methods

Highly sensitive assay of HMG-CoA reductase activity by LC-ESI-MS/MS

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Abstract **We have developed a new sensitive and specific nonradioisotope assay method to measure the activity of HMG-CoA reductase, the rate-controlling enzyme in the cholesterol biosynthetic pathway. This method was based upon a stable isotope dilution technique by liquid chromatography-tandem mass spectrometry using electrospray ionization in positive mode. Mevalonic acid, the product of HMG-CoA reductase, was converted to mevalonolactone (MVL) in an incubation mixture, extracted by a salting-out procedure, derivatized into the mevalonyl-(2-pyrrolidin-1-yl-ethyl)-amide, and then purified using a disposable silica cartridge. The resulting mevalonylamide was quantified by selected reaction monitoring using the positive electrospray ionization mode. The detection limit of this mevalonylamide was found to be 240 amol (signal-to-noise ratio = 3), ∼833 times more sensitive than that of MVL measured by a conventional radioisotope (RI) method (200 fmol). The variances between sample preparations and between measurements by this method were analyzed by one-way layout and calculated to be 3.2% and 1.8%, respectively. The recovery experiments were performed using incubation mixtures spiked with 0.77–2.31 nmol MVL/mg protein and were validated by a polynomial equation. These results showed that the estimated concentration within a 95% confidence limit was 0.47 ± 0.07 nmol/mg protein, which coincided completely with the observed $\overline{X}_0$ nmol/mg protein with a mean recovery of 94.6%. This method made it possible to measure HMG-CoA reductase activity with a high degree of reproducibility and reliability, and especially with sensitivity superior to that of the conventional RI method.**—Honda, A., Y. Mizokami, Y. Matsuzaki, T. Ikegami, M. Doy, and H. Miyazaki. **Highly sensitive assay of HMG-CoA reductase activity by LC-ESI-MS/MS.** ***J. Lipid Res.*** **2007.** 48: **1212–1220.**

Supplementary key words cholesterol biosynthesis • mevalonic acid • mevalonolactone • liquid chromatography-electrospray ionization-tandem mass spectrometry • 3-hydroxy-3-methylglutaryl-coenzyme A reductase

The activity of HMG-CoA reductase, the rate-limiting enzyme in the cholesterol biosynthetic pathway, is regulated at the levels of protein synthesis, degradation, and phosphorylation (1). Recent advances in molecular biological research have provided new insights into the regulation of HMG-CoA reductase activity. Sterol-regulatory element binding proteins (SREBPs) are positive transcription factors for the HMG-CoA reductase gene that are synthesized in the endoplasmic reticulum and released to the nucleus by proteolysis (2). This proteolysis is controlled by sterols and the SREBP processing system, which consists of SREBP cleavage-activating protein and insulin-induced genes (3). Although the methods for determining mRNA and protein expression levels in each regulatory step are well established, the only way to determine the overall consequence(s) of regulation by all factors is to measure HMG-CoA reductase activity.

The primary method for assaying HMG-CoA reductase activity is the radioisotope (RI) technique that measures the radioactivity in [^{14}C]mevalonic acid (MVA) produced from [^{14}C]HMG-CoA (4–6). This method is simple but requires the handling of radiolabeled materials. To overcome this disadvantage, in 1978, Miyazaki et al. (7) developed a new method using gas chromatography-chemical ionization-mass spectrometry (GC-CI-MS). This method was subsequently replaced by gas chromatography-electron ionization-mass spectrometry (GC-EI-MS) (8–10) because of the troublesome nature of GC-CI-MS operations, such as frequent cleaning of the CI ion source to maintain its high sensitivity. The GC-EI-MS methods are inferior in their sensitivity than the GC-CI-MS method but are still

Abbreviations: CI, chemical ionization; EI, electron ionization; GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MVA, mevalonic acid; MVL, mevalonolactone; MV-PLEA, mevalonyl-(2-pyrrolidin-1-yl-ethyl)-amide; P-ESI, electrospray ionization in positive mode; RI, radioisotope; S/N, signal-to-noise ratio; SREBP, sterol-regulatory element binding protein; SRM, selected reaction monitoring.

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more sensitive than the RI technique. In fact, GC-EI-MS methods have been used to quantify plasma (11) and urinary (12, 13) MVA that cannot be determined using the RI technique. However, contrary to our expectation, HMG-CoA reductase activity has not been assayed extensively using GC-EI-MS methods because of the laborious sample preparation and the need for a long analytical process to eliminate interfering peaks.

To analyze relatively polar compounds, such as MVA or mevalonolactone (MVL), liquid chromatography-mass spectrometry (LC-MS) or liquid chromatography-tandem mass spectrometry (LC-MS/MS) have been used more readily than GC-MS, because LC-MS and LC-MS/MS do not require a derivatization step. Park et al. (14) and Ndong-Akoume et al. (15) assayed HMG-CoA reductase activity by measuring MVL, the lactonized form of MVA, with LC-MS and LC-MS/MS, respectively, using the positive electrospray ionization (P-ESI) mode. Plasma and urinary MVA were quantified by LC-P-ESI-MS/MS after conversion into MVL (16) as well as directly by LC-negative ESI-MS/MS without lactonization (17, 18). However, there is no evidence that the detection limits of MVA or MVL by any LC-MS or LC-MS/MS method can surpass the detection limit of the RI technique using [^{14}C]MVL.

The aim of this study was to develop an LC-P-ESI-MS/MS method that was more sensitive and reliable than the conventional RI technique to measure HMG-CoA reductase activity.

