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Determination of 7α-hydroxy-4-cholesten-3-one level in plasma using isotope-dilution mass spectrometry and monitoring its circadian rhythm in human as an index of bile acid biosynthesis

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Abstract

A highly sensitive and specific method has been developed for determination of the level of 7α-hydroxy-4-cholesten-3-one in plasma. This method is based on a stable isotope-dilution technique by gas chromatography–selected-ion monitoring mass spectrometry. 7α-Hydroxy-4-cholesten-3-one was extracted from plasma by salting-out extraction, and then purified by serial solid-phase extractions. The extract was treated with O-methylhydroxylamine hydrochloride and then dimethylethylsilylated. The resulting methyloxime–dimethylethylsilyl ether derivative was quantified by gas chromatography–selected-ion monitoring mass spectrometry with a high-resolution mode. The plasma levels of 7α-hydroxy-4-cholesten-3-one were correlated with the cholesterol 7α-hydroxylase activity to a higher degree than those of any other form of 7α-hydroxycholesterol ($r = 0.84$, $n = 16$, $p < 0.0001$). The present method was applied to monitor the circadian rhythm of 7α-hydroxy-4-cholesten-3-one levels in human plasma. It was concluded that the plasma level of 7α-hydroxy-4-cholesten-3-one is a useful index for the monitoring of bile acid biosynthesis in the human liver.

1. Introduction

Bile acid biosynthesis plays an important role in the regulation of cholesterol homeostasis. 7α-Hydroxylation of cholesterol by cholesterol 7α-hydroxylase (EC 1.14.13.7) is the first and rate-limiting step in the major pathway of bile acid biosynthesis [1–3]. However, studies concerning the regulation of this enzyme in human liver have been hampered by the need to sample liver specimens. Several studies have provided data that reflect the activity of this enzyme [4–7]. Björkhem *et al.* [4] reported that the serum levels of unesterified 7α-hydroxycholesterol correlated with the activities of cholesterol 7α-hy-

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droxylase in cholestyramine-treated patients. Axelson *et al.* [6] developed a convenient high-performance liquid chromatographic (HPLC) method to determine serum concentrations of 7α-hydroxy-4-cholesten-3-one, which is the product of the next reaction following the 7α-hydroxylation of cholesterol. They reported that the plasma concentration of 7α-hydroxy-4-cholesten-3-one is a better index for hepatic cholesterol 7α-hydroxylase activity than the level of unesterified 7α-hydroxycholesterol [7].

We have recently reported the simultaneous assay of the activities of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34), the rate-limiting enzyme of the cholesterol biosynthesis, and cholesterol 7α-hydroxylase by an isotope-dilution technique [8]. With minor modifications to this method, we also have developed a sensitive and specific method for the determination of the plasma levels of mevalonate and 7α-hydroxycholesterol [9]. Our results are similar to those of Björkhem *et al.* [4] in that the concentrations of free 7α-hydroxycholesterol were significantly correlated with the hepatic cholesterol 7α-hydroxylase activity only when the activities of cholesterol 7α-hydroxylase were much higher than normal.

The present study reports the development of a simple and convenient method for the determination of the levels of 7α-hydroxy-4-cholesten-3-one in human plasma using gas chromatography–selected-ion monitoring mass spectrometry (GC–SIM-MS) with a high resolution mode, and the relationship between the cholesterol 7α-hydroxylase activity in liver and the 7α-hydroxy-4-cholesten-3-one level in plasma. Furthermore, we describe the circadian rhythm of the 7α-hydroxy-4-cholesten-3-one level in plasma as an index of bile acid biosynthesis.

2. Experimental

2.1. Chemicals

[25,26,26,26,27,27,27 - $^2H_7$]7α - Hydroxycholesterol was prepared as described previously [8]. [4-^{14}C]Cholesterol was purchased from Du Pont (Wilmington, DE, USA). Dimethylethylsilyl imidazole (DMESI) and O-methylhydroxylamine hydrochloride (MOA-HCl) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Cartridges of Bond Elut SI (unbonded silica, 500 mg, 3 ml) and Bond Elut C_{18} (octadecyl, 500 mg, 3 ml) were obtained from Varian (Harbor City, CA, USA). Sephadex LH-20 was obtained from Pharmacia (Uppsala, Sweden). Analytical grade solvents and other reagents were used.

