassium hydroxide solution to serum samples and heating at 55°C, the concentration of 7α-hydroxycholesterol increased rapidly. Maximum concentration was reached during the initial 15 min and was unchanged for a subsequent 105 min.

In order to confirm the presence of acyl esters of 7α-hydroxycholesterol and to obtain information on the position(s) of the acyl groups, enzymatic hydrolysis of serum samples with cholesterol esterase was tried. Comparison of 7α-hydroxycholesterol levels after alkaline hydrolysis and those after the enzymatic hydrolysis is shown in **Table 1**. The values obtained after the enzymatic hydrolysis were identical to those after alkaline hydrolysis, indicating that esterified 7α-hydroxycholesterol was also hydrolyzed quantitatively with cholesterol esterase. In addition, in blank experiments for the enzymatic hydrolysis, where distilled water was added instead of cholesterol esterase solution and all other conditions were the same, amounts of 7α-hydroxycholesterol obtained after incubation were identical to those of free 7α-hydroxycholesterol determined without alkaline hydrolysis.

Purification of serum 7α-hydroxycholesterol by TLC

Our new purification method utilizing Bond-Elut silica cartridge columns was compared with the previous method with TLC (7) and results are shown in **Table 2**. The values obtained by the present purification method were similar to those obtained by the previous method. Levels of free 7α-hydroxycholesterol purified with the silica cartridge columns were slightly higher than those purified with TLC ($P<0.05$ by paired *t*-test). However, there was no statistical difference between the two purification methods as assessed by analysis of variance.

Reproducibility and recovery of serum 7α-hydroxycholesterol standard assay system

The precision of the method was evaluated by replicate measurements of five identical serum samples. Concentrations of total, esterified, and free 7α-hydroxycholesterol were 191.7 ± 5.0, 166.2 ± 4.8, and 25.5 ± 0.9 pmol/ml, respectively (mean ± SD, n = 5). The coefficient of

TABLE 1. Comparison of alkaline hydrolysis and enzymatic hydrolysis

	Serum 7α-Hydroxycholesterol					
	Alkaline Hydrolysis			Enzymatic Hydrolysis		
Sample	Total[a]	Free[c]	Ester[e]	Total[b]	Free[d]	Ester[f]
	pmol/ml			*pmol/ml*		
A	153.6	25.8	127.8	153.4	29.9	123.5
B	249.8	39.0	210.8	249.6	41.3	208.3
C	312.0	64.3	247.7	282.2	79.0	203.2
D	319.7	40.2	279.5	305.8	44.3	261.5
E	445.8	185.3	260.5	449.4	204.8	244.6

[a,b]Total 7α-hydroxycholesterol levels were analyzed after either alkaline[a] (2.5% KOH in 90% ethanol at 55°C for 45 min) or enzymatic[b] (1 unit of cholesterol esterase at 37°C for 60 min) hydrolysis.

[c]Free 7α-hydroxycholesterol levels were determined without alkaline hydrolysis.

[d]Free 7α-hydroxycholesterol levels were determined after blank incubation without cholesterol esterase.

[e,f]Esterified 7α-hydroxycholesterol levels were calculated by subtracting the amount of free form from that of total 7α-hydroxycholesterol in the respective hydrolysis method. There were no significant differences between the two methods by paired *t*-test and analysis of variance.

TABLE 2. Comparison of two different purification methods

Sample	Serum 7α-Hydroxycholesterol					
	Thin-Layer Chromatography			Silica Cartridge Column		
	Total	Free[a]	Ester[b]	Total	Free[a]	Ester[b]
	pmol/ml			*pmol/ml*		
1	98.5	30.0	68.5	96.0	35.3	60.7
2	111.3	36.5	74.8	113.5	39.7	73.8
3	307.6	57.6	250.0	297.2	61.4	235.8
4	397.3	45.4	351.9	394.0	49.5	344.5
5	410.7	130.5	280.2	415.8	144.9	270.9

Serum 7α-hydroxycholesterol levels were analyzed by GLC-SIM after purification steps using thin-layer chromatography or a Bond-Elut silica cartridge column. Total and free 7α-hydroxycholesterol levels were determined with or without alkaline hydrolysis followed by respective purification methods.

