(≥ 80) 27-hydroxycholesterol concentrations than in those with low (< 80) concentrations [+7.1 ± 3.6% (n = 16) vs. −4.0 ± 2.6% (n = 14); $P < 0.05$, significantly different by both the two-sample Student t-test and the Mann–Whitney test].

DISCUSSION

SERUM CONCENTRATIONS OF several marker sterols reflect cholesterol metabolism in the body. First, serum concentrations (relative to cholesterol) of plant sterols, sitosterol and campesterol, are positively correlated with the fractional absorption of dietary cholesterol and negatively correlated with fecal endogenous cholesterol outputs.[27,28] Second, serum concentration (relative to cholesterol) of lathosterol, a cholesterol precursor, reflects whole body cholesterol synthesis[29] or hepatic activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the cholesterol biosynthesis.[30] Third, serum concentration (relative to cholesterol) of 7α-hydroxy-4-cholesten-3-one, an intermediate in bile acid synthesis, has been used as a marker for hepatic activity of cholesterol 7α-hydroxylase (CYP7A1),[23] the rate-limiting enzyme in the classic bile acid biosynthetic pathway, and total bile acid synthesis.[31] To explore a biomarker that might predict responsiveness to cholesterol intake, we tried to measure serum baseline concentrations of the above sterols. However, they were not at all correlated with the percent change of LDL cholesterol by cholesterol loading (r_s = 0.000–0.138, P = 1.000–0.466).

Serum 27-hydroxycholesterol is another candidate for a predictor of cholesterol responsiveness. In fact, although the result did not reach statistical significance, a higher correlation coefficient was obtained between baseline 27-hydroxycholesterol concentrations and the percent change of LDL cholesterol because of cholesterol loading (r_s = 0.321, P = 0.083). This sterol is synthesized by CYP27A1 that is expressed in many tissues, including liver,[32] intestine,[32] vascular endothelium,[33] macrophages,[34] and atherosclerotic plaque.[35] CYP27A1 seems to protect the human body from cholesterol overload by at least three concurrent but separate mechanisms. The first mechanism operates via a suppression of cholesterol biosynthesis[36] by the inhibition of SREBP2 processing.[37] The second, 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid (immediate metabolite of 27-hydroxycholesterol by the same CYP27A1), are more polar than cholesterol and are transported into the liver and metabolized to bile acids more easily than cholesterol.[38] The third, 27-hydroxycholesterol, is one of the endogenous ligands for LXRα and inhibits the accumulation of cholesterol by activating this nuclear receptor.[39]

Cholesterol homeostasis in mammals is maintained by a balance between absorption from the intestine, *de novo* synthesis in the liver and extrahepatic tissues, and excretion to the bile as cholesterol or bile acids.[40] Our results that subjects with high baseline serum 27-hydroxycholesterol concentrations (≥ 80 ng/mg cholesterol) showed a higher percent change of LDL cholesterol by cholesterol loading suggest that these subjects had positive cholesterol balance in the body and less extra capacity to preserve serum LDL cholesterol concentrations after cholesterol loading. Thus serum 27-hydroxycholesterol concentrations seemed to predict to some extent the responsiveness to dietary cholesterol.

Since serum 27-hydroxycholesterol concentrations were fairly stable and not significantly affected by cholesterol loading (Fig. 3), the concentration appears to be determined by endogenous factors rather than dietary cholesterol. Therefore it may also be true that a restriction of cholesterol results in a more effective reduction of serum LDL cholesterol in subjects with high serum 27-hydroxycholesterol concentrations compared to those with low concentrations. An interesting finding in the present study is that the subjects with low 27-hydroxycholesterol concentrations (< 80 ng/mg cholesterol) were all females, and 65% of the subjects with high 27-hydroxycholesterol concentrations (≥ 80) were males (Table 2). Several studies have suggested that a low cholesterol diet reduces serum total cholesterol and LDL cholesterol concentrations more greatly in males than in females,[41,42] which may be explained in part by our idea that serum 27-hydroxycholesterol concentrations predict the effects of cholesterol restriction on serum LDL cholesterol.

The importance of serum 27-hydroxycholesterol concentrations and CYP27A1 activity in hepatic and extrahepatic tissues for the response to dietary cholesterol has also been pointed out in experiments using baboons.[43,44] However, the conclusions are completely different from humans. In baboons, baseline serum 27-hydroxycholesterol concentrations were not significantly different between high and low responders, and with a high-cholesterol diet, a significant elevation of 27-hydroxycholesterol concentrations was observed only in the low-responding baboons. Although we excluded subjects with hypocholesterolemia in our study, it may be possible that the treatment of hypocholesterolemic patients with high-cholesterol diets

shows results similar to those of the baboons because basal serum LDL cholesterol concentrations in baboons are very low (less than HDL cholesterol).

In our results, the concentrations of HDL cholesterol were significantly low in subjects with high serum 27-hydroxycholesterol concentrations (Table 2). LXRα upregulates the expression of cholesteryl ester transfer protein (CETP) and CETP transfers cholesteryl ester from HDL to other lipoproteins, so that serum HDL cholesterol levels are reduced. Thus high serum 27-hydroxycholesterol concentrations may reflect the activation of LXRα *in vivo*. A recent report by Higuchi *et al.*[45] suggests that the activation of LXRα is one of the important factors that cause nonalcoholic fatty liver disease (NAFLD) in humans. Further investigations are expected to use serum oxysterol markers for the evaluation of hepatic LXRα activity.

In summary, serum high 27-hydroxycholesterol concentrations were thought to reflect positive cholesterol balance in the body and predict, to some extent, a responsiveness to dietary cholesterol loading. A determination of serum 27-hydroxycholesterol concentrations seems to be useful in predicting tolerance to a high-cholesterol diet and the effects of cholesterol restriction therapies.

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methods

Highly sensitive quantification of key regulatory oxysterols in biological samples by LC-ESI-MS/MS[S]

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Abstract **We describe a highly sensitive and specific method for the quantification of key regulatory oxysterols in biological samples. This method is based upon a stable isotope dilution technique by liquid chromatography-tandem mass spectrometry (LC-MS/MS). After alkaline hydrolysis of human serum (5 μl) or rat liver microsomes (1 mg protein), oxysterols were extracted, derivatized into picolinyl esters, and analyzed by LC-MS/MS using the electrospray ionization mode. The detection limits of the picolinyl esters of 4β-hydroxycholesterol, 7α-hydroxycholesterol, 22R-hydroxycholesterol, 24S-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, and 24S,25-epoxycholesterol were 2–10 fg (5–25 amol) on-column (signal-to-noise ratio = 3). Reproducibilities and recoveries of these oxysterols were validated according to one-way layout and polynomial equation, respectively. The variances between sample preparations and between measurements by this method were calculated to be 1.8% to 12.7% and 2.9% to 11.9%, respectively. The recovery experiments were performed using rat liver microsomes spiked with 0.05 ng to 12 ng of oxysterols, and recoveries of the oxysterols ranged from 86.7% to 107.3%, with a mean recovery of 100.6%. This method provides reproducible and reliable results for the quantification of oxysterols in small amounts of biological samples.**— Honda, A., K. Yamashita, T. Hara, T. Ikegami, T. Miyazaki, M. Shirai, G. Xu, M. Numazawa, and Y. Matsuzaki. **Highly sensitive quantification of key regulatory oxysterols in biological samples by LC-ESI-MS/MS.** *J. Lipid Res.* **2009.** 50: **350–357.**

Supplementary key words liquid chromatography-tandem mass spectrometry • electrospray ionization • 24S,25-epoxycholesterol • 4β-hydroxycholesterol • 7α-hydroxycholesterol • 22R-hydroxycholesterol • 24S-hydroxycholesterol • 25-hydroxycholesterol • 27-hydroxycholesterol

Biological samples contain a large number of oxysterols (1), and most of them are formed from cholesterol by enzymatic oxidation (2–6) (**Fig. 1**) or autoxidation (7). By contrast, the oxysterol 24S,25-epoxycholesterol is not derived from cholesterol but is produced de novo from acetyl-CoA via a shunt in the mevalonate pathway (8).

These oxysterols are important molecules for preserving lipid homeostasis in the body. 7α-Hydroxycholesterol is a product of CYP7A1, which is the rate-limiting enzyme in the classic bile acid biosynthetic pathway. 27-Hydroxycholesterol, 24S-hydroxycholesterol, 4β-hydroxycholesterol, 22R-hydroxycholesterol, and 24S,25-epoxycholesterol are effective endogenous ligands of the nuclear receptors liver X receptor α (LXRα) and LXRβ (9–11). In addition, 27-hydroxycholesterol (12), 25-hydroxycholesterol (13), and 24S,25-epoxycholesterol (14) are known to downregulate the cholesterol biosynthetic pathway, presumably by blocking the processing of the sterol-regulatory element binding protein.

GC-MS has historically been used for the analyses of oxysterols in serum and tissues (1, 15) because the sensitivity and specificity of conventional GC with flame ionization detector is not sufficient to quantify oxysterols in biological samples. However, GC-MS is still not an ideal method, especially for the analysis of 24S,25-epoxycholesterol, because this epoxycholesterol does not survive the temperature required for GC analysis (16). Another approach to quantifying oxysterols in biological samples was HPLC with ultraviolet (UV) detection after derivatization to the Δ^4-3-ketones (16–19). This method made it possible to detect

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Abbreviations: CTX, cerebrotendinous xanthomathosis; ESI, electrospray ionization; LC-APCI-MS, liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LXRα, liver X receptor α; SRM, selected reaction monitoring; TMS, trimethylsilyl.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of three tables.