2.2. Synthesis of [4-^{14}C]7α-hydroxy-4-cholesten-3-one and [$^2H_7$]7α-hydroxy-4-cholesten-3-one

Using Stárka's method [10], [4-^{14}C]7α-hdroxycholesterol was synthesized from [4-^{14}C]-cholesterol. [25,26,26,26,27,27,27 - $^2H_7$]7α - Hydroxy-4-cholesten-3-one and [4-^{14}C]7α-hydroxy-4-cholesten-3-one were synthesized from [$^2H_7$]-7α-hydroxycholesterol and [4-^{14}C]7α-hydroxycholesterol with cholesterol oxidase, respectively [11]. Each of these standards produced a single spot on a thin-layer chromatogram.

2.3. Subjects and experimental procedure

Sixteen patients (six with cholelithiasis, ten with early cancer of the gastrointestinal tract), aged 39–72 years, were studied. None of the patients had signs of diabetes mellitus, hyperlipoproteinemia, or diseases affecting the liver, thyroid or kidney functions. At the start of the experiments (9:00 a.m.–11:00 a.m.), surgical liver biopsy specimens and venous blood were simultaneously obtained. The biopsy specimen was immediately placed in ice-cold homogenizing medium. The heparinized blood sample was immediately centrifuged at 1000 *g* for 15 min to separate plasma. To 1 ml of plasma 10 μg butylated hydroxytoluene was added and the sample was stored at −20°C until analysis.

Three normolipidemic male volunteers, aged 24–28 years, were used to monitor the circadian rhythm. Blood samples were taken every 2 h from 6:00 a.m. the first day to 6:00 a.m. the second day. The volunteers ate normal hospital diets at 8:00 a.m., 12:30 p.m., 5:30 p.m., and they slept from 9:00 p.m. to 6:00 a.m.

Informed consent was obtained from all subjects, and the experimental procedures were carried out in accordance with the ethical standards of the Helsinki Declaration.

2.4. Assay for the activities of microsomal cholesterol 7α-hydroxylase and plasma levels of 7α-hydroxycholesterol

The preparation of liver microsomes, the assay for the activity of cholesterol 7α-hydroxylase, and quantification of the plasma levels of 7α-hydroxycholesterol were performed according to the previously described method [8,9].

2.5. Determination of 7α-hydroxy-4-cholesten-3-one in human plasma

A 7-ng aliquot of [$^2H_7$]7α-hydroxy-4-cholesten-3-one was added to 200 μl of plasma, as an internal standard. After diluting the sample with 0.8 ml of distilled water, 2 ml of acetonitrile was added and the sample was mixed well. The mixture was saturated with *ca.* 500 mg of ammonium sulfate. After separation and collection of the acetonitrile layer by centrifugation at 1000 *g* for 15 min, the collected solvent was diluted with 0.5 ml of distilled water, and the mixture was applied to a Bond Elut C_{18} cartridge prewashed with 5 ml of chloroform, 5 ml of ethanol and 10 ml of distilled water. 7α-Hydroxy-4-cholesten-3-one was eluted with 3 ml of acetonitrile–ethanol (4:1, v/v). After evaporation under reduced pressure, the residue was dissolved in 500 μl of *n*-hexane–diethyl ether (9:1, v/v), and the resulting solution was applied to a Bond Elut SI cartridge prewashed with 3 ml of the solvent mixture described above. After washing with 5 ml of the same mixture and 5 ml of *n*-hexane–diethyl ether (3:1, v/v), 7α-hydroxy-4-cholesten-3-one was eluted with 5 ml of *n*-hexane–diethyl ether (1:3, v/v).

After evaporation of the resulting eluate to dryness, the residue was dissolved with 100 μl of 10% MOA-HCl–pyridine (w/v) and heated at 60°C for 120 min. The reaction mixture was treated with 50 μl of DMESI [12]. After a 60-min incubation at room temperature excess silylating reagent was removed on a Sephadex LH-20 column (60 × 6 mm I.D.) equilibrated with 3 ml of *n*-hexane–chloroform (1:1, v/v). After evaporation under reduced pressure, the residue was dissolved in 50 μl of *n*-hexane.