[a,b]Significant difference between the two purification methods ($P < 0.05$ by paired t-test). The two methods were equivalent as assessed by analysis of variance.

variation for sample preparation was about 3%. The recovery of added 7α-hydroxycholesterol ranged from 96.8% to 109.6% with a mean of 102.6% (**Table 3**).

Serum levels of 7α-hydroxycholesterol and hepatic microsomal cholesterol 7α-hydroxylase activities in surgical patients

Serum levels of free, esterified, and total 7α-hydroxycholesterol and microsomal cholesterol 7α-hydroxylase activities in each group of patients on the day of operation are summarized in **Table 4**. There were no significant differences in serum 7α-hydroxycholesterol concentrations and cholesterol 7α-hydroxylase activities between patients with cholelithiasis and those with gastric cancer. Administration of CDCA resulted in a marked decrease of both serum levels and the enzyme activities. Treatment with UDCA had no apparent effects. Correlations between cholesterol 7α-hydroxylase activities and serum 7α-hydroxycholesterol concentrations are depicted in **Fig. 2**. There were significant positive correlations between the enzyme activities and serum 7α-hydroxycholesterol concentrations [free ($r = 0.71$, n = 38, $P<0.001$, y = 16.7 + 15.4x), esterified ($r = 0.87$, n = 38, $P<0.001$, y = 40.0 + 23.6x), total ($r = 0.87$, n = 38, $P<0.001$, y = 56.6 + 29.0x)].

Relationship between concentrations of serum cholesterol and 7α-hydroxycholesterol

Serum levels of free, esterified, and total cholesterol in each group are listed in **Table 5**. There were no significant differences in cholesterol concentrations among all groups, although administration of CDCA tended to decrease serum cholesterol concentrations. Serum levels of 7α-hydroxycholesterol were not correlated with those of cholesterol [free ($r = 0.20$, n = 38, $P>0.2$), esterified ($r = 0.28$, n = 38, $P>0.05$), total ($r = 0.26$, n = 38, $P>0.1$)]. The proportion of esterified cholesterol was approximately 74% in all groups and was very close to that of 7α-hydroxycholesterol (77%, see Table 4).

DISCUSSION

The present study has shown the presence of a considerable amount of esterified 7α-hydroxycholesterol in human serum. To start with, we had to prove that the esterified 7α-hydroxycholesterol found was not an artificial product from esterified cholesterol during the sample

TABLE 3. Recovery of 7α-hydroxycholesterol

Sample (X_o + na)[a] (n = 0,1,2,3)	Amount Added	Amount Found		Estimated Amount (X_o)		Recovery[b]	
		Free[c]	Total[d]	Free	Total	Free	Total
	pmol	*pmol*		*pmol*		%	
(X_o^*)	0	21.6	147.0	23.8	143.4		
(X_o)	0	21.6	144.6				
(X_o + a)	95.8	128.3	243.4			109.1	104.4
(X_o + a)	95.8	124.5	238.3			105.1	99.1
(X_o + 2a)	191.6	209.3	349.6			96.8	107.6
(X_o + 2a)	191.6	214.1	342.5			99.3	103.9
(X_o + 3a)	287.4	317.4	433.9			102.2	101.1
(X_o + 3a)	287.4	311.5	437.3			100.1	102.3

[a]X_o, estimated amount calculated by linear regression analysis (13); na, amount of 7α-hydroxycholesterol added to 0.5 ml of serum.

[b]Recovery was calculated as follows: recovery (%) = (amount found − estimated amount)/amount added × 100.

[c,d]Serum 7α-hydroxycholesterol amount was analyzed without[c] or with[d] alkaline hydrolysis after addition of various known amounts of free 7α-hydroxycholesterol to serum sample.