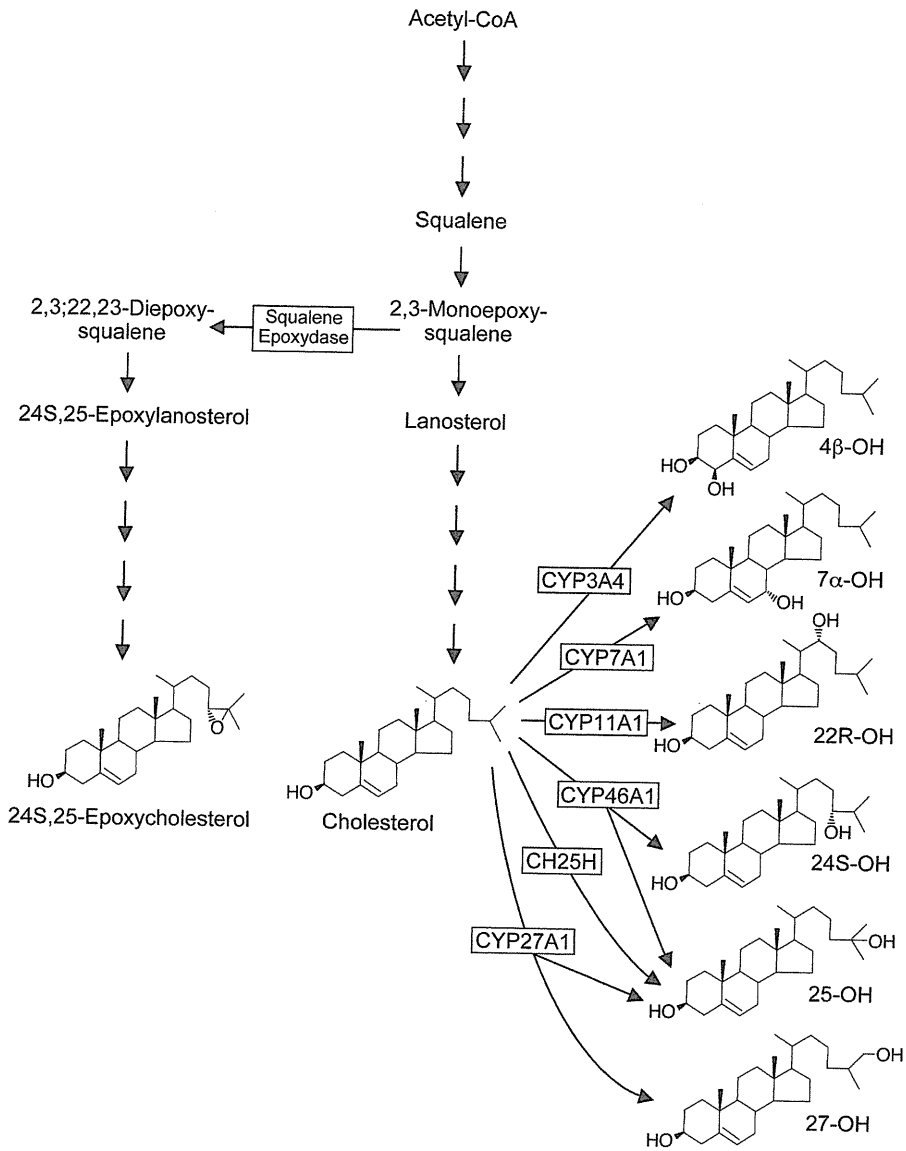

Fig. 1. Biosynthetic pathways for key regulatory oxysterols. Hydroxycholesterols are synthesized from cholesterol, whereas 24S,25-epoxycholesterol is derived from a shunt in the cholesterol biosynthetic pathway. CH25H, cholesterol 25-hydroxylase; 4β-OH, 4β-hydroxycholesterol; 7α-OH, 7α-hydroxycholesterol; 22R-OH, 22R-hydroxycholesterol; 24S-OH, 24S-hydroxycholesterol; 25-OH, 25-hydroxycholesterol; and 27-OH, 27-hydroxycholesterol.

the 24S,25-epoxycholesterol derivative as an intact form, but the lower limit of detection for the Δ^4-3-ketones of oxysterols was about 2 ng on-column (16), which was not sufficient for quantification of the oxysterols in a small amount of biological sample.

Recently, liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) was introduced as a sensitive, specific, and rapid method for the quantification of oxysterols (20, 21). In addition, LC-tandem mass spectrometry (LC-MS/MS) using electrospray ionization (ESI) has also been applied to the analysis of oxysterols (22). In general, ESI is not the best ionization method for neutral steroids because of its poor ionization efficiency. However, our recent study demonstrated that the derivatization of monohydroxysterols into picolinyl esters markedly enhanced the ionization efficiency in the ESI process, and the method was much more sensitive than the assay of native monohydroxysterols by LC-APCI-MS/MS (23). In this study, we have applied our derivatization method to dihydroxy- and epoxysterols. In each case, singly charged ions were observed as the base peaks in the positive ESI mass spectra and amol levels of these oxysterols were detectable.

MATERIALS AND METHODS

Chemicals

4β-Hydroxycholesterol (cholest-5-en-3β,4β-diol), 7α-hydroxycholesterol (cholest-5-en-3β,7α-diol), 22R-hydroxycholesterol (cholest-5-en-3β,22R-diol), 24S-hydroxycholesterol (cholest-5-en-3β,24S-diol), 25-hydroxycholesterol (cholest-5-en-3β,25-diol),

and 24S,25-epoxycholesterol (cholest-5-en-24S,25-epoxy-3β-ol) were purchased from Steraloids (Wilton, NH). [25,26,26,26,27,27,27-$^2H_7$]4β-hydroxycholesterol, [26,26,26,27,27,27-$^2H_6$]24-hydroxycholesterol, [27,27,27-$^2H_3$]25-hydroxycholesterol, and [26,26,26,27,27,27-$^2H_6$]24,25-epoxycholesterol were obtained from Avanti Polar Lipids (Alabaster, AL). 27-Hydroxycholesterol [(25R)-cholest-5-en-3β,26-diol], [25,26,26,26,27,27,27-$^2H_7$]27-hydroxycholesterol, and [25,26,26,26,27,27,27-$^2H_7$]7α-hydroxycholesterol were prepared as described previously (24).

Picolinic acid and 2-methyl-6-nitrobenzoic anhydride were purchased from Tokyo Kasei Kogyo (Tokyo, Japan), and 4-dimethylaminopyridine and triethylamine were obtained from Wako Pure Chemical Industries (Osaka, Japan). Additional reagents and solvents were of analytical grade.

Sample collection

Blood samples were collected from healthy human volunteers and from a patient with cerebrotendinous xanthomatosis (CTX). After coagulation and centrifugation at 1,500 *g* for 10 min, serum samples were stored at −20°C until analysis. Informed consent was obtained from all subjects, and the experimental procedures were conducted in accordance with the ethical standards of the Helsinki Declaration. Rat liver microsomes were prepared in our previous study (25) and had been stored at −70°C until they were used in the present experiments.

Sample preparation

[$^2H_7$]4β-hydroxycholesterol (5 ng), [$^2H_7$]7α-hydroxycholesterol (10 ng) [$^2H_6$]24-hydroxycholesterol (5 ng), [$^2H_3$]25-hydroxycholesterol (1 ng), [$^2H_7$]27-hydroxycholesterol (10 ng), and [$^2H_6$]24,25-epoxycholesterol (1 ng) as internal standards and 5 μg of butylated hydroxytoluene were added to serum (5 μl) or microsomes (1 mg protein), and saponification was carried out in 0.5 ml of 1 N ethanolic KOH at 37°C for 1 h. After the addition of 0.25 ml of distilled water, sterols were extracted twice with 1 ml of *n*-hexane, and the extract was evaporated to dryness under a stream of nitrogen. Derivatization to the picolinyl ester was performed according to our previous method (23) with minor modifications. The reagent mixture for derivatization consisted of 2-methyl-6-nitrobenzoic anhydride (100 mg), 4-dimethylaminopyridine (30 mg), picolinic acid (80 mg), pyridine (1.5 ml), and triethylamine (200 μl). The freshly prepared reagent mixture (170 μl) was added to the sterol extract, and the reaction mixture was incubated at 80°C for 60 min. After the addition of 1 ml of *n*-hexane, the mixture was vortexed for 30 s and centrifuged at 700 *g* for 3 min. The clear supernatant was collected and evaporated at 80°C under nitrogen. The residue was redissolved in 50 μl of acetonitrile, and an aliquot (1 μl) was injected into the following LC-MS/MS system.

LC-MS/MS analysis

The LC-MS/MS system consisted of a TSQ Quantum Ultra quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an H-ESI probe and a Nanospace SI-2 HPLC system (Shiseido, Tokyo, Japan). Chromatographic separation was performed using a Hypersil GOLD column (150 × 2.1 mm, 3 μm, Thermo Electron) at 40°C, and the following gradient system was used at a flow rate of 300 μl/min: initially, the mobile phase was composed of acetonitrile-methanol-water (40:40:20, v/v/v) containing 0.1% acetic acid; then it was programmed in a linear manner to acetonitrile-methanol-water (45:45:10, v/v/v) containing 0.1% acetic acid over 20 min. The final mobile phase was kept constant for an additional 20 min.

The general LC-MS/MS conditions were as follows: spray voltage, 1,000 V; vaporizer temperature, 350°C; sheath gas (nitrogen) pressure, 85 psi; auxiliary gas (nitrogen) flow, 60 arbitrary units; ion transfer capillary temperature, 350°C; collision gas (argon) pressure, 1.5 mTorr; and ion polarity, positive. Selected reaction monitoring (SRM) was conducted using the characteristic precursor-to-product ion transition under optimized collision energy, as listed in **Table 1**.

Statistics

Data are reported as the mean ± SD. Reproducibility was analyzed by one-way layout (JMP software; SAS Institute Inc., Cary, NC). Recovery was analyzed using a polynomial equation (26). Linearity of the calibration curves was analyzed by simple linear regression. Regression analysis was also used to calculate the estimated amount ±95% confidence limit in the recovery study. For all analyses, significance was accepted at the level of $P < 0.05$.

RESULTS

Selection of monitoring ions for SRM

Seven oxysterols were converted into the corresponding picolinyl ester derivatives and positive ESI-MS, MS/MS,

TABLE 1. Positive ESI-MS, MS/MS, SRM, and HPLC data of the picolinoyl ester derivative of each oxysterol

Oxysterols (Derivatives)	MS Data $[M+Na]^+$ (Relative Intensity)	MS/MS Data[a] (Collision Energy at Maximum Intensity)	SRM Data[b] Collision Energy	SRM Data[b] Precursor to Product	SRM Data[b] S/N[c]	HPLC Data[b] (RRT[d])
	m/z (%)	*m/z* (*V*)	*V*	*m/z*		
4β-Hydroxycholesterol (cholest-5-en-3β,4β-dipicolinates)	635 (100)	146 (22) 512 (20)	22	635 → 146	200	0.77
7α-Hydroxycholesterol (cholest-5-en-3β,7α-dipicolinates)	635 (100)	146 (15) –[e]	15	635 → 146	200	0.62
22R-Hydroxycholesterol (cholest-5-en-3β,22R-dipicolinates)	635 (100)	146 (26) 512 (22)	22	635 → 512	40	0.45
24S-Hydroxycholesterol (cholest-5-en-3β,24S-dipicolinates)	635 (100)	512 (22) 146 (31)	22	635 → 512	80	0.48
25-Hydroxycholesterol (cholest-5-en-3β,25-dipicolinates)	635 (100)	512 (19) 146 (28)	22	635 → 512	40	0.51
27-Hydroxycholesterol (cholest-5-en-3β,27-dipicolinates)	635 (100)	512 (12) 146 (33)	22	635 → 512	80	0.56
24S,25-Epoxycholesterol (cholest-5-en-24S,25-epoxy-3β-picolinate)	528 (100)	146 (20) —[e]	20	528 → 146	80	0.41

ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry; RRT, relative retention time; S/N, signal-to-noise ratio; SRM, selected reaction monitoring.