Gas chromatography–mass spectrometry (GC–MS) was performed using a JMS-SX102 instrument equipped with a DA-7000 data processing system (JEOL, Tokyo, Japan). The column used was an Ultra Performance 1 capillary column (25 m × 0.32 mm I.D., 0.17 μm film thickness) coated with methylsilicone (Hewlett-Packard, CA, USA). The flow-rate of the carrier gas (helium) was 1.0 ml/min. The operating conditions were as follows: the column oven was programmed from 150°C to 280°C at 30°/min after a 1-min delay at the start; the accelerating voltage was 10 kV; the injector temperature was 300°C; the separator and ion-source temperature were 230°C; the ionization energy was 70 eV; the trap current was 600 μA. Selected-ion monitoring (SIM) in the high-resolution mode was performed using the data system. The mass spectral resolution was greater than 10 000.

2.6. Statistical analysis

The absolute recoveries of 7α-hydroxy-4-cholesten-3-one in each step of the procedure were studied by adding [4-^{14}C]7α-hydroxy-4-cholesten-3-one (1.3 kBq) to 1 ml of plasma, and determination of the radioactivity in each step by liquid scintillation counting. The absolute recovery of cholesterol in each step was determined by a colorimetric enzyme method. Using GC–SIM-MS, we studied the reproducibility of the method by analyzing four samples in triplicate, and analyzing the results by analysis of variance [13]. For the recovery study, known amounts of 7α-hydroxy-4-cholesten-3-one were spiked to four groups of duplicate samples. The recovery was calculated by dividing the amount recovered by the amount of 7α-hydroxy-4-cholesten-3-one added. The estimated values were calculated by linear regression analysis [14]. Regression analysis was also used to verify the correlations between the enzyme activity and the plasma levels of 7α-hydroxy-4-cholesten-3-one,

and between the enzyme activity and those of 7α-hydroxycholesterol. Plasma concentrations of 7α-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one were fitted to a cosine curve to estimate the amplitude and acrophase of the circadian rhythm [15]. We used the method of Nelson *et al.* to estimate the zero-amplitude test of the circadian rhythm [15].

3. Results

3.1. Absolute recovery and elimination rate of cholesterol

The recovery of 7α-hydroxy-4-cholesten-3-one in each step of the purification procedure was checked by adding 1.3 kBq of [4-^{14}C]7α-hydroxy-4-cholesten-3-one to 1 ml of plasma. The recovery of cholesterol in each step was checked by the colorimetric enzyme method. Table 1 shows the absolute recoveries of 7α-hydroxy-4-cholesten-3-one and the elimination rate of cholesterol. The overall recovery was found to be 85.4 ± 5.9%. In this procedure, 94.9 ± 1.5% of cholesterol was eliminated from plasma.

3.2. Representative SIM

Fig. 1 shows the mass spectrum of methyloxime dimethylethylsilyl (MO-DMES) ether derivatives obtained by GC–MS in the EI mode.

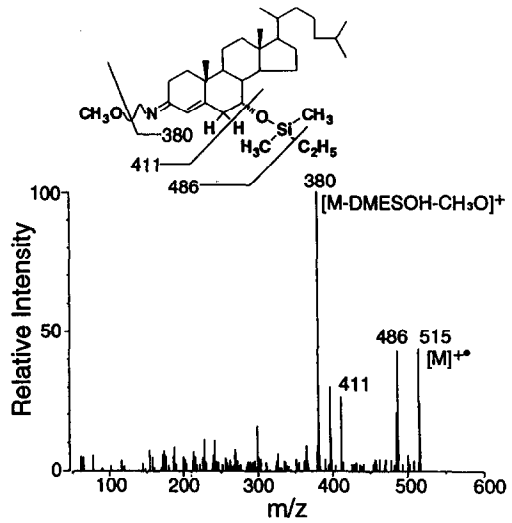

Fig. 1. Electron impact ionization spectrum of methyloxime dimethylethylsilyl ether derivative of 7α-hydroxy-4-cholesten-3-one obtained during GC–MS analysis.