TABLE 4. Serum 7α-hydroxycholesterol concentrations and hepatic microsomal cholesterol 7α-hydroxylase activities

Patients	n	Serum 7α-Hydroxycholesterol				Cholesterol 7α-Hydroxylase
		Total	Free	Ester	(% Ester[a])	
		pmol/ml				*pmol/min/mg protein*
Cholelithiasis						
Untreated	12	246.3 ± 105.9	48.3 ± 19.8	198.0 ± 90.3	(79.5 ± 6.1)	7.01 ± 3.52
Treated with CDCA	9	85.4 ± 40.8[b]	20.5 ± 11.1[b]	64.9 ± 33.6[b]	(75.0 ± 9.7)	1.55 ± 0.70[b]
Treated with UDCA	9	273.1 ± 112.2	58.6 ± 30.1	214.4 ± 91.1	(78.0 ± 6.9)	6.24 ± 3.54
Gastric cancer	8	239.1 ± 115.9	55.9 ± 31.8	183.2 ± 94.3	(75.4 ± 7.6)	6.31 ± 2.88

The values are given as mean ± SD.
[a] % Ester, ester/total × 100.
[b] $P < 0.01$, significantly different from the values of untreated gallstone patients by Student's *t*-test.

preparation steps. It has been reported that cholesterol is a labile molecule and is susceptible to autooxidation (15–18). Manipulation of labeled cholesterol in air or exposure of cholesterol to ultraviolet light easily converts cholesterol into various types of oxygenated cholesterols (15–18). 7-Ketocholesterol and 7β-hydroxycholesterol are reported to be the two major products and a lesser amount of 7α-hydroxycholesterol is also formed (15–18). As shown in Tables 4 and 5, the concentration of serum cholesterol was approximately 20,000-fold that of 7α-hydroxycholesterol and minimal autooxidation of cholesterol during the sample preparation might result in an appreciable increase of 7α-hydroxycholesterol. In the present study, however, concentration of total (free and hydrolyzed) 7α-hydroxycholesterol increased rapidly and the equilibrium once established was not changed during alkaline hydrolysis. This strongly suggested that there is another form of 7α-hydroxycholesterol in human serum which is readily converted to free 7α-hydroxycholesterol under alkaline conditions and that autooxidation of cholesterol (15–18) and destruction of 7α-hydroxycholesterol once formed (19) do not seem to be taking place during the alkaline hydrolysis step. This result was consistent with that of Koopman et al. (10), who reported the stability of 7α-hydroxycholesterol during alkaline hydrolysis. The levels of total 7α-hydroxycholesterol after the enzymatic hydrolysis were identical with those after alkaline hydrolysis. Blank experiments lacking only cholesterol esterase in the incubation system resulted in 7α-hydroxycholesterol levels that were similar to those without hydrolysis (Table 1), indicating that no detectable amount of autooxidation product was formed during the incubation. Considering very limited substrate specificity of the purified enzyme used, we conclude that: *1*) there exists not only free (or unesterified) 7α-hydroxycholesterol but also a considerable amount of esterified 7α-hydroxycholesterol in human serum; *2*) both alkaline and enzymatic hydrolyses quantitatively release free 7α-hydroxycholesterol; *3*) fatty acids (species still unknown) should be bound to the oxygenated sterol; and *4*) the position of acylation is probably at the 3β hydroxyl group.

Björkhem et al. (7) reported that no significant part of 7α-hydroxycholesterol in serum was esterified. Major differences from the present study were in the internal standard used and the sample purification method. Each internal standard seemed to work in an equivalent way in the respective methods as long as free 7α-hydroxycholesterol was to be analyzed (8). We therefore tried the sample purification with TLC used by Björkhem et al. (7), which resulted in 7α-hydroxycholesterol levels similar to those quantified after purification with silica cartridge columns (Table 2). It is possible that Björkhem et al. (7) could not detect significant amounts of esterified 7α-hydroxycholesterol in human serum because they performed alkaline hydrolysis at a very low level of serum 7α-hydroxycholesterol (14 ± 1 ng/ml). Another possibility is that the conditions of alkaline hydrolysis (details were not given in reference 7) were too mild to hydrolyze esterified 7α-hydroxycholesterol.

Esterified 7α-hydroxycholesterol was first identified by Boyd and Mawer (20) as early as 1961. Although they had very limited methodology, i.e., paper chromatography, infrared analysis, and colorimetric methods, they showed the presence of 3β-esters of 7α-hydroxycholesterol in both serum (20, 21) and liver microsomes (22). Because of the unavailability of quantitative methods at that time, they could not show whether there was a significant amount of esterified 7α-hydroxycholesterol in serum. The present study has shown conclusively the presence of 3β-acyl esters of 7α-hydroxycholesterol and revealed the predominance of the esters over the free form in human serum.