MATERIALS AND METHODS

Chemicals

Unlabeled MVL and HMG-CoA were purchased from Sigma-Aldrich (St. Louis, MO), and 3,5-dihydroxy-[3-$^2H_3$]methyl-[4,4,5,5-$^2H_4$]valerolactone ([$^2H_7$]MVL) was from Merck Frosst Canada (Montreal, Canada). *RS*-[5-^{3}H]MVL (888.0 GBq/mmol) and 3-hydroxy-3-methyl-[3-^{14}C]glutaryl-CoA (2.15 GBq/mmol) were obtained from New England Nuclear (Boston, MA) and Amersham (Aylesbury, UK), respectively. 1-(2-Aminoethyl)piperidine, 1-(2-aminoethyl)piperazine, 4-(2-aminoethyl)morpholine, 1-(2-aminoethyl)pyrrolidine, 4-(2-aminoethyl)pyridine, *N,N*-dimethylethylenediamine, and 4-dimethylaminobenzylamine dihydrochloride were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Additional reagents and solvents were of analytical grade.

Procedure for the synthesis of mevalonyl-alkylamide derivatives

A 100 μl volume of toluene and 20 μl of each of the alkylamines were added to MVL, and the mixture was incubated at 75°C for 60 min. After evaporating the solvent by heating at 55–100°C under a stream of nitrogen, the residue was dissolved in methanol-water (1:1, v/v). 4-Dimethylaminobenzylamine was prepared from 4-dimethylaminobenzylamine dihydrochloride by the addition of an equimolar NaOH solution and extraction with diethyl ether.

Preparation of rat liver microsomes

Male Sprague-Dawley rats were purchased from Charles River Japan (Yokohama, Japan). They were euthanized between 1:00 and 2:00 PM under diethyl ether anesthesia. Livers were excised and frozen immediately in liquid N_2 and stored at −70°C until later use. The animal protocol was approved by the Institutional Animal Care and Use Committee. Microsomes were prepared from livers by differential ultracentrifugation (19), and the protein concentrations were determined by the method of Bradford (20).

Conventional method for the measurement of HMG-CoA reductase activity using RI

The conventional method for the measurement of microsomal HMG-CoA reductase activity using the RI technique was based on the methods of Shefer et al. (6) and Nguyen et al. (21) with some modifications. Microsomes (100 μg of protein) were incubated for 30 min at 37°C in a total 150 μl volume of 100 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 50 mM KCl, 10 mM DTT, a NADPH generating system (34 mM NADPH, 30 mM glucose-6-phosphate, and 1 unit of glucose-6-phosphate dehydrogenase), and 30 nmol of [^{14}C]HMG-CoA (diluted with unlabeled HMG-CoA to give a specific activity of 30 dpm/pmol). The reaction was terminated with the addition of 20 μl of 6 N HCl, and the tubes were allowed to stand at room temperature for 10 min to ensure lactonization of the biosynthetic MVA at pH < 1. A 200 μl volume of ethanol containing 1 mg of [^{3}H]MVL (diluted with unlabeled MVL to give a specific activity of 40,000 dpm/mg), 1 ml of diethyl ether, 0.2 g of ammonium sulfate, and 50 μl of water was added to each tube in order. The tubes were vortexed for 1 min and centrifuged at 1,000 *g* for 2 min. The ether-phase supernatant was collected, and 1 ml of diethyl ether was again added to the residual fraction. After the same extraction procedure, the combined ether fraction was evaporated to dryness under nitrogen. The residue was redissolved in 50 μl of acetone, applied to a thin-layer chromatography plate, and developed with benzene-acetone (1:1, v/v). The MVL band was isolated from the plate, and the radioactivity was measured for 10 min by dual-label liquid scintillation counting.

Measurement of HMG-CoA reductase activity by LC-P-ESI-MS/MS

The present method for the measurement of HMG-CoA reductase activity by LC-P-ESI-MS/MS was carried out as follows. The incubation of microsomes and extraction of biosynthetic MVA were performed by the same method described above for the RI assay, except that unlabeled HMG-CoA and [$^2H_7$]MVL (10 ng) were used instead of [^{14}C]HMG-CoA and [^{3}H]MVL, respectively. A 100 μl volume of toluene and 20 μl of 1-(2-aminoethyl)-pyrrolidine were added to the residue of the ether extract and incubated at 55°C for 60 min. After the addition of 2 ml of toluene, the mixture was applied to a Bond Elut SI cartridge (100 mg; Varian, Harbor City, CA) preconditioned with 1 ml of toluene. The cartridge was washed with 2 ml of toluene-ethyl acetate (1:1, v/v), and the mevalonyl-(2-pyrrolidin-1-yl-ethyl)-amide (MV-PLEA) was eluted with 2 ml of ethyl acetate-methanol (4:1, v/v). After evaporation, the residue was redissolved in 100 μl of methanol-water (5:95, v/v) containing 0.1% acetic acid, and an aliquot (1 μl) was injected into the LC-P-ESI-MS/MS system described below.

