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Simultaneous determination of mevalonate and 7α-hydroxycholesterol in human plasma by gas chromatography–mass spectrometry as indices of cholesterol and bile acid biosynthesis

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ABSTRACT

A very sensitive and specific method for the simultaneous determination of mevalonate and 7α-hydroxycholesterol in human plasma is described. The assay is based on isotope dilution mass spectrometry: the extracts from plasma were treated with benzylamine followed by dimethylethylsilylimidazole, then the resulting dimethylethylsilyl ether derivatives of mevalonylbenzylamide and 7α-hydroxycholesterol were determined by gas chromatography–mass spectrometry using high-resolution selected-ion monitoring. Simple regression analysis revealed significant correlations between the plasma level of mevalonate and the hepatic activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (EC 1.1.1.34) ($r = 0.83$, $P < 0.01$) and between the plasma level of free 7α-hydroxycholesterol and the hepatic activity of cholesterol 7α-hydroxylase (EC 1.14.13.7) ($r = 0.76$, $P < 0.05$).

INTRODUCTION

It is well known that the rate-limiting step in cholesterol biosynthesis is the conversion of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) into mevalonate (MVA) by HMG-CoA reductase (EC 1.1.1.34) [1,2], and that the first and rate-limiting step in the major pathway for conversion of cholesterol into bile acids is catalysed by cholesterol 7α-hydroxylase (EC 1.14.13.7) [3–5]. Consequently, cholesterol homeostasis in the human body is preserved mainly through the

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modulation of the activities of these two key enzymes. However, research on the regulation of cholesterol homeostasis in humans has been hampered by the difficulty of obtaining the liver specimens.

Einarsson *et al.* [6] and Björkhem *et al.* [7] reported that the serum levels of free 7α-hydroxycholesterol correlated well with the activities of cholesterol 7α-hydroxylase in cholelithiasis patients treated with cholestyramine. Recently, Oda *et al.* [8] reported that the cholesterol 7α-hydroxylase activities correlated higher with the serum level of the esterified 7α-hydroxycholesterol than that of the free form.

On the other hand, the plasma levels of MVA were first investigated by Hagenfeldt and Hellström [9] using gas chromatography–mass spectrometry (GC–MS). Parker *et al.* [10] and Popják *et al.* [11] found that plasma levels of MVA were positively correlated with the capacity of cholesterol synthesis in the rat using radioenzymatic assay [10–12]. Recently, Del Puppo *et al.* [13] described a method for the determination of MVA in human plasma using isotope dilution mass spectrometry in the electron impact (EI) ionization mode. However, their method has the disadvantage that it is difficult to determine exactly trace amounts of MVA in plasma owing to the lack of its selectivity and sensitivity. Scoppola *et al.* [14] extended this approach and developed a highly sensitive and specific method for the determination of MVA using capillary gas chromatography–mass spectrometry with electron-capture detection (GC–MS–ECD). However, their GC–MS–ECD method also has the disadvantage that it requires a tedious maintenance procedure to retain its high sensitivity.

In a previous paper [15], we reported for the simultaneous assay of the activity of HMG-CoA reductase and cholesterol 7α-hydroxylase by use of isotope dilution mass spectrometry. This paper describes a highly sensitive and selective method for the simultaneous determination of MVA and 7α-hydroxycholesterol in human plasma using the previous method [15] with some modification. Further, the relationships between the plasma levels of these products and the hepatic activities of the corresponding enzymes were studied in patients with cholelithiasis or early cancer of the gastrointestinal tract.

EXPERIMENTAL

Chemicals

Heptadeutero-7α-hydroxycholesterol, mevalonolactone (MVL)-d_7 and HMG-d_3-CoA were prepared as described previously [15]. Glucose-6-phosphate (G-6-P), G-6-P dehydrogenase and NADPH were purchased from Oriental Yeast (Tokyo, Japan), dithiothreitol (DTT) and butylated hydroxytoluene (BHT) from Wako (Osaka, Japan) and disodium ethylenediaminetetraacetate (EDTA) from Sigma (St. Louis, MO, USA).

Solvents and other reagents were of analytical-reagent grade. Bond Elut CN (cyanopropyl, 500 mg), SAX (quaternary amine, 500 mg) SI (non-bonded silica, 500 mg) and Mega Bond Elut C_{18} (octadecyl, 1000 mg) cartridges were obtained from Varian (Harbor City, CA, USA), Sephadex LH-20 from Pharmacia (Uppsala, Sweden) and dimethylethylsilylimidazole (DMESI) [16] from Tokyo Kasei Kogyo, (Tokyo, Japan).