The ion [M − 135] that resulted from elimination of the DMES-OH and–OCH_3 groups from the molecular ion was observed as a major peak in the mass spectrum of the MO-DMES ether derivative. The fragmentation patterns of the *syn* and *anti* isomers of the MO derivatives were very similar. Fig. 2 shows the high-resolution selected-ion recordings of 7α-hydroxy-4-cholesten-3-one from human plasma. The multiple-ion detector was focused on *m/z* 380.3317 for the

Table 1
Absolute recovery of 7α-hydroxy-4-cholesten-3-one and elimination rate of cholesterol from plasma[a]

Step		Absolute recovery	Elimination rate of cholesterol[b] (%)
I	Salting-out extraction	100.7 ± 3.5	< 0.1
II	Bond Elut C_{18}	91.0 ± 2.1	92.0 ± 0.6
III	Bond Elut SI	93.2 ± 4.2	2.9 ± 1.2
	Overall	85.4 ± 5.9	94.9 ± 1.5

[a]Values are mean ± S.D., $n = 5$.
[b]The elimination rate of cholesterol was calculated as follows: elimination rate of cholesterol = 100.0 − absolute recovery of cholesterol (%).

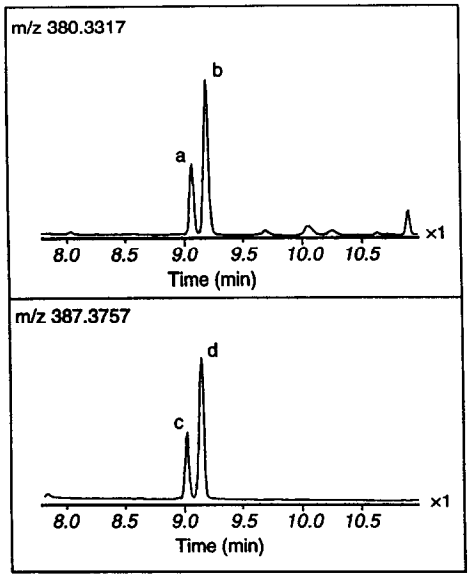

Fig. 2. High-resolution selected-ion recordings of 7α-hydroxy-4-cholesten-3-one and its [$^2H_7$]methyloxime dimethylethylsilyl ether derivatives in the extract from plasma of healthy volunteers (sample A, Table 2): (a) and (b) correspond to the *syn* and *anti* isomers of 7α-hydroxy-4-cholesten-3-one, and (c) and (d) correspond the *syn* and *anti* isomers of [$^2H_7$]7α-hydroxy-4-cholesten-3-one.

non-labelled form ([$^2H_0$]) and on *m/z* 387.3757 for its [$^2H_7$] variant. The peaks of the *syn* and *anti* isomers were completely separated and the ratios peak areas of the former and the latter were practically constant.

3.3. Calibration curve

The peak areas of [$^2H_0$] and the [$^2H_7$] variant were calculated as the sum of the *syn* and *anti* isomer peak areas. Good linearity was obtained in the range 10 pg–12 ng, and the detection limit of 7α-hydroxy-4-cholesten-3-one in human plasma was found to be 5 pg/ml.

3.4. Reproducibility of the method and recovery of 7α-hydroxy-4-cholesten-3-one

We studied the precision of the method by analyzing four samples in triplicate by GC–SIM-MS. The inter-assay coefficients of variation of the present method were found to be 5.16% for the between-sample variation and 2.54% for the within-sample variation, respectively. For the recovery experiment, known amounts of 7α-hydroxy-4-cholesten-3-one were spiked to four groups of duplicate samples. These samples were subjected to the clean-up procedure and SIM-MS was carried out in triplicate for each sample. The recoveries of the spiked known amounts of 7α-hydroxy-4-cholesten-3-one ranged from 93.6% to 100.4% (Table 2).