The 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 of MV-PLEA was performed using a Hypersil GOLD aQ column (150 × 2.1 mm, 3 μm; Thermo Fisher Scientific) maintained at 40°C. The mobile phase consisted of methanol-water (5:95, v/v) containing 0.1% acetic acid and was used at a flow rate of 200 μl/min. The general LC-MS/MS conditions were as follows: spray voltage, 1,000 V; vaporizer temperature, 350°C; sheath gas (nitrogen) pressure, 50 p.s.i.; auxiliary gas (nitrogen) flow, 50 arbitrary units; ion transfer capil-

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lary temperature, 350°C; collision gas (argon) pressure, 1.5 mTorr; collision energy, 13 V; and ion polarity, positive. Selected reaction monitoring (SRM) was conducted using m/z 245 → m/z 227 for the MV-PLEA and m/z 252 → m/z 234 for the $^{2}H_7$ variant.

Evaluation of the extraction and derivatization processes

The absolute recovery of MVL in the salting-out extraction process was confirmed by adding 10 ng of MVL to the assay mixture without incubation. After diethyl ether extraction, followed by the addition of [$^{2}H_7$]MVL as an internal standard, MVL and its $^{2}H_7$ variant were derivatized, purified, and quantified by LC-P-ESI-SRM.

The recovery of MV-PLEA from the Bond Elut SI cartridge was determined by the addition of MV-PLEA (10 ng) to the cartridge with a derivatizing mixture, which was prepared from the assay mixture without incubation. After the purification steps, [$^{2}H_7$]MV-PLEA (10 ng) was added to the eluate as an internal standard, and both MV-PLEA and its $^{2}H_7$ variant were quantified by LC-P-ESI-SRM.

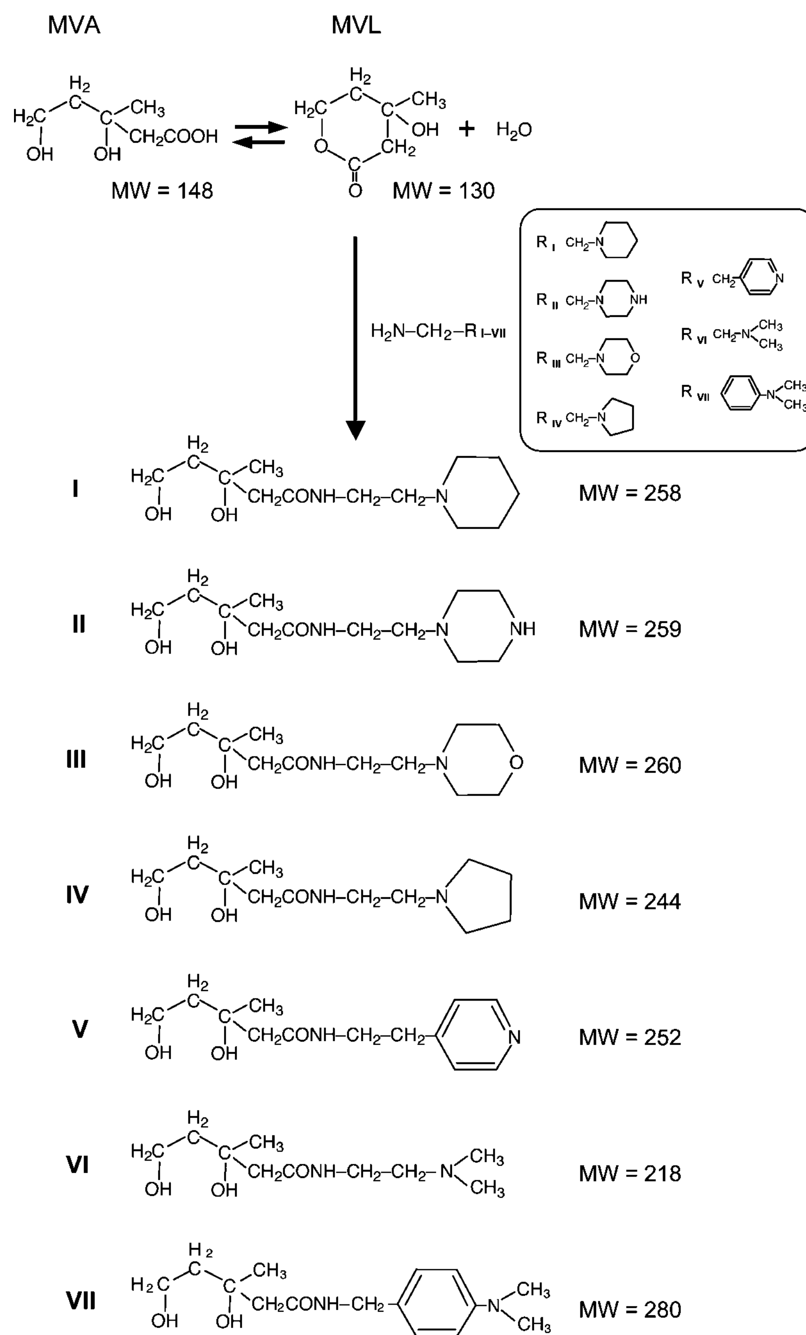

Fig. 1. Structures of mevalonic acid (MVA), mevalonolactone (MVL), and the alkylamide derivatives used in this study: I, mevalonyl-(2-piperidin-1-yl-ethyl)-amide; II, mevalonyl-(2-piperazin-1-yl-ethyl)-amide; III, mevalonyl-(2-morpholin-4-yl-ethyl)-amide; IV, mevalonyl-(2-pyrrolidin-1-yl-ethyl)-amide (MV-PLEA); V, mevalonyl-(2-pyridyl-4-yl-ethyl)-amide; VI, mevalonyl-(2-dimethylamino-ethyl)-amide; VII, mevalonyl-dimethylamino-benzylamide. MW, molecular weight.