Subjects

Ten patients (four males and six females) were studied. Three patients had cholelithiasis (one case of cholesterol stone and two cases of pigment stone) and seven patients had early cancer of the gastrointestinal tract. Their ages ranged from 39 to 72 (53.9 ± 10.8, mean ± S.D.) years. They had no evidence of abnormal lipid metabolism, liver dysfunction or overconsumption of alcohol. Informed consent was obtained from all patients and the study procedures were carried out in accordance with the ethical standards of the Helsinki Declaration.

Experimental procedure

The patients were admitted to hospital at least seven days prior to operation and were given a regular hospital diet. Surgical liver biopsy specimens (50–200 mg) and 2 ml of venous blood were obtained at the start of the operations, which were performed from 9 to 12 a.m. after a 14-h fast.

Preparation of liver microsomes

The specimen was immediately placed in ice-cold 50 m*M* Tris–HCl buffer (pH 7.4) containing 0.3 *M* sucrose, 10 m*M* DTT, 10 m*M* EDTA and 50 m*M* NaF. The microsomal fraction was prepared according to the procedure described previously [15]. The resulting microsomal fraction was suspended in 50 m*M* Tris HCl buffer (pH 7.4) containing 0.3 *M* sucrose, 5 m*M* DTT and 1 m*M* EDTA. The microsomal content of protein was determined by the method of Bradford [17].

Assay for the activities of microsomal cholesterol 7α-hydroxylase and HMG-CoA reductase

The microsomal fraction (50–200 mg of protein) was preincubated for 60 min at 37°C in a total volume of 225 μl containing 50 m*M* Tris–HCl buffer (pH 7.4), 0.3 *M* sucrose, 5 m*M* DTT, 1 m*M* EDTA, 12 m*M* G-6-P and 0.1 m*M* HMG-d_3-CoA. The assay was initiated with the addition of 1 U G-6-P dehydrogenase and 750 nmol of NADPH dissolved in 25 μl of preincubation buffer, making up to 3 m*M* NADPH solution. The incubation was carried out in duplicate for 15 min at 37°C and was stopped by the addition of 50 μl of 1 *M* NaOH. Sample purification by solid extraction and selected-ion monitoring (SIM) were performed according to the method described previously [15]. Activity of cholesterol 7α-hydroxylase was calculated by subtracting the amounts found in the zero-time assay from those produced at the end of incubation, and was expressed as pmol/min per mg protein.

Determination of MVA and free 7α-hydroxycholesterol in human plasma

The venous blood was transferred into tubes containing dry heparin and packed in ice. The chilled and heparinized blood was immediately centrifuged at 1000 *g* for 15 min. After centrifugation, 10 μg of BHT per ml of plasma were added. In the standard procedure, plasma was stored at −20°C until analysed.

For the MVA fraction, an 8-ng aliquot of MVL-d_7 in 25 μl of acetonitrile as an internal standard and 500 μl of acetonitrile were added to 500 μl of plasma. After ultrasonication for 15 min, the organic phase was collected. After addition of 50 μl of 1 *M* NaOH and 950 μl of distilled water, the mixture was applied to a Bond Elut SAX cartridge prewashed with 3 ml of 0.6 *M* HCl and 20 ml of distilled water, and the cartridge was washed with 3 ml of 10 m*M* phosphate buffer (pH 8.5). MVA was eluted with 1 ml of 0.6 *M* HCl. The eluate was kept at room temperature for 15 min to lactonize the resulting MVA. After complete lactonization, this fraction was applied to a Mega Bond Elut C_{18} (1000 mg) cartridge prewashed with 10 ml of chloroform, ethanol and distilled water, and the cartridge was eluted with 3 ml of acetonitrile. The eluate was evaporated to dryness under reduced pressure, and the resulting residue was dissolved in 2 ml of benzene. The solution was applied to a Bond Elut CN cartridge prewashed with 3 ml of dichloromethane and 3 ml of benzene, and the cartridge was washed with 3 ml of benzene–dichloromethane (1:1, v/v). MVL was eluted with 5 ml of dichloromethane. After evaporation to dryness under reduced pressure, the resulting residue was dissolved in 100 μl of benzene and 40 μl of benzylamine. The reaction mixture was kept at room temperature for 20 min, and 5 ml of benzene were added. The mixture was applied to a Bond Elut CN cartridge prewashed with 3 ml of benzene, and the cartridge was washed with 5 ml of benzene to remove the excess of benzylamine. The resulting mevalonylbenzylamide was eluted with 5 ml of ethyl acetate.