[a] $[M+Na]^+$ was used as a precursor ion for each MS/MS analysis. Major product ions were arranged in the order of abundance from left to right.

[b] The same HPLC column and flow rate described in Materials and Methods were employed.

[c] S/Ns were determined by injecting 100 fg of each derivative.

[d] RRTs are expressed relative to the retention time of cholesterol 3β-picolinate.

[e] Intense ion (>5% of base peak) was not observed.

SRM, and HPLC data were obtained for each of them (Table 1). All picolinyl ester derivatives exhibited $[M+Na]^+$ ions as the base peaks. The fragmentation pattern of the base peak ion of each derivative was examined under various levels of collision energy, and [M+Na−picolinic acid $(C_6H_5NO_2)]^+$ (m/z = 512) or [picolinic acid $(C_6H_5NO_2)$+ $Na]^+$ (m/z = 146) ions were observed as the most-abundant product ions, so that they were selected as monitoring ions for authentic oxysterols by SRM. The monitoring ions and optimal collision energies for deuterated internal standards were m/z 642 → 146 (22 V) for 3β,4β-dipicolinates of [$^2H_7$] 4β-hydroxycholesterol, m/z 642 → 146 (15 V) for 3β,7α-dipicolinates of [$^2H_7$]7α-hydroxycholesterol, m/z 641 → 518 (22 V) for 3β,24-dipicolinates of [$^2H_6$]24-hydroxycholesterol, m/z 638 → 515 (22 V) for 3β,25-dipicolinates of [$^2H_3$]25-hydroxycholesterol, m/z 642 → 519 (22 V) for 3β,27-dipicolinates of [$^2H_7$]27-hydroxycholesterol, and m/z 534 → 146 (20 V) for 3β-picolinate of [$^2H_6$]24,25-epoxycholesterol.

Sensitivity of the present method

To determine the sensitivity of our SRM method, the standard mixture solution of the seven oxysterol derivatives was diluted and injected into the LC-MS/MS system. The limit of detection (signal-to-noise ratio of 3) of each steroid was 2 fg (5 amol) on-column for 4β-hydroxycholesterol and 7α-hydroxycholesterol, 5 fg (12.5 amol) on-column for 24S-hydroxycholesterol, 27-hydroxycholesterol, and 24S,25-epoxycholesterol, and 10 fg (25 amol) on-column for 22R-hydroxycholesterol and 25-hydroxycholesterol.

Calibration curves

A calibration plot was established for each oxysterol. Different amounts of authentic oxysterol were mixed with deuterated internal standard, derivatized to the picolinyl ester, and quantified as described in the Materials and Methods. The weight ratio of each oxysterol, relative to the corresponding deuterated internal standard, was plotted on the abscissa, and the peak area ratio of the picolinyl ester of the authentic oxysterol to the deuterated variant measured by SRM was plotted on the ordinate. Because deuterium-labeled 22R-hydroxycholesterol was not available, [$^2H_6$]24-hydroxycholesterol was used as an internal standard for 22R-hydroxycholesterol. The linearity of the standard curves, as determined by simple linear regression, was excellent, as shown in **Table 2.**

Representative SRM

The separation of various authentic oxysterol picolinates by SRM is shown in **Fig. 2A.** All oxysterol picolinates tested were successfully separated. 7β-Hydroxycholesterol, an autoxidation product of cholesterol, gave a peak just before 7α-hydroxycholesterol (not shown in the figure), and the retention times (relative to cholesterol) of these oxysterols (as picolinates) were 0.61 and 0.62, respectively. Figure 2B–D shows typical SRM chromatograms obtained from 1 mg of protein from rat liver microsomes (Fig. 2B) and 5 μl of sera from a control subject (Fig. 2C) and a CTX patient (Fig. 2D). In rat liver microsomes, a significant amount of 24S,25-epoxycholesterol was detected, whereas only a trace amount of 24S-hydroxycholesterol was observed. In contrast, human serum contained a very low concentration of 24S,25-epoxycholesterol, but a significant amount of 24S-hydroxycholesterol was present. When serum oxysterol profiles were compared between controls and CTX, markedly reduced serum 25- and 27-hydroxycholesterol concentrations were observed.

Precision and accuracy of the present method

The following studies were performed to determine the precision and accuracy of the present method using rat liver microsomes. Reproducibility was investigated by analyzing four samples in triplicate by LC-MS/MS (**Table 3**). The results were analyzed by a one-way layout, in which the analytical errors were divided into two sources: sample preparation and SRM measurement. The variances were not considered to be attributable to the sample preparation, because the errors during sample preparation were not significantly larger than those between the measurements (see supplementary Tables I, II). The inter-assay coefficients of variation for the between- and within-sample variations were 1.8% to 12.7% and 2.9% to 11.9%, respectively.

For the recovery experiment, known amounts of oxysterols (a, 2a, 3a; a = 0.05–4.0 ng) were spiked into 1 mg of rat liver microsomal protein (n = 2). After alkaline hydrolysis and derivatization, LC-MS/MS was carried out in triplicate for each sample. The recoveries of the known spiked amounts of the oxysterols ranged from 86.7% to 107.3%, with a mean of 100.6% (**Table 4**). In addition, the amounts of each endogenous oxysterol found in 1 mg of unspiked microsomal protein were within the 95% confidence limit for the estimated amount of each

TABLE 2. Linearities of calibration plots for each oxysterol

Oxysterol	Range (n)	Linear Regression Equation[a]	Correlation Coefficient (r)
	ng		
4β-Hydroxycholesterol	0.05 – 10 (7)	$Y = 0.436X - 0.009$	0.999
7α-Hydroxycholesterol	0.1 – 20 (7)	$Y = 1.075X - 0.011$	1.000
22R-Hydroxycholesterol	0.05 – 5 (6)	$Y = 0.084X - 0.000$	0.993
24S-Hydroxycholesterol	0.05 – 5 (6)	$Y = 0.615X - 0.010$	0.996
25-Hydroxycholesterol	0.01 – 1 (6)	$Y = 0.935X - 0.007$	1.000
27-Hydroxycholesterol	0.1 – 10 (6)	$Y = 1.400X - 0.020$	0.998
24S,25-Epoxycholesterol	0.01 – 2 (7)	$Y = 0.444X - 0.004$	0.998

[a] X is the weight ratio of each oxysterol to the corresponding deuterated internal standard, and Y is the peak area ratio calculated as the peak area of the oxysterol-picolinate(s) divided by that of deuterated oxysterol-picolinate(s) (internal standard). [$^2H_6$]24-hydroxycholesterol was used as an internal standard for 22R-hydroxycholesterol.