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The effects of incubation temperature and duration on the (2-pyrrolidin-1-yl-ethyl)-amidation of MVL were examined. A 4 μg aliquot of MVL was incubated with 20 μl of 1-(2-aminoethyl)-pyrrolidine in 100 μl of toluene. After the addition of [$^2H_7$]MV-PLEA as an internal standard and purification by a Bond Elut SI cartridge, MV-PLEA and its $^2H_7$ variant were quantified by LC-P-ESI-SRM.

Statistics

Data are reported as means ± SD. Reproducibility was analyzed by one-way layout (JMP software; SAS Institute, Inc., Cary, NC). Recovery was analyzed using a polynomial equation (22). Linearity of the calibration curve, correlation between the amount of microsomal protein and the formation of MVL, and correlation between enzyme activities determined by LC-P-ESI-SRM and the RI methods were 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 $P < 0.05$.

RESULTS

Selection of the best derivative for the quantification of MVL

To find the best derivative for MVL, we synthesized seven kinds of mevalonyl-alkylamides (**Fig. 1**), and their P-ESI mass spectral and tandem mass spectral data were compared. As shown in **Table 1**, all mevalonyl-alkylamide derivatives exhibited protonated molecular ions ($[M+H]^+$) as the base peaks. The fragmentation pattern of the protonated molecular ion of each derivative under various levels of collision energy was examined, and the most abundant product ion was selected as a monitoring ion for SRM. The signal-to-noise ratios (S/Ns) of these derivatives determined by injecting 100 fg of each derivative were as follows: VI (20) > V (15) > IV (10) = III (10) > I (5) = II (5) = VII (5). However, additional factors, such as the lower boiling point of the remaining alkylamines, the convenience of the solid-phase purification procedure, and an improved symmetric shape of the peak in the chromatogram finally determined that MV-PLEA (derivative IV) was the best derivative for the quantification of MVL in the incubation mixture. The representative P-ESI mass spectrum and MS/MS spectrum of the MV-PLEA are shown in **Fig. 2**.

Evaluation of the extraction and derivatization processes

The absolute recovery of MVL from the incubation mixture by the salting-out extraction procedure was calculated as 88.1 ± 2.8% (n = 4). The recovery of MV-PLEA from the Bond Elut SI cartridge was found to be 98.1 ± 3.1% (n = 4). The derivatization of MVL to MV-PLEA achieved a maximum efficiency at 55°C for 60 min. Higher incubation temperature or longer incubation time did not result in increased formation of MV-PLEA. When the derivatizing mixture was incubated at room temperature (20°C) for 5, 10, 30, and 60 min, the derivatization was 8, 14, 81, and 94%, respectively.

Calibration curve

A calibration curve was established for MVL (**Fig. 3**). Different quantities (0.04, 0.1, 0.4, 1, 4, 10, 40, and 100 ng) of authentic MVL were each mixed with 10 ng of [$^2H_7$]MVL, derivatized to the MV-PLEA, and purified using the Bond Elut SI cartridge, as described in Materials and Methods. The weight ratio of MVL, relative to the corresponding deuterated internal standard, was compared with the peak area ratio of the MV-PLEA to the $^2H_7$ variant measured by LC-P-ESI-SRM. The linearity of the standard curve, as determined by simple linear regression, was excellent

TABLE 1. Positive ESI-MS, MS/MS, and SRM data of the mevalonyl-alkylamides

Derivatives[a]	MS Data $[M+H]^+$ (Relative Intensity)	MS/MS Data[b] (Collision Energy at Maximum Intensity)				SRM Data[c] Collision Energy	SRM Data[c] Precursor to Product	SRM Data[c] S/N[d]
	m/z (%)	*m/z* (V)				V	*m/z*	
I	259 (100)	241 (13)	174 (18)	86 (23)	112 (17)	13	259→241	5
II	260 (100)	224 (13)	113 (20)	86 (21)	138 (22)	13	260→224	5
III	261 (100)	243 (13)	114 (18)	138 (20)	86 (21)	13	261→243	10
IV	245 (100)	227 (13)	98 (19)	86 (20)	138 (24)	13	245→227	10
V	253 (100)	106 (30)	112 (15)	77 (51)	–[e]	30	253→106	15
VI	219 (100)	91 (31)	202 (13)	86 (20)	–[e]	31	219→91	20
VII	281 (100)	134 (29)	118 (48)	91 (54)	–[e]	29	281→134	5

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

[a] Mevalonolactone (MVL) was derivatized to I, mevalonyl-(2-piperidin-1-yl-ethyl)-amide; II, mevalonyl-(2-piperazin-1-yl-ethyl)-amide; III, mevalonyl-(2-morpholin-4-yl-ethyl)-amide; IV, mevalonyl-(2-pyrrolidin-1-yl-ethyl)-amide (MV-PLEA); V, mevalonyl-(2-pyridyl-4-yl-ethyl)-amide; VI, mevalonyl-(2-dimethylamino-ethyl)-amide; and VII, mevalonyl-dimethylamino-benzylamide.

[b] $[M+H]^+$ 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.

[c] The same HPLC column and flow rate described in Materials and Methods were used, but the following gradient system was used for the elution of all mevalonyl-alkylamides from the column: for the first 2 min, the mobile phase consisted of methanol-water (1:9, v/v) containing 0.1% acetic acid; then it was programmed in a linear manner to methanol-water (1:1, v/v) containing 0.1% acetic acid over 4 min. The final mobile phase was kept constant for 2 additional min. The retention times of derivatives I–VII under this gradient HPLC condition were 2.9 min (II), 2.9 min (III), 3.0 min (IV), 3.2 min (V), 3.8 min (I), 4.6 min (VI), and 7.0 min (VII).