3.5. Correlation between plasma levels of 7α-hydroxy-4-cholesten-3-one and hepatic cholesterol 7α-hydroxylase activities

Simple regression analysis was done to study the correlation between the cholesterol 7α-hydroxylase activity and the levels of 7α-hydroxy-4-cholesten-3-one or unesterified, esterified, and total 7α-hydroxycholesterol using the same samples. Fig. 3 shows the highly significant correlation between the hepatic activity of cholesterol 7α-hydroxylase and the plasma levels of 7α-hydroxy-4-cholesten-3-one. There was also a significant correlation between the enzyme activity and the levels of free 7α-hydroxycholesterol ($r = 0.73$, $n = 16$, $p < 0.01$, $y = 15.84 + 0.52x$). However, we found no significant correlation between the enzyme activity and the plasma levels of total and esterified 7α-hydroxycholesterol (total: $r = 0.42$, $n = 16$; esterified: $r = 0.36$, $n = 16$).

3.6. The circadian rhythms of the levels of 7α-hydroxy-4-cholesten-3-one and 7α-hydroxycholesterol in human plasma

The circadian rhythms of the levels of 7α-hydroxy-4-cholesten-3-one and 7α-hydroxycholesterol are shown in Fig. 4A–D. The acrophase and amplitude of the circadian rhythms are shown in Table 3. The amplitude of the rhythms of 7α-hydroxy-4-cholesten-3-one level and free 7α-hydroxycholesterol were shown to be significant by a zero-amplitude test [15] ($p < 0.005$), whereas those of the total and esterified 7α-hydroxycholesterol were not found to be

Table 2
Precision and accuracy of quantitation of 7α-hydroxy-4-cholesten-3-one in human plasma

Sample ($X_0 + na$)[a]	Amount added (ng/ml)	Amount found (ng/ml)			Recovery[b] (mean ± S.D.) (%)	Estimated amount ± 95% confidence limit[c] (ng/ml)
A X_0	0	8.95	9.38	9.16		
B X_0	0	9.33	9.18	9.42	[$\bar{X}_0 = 9.24 \pm 0.18$]	8.31 ± 1.15
C $X_0 + a$	9	18.51	18.31	17.39		
D $X_0 + a$	9	17.13	17.76	16.88	93.63 ± 7.23	
E $X_0 + 2a$	18	26.08	25.89	26.75		
F $X_0 + 2a$	18	25.88	26.56	27.03	95.15 ± 2.69	
G $X_0 + 3a$	27	35.98	34.9	38.15		
H $X_0 + 3a$	27	36.07	36.5	36.5	100.4 ± 3.92	

[a] $a = 9.00$ (ng/ml), $n = 0, 1, 2, 3$.
[b] Recovery (%) = (amount found − $\bar{X}_0$)/amount added × 100.
[c] The estimated amount was calculated by linear regression analysis.

significant. The rhythm of the 7α-hydroxy-4-cholesten-3-one level was more smooth than that of the free 7α-hydroxycholesterol (Figs. 4A and B), indicating that the former is a better index than the latter. The fitting curves of 7α-hydroxy-4-cholesten-3-one and 7α-hydroxycholesterol are shown in Fig. 5. The curves for 7α-hydroxy-4-cholesten-3-one and free 7α-hydroxycholesterol are very similar, although the amplitude for 7α-hydroxy-4-cholesten-3-one was larger than that for free 7α-hydroxycholesterol.

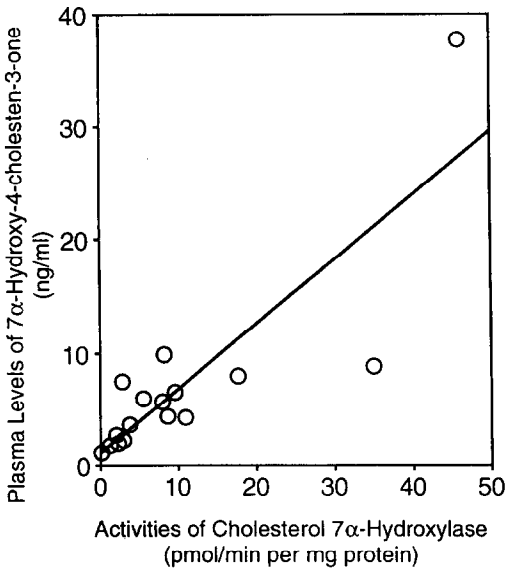

Fig. 3. Correlation between cholesterol 7α-hydroxylase activity and plasma levels of 7α-hydroxy-4-cholesten-3-one in patients with cholelithiasis or early gastrointestinal cancer. A significant correlation was found: $y = 1.17 + 0.56x$, $r = 0.84$, $p < 0.0001$, $n = 16$.