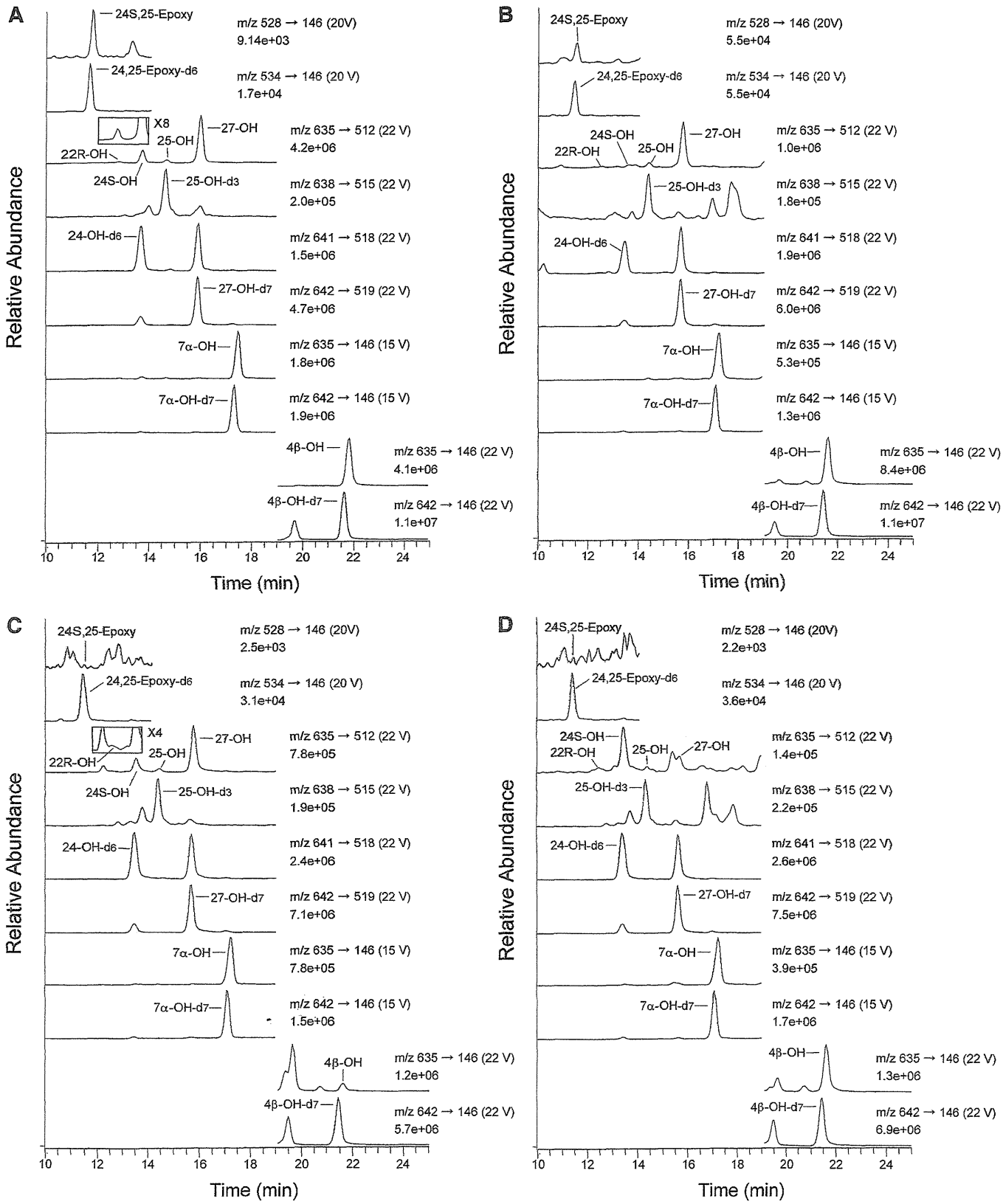

Fig. 2. Comparison of selected reaction monitoring chromatograms obtained from authentic oxysterols (A), 1 mg protein of the microsomal fraction from a normal rat liver (B), and 5 μl of sera from a normal volunteer (C) and a patient with CTX (D). The quantities of each peak (in A) of authentic oxysterol standards are: ~200 pg for 7α-hydroxycholesterol (7α-OH), [$^2H_7$]7α-OH (7α-OH-d_7), 27-hydroxycholesterol (27-OH), and [$^2H_7$]27-OH (27-OH-d_7), ~100 pg for 4β-hydroxycholesterol (4β-OH), [$^2H_7$]4β-OH (4β-OH-d_7), 24S-hydroxycholesterol (24S-OH), and [$^2H_6$]24-OH (24-OH-d_6), and ~20 pg for 25-hydroxycholesterol (25-OH), [$^2H_3$]25-OH (25-OH-d_3), 22R-hydroxycholesterol (22R-OH), 24S,25-epoxycholesterol (24S,25-Epoxy), and [$^2H_6$]24,25-Epoxy (24,25-Epoxy-d_6). The numbers on the right side of each chromatogram represent the full scale of the chromatogram.

TABLE 3. Reproducibility of the quantification of each oxysterol in rat liver microsomes

Oxysterol	Mean ± SD (n = 12)	Relative SD	
		Sample Preparation	Error (SRM)
	ng	%	
4β-Hydroxycholesterol	5.56 ± 0.28	3.3	5.6
7α-Hydroxycholesterol	4.22 ± 0.13	3.7	2.9
22R-Hydroxycholesterol	0.107 ± 0.013	12.7	11.9
24S-Hydroxycholesterol	0.104 ± 0.007	8.7	5.8
25-Hydroxycholesterol	0.64 ± 0.02	1.8	3.7
27-Hydroxycholesterol	3.16 ± 0.23	8.1	6.9
24S,25-Epoxycholesterol	1.11 ± 0.08	5.1	8.4

Each oxysterol was quantified in 1 mg protein from normal rat liver microsomes. Four samples were prepared and quantified in triplicate by liquid chromatography-tandem mass spectrometry. The results were analyzed by a one-way layout, in which the analytical errors were divided into two sources: sample preparation and SRM measurement.

oxysterol calculated by linear regression analysis; this also constituted an index for the precision and accuracy of the method (see supplementary Table III).

DISCUSSION

Neutral monohydroxysterols are poorly ionized by electrospray. To overcome this disadvantage, we have developed a new method for the enhancement of the ionization efficiency by derivatizing into picolinyl esters (23, 27). Dihydroxy- or epoxysterols are more efficiently ionized by electrospray, and their limit of detection (5–60 fmol on-column) was reported to be more than 10 times lower than that of monohydroxysterols (175–2,000 fmol on-column)

TABLE 4. Recovery of each oxysterol from rat liver microsomes

Oxysterol	Amount Added	Average Recovery[a] (Mean ± SD) (n = 6)
	ng	%
4β-Hydroxycholesterol	2.00	102.7 ± 8.7
	4.00	98.5 ± 9.9
	6.00	104.3 ± 11.7
7α-Hydroxycholesterol	4.00	89.5 ± 7.1
	8.00	86.7 ± 6.9
	12.00	90.8 ± 8.8
22R-Hydroxycholesterol	0.05	103.0 ± 15.5
	0.10	105.2 ± 6.9
	0.15	99.8 ± 5.6
24S-Hydroxycholesterol	0.05	107.3 ± 14.0
	0.10	100.3 ± 8.4
	0.15	102.0 ± 9.0
25-Hydroxycholesterol	0.20	106.6 ± 12.7
	0.40	100.1 ± 6.8
	0.60	103.1 ± 5.3
27-Hydroxycholesterol	1.00	98.2 ± 15.0
	2.00	102.6 ± 4.8
	3.00	103.7 ± 2.2
24S,25-Epoxycholesterol	0.40	97.5 ± 15.2
	0.80	107.2 ± 18.5
	1.20	104.2 ± 7.5

Known amounts of each oxysterol were spiked into 1 mg protein from normal rat liver microsomes before sample preparation.

[a] Recovery (%) = (amount found $-X0$)/amount added × 100. $X0$ value was obtained from TABLE 3. (See Table 5 in ref. 35.)

(22). In this paper, we have studied the usefulness of our derivatization method on dihydroxy- and epoxysterols that are key regulatory oxysterols in biological samples. The detection limits of oxysterol dipicolinates and epoxycholesterol picolinate were 5–25 amol on-column, which was about 1,000-fold more sensitive than those with the underivatized ESI method (22). We also determined the detection limits of native dihydroxy- and epoxysterols by LC-APCI-MS/MS analysis, and they were about 10 fmol on-column (data not shown). Thus, highly sensitive LC-MS/MS analysis after picolinyl ester derivatization can be used not only for monohydroxysterols but also for dihydroxy- and epoxysterols.

A few derivatization methods that are suitable for LC-ESI-MS/MS analysis of dihydroxysterols have been reported. Griffiths et al. (28) converted oxysterols with a 3β-hydroxy-Δ^5 structure into 3-oxo-Δ^4 steroids by using cholesterol oxidase, and then derivatized with the Girard P reagent to Girard P hydrazone. This method improved the sensitivity by enhancing ionization and was successfully applied to the identification of oxysterols in the brain (29). However, this method has several disadvantages for simple and highly sensitive quantification of oxysterols in biological samples. First, two steps are needed to convert 3β-hydroxysterols into Girard P hydrazone derivatives. Second, the derivatization gives *syn* and *anti* forms with different retention times. Third, 3β-hydroxysterols with an oxo group are converted to the mono- and bis-Girard P hydrazone derivatives. Finally, this method produces the same derivative from 7α-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one, which are important intermediates in the hepatic bile acid biosynthetic pathway.

Recently, Jiang, Ory, and Han (30) reported another derivatizing method that converted oxysterols into dimethylglycine esters. This method appears to have overcome the weaknesses of the above Girard P hydrazone derivatives. However, overnight incubation at 50°C was necessary to make the dimethylglycine esters, and the formed dimethylglycine diesters provided a doubly protonated ion. MS/MS spectra of doubly protonated ions are more complicated than those of singly protonated ions. Therefore, singly charged ions are preferable as precursor ions for simple and highly sensitive MS/MS analysis.

In our picolinyl ester derivatization, Yamashita et al. (31) reported in a recent study that estradiol dipicolinates gave singly charged ions in the positive ESI mass spectrum. In the present study, oxysterols with two hydroxyl groups were also derivatized to picolinyl diesters showing singly charged ions in the positive ESI mass spectra, which appears to be a general characteristic of the picolinyl ester derivatization of steroids with two hydroxyl groups. Because of the better ionizing efficiency due to the double picolinyl moieties and a simple MS/MS spectra, the detection limits of dihydroxysterols (5–25 amol on-column) were about 100 times lower than those of monohydroxysterols (260–2,600 amol on-column) (23).

In addition, our method made it possible to quantify 24S,25-epoxycholesterol in biological samples with high sensitivity (12.5 amol on-column) and specificity. Although

this epoxycholesterol appears to be one of the most important regulatory oxysterols for cholesterol homeostasis (10, 14), the concentrations in biological samples have not been determined widely because of instability during GC-MS analysis and insufficient sensitivity by HPLC with UV detection (16). In fact, we have measured this epoxycholesterol concentration in hepatic tissues by high-resolution GC-MS after trimethylsilyl (TMS) ether derivatization (32). However, the derivative became decomposed during GC separation, giving several peaks with similar mass spectra, and 100 fmol of 24S,25-epoxycholesterol was barely detectable on-column. Although this sensitivity exceeded that obtained by the HPLC-UV method (16), it was still not sufficient to quantify this epoxycholesterol in small amounts of biological samples.

Another merit of highly sensitive quantification is that the loading amount on the HPLC column can be minimized, so that the solid-phase extraction/purification step was omitted in our assay. In human serum analysis, less than 20 pg of oxysterol picolinates was injected on the column with approximately 200 ng of cholesterol picolinate. Under our HPLC conditions, this amount of cholesterol picolinate was easily trapped in the column and eluted around 29 min, which was well separated from oxysterols and did not affect the separation or elution of each oxysterol picolinate. HPLC column separation was very important in the present method because many oxysterols have the same molecular weight and MS spectrum. By changing the collision energies, the specific MS/MS spectrum of each oxysterol was observed to some extent, but we selected less-specific SRM ion pairs rather than more-specific ones because the former showed higher sensitivities and better signal-to-noise ratios compared with the latter.

The procedure for picolinyl ester derivatization was essentially the same as that in our previous report (23), but a few modifications were made. First, the reagent mixture was prepared by using pyridine instead of tetrahydrofuran, and the incubation was performed at 80°C for 60 min. Usually, this esterification progresses easily at room temperature, but the only hydroxyl at the C-25 position of 25-hydroxycholesterol was resistant to picolinyl ester formation. However, complete esterification of this C-25 position was achieved by heating at 80°C for 60 min. After the derivatization step, excess reagents were precipitated by the addition of *n*-hexane, and picolinyl ester derivatives were recovered in the supernatant.