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

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

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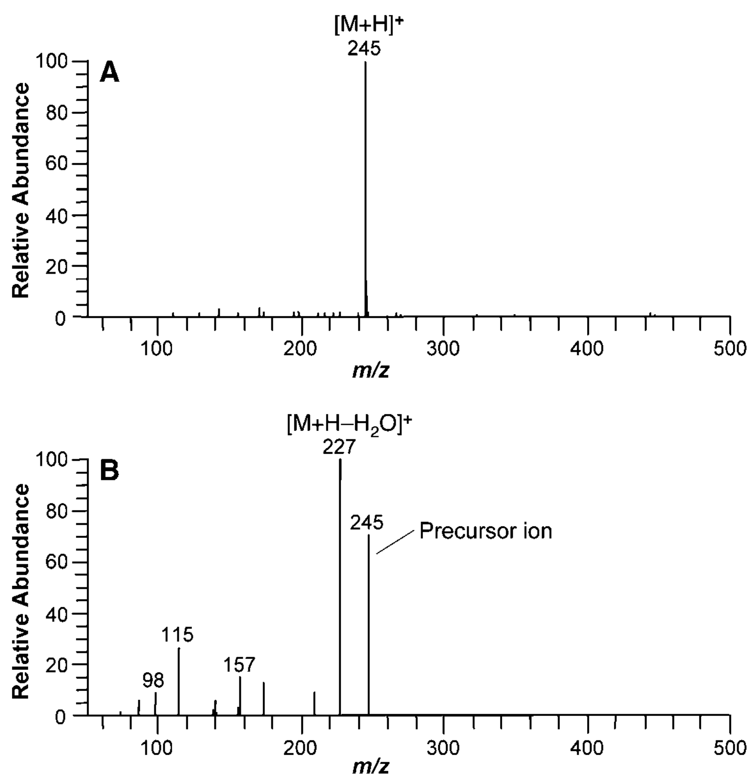

Fig. 2. Typical electrospray ionization in positive mode (P-ESI) mass spectrum (A) and product ion mass spectrum using *m/z* 245 as a precursor ion (B) of the MV-PLEA. The general liquid chromatography-tandem mass spectrometry (LC-MS/MS) conditions were as follows: introducing solvent, methanol-water (5:95, v/v) containing 0.1% acetic acid; flow rate, 200 μl/min; spray voltage, 1,000 V; collision energy, 13 V.

for weight ratios between 0.004 and 10 (n = 8, r = 1.000, $P < 0.0001$).

Representative recordings of LC- P-ESI-SRM

Figure 4 presents typical LC-P-ESI-SRM chromatograms obtained by analysis of a standard incubation mixture using 100 μg of protein of microsomes from normal rat liver. The peak for MV-PLEA at *m/z* 245 → *m/z* 227 in Fig. 4B reflects the amount of MVL after incubation and corresponds to ∼90 pg (∼23 pmol/min/mg protein). As shown in Fig. 4A, endogenous MV-PLEA was detected at zero time, but the amount did not significantly affect the assay of the enzyme activity.

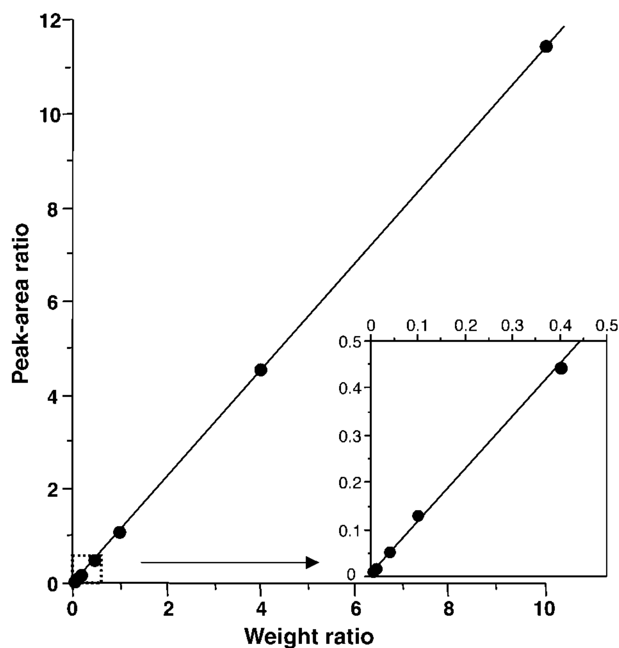

Fig. 3. Calibration curve for the weight ratio of MVL to the corresponding deuterated internal standard. Linearity was checked by simple linear regression, and the equation for the line of best fit was y = 1.144x − 0.004 (n = 8, r = 1.000, $P < 0.0001$).

Precision and accuracy of this method

The precision and accuracy of our method was determined using the same microsomes obtained from a normal rat liver. Reproducibility was investigated by analyzing four samples in triplicate by LC-P-ESI-SRM (**Tables 2**, **3**). The results were analyzed by one-way layout, in which the analytical errors were divided into two sources: sample preparation and LC-P-ESI-SRM measurement. The variances were considered to be attributable to the measurement, because the errors during sample preparation were negligible. The interassay coefficients of variation for the between- and within-sample variations were 3.2% and 1.8%, respectively.