4. Discussion

To quantify trace amounts of 7α-hydroxy-4-cholesten-3-one in biological specimens using GC–MS, we had to solve the following problems: the instability of 7α-hydroxy-4-cholesten-3-one in alkaline or acidic media, and the coexistence of large amounts of cholesterol in plasma required tedious and time-consuming purification operations such as the combined use of thin-layer chromatography and HPLC [16]. Axelson *et al.* solved these problems using a reversed-phase column and determination by HPLC [6,7]. However, in their method the column must be maintained at 64°C for extraction from plasma, and [^{3}H]-labelled 25-hydroxyvitamin D_3 was used as internal standard for the HPLC quantification. In the method presented here, [$^2H_7$]7α-hydroxy-4-cholesten-3-one was added to plasma as an ideal internal standard for quantification by GC–MS. Acetonitrile was added to the diluted plasma, and then 7α-hydroxy-4-cholesten-3-one was extracted completely into the acetonitrile

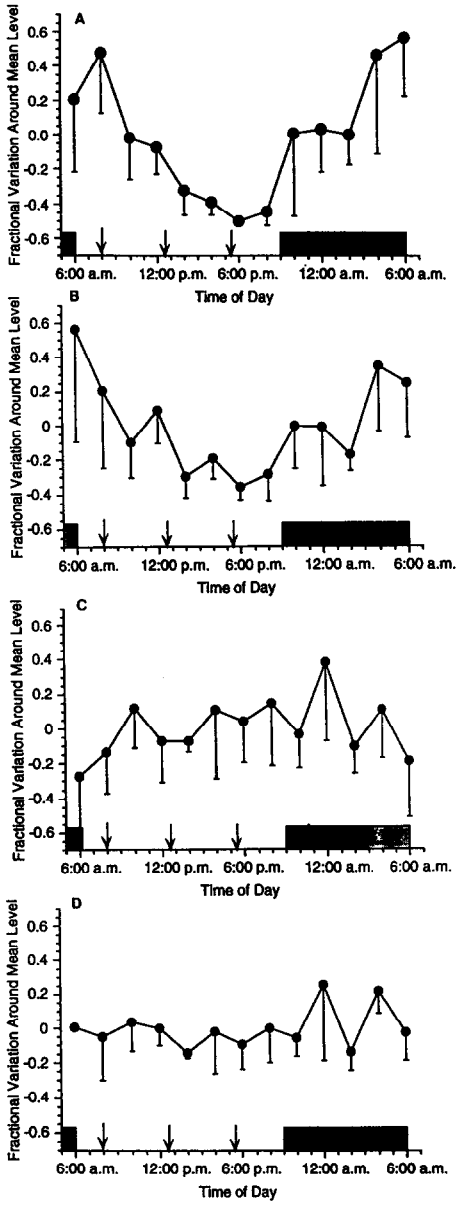

Fig. 4. The circadian rhythm of the plasma levels of 7α-hydroxy-4-cholesten-3-one and 7α-hydroxycholesterol in three normal volunteers. Blood samples were taken every 2 h from 6:00 a.m. on the first day to 6:00 a.m. on the second day. The volunteers ate normal hospital diets at 8:00 a.m., 12:30 p.m., and 5:30 p.m. (shown by arrows), and slept from 9:00 p.m. on the first day to 6:00 a.m. on the second day (shown by shaded box). The values are expressed as the fractional variations around the mean levels. Each bar represents mean ± S.D. at the indicated time of the day. (A) 7α-Hydroxy-4-cholesten-3-one; (B) free 7α-hydroxycholesterol; (C) esterified 7α-hydroxycholesterol; (D) total 7α-hydroxycholesterol. S.D. = standard deviation.

layer by salting-out extraction with ammonium sulfate [17]. The separated acetonitrile solution was diluted to 60–80% acetonitrile and then applied to a Bond Elut C_{18} cartridge at room temperature, and eluted by a acetonitrile–ethanol (4:1, v/v) solution. 7α-Hydroxy-4-cholesten-3-one was further purified by a Bond Elute SI cartridge in the normal-phase mode. More than 90% of the cholesterol was eliminated from the plasma by this procedure.