Serum total (free + esterified) oxysterol concentrations in 19 normal volunteers were measured by our LC-ESI-MS/MS method (**Table 5**), and the concentrations of 4β-hydroxycholesterol, 7α-hydroxycholesterol, 22R-hydroxycholesterol, 25-hydroxycholesterol, and 24S,25-epoxycholesterol looked higher than those determined by previous methods. However, 7α-hydroxycholesterol levels determined by our method did not differ significantly ($P > 0.05$) from those by the GC-MS method (33), and 22R-hydroxycholesterol and 24S,25-epoxycholesterol levels appeared to be less than the detection limits by the HPLC method (34). We cannot exclude the possibility that some 25-hydroxycholesterol was produced by cholesterol autoxidation, but it is also possible that the concentration was not quantified accurately by the low-resolution GC-MS method. This is because the TMS ether derivative of 25-hydroxycholesterol did not give an ideal mass spectrum in the high mass region and *m/z* 131 was used for the quantification by selected ion monitoring. In general, high background noise is expected when a low mass number is selected as a monitoring ion for GC-MS analysis of biological samples. We have measured 25-hydroxycholesterol and 4β-hydroxycholesterol concentrations by using different SRM ion pairs [*m/z* 635 → 146 (22 V) and *m/z* 635 → 512 (20 V), respectively], and virtually the same results have been obtained.

A recent study using Cyp27a1 knockout mice demonstrated that 25-hydroxycholesterol was also synthesized by CYP27A1 (6). Our results showed that not only 27-hydroxycholesterol but also 25-hydroxycholesterol concentrations were markedly lower in serum from a patient with CTX, CYP27A1 deficiency, compared with that from a control subject (Fig. 2C, D), which lends support to the idea that a portion of the 25-hydroxycholesterol circulating in human serum is derived from CYP27A1.

In summary, we have developed a very sensitive and specific method for the quantification of key regulatory oxysterols in biological samples. Derivatization of dihydroxy- and epoxysterols into the picolinyl esters allowed them to be quantified by LC-ESI-MS/MS with excellent sensitivity and reliability. This method is useful for the study of

TABLE 5. Concentrations of total (free + esterified) oxysterols in normal human serum: comparison with previous methods

	Present Method	Previous Methods	
Oxysterol	Mean ± SD (n = 19)	Mean ± SD (n)	Method (Reference)
	ng/ml	*ng/ml*	
4β-Hydroxycholesterol	77 ± 40	29 ± 10 (125)	GC-MS (2)
7α-Hydroxycholesterol	145 ± 82	99 ± 43 (12) 43 ± 48 (31)	GC-MS (33) GC-MS (1)
22R-Hydroxycholesterol	10 ± 18	ND (2)	HPLC (34)
24S-Hydroxycholesterol	51 ± 12	64 ± 24 (31) 64 ± 14 (22)	GC-MS (1) LC-APCI-MS (20)
25-Hydroxycholesterol	31 ± 11	2 ± 3 (22)	GC-MS (1)
27-Hydroxycholesterol	117 ± 35	154 ± 43 (31) 120 ± 30 (22)	GC-MS (1) LC-APCI-MS (20)
24S,25-Epoxycholesterol	2 ± 2	ND (2)	HPLC (34)

LC-APCI-MS, liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry; ND, not detectable.

lipid metabolism controlled by oxysterols as well as the screening and diagnosis of metabolic disorders in oxysterols.

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Original Article

Impact of determination of hepatitis B virus subgenotype and pre-core/core-promoter mutation for the prediction of acute exacerbation of asymptomatic carriers

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Aim: A large cohort study in Japan revealed that the specific viral profile may influence the fulminant outcome in acute hepatitis B virus (HBV) infections, while the genetic influence on outcome has not been clarified in patients with acute exacerbation of chronic liver disease caused by HBV. We experienced a case of fatal liver failure that developed as the result of chronic HBV infection. To determine possible genetic factor involving acute exacerbation, genetic analysis of serum from the patient and his siblings was performed.

Methods: HBV subgenotype as well as pre-core/core-promoter mutations of samples mentioned above were determined.

Results: Patient had HBV-Bj with pre-core (1896/1899) and core-promoter (1762/1764) mutations, the genomic profile frequently seen in fulminant hepatitis caused by acute HBV infection.

Conclusion: This result suggests that determination of the HBV subgenotype and pre-core/core promoter mutations could provide a rationale for development of a treatment strategy in asymptomatic HBV carriers.

Key words: hepatits B virus (HBV), fulminant hepatitis, chronic hepatitis, subgenotype, pre-core/core promoter mutations

INTRODUCTION

A RECENT REPORT has suggested that genotype and pre-core mutations may influence the fulminant outcome of acute hepatitis B virus (HBV) infection.[1] The report suggests that fulminant hepatitis was frequently associated with a Bj subgenotype and a lack of HBeAg, as well as high replication due to a pre-core mutation (PC) in patients with acute HBV infection. Further, 12 of 22 patients (55%) infected with HBV-Bj developed fulminant hepatitis, whereas no patients infected with HBV-Ae did, and that both PC (G1896A) and core-promoter (CP; A1762T/G1764A) mutations were significantly more frequent in patients with fulminant hepatitis compared to those with acute self-limiting hepatitis.[1]

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The genetic influence on outcome has not been clarified in patients with acute exacerbation of chronic liver disease caused by HBV. We experienced a case of fatal liver failure that developed from chronic infection of HBV. To determine possible genetic factor involving acute exacerbation, we performed genetic analysis of serum from the patient and his siblings and obtained interesting findings. Although accumulation of data among a large number of asymptomatic carriers are needed, it is worthwhile to mention the determination of subgenotype and PC or CP mutations in asymptomatic HBV carriers may give us a rationale to predict future exacerbation of hepatitis and in turn, provide a better outcome for these patients.

CLINICAL CASE

THE PATIENT WAS a 41-year-old man, born in Okinawa prefecture. He initially presented with general fatigue, and a physical examination and blood tests showed liver dysfunction and jaundice. He did not have any history of hospitalization. No abnormal

Table 1 Laboratory data on admission

Blood Cell Count: WBC 5850 (Stab 4.0, Seg 78.0, Lym 11.0, Mono 7.0), RBC 429 ¥ 10^4, Hb 14.4 g/dL, Plt 11.0 ¥ 10^4
Coagulation Tests: PT 9.8% (INR 6.88), Fibrinogen 87 mg/dL, FDP 9.3 mg/mL
Biochemistry: Na/K/Cl 133/3.8/99 mM, TP 5.7 g/dL, Alb 3.2 g/dL
BUN 7.5 mg/dL, s-Cre 0.6 mg/dL
AST 910 U/l, ALT 1752 U/l, LDH 511 U/l,
ALP 708 U/l, -GTP 150 U/L, ChE 170 U/L
T-Bil 18.1 mg/dL, D-Bil 7.7 mg/dL (D/T = 0.425)
CRP 0.7 mg/dL, NH_3 212 mg/dL
Viral Markers: HBsAg (+) (EIA titer 184.83)
IgM-HBc Ab (-), HBc Ab/CLIA (+)
HBeAg (-), HBeAb (+) (99.8% inhibition)
HBV-DNA/TMA 6.6 LGE/mL, HDV-DNA (-)
HCV-Ab (-), IgM-HA-Ab (-), HEV-DNA (-)
CMV-Ab IgG 10>, CMV-IgM 10>
EBV-anti VCA IgG ¥ 80?IgM 10>
Others: ANA 20.2, anti DNA antibody 80,
Anti Mitochondria Ab 20>

findings were noted at annual physical check-up. He was considered to be an asymptomatic HBV carrier. The patient was hospitalized on the day of his first visit. Follow-up tests showed worsening of liver enzyme levels and an alteration in consciousness level was noticed after 2 days. The patient was then referred to Tsukuba Gakuen Hospital as the development of acute liver failure was of concern.

Laboratory data on arrival are shown in Table 1. Elevation of serum ALT (1754 IU/L) and hyperbililubinemia (T-Bil, 18.1 mg/dL) were observed. The patient was positive for HBVs antigen and HBe antibody, but markers for other types of viral hepatitis, including hepatitis A virus, hepatitis C virus, hepatitis D virus and hepatitis E virus were all negative. The HBV-DNA level was found to be more than 6.6 log genome equivalents (LGE)/mL using transcription-mediated amplification. Prothrombin time was markedly prolonged (PT% = 20) and an atrophic liver was apparent on abdominal ultrasonography. The patient showed disorientation and pronounced confusion on the day of arrival, and thus was diagnosed with grade III hepatic encephalopathy. He was immediately placed on artificial liver support (ALS) comprising plasma exchange and hemodiafiltration[2] with a combination of interferon beta and lamivudine, only nucleic acid analog approved by Japanese National health Insurance system at this time, as antiviral therapy (Fig. 1). A large dose of methylpredonisolone was also administered to suppress a presumably enhanced host immune response.[3]

In parallel, we started to discuss liver transplantation with his family. The patient had four brothers, and three were considered as possible donors for orthopedic liver transplantation. However, screening by blood tests and ultrasonography showed that the three brothers were positive for HBs antigen, and therefore all were excluded as donor candidates. A cadaveric liver was unavailable and thus we had to give up on liver transplantation. Despite intensive care, the patient complicated with acute respiratory distress syndrome (ARDS) and acute renal failure, and died on the 8th day of admission.

GENETIC ANALYSIS OF HBV

WITH THE PERMISSION of the family members, genetic analysis of HBV in serum acquired from the patient and family members was performed. HBV DNA sequences spanning the S gene were determined by real-time PCR according to the method of Abe *et al.*,[4] with a detection limit of 100 copies/mL. HBV DNA sequences bearing the CP, PC region, and core gene were amplified by PCR using hemi-nested primers, as described previously.[4] The neighbor-joining method[5] was used for phylogenetic analysis of the S region to classify HBV into subgenotypes. To confirm the reliability of the phylogenetic tree, bootstrap re-sampling tests were performed 1000 times.

Only the third sibling ("sibling C" in Table 2) showed an elevated ALT level (50 IU/L), with ALT levels in the

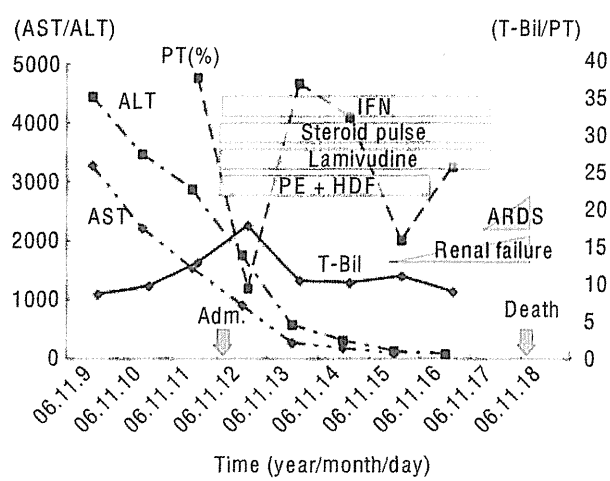

Figure 1 Clinical Course of the patient. ARDS, acute respiratory distress syndrome; HDF, hemodiafiltration; IFN, interferon beta; PE, plasma exchange; PT, prothrombin time.