For the recovery experiment (**Table 4**), known quantities of MVL (10–30 ng, 0.77–2.31 nmol/mg protein) were spiked into the mixture at the end of the incubation. After the extraction and clean-up procedures, LC-P-ESI-SRM was conducted in triplicate for each sample. The recoveries of the known spiked amounts of MVL ranged from 92.6% to 96.2%, with a mean of 94.6%. In addition, the amount of MVL found in the unspiked incubation mixture was within the 95% confidence limit for the estimated amount of MVL, as calculated by linear regression analysis; this also constituted an index for the precision and accuracy of the method.

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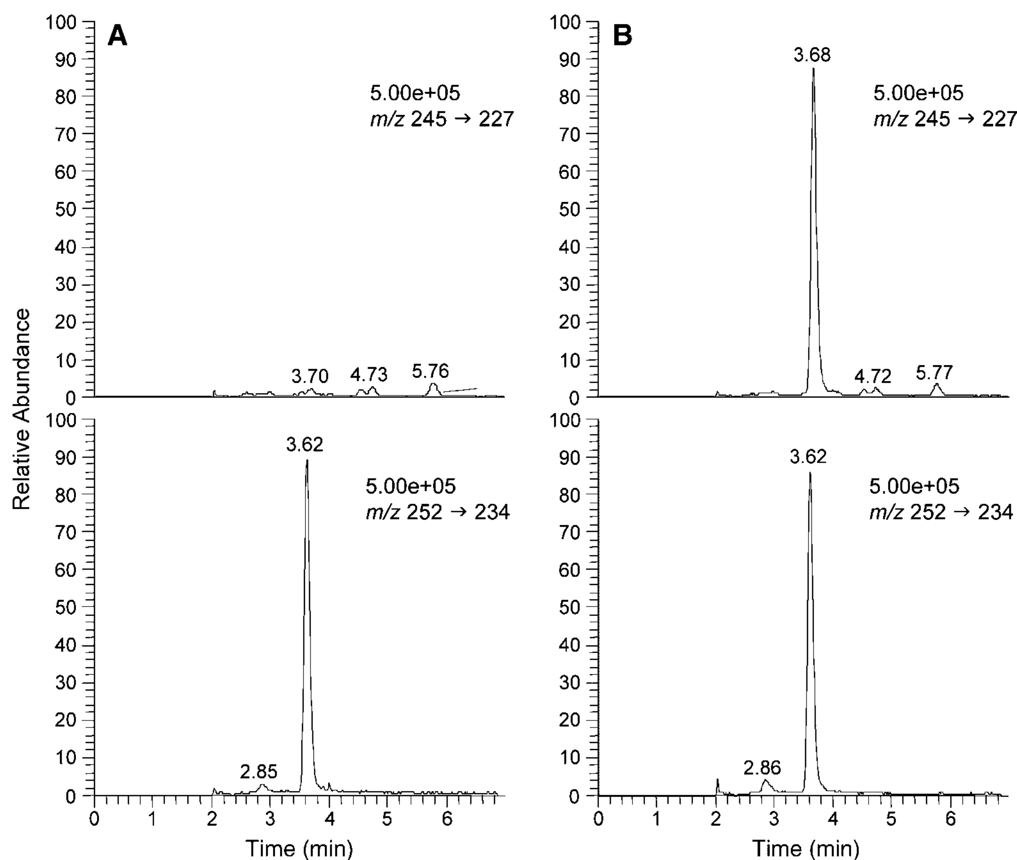

Fig. 4. Representative LC-selected reaction monitoring (SRM) chromatograms of MV-PLEA and its $^{2}H_{7}$ variant (internal standard) in extracts obtained from the standard incubation mixture. A: Zero time. B: Incubated with 100 μg of protein of rat liver microsomes for 30 min. The peak of MV-PLEA in B corresponds to ~90 pg (~23 pmol/min/mg protein).

Application to a microscale assay

We studied the effect of reduced levels of microsomal protein on the quantification of MVL amount and enzyme activity. Different quantities (5, 10, 25, 50, and 100 μg of protein) of normal rat liver microsomes were used for the enzyme assay, and extraction and quantification of MVL were conducted as described in Materials and Methods. As shown in **Fig. 5**, proportionality was observed when the quantity of microsomal protein ranged from 5 μg up to at least 100 μg for the production of MVL.

Correlation between our method and the RI method

Hepatic HMG-CoA reductase activities were measured by both RI and positive LC-P-ESI-SRM methods in 11 normal rats. The HMG-CoA reductase activities obtained by this method correlated well with those obtained by the RI method ($r = 0.930$, $P < 0.0001$) (**Fig. 6**).

DISCUSSION

We have developed and optimized various derivatization methods that are appropriate for enhancing the sensitivity

TABLE 2. Reproducibility in the measurement of HMG-CoA reductase activity: analytical data

Sample	Individual Values			Mean ± SD
	pmol/min/mg protein			
A	15.7	16.1	15.2	15.7 ± 0.4
B	16.0	15.9	16.0	16.0 ± 0.1
C	15.3	15.4	15.3	15.4 ± 0.1
D	15.3	15.0	15.7	15.4 ± 0.4
Mean ± SD				15.6 ± 0.4

HMG-CoA reductase activity was measured using 100 μg of protein of rat microsomes.