For the free 7α-hydroxycholesterol fraction, an 8-ng aliquot of 7α-hydroxycholesterol-d_7 in 25 μl of acetonitrile was added to 100 μl of plasma as an internal standard. After addition of 1.8 ml of absolute ethanol and 1.1 ml of distilled water, the mixture was extracted three times with 3 ml of *n*-hexane [8]. The *n*-hexane phase was evaporated to dryness under reduced pressure and the residue was dissolved in 0.3 ml of *n*-hexane–2-propanol (97:3, v/v), and applied to a Bond Elut SI (500 mg) cartridge prewashed with 3 ml of the above *n*-hexane–2-propanol solution. After washing with 6 ml of the *n*-hexane–2-propanol solution, 7α-hydroxycholesterol was eluted with 3 ml of *n*-hexane–2-propanol (80:20, v/v).

The resulting eluates of both fractions were mixed and evaporated to dryness. The residue was dissolved in 100 μl of distilled pyridine and the solution was treated with 30 μl of DMESI. The reaction mixture was kept at 80°C for 60 min and the excess of silylating reagent was removed using a Sephadex LH-20 column (60 mm × 6 mm I.D.) equilibrated with *n*-hexane–chloroform (1:1, v/v). The DMES ether derivatives of MVL-benzylamide and 7α-hydroxycholesterol were recovered in the first 3 ml of the eluate.

GC–MS was performed using a JMS-SX102 instrument equipped with a data processing system DA-6000 (JEOL, Tokyo, Japan). The column used was an Ultra Performance capillary column (25 × 0.32 mm I.D.) coated with methylsilicone (Hewlett-Packard, Avondale, PA, USA). The flow-rate of the carrier gas (helium) was 1.0 ml/min. The operating conditions were as follows: the column oven temperature, programmed from 160 to 194°C at 2°C/min after a 1-min delay from the start time, and from 194 to 280°C at 30°C/min; accelerating voltage, 10 kV; injector temperature, 300°C; separator and ion source temperature, 230°C; ionization energy, 70 eV; and trap current, 600 μA. SIM in the high-resolution mode was carried out with aid of the data system. The mass spectral resolution was more than *ca.* 10 000.

Determination of total 7α-hydroxycholesterol in human plasma

An 8-ng aliquot of 7α-hydroxycholesterol-d_7 in 25 μl of acetonitrile was added to 100 μl of plasma as an internal standard. Alkaline hydrolysis of plasma was performed with a mixture of 60 μl of 8.9 *M* KOH and 1 ml of absolute ethanol at 37°C for 60 min. After addition of 1.1 ml of distilled water and 0.8 ml of absolute ethanol, the mixture was extracted with *n*-hexane. The purification procedure was performed in the same way as for the free form of 7α-hydroxycholesterol.

The amount of esterified 7α-hydroxycholesterol was calculated by subtracting that of free forms from that of total 7α-hydroxycholesterol.

RESULTS

Representative SIM

Fig. 1 shows representative high-resolution selected-ion recordings obtained from human plasma. The monitoring ions used were at *m/z* 380.2077 and 387.2517 for MVL and its $^2H_7$ variant and *m/z* 470.3944 and 477.4383 for 7α-hydroxycholesterol and its $^2H_7$ variant.

GC–MS indicated that the contents of non-labelled 7α- and 7β-hydroxycholesterol and [$^2H_7$]7β variant in this deuterated internal standard were 1.05, 0.41 and 1.96%, respectively. Consequently, the peaks that appeared at 25.4 min on the selected-ion recording by monitoring at *m/z* 477 were attributed to endogenous substances in the plasma.