Oda *et al.* [5] reported that the levels of esterified and/or total 7α-hydroxycholesterol were better indices for the enzyme activity than the free forms. We have previously noted that the discrepancy between our results [9] and those of Oda *et al.* [5] leave some problems to be resolved. One of the problems may be caused by the difference in subjects. Oda *et al.* obtained blood samples and liver specimens at different times during the day: blood samples were obtained between 7:00 a.m. and 8:00 a.m.; liver specimens were taken between 9:00 a.m. and 10:00 a.m. [5]. The plasma levels of free 7α-hydroxycholesterol are decreased from 8:00 a.m. to 10:00 a.m., whereas the concentrations of esterified and total 7α-hydroxycholesterol were constant, according to our results. Furthermore, the patients studied by Oda *et al.* were treated with chenodeoxycholic acid (CDCA) and with cholestyramine for two weeks [5]. Pooler and Duane reported that CDCA inhibits total bile acid synthesis and delays the acrophase of the rhythm of bile acid synthesis [18]. In our study, we avoided these problems by studying only patients treated with drugs that do not affect the cholesterol 7α-hydroxylase activity, and by simultaneously sampling blood and collecting liver specimens.

It is widely known that bile acid biosynthesis has a circadian rhythm [18,19]. Pooler and Duane have reported that bile acid biosynthesis has a circadian rhythm and found the acrophase of this rhythm to be from 6:00 a.m. to 7:00 a.m. as determined by a tracer kinetics method [18]. Our study reveals that there was neither a significant acrophase nor an amplitude of the rhythms of the total and esterified 7α-hydroxycholesterol levels. This result indicates that the

Table 3
Fitting curve parameters of circadian rhythms of 7α-hydroxy-4-cholesten-3-one levels and 7α-hydroxycholesterol levels in plasma

Fitting curve[a] parameter	7α-Hydroxy-4-cholesten-3-one	7α-Hydroxycholesterol		
		Free	Esterified	Total
Acrophase (time of day)	5:35 a.m.	5:39 a.m.	8:17 p.m.	2:28 p.m.
Amplitude	0.45	0.32	0.12	0.07
ϕ	-1.46	-1.48	-5.31	-0.65
Zero-amplitude test[b] (F)	21.62[c]	9.28[c]	2.28	1.33

[a] $f(t) = A\cos(\omega t + \phi)$, A = amplitude; ω = angular frequency ($= 2\pi/24$ h); ϕ = acrophase.
[b] Zero-amplitude test was estimated by Nelson *et al.* [15].
[c] $p < 0.005$.

concentrations of these forms of 7α-hydroxycholesterol did not reflect the rhythm of bile acid biosynthesis. Under the above experimental conditions, the concentration of 7α-hydroxy-4-cholesten-3-one much better reflects the activity of cholesterol 7α-hydroxylase than that of the free 7α-hyroxycholesterol, since the coefficient of correlation between the level of 7α-hydroxy-4-cholesten-3-one and the enzyme activity was higher than that between the level of free 7α-hyroxycholesterol and the enzyme activity. In addition, the circadian rhythm of 7α-hydroxy-4-cholesten-3-one was much smoother than that of free 7α-hydroxycholesterol as shown in Fig. 4A–D and in Table 3.

In conclusion, a highly sensitive and selective method for the determination of 7α-hydroxy-4-cholesten-3-one in human plasma was developed. The plasma levels of 7α-hydroxy-4-cholesten-3-one reflect the hepatic activity of cholesterol 7α-hydroxylase without delay. The plasma concentrations of 7α-hydroxy-4-cholesten-3-one may be used as a highly reliable index for bile acid biosynthesis.