Table 2 Profile of each sibling

	Age	Sex	AST/ALT	HBV-DNA	HBeAg	1896A/1899A/CP
Patient	41	M	910/1752	6.02 log copy/mL	–	+/+/+
Sibling A	45	M	36/35	5.25 log copy/mL	–	+/-/-
Sibling B	36	M	28/32	4.68 log copy/mL	–	+/-/-
Sibling C	31	M	31/50	<2.6 log copy/mL	–	+/+/-

other siblings within the normal range (about 40 IU/L). None of the siblings were HBeAg-positive. HBV-DNA loads were highest in the patient, relatively lower in sibling A and B, and under the detectable range in sibling C (Table 2). The HBV genotype was determined based on analysis of the S region. All siblings were infected with HBV-Bj, a subgenotype that is predominantly detected in patients who reside in a specific geographical location (including Okinawa prefecture) in Japan. HBV-DNA of patient and all siblings formed same cluster, thus it is speculated that they were infected from same source. In fact, the presence of family history of hepatic disease (their mother died by it) strongly suggests the vertical infection (Fig. 2). Mutations in the PC (G1896A) and CP (A1762T/G1764A) were also discovered. As shown in Figure 3, the patient and all siblings had the 1896A mutation, which is consistent with the absence of HBeAg, and the patient had both the G1899A and CP mutations (A1762T/G1764A). Sibling C had the G1899A mutation but not the CP mutation, and the other siblings had none of these mutations.

DISCUSSION

FULMINANT VIRAL HEPATITIS is thought to occur as a result of immunoreactions against enhanced viral replication. Findings associated with immunoresponses of hosts in fulminant cases are limited, probably due to the absence of definitive methodology for determination of individual immunoresponses. However, factors associated with viral replication have been investigated, and an HBV subgenotype and PC and CP mutations have been linked to high replication rates in acute HBV infection and in turn to fulminant outcome.[1] Besides fulminant hepatitis caused by acute infection of HBV, fatal acute liver failure may also emerge from a previously unrecognized chronic infection of HBV, but little is known about the viral factors involved in acute exacerbation in chronic HBV carriers.

In the current case, the patient was infected with the HBV/Bj subgenotype, was HBeAb-positive, and had PC and CP mutations. Although the patient was already an HBV carrier at the time of onset, his genomic profiles matched the pattern frequently seen in fulminant hepatitis caused by acute HBV infection.[1] Among the population of HBV carriers, patients infected with HBV/Ce and HBV/Bj are predominant in Japan.[6–9] HBV/Bj is a specific subgenotype that is present in less than 10% of HBV carriers, but has a higher prevalence in locations such as the Northeastern (Tohoku) district of Honshu and in Okinawa. Patients with HBV/C infection tend to have chronic sustained inflammation that progresses to liver cirrhosis and hepatocellular carcinoma, whereas HBV/Bj more frequently induces HBe seroconversion via a PC mutation, which results in a lower viral load and reduced disease severity.[10]

Sugiyama *et al.* determined the intracellular and extracellular HBV DNA levels in Huh-7 cells transfected with a plasmid carrying different genotypes/subgenotypes of the HBV genome without a CP/PC mutation.[11] HBV DNA levels in cell lysates were highest for HBV/C, followed by Bj/Ba and D/Ae, and lowest for Aa; whereas in culture media these levels were highest for Bj, with much lower levels for Ba/C/D, and still lower levels for Ae/Aa.[11] It was speculated that the strong tendency of Bj for extracellular virion secretion may endow a high infectious capacity to blood from individuals infected with this subgenotype, and that this may trigger strong immune responses in hosts.[11] This characteristic of HBV/Bj is thought to be associated with earlier seroconversion from hepatitis Be antigen (HBeAg) to the corresponding antibody (anti-HBe) and with lower histological activity.

Acute exacerbation of chronic liver disease (CLD) has been seen in some patients with chronic HBV/Bj infection post-seroconversion, with a fatal outcome similar to that in the current case. In a study of 592 patients, no significant difference in the frequency of genotype B was found between patients with CLD and those with acute exacerbation of CLD (62/531 [11.7%] versus 4/19 [21.1%]; NS),[12] but acute exacerbation was seen more frequently in CLD patients with HBV/B infection (4/62, 6.5%) compared to those with HBV/C infection (13/459, 2.8%).[12] The PC stop codon mutation (G1896A)

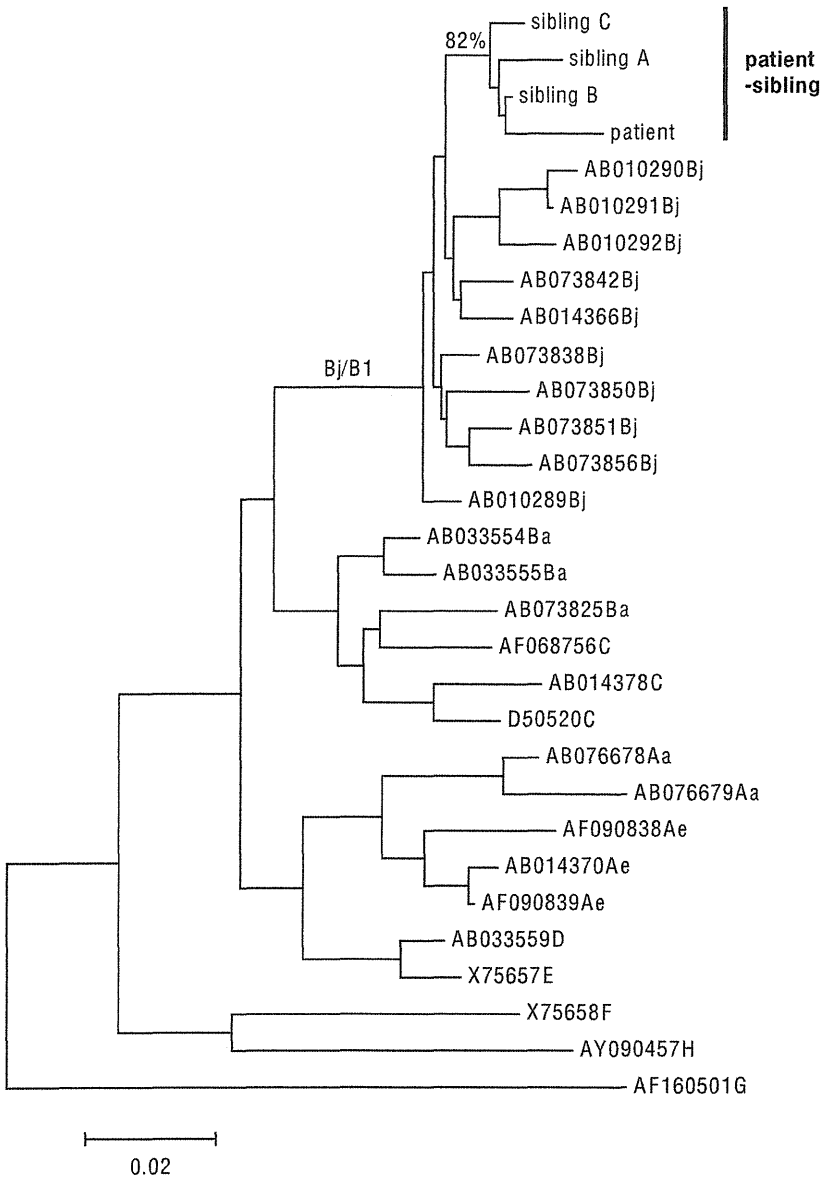

Figure 2 Subgenotyping of hepatitis B virus (HBV). HBV subgenotype was determined by sequencing of the S gene. Result of phylogenetic analysis of sequences from the pre-S region of HBV in the patient and siblings and reference strains from a database was shown. Reference strains were shown by accession number. The scale bar indicates genetic distance.

and the CP double mutation (A1762T/G1764A) were detected more frequently in HBV/Bj-infected patients with fulminant hepatitis compared to those with acute-self limited hepatitis (56% vs. 0%, and 67% vs. 0%).[1] These findings suggest that the replication potential of HBV/Bj might be stronger in the presence of CP/PC mutations than in the wild type virus, and Ozasa *et al.* demonstrated enhanced replication capacity of HBV/Bj with PC or CP mutations compared to wild type *in vitro*.[1]

Although HBV/Bj may show highly potent replication, this may not be the cause of enhanced immunoresponsiveness in patients with chronic infection of HBV/Bj. Rather, CP/PC mutation in HBV/Bj post-seroconversion may be the factor that facilitates potent viral replication and acute exacerbation of CLD. The host-associated factor that influences outcome in patients with chronic HBV/Bj infection has not been identified, but these viral characteristics may be predictive factors for future exacerbation. In the current study, the patient had both PC (G1896A/G1899A) and CP mutations and a G1896A/G1899A mutation was found in one sibling. Although HBV-DNA load in this sibling is under detectable range right now, a CP mutation in the future on this sibling may lead to a fatal outcome.

Figure 3 Nucleotide sequence of the core promoter/pre core region. Core promoter and pre-core region of hepatitis B virus (HBV) in the patient and siblings were determined. Mutation in core promoter (A1762T, G1764A) and precore (G1896A, G1899A) are indicated in boxes. The sequence of the reference HBV/Bj (AB014366Bj) is shown at the top of the figure.

Therefore, this sibling requires intensive follow-up and possible early administration of anti-viral drugs especially under the condition with immunosuppression, which can facilitate viral replication.

In conclusion, we suggest that analysis of the HBV subgenotype and CP/PC mutations should be performed during observation of asymptomatic HBV carriers to provide a more accurate understanding of the clinical presentation and to build an appropriate treatment strategy.