The purities of the MVL peaks in the selected-ion recordings were examined by utilizing an intensity-matching technique [18,19]. The peak-area ratios of the fragment ion $[M - C_2H_5]^+$ to the molecular ion $[M]^{+\cdot}$ corresponding to MVL-d_0 and its $^2H_7$ variant in the plasma and authentic compound were calculated as shown in Table I. The ratio of MVL-d_0 in the plasma was 1.23 times significantly higher than that in the authentic compound ($P < 0.01$, unpaired *t*-test). On the other hand, the ratio of MVL-d_7 in the plasma was also 1.12 times significantly higher than that in authentic compound ($P < 0.05$, unpaired *t*-test).

Reproducibility and recovery of MVA and 7α-hydroxycholesterol

Reproducibilities were investigated by analysing four samples in triplicate by SIM. The data were analysed according to a one-way layout [20], where the analytical errors were divided into two sources of sample preparation and SIM measurement. Tables II and III give the analytical data and analysis of variances for MVA and 7α-hydroxycholesterol. The latter variances were thought to be attributable to the SIM measurement, because the errors during sample preparation were negligible. The inter-assay coefficients of variation were found to be 5.6% for the between-sample preparations and 2.8% for the

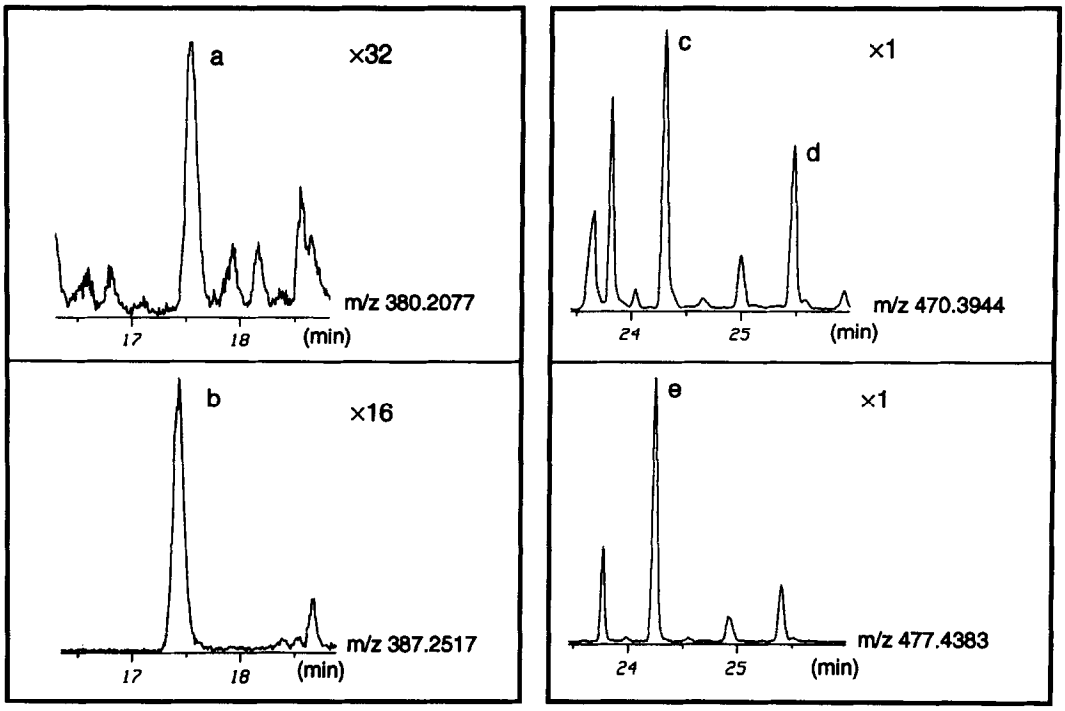

Fig. 1. High-resolution selected-ion recordings of the mevalonylbenzylamide DMES ether derivative and the 7α-hydroxycholesterol DMES ether derivative in the extracts from plasma of healthy volunteers (sample A, Table II): (a) MVL-d_0; (b) MVL-d_7; (c) 7α-hydroxycholesterol-d_0; (d) 7β-hydroxycholesterol-d_0; (e) 7α-hydroxycholesterol-d_7.

within-sample preparations of MVA, and 2.6% for the between-sample preparations and 4.2% for the within-sample preparations of 7α-hydroxycholesterol.

For the recovery experiment, known amounts of MVL and 7α-hydroxycholesterol were added to four groups of duplicate samples. These samples were subjected to the clean-up procedure,

TABLE I

INTENSITY RATIOS OF MEVALONOLACTONE IN AUTHENTIC STANDARD AND PLASMA SAMPLES

Intensity ratio = intensity of $[M - C_2H_5]^+$ peak / intensity of $[M]^{+\cdot}$ peak.