Sakamoto (11) suggested that the cholesterol esterifying enzyme [acyl-coenzyme A:cholesterol acyltransferase (ACAT)] in liver microsomes and hydrolyzing enzyme (cholesterol esterase) in liver soluble fraction may catalyze the esterification of free 7α-hydroxycholesterol and hydrolysis of esterified 7α-hydroxycholesterol, respectively.

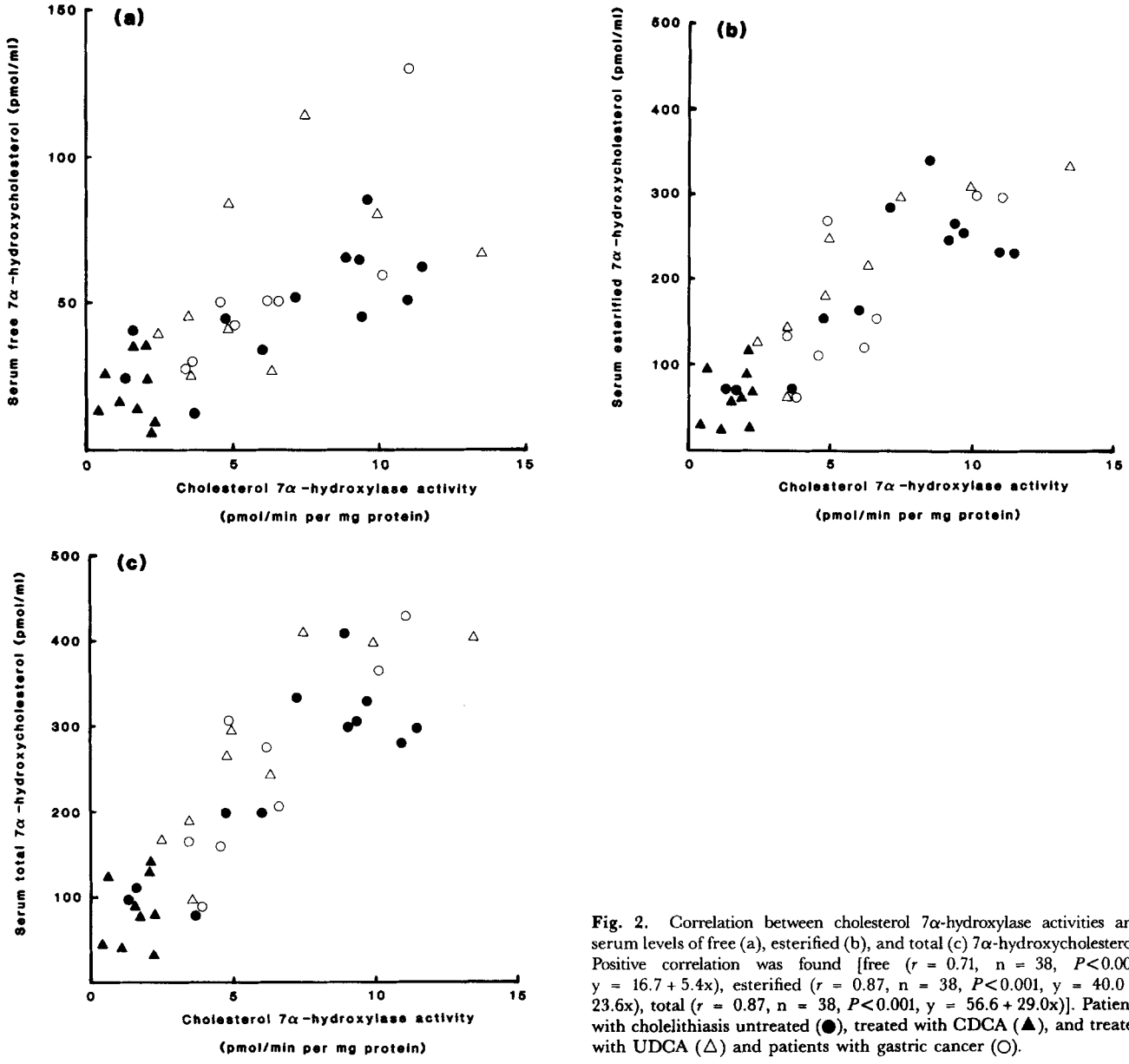


Fig. 2. Correlation between cholesterol 7α-hydroxylase activities and serum levels of free (a), esterified (b), and total (c) 7α-hydroxycholesterol. Positive correlation was found [free (r = 0.71, n = 38, $P<0.001$, y = 16.7 + 5.4x), esterified (r = 0.87, n = 38, $P<0.001$, y = 40.0 + 23.6x), total (r = 0.87, n = 38, $P<0.001$, y = 56.6 + 29.0x)]. Patients with cholelithiasis untreated (●), treated with CDCA (▲), and treated with UDCA (△) and patients with gastric cancer (○).

Some part of 7α-hydroxycholesterol synthesized in liver microsomes may be esterified with fatty acids by the esterifying enzyme, which is located in the same subcellular fraction. It is reasonable to assume that 3β-acyl esters of 7α-hydroxycholesterol would not be converted to 7α-hydroxy-4-cholesten-3-one, the next intermediate in bile acid biosynthesis, because the 3β-hydroxyl group is protected from being oxidized by 3β-hydroxysteroid dehydrogenase.