TABLE 3. Reproducibility in the measurement of HMG-CoA reductase activity: ANOVA

Source	S	f	V	F_0	Relative SD
					%
Sample preparation	0.742	3	0.247	3.04	3.2
Error (SRM)	0.650	8	0.081		1.8
Total	1.392	11			
			$F(3,8,0.05) = 4.07$		

S, residual sum of squares; f, number of degrees of freedom; f_1, $f_{\text{sample preparation}}$; f_2, f_{error}; V, unbiased variance; F_0, observed value after F distribution variance ratio ($V_{\text{sample preparation}}/V_{\text{error}}$); $F(f_1, f_2, \alpha)$, density function of F distribution with f_1 and f_2 degrees of freedom.

TABLE 4. Recovery of MVL from the incubation mixture

Sample (X_0 + na) (n = 0,1,2,3)	Amount Added	Amount Found			Recovery[a] (mean ± SD)	Estimated Amount ± 95% Confidence Limit[b]
		nmol/mg protein			*%*	*nmol/mg protein*
X_0	0	$\overline{X}_0$ ± SD = 0.47 ± 0.01[c]				0.47 ± 0.07
X_0 + a	0.77	1.24	1.23	1.20		
X_0 + a	0.77	1.19	1.20	1.19	96.2 ± 2.6	
X_0 + 2a	1.54	1.89	1.88	1.91		
X_0 + 2a	1.54	1.86	1.88	1.94	92.6 ± 1.7	
X_0 + 3a	2.31	2.70	2.76	2.60		
X_0 + 3a	2.31	2.70	2.65	2.55	95.1 ± 3.3	

Known amounts of MVL were spiked into the mixture at the end of the incubation.
[a] Recovery (%) = (amount found − $\overline{X}_0$)/amount added × 100.
[b] The estimated amount was calculated by a polynomial equation.
[c] This value was obtained from Table 2.

of the microanalysis of endogenous substances in biological specimens by GC-MS. As described in our previous investigations (7, 10, 11), MVA was lactonized easily to MVL and the resulting MVL was converted to the corresponding alkylamides with the primary alkylamines under mild conditions without any catalysts. This amidation via MVL from MVA was a characteristic reaction for γ-hydroxy fatty acids such as MVA, and the resulting alkylamides were further converted to the final derivatives suitable for the detection of ionization. Thus, these derivatives provided excellent GC-MS properties in their separation, specificity, and sensitivity and enabled us to quantify trace amounts of MVA in biological specimens.

LC-MS methods, using ESI or atmospheric pressure CI, have been used to quantify trace amounts of biologically important fatty acids after enhancing their detection sensitivity through derivatization, such as pentafluorobenzylation (23–25). Although several methods for quantifying MVA or MVL in biological specimens by LC-ESI-MS or LC-ESI-MS/MS have been described, there have been no methods to enhance their detection sensitivity through derivatization. The negative and positive modes for ESI have been used for the quantification of MVA and MVL, respectively. We used the positive mode for this quantification because it provides more abundant ions than the negative mode (26). To enhance the sensitivity of detection, the amidation reaction described above, which is characteristic for MVA, was conducted using seven kinds of primary alkylamines with a tertiary amine moiety to promote protonation. Thus, the mevalonyl-alkylamides synthesized in this study were all versatile derivatives. The resulting mass spectra exhibited $[M+H]^+$ as the base peaks, which is suitable for the highly sensitive detection of MVL using LC-P-ESI-MS/MS. Collision of $[M+H]^+$ for derivatives I–IV under relatively low energy (<14 V) produced the characteristic product ions related to the

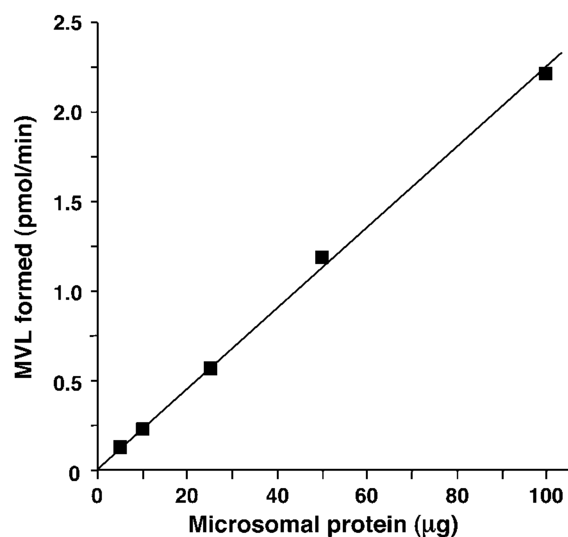

Fig. 5. Effects of reduced microsomal protein on the determination of HMG-CoA reductase activity. Linearity was checked by simple linear regression, and the equation for the line of best fit was y = 0.022x + 0.026 (n = 5, r = 0.999, P < 0.0001).

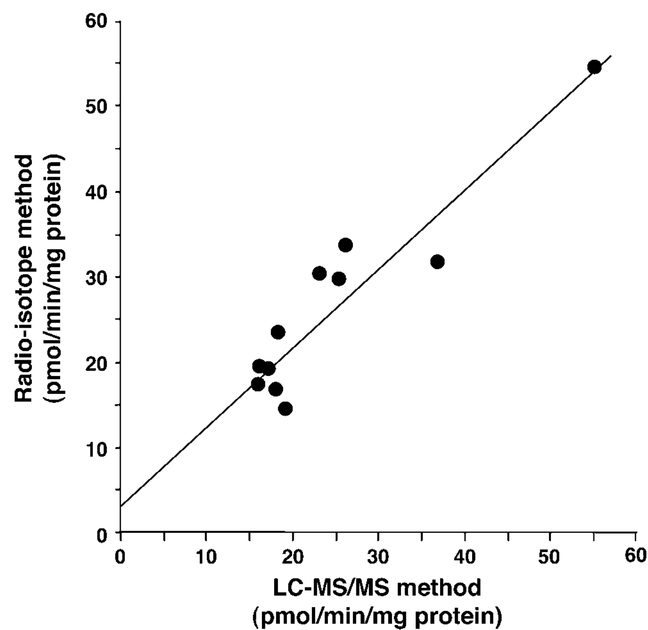

Fig. 6. Correlation between our LC-MS/MS method and the conventional radioisotope method for the assay of HMG-CoA reductase activity. Linearity was checked by simple linear regression, and the equation for the line of best fit was y = 0.905x + 3.899 (n = 11, r = 0.930, P < 0.0001).