Sample	Authentic standard		Plasma	
	MVL-d_0	MVL-d_7	MVL-d_0	MVL-d_7
1	5.19	4.66	7.49	5.31
2	6.11	5.33	7.37	6.35
3	6.76	5.41	8.18	6.24
4	7.09	5.63	7.22	5.98
5	5.21	4.98	6.98	5.21
Mean ± S.D.	6.07 ± 0.87	5.20 ± 0.38	7.45 ± 0.45[a]	5.82 ± 0.53[b]

[a] Different from MVL-d_0 in authentic standard at $P < 0.01$.

[b] Different from MVL-d_7 in authentic standard at $P < 0.05$.

TABLE II

REPRODUCIBILITIES IN THE DETERMINATION OF MEVALONATE AND 7α-HYDROXYCHOLESTEROL IN PLASMA OF HEALTHY VOLUNTEERS: ANALYTICAL DATA

Sample	Mevalonate (ng/ml)		7α-Hydroxycholesterol (ng/ml)	
	Individual values	Mean ± S.D.	Individual values	Mean ± S.D.
A	5.43, 5.91, 5.78	5.91 ± 0.06	17.27, 16.12, 16.38	16.85 ± 1.13
B	5.55, 5.50, 5.36	5.47 ± 0.10	17.54, 16.49, 16.46	16.59 ± 1.13
C	5.98, 5.89, 5.86	5.71 ± 0.25	17.32, 17.41, 16.89	17.21 ± 0.60
D	5.87, 5.60, 5.91	5.79 ± 0.17	18.09, 16.57, 15.88	16.83 ± 0.28
Mean ± S.D.		5.72 ± 0.22		16.87 ± 0.66

TABLE III

REPRODUCIBILITIES IN THE DETERMINATION OF MEVALONATE AND 7α-HYDROXYCHOLESTEROL IN PLASMA OF HEALTHY VOLUNTEERS: ANALYSIS OF VARIANCE

S = residual sum of squares; f = number of degrees of freedom; V = unbiased variance; F_0 = observed value following F distribution variance ratio ($V_{\text{sample preparation}}/V_{\text{error}}$); C.V. = coefficient of variation.

Source	Mevalonate					7α-Hydroxycholesterol				
	S	f	V	F_0	C.V. (%)	S	f	V	F_0	C.V. (%)
Sample preparation	0.313	3	0.104	4.01	5.6	0.582	3	0.194	0.370	2.6
Error (SIM)	0.207	8	0.026		2.8	4.20	8	0.524		4.2
Total	0.520	11				4.78	11			

TABLE IV

RECOVERIES OF MEVALONOLACTONE AND 7α-HYDROXYCHOLESTEROL FROM PLASMA

Sample ($X_0 + na$) (n = 0, 1, 2, 3)	Mevalonolactone					7α-Hydroxycholesterol				
	Amount added (ng/ml)	Amount found (ng/ml)			Recovery[a] (mean ± S.D.) (%)	Amount added (ng/ml)	Amount found (ng/ml)			Recovery[a] (mean ± S.D.) (%)
A X_0	0.0	5.4	5.9	5.8		0.0	17.3	16.1	16.4	
B X_0	0.0	5.6	5.5	5.4	(X_0 = 5.6 ± 0.21)	0.0	17.5	16.5	16.5	(X_0 = 16.7 ± 0.56)
C $X_0 + a$	6.8	11.8	11.9	11.7		14.8	29.0	31.0	31.1	
D $X_0 + a$	6.8	12.0	11.1	11.9	91.0 ± 5.0	14.8	30.0	30.9	31.4	93.3 ± 6.1
E $X_0 + 2a$	13.6	18.8	18.1	18.3		29.7	44.5	44.1	43.7	
F $X_0 + 2a$	13.6	18.9	18.7	18.5	95.8 ± 2.4	29.7	44.6	45.3	44.9	93.8 ± 1.9
G $X_0 + 3a$	20.3	23.8	24.0	25.6		44.5	57.7	58.5	57.0	
H $X_0 + 3a$	20.3	25.5	25.3	25.9	95.6 ± 4.4	44.5	60.4	60.4	60.5	95.3 ± 3.5

[a] Recovery (%) = [(amount found − X_0)/amount added] × 100.

and SIM was carried out in triplicate for each sample. The recoveries of the spiked MVL ranged from 91.0 to 95.8% with a mean of 94.1%, and those of spiked 7α-hydroxycholesterol from 93.3 to 95.3% with a mean of 94.1%, as shown in Table IV.