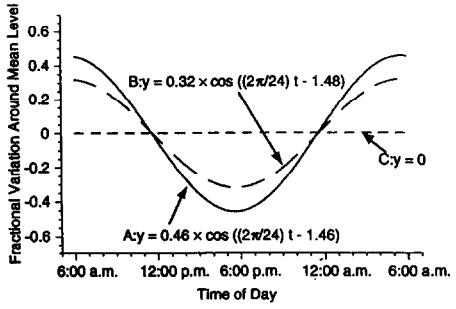

Fig. 5. The fitting curves of 7α-hydroxy-4-cholesten-3-one and 7α-hydroxycholesterol. The curve of 7α-hydroxy-4-cholesten-3-one and that of free 7α-hydroxycholesterol were very similar to each other. However, the amplitude of 7α-hydroxy-4-cholesten-3-one was higher than that of free 7α-hydroxycholesterol. (A) 7α-Hydroxy-4-cholesten-3-one, $y = 0.46\cos((2\pi/24)t - 1.46)$; (B) free 7α-hydroxycholesterol, $y = 0.32\cos((2\pi/24)t - 1.48)$; (C) esterified and total 7α-hydroxycholesterol, $y = 0$.

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6. References

[1] N.B. Myant and K.A. Mitropoulos, *J. Lipid Res.*, 18 (1977) 135–153.

[2] H. Danielsson, K. Einarsson and G. Johansson, *Eur. J. Biochem.*, 2 (1967) 44–49.

[3] H. Danielsson, *Steroids*, 20 (1972) 63–72.

[4] I. Björkhem, E. Reihnér, B. Angelin, S. Ewerth, J.-E. Åkerlund and K. Einarsson, *J. Lipid Res.*, 28 (1987) 889–894.

[5] H. Oda, H. Yamashita, K. Kosahara, S. Kuroki and F. Nakayama, *J. Lipid Res.*, 31 (1990) 2209–2218.

[6] M. Axelson, A. Aly and J. Sjövall, *FEBS Lett.*, 239 (1988) 324–328.

[7] M. Axelson, I. Björkhem, E. Reihnér and K. Einarsson, *FEBS Lett.*, 284 (1991) 216–218.

[8] A. Honda, J. Shoda, N. Tanaka, Y. Matsuzaki, T. Osuga, N. Shigematsu, M. Tohma and H. Miyazaki, *J. Chromatogr.*, 565 (1991) 53–66.

[9] T. Yoshida, A. Honda, N. Tanaka, Y. Matsuzaki, B. He, T. Osuga, N. Kobayashi, K. Ozawa and H. Miyazaki, *J. Chromatogr.*, 613 (1993) 185–193.

[10] L. Stárka, *Collect. Czech Chem. Commun.*, 26 (1961) 2454–2455.

[11] A. Shimasue, *Hiroshima J. Med. Sci.*, 23 (1974) 265–272.

[12] T. Eguchi, H. Miyazaki and F. Nakayama, *J. Chromatogr.*, 525 (1990) 25–42.

[13] G. Taguchi, *Introduction to Quality Engineering–Designing Quality into Products and Process*, Asian Productivity Organization, Tokyo, 1986.

[14] G.W. Snedecor and W.G. Cochran, *Statistical Methods*, The Iowa State University Press, Ames, 1967.

[15] W. Nelson, Y.L. Tong, J.-K. Lee and F. Halberg, *Chronobiologia*, 6 (1979) 305–323.

[16] I. Björkhem, S. Skrede, M.S. Buchmann, C. East and S. Grundy, *Hepatology*, 7 (1987) 266–271.

[17] W.G. Stillwell, A. Hung, M. Stafford and M.G. Horning, *Anal. Lett.*, 6 (1973) 407.

[18] P.A. Pooler and W.C. Duane, *Hepatology*, 8 (1988) 1140–1146.

[19] W.C. Duan, D.G. Levitt and S.M. Muller, *J. Clin. Invest.*, 72 (1983) 1930–1936.