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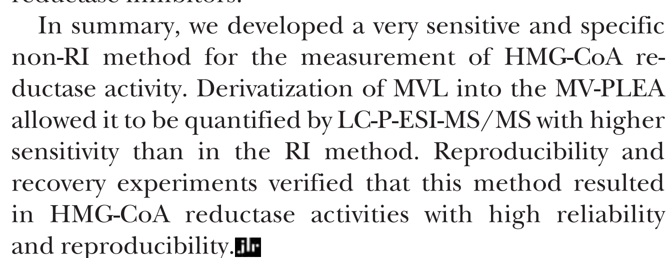
$[M+H-H_2O]^+$ or $[M+H-2H_2O]^+$. In contrast, the increase of collision energy (>14 V) resulted in the formation of product ions with low mass numbers from all seven derivatives.

All of these seven derivatives exhibited excellent LC-P-ESI-MS/MS properties with extremely high sensitivity, as shown in Table 1. However, MV-PLEA was selected as the best derivative for our purpose for the following reasons. First, the boiling point of 1-(2-aminoethyl)pyrrolidine is the second lowest, after *N,N*-dimethylethylenediamine, which is advantageous for evaporating contaminated alkylamines after solid-phase purification. Second, mevalonyl-2-(dimethylamino)ethylamide (VI) exhibited a better S/N peak than MV-PLEA, but the peak shape of derivative VI was considerably broader compared with that of derivative IV (0.3 vs. 0.7 min). Third, MV-PLEA was successfully purified from the derivatizing mixture with almost complete elimination of excess 1-(2-aminoethyl)pyrrolidine using a small Bond Elut SI cartridge. Because 1-(2-aminoethyl)pyrrolidine can be evaporated by heating at 55°C under a nitrogen stream, the solid-phase extraction step was not necessary to quantify MV-PLEA by LC-SRM. However, this step was successful not only in eliminating the excess alkylamine but also for cleaning up the extracts with diethyl ether, which reduced the load on the LC-MS/MS system.

In the conventional RI method, the specific activity of radiolabeled HMG-CoA was 30 dpm/pmol, which was equivalent to 33.3 fmol/dpm. When the standard deviation of background noise is 2 dpm, the signal would be 6 dpm when the S/N = 3. Therefore, the detection limit of the conventional RI method was calculated to be 200 fmol (26 pg) (S/N = 3). Although it may be not practical, if carrier-free $[^{14}C]$HMG-CoA without dilution with unlabeled HMG-CoA were used, the minimum detection limit of the RI method would be 46.5 fmol (6.0 pg) (S/N = 3), because the specific activity of the carrier-free $[^{14}C]$HMG-CoA is 2.15 GBq/mmol = 2.15 Bq/pmol = 129 dpm/pmol = 7.75 fmol/dpm. Clearly, the measurement of HMG-CoA reductase activity by LC-MS (14) and LC-MS/MS (15) is more specific than by the RI method, in which radioactivities are counted on thin-layer chromatography plates. However, the detection limit of underivatized MVL by LC-MS was 50 fmol (6.5 pg) (S/N = 3) (14), which was ~100,000 times more sensitive than that obtained by HPLC-ultraviolet (5.7 nmol [740 ng]) (27) but did not apparently exceed that obtained by the RI method. In comparison, the present LC-P-ESI-MS/MS method made it possible to detect only 240 amol (31 fg) (S/N = 3) of MVL. Therefore, even if a sufficient quantity of microsomes were not available, this method would still enable us to measure HMG-CoA reductase activity (Fig. 5). In addition to the high sensitivity, the use of $[^2H_7]$MVL as an ideal internal standard contributed to the development of this highly reproducible method.

In this method, we followed the incubation and extraction procedures of the conventional RI method, with the exception that unlabeled HMG-CoA and $[^2H_7]$MVL were used instead of $[^{14}C]$HMG-CoA and $[^3H]$MVL, respectively. The subsequent derivatization and purification steps are very simple, and the HPLC system operates isocratically. Therefore, it would be easy to switch from the conventional RI method to the present non-RI method if an LC-MS/MS system were available. In addition, the data obtained by this non-RI method are comparable with previous data obtained by the RI method, because both methods obtained very similar results (Fig. 6). Furthermore, this method can be used as a high-throughput screening method for HMG-CoA reductase inhibitors.



In summary, we developed a very sensitive and specific non-RI method for the measurement of HMG-CoA reductase activity. Derivatization of MVL into the MV-PLEA allowed it to be quantified by LC-P-ESI-MS/MS with higher sensitivity than in the RI method. Reproducibility and recovery experiments verified that this method resulted in HMG-CoA reductase activities with high reliability and reproducibility.

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