Although amounts of the MVA and 7α-hydroxycholesterol derivatives less than 1 pg could be detected by this method, the practical detection limits in plasma samples were 180 pg/ml for MVA and 600 pg/ml for 7α-hydroxycholesterol.

Correlation between plasma levels of MVA and the hepatic HMG-CoA reductase activity

Fig. 2 shows the results of regression analysis for clarifying the correlation between the plasma levels of MVA and the hepatic activities of MHG-CoA reductase. There was a highly significant correlation between the enzyme activities and the levels of MVA ($r = 0.83$, $P < 0.01$).

Correlations between plasma levels of 7α-hydroxycholesterol and the cholesterol 7α-hydroxylase activity

Fig. 3 shows the correlation between the plasma levels of free 7α-hydroxycholesterol and the cholesterol 7α-hydroxylase activities. The levels of free 7α-hydroxycholesterol correlated significantly with the activities of cholesterol 7α-hydroxylase ($r = 0.76$, $p < 0.05$). On the other hand, there were no statistically significant correlations between the enzyme activities and the plasma levels of either total ($r = 0.51$, $p > 0.05$) or esterified ($r = 0.45$, $p > 0.05$) 7α-hydroxycholesterol, although tendencies of correlations were observed.

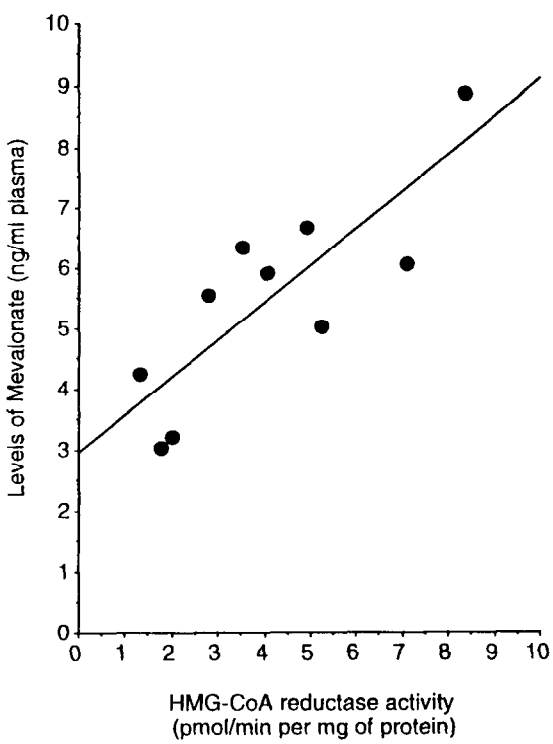

Fig. 2. Correlation between HMG-CoA reductase activities and plasma levels of MVA in the patients with cholelithiasis or early gastrointestinal cancer. A significant correlation was found: $y = 0.62x + 2.96$, $r = 0.83$, $p < 0.01$, $n = 10$.

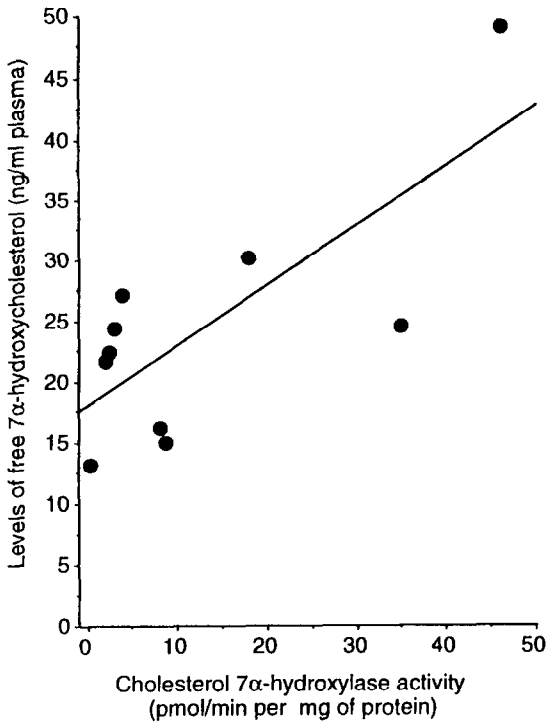

Fig. 3. Correlation between cholesterol 7α-hydroxylase activities and plasma levels of free 7α-hydroxycholesterol in the patients with cholelithiasis or early gastrointestinal cancer. A significant correlation was found: $y = 0.49x + 18.1$, $r = 0.76$, $p < 0.05$, $n = 10$.