Accumulation of 7α-hydroxycholesterol in liver microsomes was suggested by Björkhem et al. (23). They reported that the concentration of 7α-hydroxycholesterol in human liver microsomes was considerably higher than those of other intermediates in bile acid synthesis in spite of the fact that cholesterol 7α-hydroxylase is the rate-limiting enzyme in the overall biosynthesis of bile acids from cholesterol. The reason why 7α-hydroxycholesterol can accumulate in microsomes has not been elucidated. The accumulation of 7α-hydroxycholesterol may lead to leakage of the sterol from hepatocytes into the systemic circulation under physiologic conditions.

Decreased activity of 7α-hydroxylase in patients with cholesterol gallstones has been reported by several investigators (24, 25). In the present study, however, levels of serum 7α-hydroxycholesterol and cholesterol 7α-hydroxylase activities in patients with cholelithiasis were almost the same as those in patients without hepatobiliary diseases. Administration of CDCA significantly inhibited

TABLE 5. Serum cholesterol concentrations

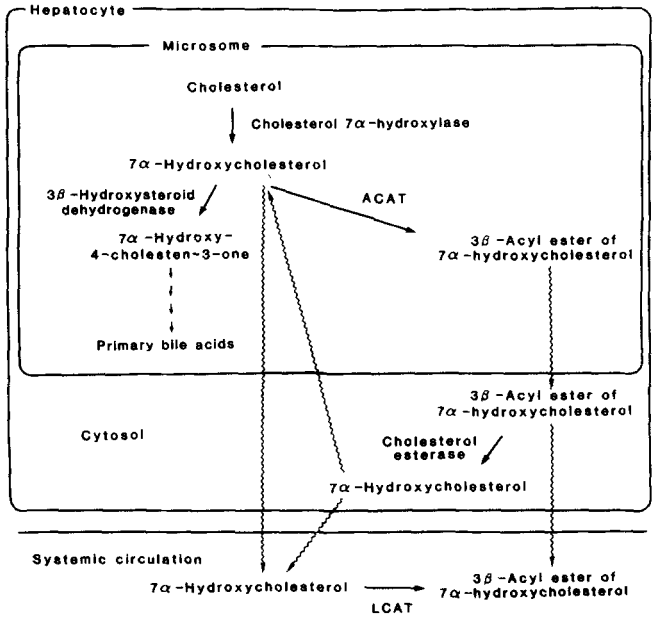
Patients	n	Serum Cholesterol			
		Total	Free	Ester	(% Ester[a])
		μmol/ml			
Cholelithiasis					
Untreated	12	3.95 ± 1.41	1.02 ± 0.42	2.93 ± 1.04	(74.1 ± 4.1)
Treated with CDCA	9	3.37 ± 1.41	0.89 ± 0.38	2.48 ± 1.04	(73.7 ± 2.5)
Treated with UDCA	9	4.09 ± 2.09	1.08 ± 0.53	3.01 ± 1.57	(73.7 ± 2.2)
Gastric cancer	8	4.15 ± 1.50	1.12 ± 0.37	3.03 ± 1.16	(72.7 ± 2.6)