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methods

Highly sensitive quantification of serum malonate, a possible marker for de novo lipogenesis, by LC-ESI-MS/MS

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Abstract We describe a new sensitive and specific method for the quantification of serum malonate (malonic acid, MA), which could be a new biomarker for de novo lipogenesis (fatty acid synthesis). This method is based upon a stable isotope-dilution technique using LC-MS/MS. MA from 50 μl of serum was derivatized into di-(1-methyl-3-piperidinyl)malonate (DMP-MA) and quantified by LC-MS/MS using the positive electrospray ionization mode. The detection limit of the DMP-MA was approximately 4.8 fmol (500 fg) (signal-to-noise ratio = 10), which was more than 100 times more sensitive compared with that of MA by LC-MS/MS using the negative electrospray ionization mode. The relative standard deviations between sample preparations and measurements made using the present method were 4.4% and 3.2%, respectively, by one-way ANOVA. Recovery experiments were performed using 50 μl aliquots of normal human serum spiked with 9.6 pmol (1 ng) to 28.8 pmol (3 ng) of MA and were validated by orthogonal regression analysis. The results showed that the estimated amount within a 95% confidence limit was 14.1 ± 1.1 pmol, which was in complete agreement with the observed $\bar{X}_0$ = 15.0 ± 0.6 pmol, with a mean recovery of 96.0%. This method provides reliable and reproducible results for the quantification of MA in human serum.—Honda, A., K. Yamashita, T. Ikegami, T. Hara, T. Miyazaki, T. Hirayama, M. Numazawa, and Y. Matsuzaki. **Highly sensitive quantification of serum malonate, a possible marker for de novo lipogenesis, by LC-ESI-MS/MS.** *J. Lipid Res.* **2009.** 50: **2124–2130.**

Supplementary key words acetyl-CoA carboxylase • carnitine palmitoyl transferase 1 • fatty acid synthase • liquid chromatography-electrospray ionization-tandem mass spectrometry • malonic acid • malonyl-CoA • malonyl-CoA decarboxylase

Acetyl-CoA carboxylase (ACC) is the rate-controlling enzyme in the fatty acid biosynthetic pathway, and catalyzes the formation of malonyl-CoA from acetyl-CoA plus bicarbonate. Malonyl-CoA is not only substrate for fatty acid synthase (FAS) but is also a potent inhibitor of carnitine palmitoyl transferase 1 (1), the rate-limiting enzyme of fatty acid β-oxidation. Therefore, malonyl-CoA is a key molecule that controls fatty acid metabolism in the body. In addition, recent studies have shown that the level of hypothalamic malonyl-CoA is dynamically regulated by fasting and feeding and that it alters subsequent feeding behavior (2).

To determine ACC activity in tissues, an invasive tissue biopsy is necessary. However, whole body synthesis of fatty acid may be evaluated by the quantification of serum malonyl-CoA metabolites. This concept originates from our previous studies, which showed that serum concentrations of the immediate products of the rate-controlling enzymes in cholesterol and bile acid biosynthetic pathways reflected the activities of the rate-controlling enzymes and whole body cholesterol and bile acid biosynthesis (3). Furthermore, patients with malonyl-CoA decarboxylase (MCD) deficiency, who must have increased tissue malonyl-CoA concentrations, are characterized by markedly elevated urinary malonic acid (MA), called "malonic aciduria" (4). This phenomenon suggests that malonyl-CoA is easily hydrolyzed into MA by an unidentified tissue thioesterase(s). Therefore, we thought that serum MA concentrations might well reflect total body FAS.

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Abbreviations: ACC, acetyl-CoA carboxylase; DMP-MA, Di-(1-methyl-3-piperidinyl)malonate; FAS, fatty acid synthase; MA, malonic acid (malonate); MCD, malonyl-CoA decarboxylase; MMA, methylmalonic acid (methylmalonate); N-ESI, ESI in negative mode; P-ESI, ESI in positive mode; SA, succinic acid (succinate); SRM, selected reaction monitoring.

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Although methodological reports for the quantification of serum MA are not available, there have been some reports that describe the methods for the determination of urinary MA levels in patients with MCD deficiency by gas chromatography (5, 6) or gas chromatography-mass spectrometry (7). In these methods, urinary organic acids were extracted with ethyl acetate and converted into trimethylsilyl derivatives before analysis. Alternatively, blood malonylcarnitine has been measured for the diagnosis of MCD deficiency using liquid chromatography-tandem mass spectrometry coupled with electrospray ionization mode (LC-ESI-MS/MS) (8). However, because all of these methods were developed to diagnose markedly elevated MA levels in patients with MCD deficiency, the authors did not pay significant attention to the sensitivities of the methods.

The aim of this study was to measure serum MA concentrations in normal human subjects with sufficient sensitivity and specificity. For this purpose, serum MA was derivatized into di-(1-methyl-3-piperidinyl)malonate (DMP-MA) and quantified using positive LC-ESI-MS/MS (LC-P-ESI-MS/MS).

MATERIALS AND METHODS

Chemicals

MA and [$^{13}C_3$]MA were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). 3-Hydroxy-1-methylpiperidine and 2-methyl-6-nitrobenzoic anhydride were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and 4-dimethylaminopyridine and formic acid were obtained from Wako Pure Chemical Industries (Osaka, Japan). Additional reagents and solvents were of analytical grade.

Sample collection

Blood samples were collected from healthy human volunteers. After coagulation and centrifugation at 1,500 *g* for 10 min, serum samples were stored at −20°C until analysis. Informed consent was obtained from all subjects, and the experimental procedures were conducted in accordance with the ethical standards of the Helsinki Declaration. Rat serum was prepared in our previous study (9) and had been stored at −20°C until it was used in the present experiments.

Sample preparation

Fifty μl of serum was placed in a microcentrifuge tube (1.5 ml, Eppendorf, Hamburg, Germany), and 19.2 pmol (2 ng) of [$^{13}C_3$] MA in 100 μl of acetonitrile as an internal standard. The sample tube was vortexed for 1 min and centrifuged at 2,000 *g* for 1 min. The solution of internal standard in acetonitrile led to deproteinization of the sample and the liquid phase was collected and evaporated to dryness at 80°C under a nitrogen stream. Derivatization of MA into DMP-MA was performed according to the Shiina method for the synthesis of carboxylic esters (10) with some modifications. The reagent mixture for derivatization consisted of 2-methyl-6-nitrobenzoic anhydride (67 mg), 4-dimethylaminopyridine (20 mg), pyridine (900 μl), and 3-hydroxy-1-methylpiperidine (100 μl). The freshly prepared reagent mixture (100 μl) was added to the serum extract and the reaction mixture was allowed to stand at room temperature for 30 min. After the addition of 2 ml of n-hexane, the mixture was vortexed for 30 s and centrifuged at 700 *g* for 2 min. The clear supernatant was collected and evaporated at 80°C under nitrogen. The residue was redissolved in 50 μl of 1% formic acid in water and an aliquot (1 μl) was injected into the following LC-MS/MS system.

Determination of DMP-MA by LC-P-ESI-MS/MS

The LC-MS/MS system consisted of a TSQ Quantum Ultra quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an H-ESI probe and a Nanospace SI-2 HPLC system (Shiseido, Tokyo, Japan). Chromatographic separation was performed using a Hypersil GOLD aQ column (150 × 2.1 mm, 3 μm, Thermo Fisher Scientific) at 40°C. Initially, the mobile phase was comprised of 0.2% formic acid in water and was used at a flow rate of 200 μl/min for 5 min, and it was then switched to 0.2% formic acid in acetonitrile at a flow rate of 300 μl/min for an additional 3.5 min. The general LC-MS/MS conditions were as follows: spray voltage, 1000 V; vaporizer temperature, 350°C; sheath gas (nitrogen) pressure, 50 psi; auxiliary gas (nitrogen) flow, 40 arbitrary units; ion transfer capillary temperature, 350°C; collision gas (argon) pressure, 1.5 mTorr; collision energy, 15 V; and ion polarity, positive.

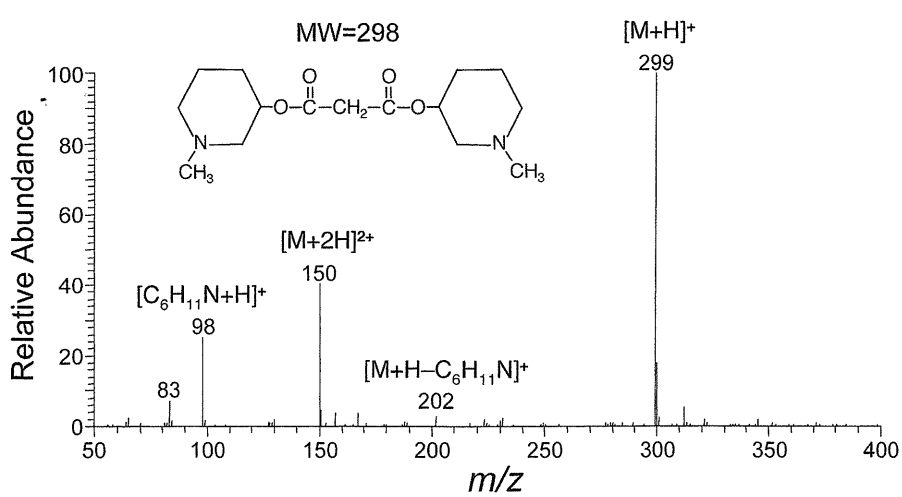

Fig. 1. Typical P-ESI mass spectrum of the DMP-MA. The general LC-MS/MS conditions were as described in Materials and Methods.

Determination of MA by LC-N-ESI-MS/MS

LC-negative (N)-ESI-MS/MS analysis of MA was carried out using the same LC-MS/MS instrument described above. Hypersil GOLD column (150 × 2.1 mm, 3 μm, Thermo Fisher Scientific) was used at 40°C. The mobile phase consisted of methanol-water (5:95, v/v) containing 0.2% formic acid and was used at a flow rate of 200 μl/min. The general LC-MS/MS conditions were as follows: spray voltage, 4000 V; vaporizer temperature, 350°C; sheath gas (nitrogen) pressure, 50 psi; auxiliary gas (nitrogen) flow, 30 arbitrary units; ion transfer capillary temperature, 300°C; collision gas (argon) pressure, 1.5 mTorr; collision energy, 15 V; and ion polarity, negative.

Statistics

Data are reported as the mean ± SD. Linearity of the calibration curve was analyzed by simple linear regression. Reproducibility was analyzed by one-way ANOVA (JMP software, SAS Institute, Inc., Cary, NC). The estimated amount ± 95% confidence limit was obtained as an index of precision (11). To calculate the values, orthogonal regression analysis was performed in the recovery study by using JMP software. For all analyses, significance was accepted at the level of $P < 0.05$.