DISCUSSIONS

In a previous paper [15], we reported a method for the simultaneous assay of the activities of HMG-CoA reductase and cholesterol 7α-hydroxylase by GC–SIM. In this study, the previous purification procedure was modified. Prior to applying the plasma sample to a Bond Elut SAX cartridge, acetonitrile was added to the plasma to extract MVA from plasma, and then sodium hydroxide solution was added to the solution diluted with distilled water. The resulting mixture was applied to a Bond Elut SAX cartridge, which is a strong anion exchanger, and MVA on the cartridge was eluted with 0.6 *M* HCl. In the previous method, a tedious and time-consuming operation was required to remove excess of benzylamine after amidation of MVL. This disadvantage was eliminated by using a Bond Elut CN cartridge: benzylamine was almost completely washed out from this cartridge with benzene, and MVL-benzylamide was eluted with 5 ml of ethyl acetate. The recoveries of the process through the benzylamidation of MVL and this purification by the solid cartridge were checked by the use of ^{14}C-labelled MVL (130 ng, 1.8 kBq) and found to be 80.8 ± 1.49% (n = 5).

In the present method, MVA and 7α-hydroxycholesterol were determined simultaneously from a small amount of the plasma. The determination of MVA in the human plasma by GC–SIM has been already reported by Del Puppo *et al.* [13] and Scoppola *et al.* [14]. However, it is difficult to determine trace amounts of MVA in plasma, *e.g.*, in patients treated with HMG-CoA reductase inhibitors, using the method reported previously [13], owing to interference by peaks from endogenous substances at the low mass used to monitor the trimethylsilyl derivative of MVA (m/z 145). In the method described by Scoppola *et al.* [14], the masses of the monitored ions are higher, resulting in very high sensitivity and selectivity. However, this GC–MS–ECD method requires a tedious procedure to maintain high sensitivity. The present method, using EI-MS at high resolution and monitoring of ions in a higher mass region, permits simultaneous determinations of trace amounts of MVA and 7α-hydroxycholesterol in plasma.

The validity of the determination of MVA in plasma by the present method was examined by using an intensity-matching technique [18,19]. The $[M - C_2H_5]^+/[M]^{+\cdot}$ ratios of MVL-d_0 and its $^2H_7$ variant in the plasma were significantly higher than those of authentic compounds, indicating that very small amounts of impurities originating from the plasma were contaminants in these peaks. However, these overestimations were reduced to less than 10%, because the determination was carried out by using the peak-area ratios of the MVL-d_0 to its $^2H_7$ variant on the selected-ion recordings. This indicates that this overestimation may be almost negligible.

Several papers concerning convenient indices for the activities of HMG-CoA reductase have been published. Parker and co-workers [10,11] reported first that the plasma levels of MVA in the rat were correlated with the activity of hepatic HMG-CoA reductase and they discussed the use of plasma MVA as an index of enzyme activity. The present study revealed that the levels of MVA in human plasma were significantly correlated with the hepatic activities of HMG-CoA reductase. The above findings led us to conclude that the plasma level of MVA is a useful index for HMG-CoA reductase activity also in humans.

As cholestrol is susceptible to autoxidation [21], we studied the influence of alkaline hydrolysis on the levels of 7α-hydroxycholesterol using BHT as an antioxidant at a level of 10 or 50 μg/ml in plasma and deoxygenation by bubbling argon into the reaction mixture. The levels of 7α- and 7β-hydroxycholesterol did not change significantly under these different conditions. A study of the time course of the hydrolysis showed that a maximum of 7α-hydroxycholesterol was reached after 30 min and remained unchanged until 150 min. These results indicate that there was no autoxidation of cholesterol during alkaline hydrolysis by the present method.