The values are mean ± SD.
[a]% Ester, ester/total × 100.

the activity of hepatic microsomal cholesterol 7α-hydroxylase. This was in good agreement with the previous reports (5, 7, 26–29). The effect on bile acid synthesis by oral administration of UDCA is still controversial (29–34). In a recent study, Reihnér et al. (5), using isotope-dilution mass spectrometry, found no significant changes in 7α-hydroxylase activity when 15 mg/kg per day of UDCA was fed for 3 to 4 weeks. The results of the present study and those of Reihnér et al. (5) suggested that UDCA has little inhibitory effect on bile acid synthesis at a dose of about 15 mg/kg per day.

In all groups studied, strong correlations were found between the activities of cholesterol 7α-hydroxylase and serum levels of 7α-hydroxycholesterol. Better correlation was observed when total or esterified 7α-hydroxycholesterol concentration was compared with the enzyme activity. More than 70% of the 7α-hydroxycholesterol was present in serum in the esterified form and the relative abundance of the esters may be the reason for the higher correlation. Tilvis and Miettinen (35) reported that sterols without methyl group(s) at position 4 of the steroid nucleus may be esterified by lecithin:cholesterol acyltransferase (LCAT) in serum, while those having methyl group(s) may not be the substrates of the enzyme. We thus speculate that 7α-hydroxycholesterol is also esterified by LCAT in serum. A similar explanation has been given for the correlation between serum levels of some cholesterol precursors and the activities of hepatic microsomal 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the cholesterol biosynthesis (36, 37). Methyl sterols, which are esterified by ACAT (38) but not by LCAT (35), are present in serum mainly in the free form, which correlates better with HMG-CoA reductase activity than the esterified form (36, 37). On the other hand, demethylated sterols, which are esterified by both enzymes (39), are present mainly in the esterified form, which correlates better with the enzyme activity than the free form (36, 37). Björkhem et al. (7) concluded that the serum level of (free) 7α-hydroxycholesterol correlated with cholesterol 7α-hydroxylase activity only in the cases of increased biosynthesis of bile acids. The present study has clearly shown that levels of esterified and/or total 7α-hydroxycholesterol reflect the enzyme activity even when bile acid synthesis is decreased by treatment with CDCA.

A possible metabolism of 7α-hydroxycholesterol and its ester(s) in hepatocytes and in serum is depicted in **Scheme 1**. In liver microsomes, newly synthesized cholesterol is preferably hydroxylated at the 7α-position to form 7α-hydroxycholesterol (40–42). Most of 7α-hydroxycholesterol formed is immediately oxidized to 7α-hydroxy-4-cholesten-3-one (43) and a small part, which escapes oxidation, may be esterified with fatty acids by microsomal ACAT (11). Esterified 7α-hydroxycholesterol



Scheme 1. Possible metabolism of 7α-hydroxycholesterol in hepatocyte and in serum.

may leave microsomes into the cytosol and be hydrolyzed partly by cholesterol esterase (11). Free and esterified 7α-hydroxy-cholesterol thus formed may be released into the systemic circulation. Free 7α-hydroxycholesterol in serum may well be esterified by LCAT as described above. At present, nothing is known about the further metabolism of serum 7α-hydroxycholesterol.

In conclusion, we have shown the presence of fatty acid acyl esters of 7α-hydroxycholesterol in a considerable amount in human serum. Both alkaline hydrolysis and the enzymatic hydrolysis with cholesterol esterase quantitatively released free (hydrolyzed) 7α-hydroxycholesterol. There were highly significant positive correlations between the activity of hepatic microsomal cholesterol 7α-hydroxylase and levels of serum esterified and total 7α-hydroxycholesterol. A simple blood sampling followed by analysis of serum esterified and/or total 7α-hydroxycholesterol concentration may allow the assessment of endogenous bile acid synthesis, which otherwise is quite difficult to evaluate.

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