RESULTS

Selected reaction monitoring

A typical ESI positive mass spectrum of the DMP-MA is shown in **Fig. 1**. This DMP ester derivative exhibited $[M+H]^+$ ion at *m/z* 299 as the base peak. In the MS/MS spectrum using *m/z* 299 as a precursor ion, the $[C_6H_{11}N+H]^+$ ion was observed at *m/z* 98 as the most prominent peak. The selected reaction monitoring (SRM) was conducted using *m/z* 299 → *m/z* 98 for the DMP-MA and *m/z* 302 → *m/z* 98 for the $[^{13}C_3]$ variant. We also monitored *m/z* 299 → *m/z* 202, a product ion containing the MA molecule

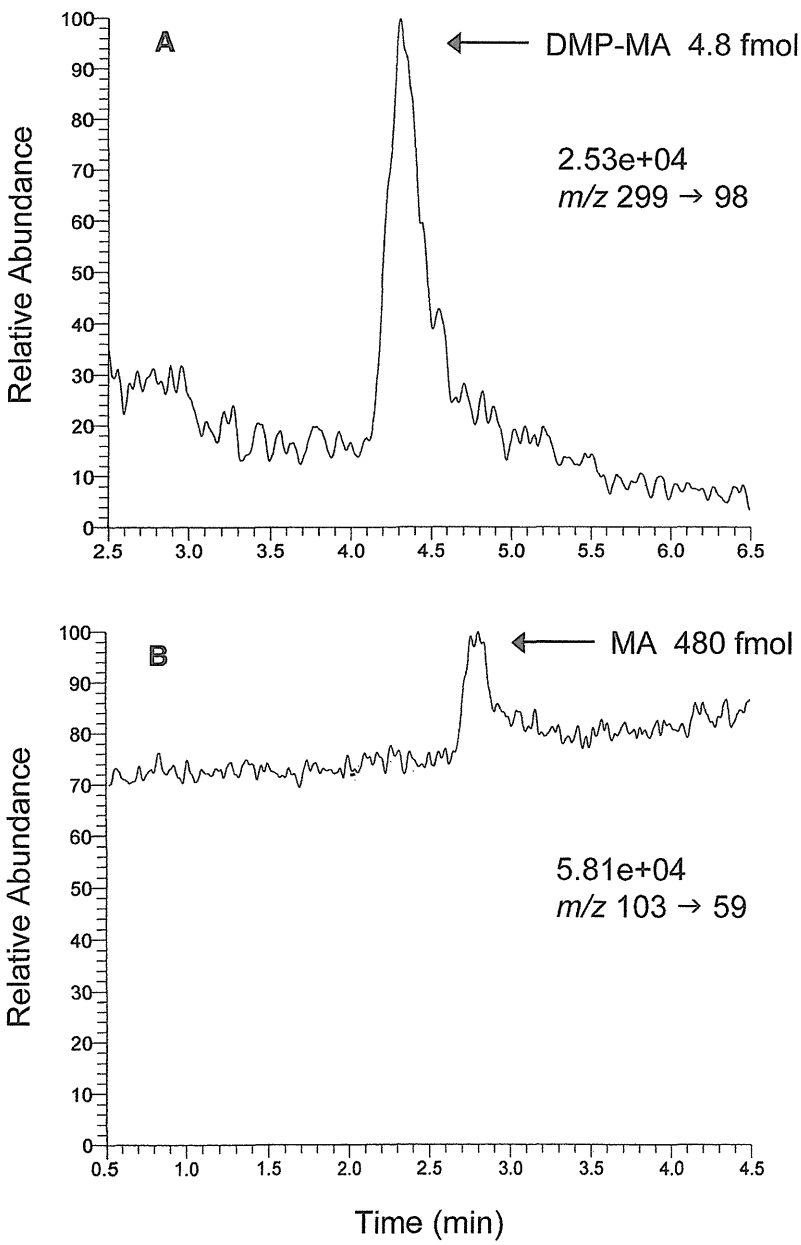

Fig. 2. Comparison of the detection limit of DMP-MA by LC-P-ESI-MS/MS at *m/z* 299 → *m/z* 98 (A) with that of MA by LC-N-ESI-MS/MS at *m/z* 103 → *m/z* 59 (B). Authentic standard of DMP-MA (4.8 fmol) or MA (480 fmol) was injected into the HPLC. The numbers written above the SRM ion pair represent the full scale of the chromatogram.

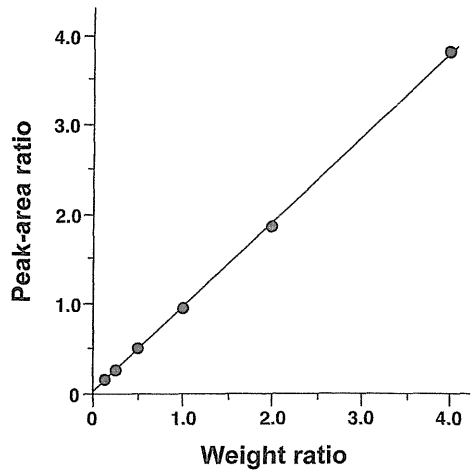

Fig. 3. Calibration curve for the weight ratio of MA to the corresponding deuterated internal standard. Linearity was checked by simple linear regression and the equation for the line of best fit was $y = 0.948x + 0.021$ ($n = 6$; $r = 1.000$; $P < 0.0001$).

but the former showed much better signal-to-noise ratio than the latter.

By N-ESI mode, authentic MA exhibited $[M-H]^-$ ion at m/z 103 as the base peak. In the MS/MS spectrum, the CH_3COO^- ion was observed at m/z 59 as the most prominent peak. The SRM was conducted using m/z 103 → m/z 59 for the MA.

Comparison of the sensitivities between P-ESI and N-ESI methods

To compare the sensitivity of DMP-MA by LC-P-ESI-MS/MS with that of MA by LC-N-ESI-MS/MS, the standard DMP-MA or MA solution was diluted and injected into the LC-MS/MS system. As shown in **Fig. 2A**, the DMP-MA was easily detected to 4.8 fmol by LC-P-ESI-MS/MS, with a signal-to-noise ratio of 10, whereas the conventional LC-N-ESI-MS/MS was barely able to detect 480 fmol of MA (Fig. 2B).

Calibration curve

A calibration curve was established for MA (**Fig. 3**). Each of different amounts (2.4, 4.8, 9.6, 19.2, 38.5, and 76.9 pmol) of authentic MA was mixed with 19.2 pmol of $[^{13}C_3]$MA, derivatized to the DMP ester and quantified as described in the Materials and Methods. The weight ratio of MA, relative to the corresponding ^{13}C-labeled internal standard, was plotted on the abscissa and the peak-area ratio of the DMP-MA to the $[^{13}C_3]$ variant measured by LC-P-ESI-MS/MS was plotted on the ordinate. The linearity of the standard curve, as determined by simple linear regression, was excellent for weight ratios between 0.125 and 4.0 ($n = 6$; $r = 1.000$; $P < 0.0001$).

Representative SRM

Figure 4 shows typical SRM chromatograms for DMP-MA and the $[^{13}C_3]$ variant obtained with 50 μl sera from a normal human (A) and a control rat (B). The peak-area ratio of the DMP-MA to the $[^{13}C_3]$ variant was calculated from the chromatograms, and MA amount was determined by applying the ratio to the calibration curve. The peaks of DMP-MA in chromatograms A and B correspond to ~0.66 pmol (0.66 μM) and ~4.43 pmol (4.43 μM), respectively.

Precision and accuracy of the LC-P-ESI-MS/MS method

The following studies were performed to determine the precision and accuracy of the present method using the same serum obtained from a normal human subject. Reproducibility was investigated by analyzing four samples in triplicate by LC-P-ESI-MS/MS (**Table 1**). The results were analyzed by a one-way ANOVA in which the analytical errors were divided into two sources, sample preparation

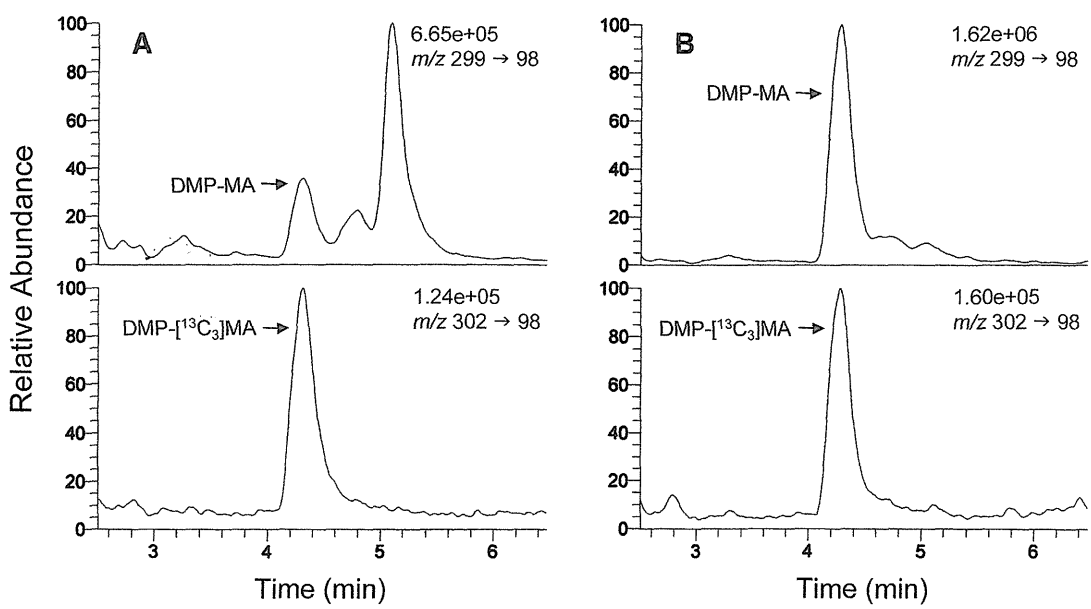

Fig. 4. Representative SRM chromatograms of DMP-MA and its $^{13}C_3$ variant (internal standard) obtained from 50 μl sera of a normal human (A) and a rat (B). The peaks of DMP-MA in chromatograms A and B correspond to ~0.66 pmol (0.66 μM) and ~4.43 pmol (4.43 μM), respectively. The numbers written above the SRM ion pair represent the full scale of the chromatogram.