As reported by others [6–8], the level of free 7α-hydroxycholesterol was positively correlated with the activity of cholesterol 7α-hydroxylase. The levels of total or esterified 7α-hydroxychol-

esterol did not show this correlation with enzyme activity, although a similar tendency was observed. These results are in contrast to those reported by Oda *et al.* [8], who found that the levels of esterified and total 7α-hydroxycholesterol gave a more significant positive correlation with the enzyme activity of cholesterol 7α-hydroxylase than did the level of the free form. The reason for this discrepancy remains to be further investigated. Bascoul *et al.* [22] reported that the half-life of total plasma 7α-hydroxycholesterol was a good indicator for that of low-density lipoproteins. They did not determine relationships between plasma levels of 7α-hydroxycholesterol and enzyme activities.

In conclusion, a highly sensitive and selective method for the simultaneous determination of MVA and 7α-hydroxycholesterol in human plasma was developed, and the plasma levels of MVA and free 7α-hydroxycholesterol may be used as excellent indices for the activities of HMG-CoA reductase and cholesterol 7α-hydroxylase in human liver.

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REFERENCES

1 J. M. Dietschy and M. S. Brown, *J. Lipid Res.*, 15 (1974) 508–516.
2 V. W. Rodwell, J. L. Nordstrom and J. J. Mitschelen, *Adv. Lipid Res.*, 14 (1976) 1.
3 N. B. Myant and K. A. Mitropoulos, *J. Lipid Res.*, 18 (1977) 135–153.
4 H. Danielsson, K. Einarsson and G. Johansson, *Eur. J. Biochem.*, 2 (1967) 44–49.
5 H. Danielsson, *Steroids*, 20 (1972) 63–72.
6 K. Einarsson, B. Angelin, S. Ewerth, K. Nilsell and I. Björkhem, *J. Lipid Res.*, 27 (1986) 82–88.
7 I. Björkhem, E. Reihnér, B. Angelin, S. Ewerth, J.-E. Åkerlund and K. Einarsson, *J. Lipid Res.*, 28 (1987) 889–894.
8 H. Oda, H. Yamashita, K. Kosahara, S. Kuroki and F. Nakayama, *J. Lipid Res.*, 31 (1990) 2209–2218.
9 L. Hagenfeldt and K. Hellström, *Life Sci.*, 11 (1972) 669–676.
10 T. S. Parker, D. J. McNamara, C. Brown, O. Garrigan, R. Kolb, H. Batwin and E. H. Ahrens, Jr., *Proc. Natl. Acad. Sci U.S.A.*, 79 (1982) 3037–3041.
11 G. Popják, G. Boehm, T. S. Parker, J. Edmond, P. A. Edwards and A. M. Fogelman, *J. Lipid Res.*, 20 (1979) 716–728.
12 R. R. Kopito, S. B. Weinstock, L. E. Freed, D. M. Murray and H. Brunengraber, *J. Lipid Res.*, 23 (1982) 577–583.
13 M. Del Puppo, G. Cighetti, M. Galli Kienle and L. De Angelis, *Biomed. Environ. Mass Spectrom.*, 13 (1989) 174 176.
14 A. Scoppola, V. M. G. Maher, G. R. Thompson, N. B. Rendell and G. W. Taylor, *J. Lipid Res.*, 32 (1991) 1057–1060.
15 A. Honda, J. Shoda, N. Tanaka, Y. Matsuzaki, T. Osuga, N. Shigematsu, M. Tohma and H. Miyazaki, *J. Chromatogr.*, 565 (1991) 53–66.
16 H. Miyazaki, M. Ishibashi, M. Itoh and T. Nambara, *Biomed. Mass Spectrom.*, 4 (1977) 23–35.
17 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248–254.
18 H. Miyazaki, M. Ishibashi, G. Idzu and T. Izawa, *Chem. Pharm. Bull.*, 23 (1975) 1806–1813.
19 H. Miyazaki, M. Ishibashi and K. Yamashita, *Biomed. Mass Spectrom.*, 5 (1978) 469–476.
20 G. Taguchi, *Introduction to Quality Engineering – Designing Quality into Products and Process*, Asian Productivity Organization, Tokyo, 1986.
21 L. L. Smith, *Cholesterol Autooxidation*, Plenum Press, New York, 1977.
22 J. Bascoul, C. Gozé, N. Domergue and A. Crastes de Paulet, *Biochim. Biophys. Acta*, 1044 (1990) 357–360.