methods

Validation of the LC-MS/MS method for the quantification of mevalonic acid in human plasma and determination of the matrix effect

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Abstract A simple, specific, and sufficiently sensitive liquid chromatography-tandem mass spectrometry (negative-ion electrospray ionization) methodology to determine mevalonic acid (MVA) in human plasma is described, and its application to the analysis of rat plasma MVA levels after rosuvastatin administration is demonstrated. The method was validated over the linearity range of 0.5–50.0 ng/ml ($r^2 > 0.99$) using deuterated MVA as an internal standard. The lower limit of quantification was 0.5 ng/ml. The assay procedure involved the isolation of MVA from plasma samples using solid-phase extraction. Chromatographic separation was achieved on a HyPurity Advance column with a mobile phase consisting of ammonium formate buffer (10 mM, pH 8.0) and acetonitrile (70:30, v/v). Excellent precision and accuracy were observed. MVA and deuterated mevalonolactone were stable in water and plasma under different storage and processing conditions. The recovery observed was low, which was attributable to a significant matrix effect. A significant decrease (30–40%; $P < 0.05$) was observed in rat plasma MVA levels after rosuvastatin administration.—Saini, G. S., T. A. Wani, A. Gautam, B. Varshney, T. Ahmed, K. S. Rajan, K. K. Pillai, and J. K. Paliwal. **Validation of the LC-MS/MS method for the quantification of mevalonic acid in human plasma and determination of the matrix effect.** ***J. Lipid Res.*** **2006.** 47: **2340–2345.**

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In the biosynthesis of cholesterol, the conversion of HMG-CoA to mevalonic acid (MVA) by HMG-CoA reductase is an early and rate-limiting step (1–3). The statin class of drugs, such as simvastatin, atorvastatin, and rosuvastatin, act on HMG-CoA reductase, resulting in the inhibition of MVA biosynthesis (**Fig. 1**) (4, 5). Understanding the reason for increased cholesterol levels and interindividual variability in response to statin therapy can lead to better and monitored pharmacotherapy (6). Because the reduction of MVA levels is an indirect measure of decreased cholesterol levels, MVA can be used as a biomarker to measure the extent of statin activity.

A large variety of methods have been published for MVA estimation in urine and plasma. These involve enzyme immunoassay (7), radioimmunoassay (2), and GC-MS methods (8–10). However, there are very few methods reported for liquid chromatography-tandem mass spectrometry (LC-MS/MS) (11, 12).

The main challenge in developing and validating a method for determining MVA in human plasma was that MVA is a polar, endogenous moiety that circulates in the blood stream at nanogram levels. In most methods, the extraction of MVA from plasma was carried out using ion-exchange resins in the form of mevalonolactone (MVAL) (11, 12). Complicated procedures such as column switching and gradient flow with long run times were followed (11). In a modified assay procedure, a polar-end-capped reverse-phase liquid chromatography column was used for the quantification of plasma MVA over a calibration range of 0.5–50 ng/ml in human plasma (12). This assay had the advantages of shorter run time and isocratic flow.

These methods have reported recovery to be 50–87%. The procedure followed does not capture the effect of any constant impurity/substance that may suppress ionization. The exact recovery can be obtained by comparing the response of processed spiked plasma with that of aqueous samples at the same concentration. The matrix effect can be evaluated by comparing spiked processed plasma blanks with aqueous samples at the same concentration. By knowing the recovery and matrix effect, the sensitivity of the method can be improved.

A specific and sufficiently sensitive method was required for the quantification of plasma MVA levels in clinical trials. The reported normal range of human plasma MVA

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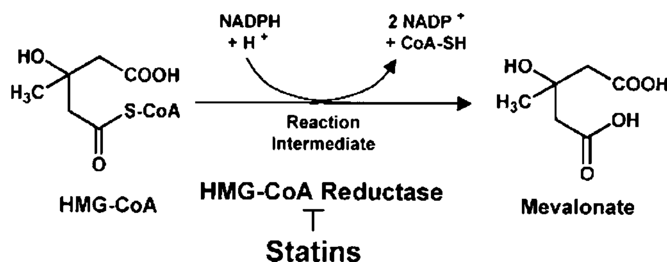

Fig. 1. Conversion of HMG-CoA to mevalonate.

levels is 1.5–11.8 ng/ml (13). After statin treatment even at the highest dose, the percentage decrease in plasma MVA levels has been shown to be 30–50% (11, 14, 15). Therefore, the lowest limit of quantification was kept at 0.5 ng/ml and the upper limit was 50 ng/ml. The use of surrogate matrix (water) has been reported (11, 12) and is necessary to achieve lower levels of quantification.

In this study, a simple, robust, and reproducible method has been developed and validated to estimate MVA concentrations in human plasma. Equilibration time for the conversion of MVA to MVAL under acidic pH was further optimized. An approach to differentiate the matrix effect from recovery was performed under validation. The validated method was applied to quantify plasma MVA concentrations in rat to determine the effect of rosuvastatin on plasma MVA levels.

EXPERIMENTAL PROCEDURES

Materials

MVAL (97%) was obtained from Sigma-Aldrich (Poole, Dorset, UK), and the internal standard (IS), deuterated MVAL (D7-MVAL), was from CDN Isotopes (Pointe-Claire, Quebec, Canada). The cartridges (IST ENV+; 100 mg/3 ml) were procured from International Sorbent Technology (Mid Glamorgan, UK). All solvents and other reagents were of analytical grade. Control human plasma (lithium heparin anticoagulant) for the preparation of quality control (QC) samples was obtained from a blood bank and stored at −70°C before use.

Column liquid chromatography

The column was a HyPurity Advance column (50 mm × 4.6 mm, 5 μm particle size; Thermo Electron Corp.). The column was kept at ambient temperature. The mobile phase consisted of ammonium formate buffer (10 mM, pH 8.0, adjusted with liquid ammonia) and acetonitrile (70:30, v/v). The flow rate was 0.8 ml/min, and the total run time was 3 min.

Mass spectrometry

The liquid chromatograph (Agilent 1100; Agilent Technologies, Inc., Palo Alto, CA) was coupled to a mass spectrometer with a turbo electrospray ion source (4000 Qtrap; Applied Biosystems, Foster City, CA) and was used in negative ionization mode with the following source settings. The turbo ion-spray interface was maintained at 530°C with zero air nebulization. The zero air was kept at a pressure of 70 p.s.i. The turbo ion-spray drying gas (zero air) was kept at a pressure of 70 p.s.i. The collision-activated dissociation gas pressure was 7 p.s.i., and the curtain gas pressure was 30 p.s.i.; turbo ion-spray voltage was −3,500 V. Declustering potential was −35 V; entrance potential was −10 V; collision energy was −20 V; collision cell exit potential was −1 V; and channel electron multiplier was 2,600 V. The multiple reaction monitoring pair monitored was m/z 147 → 59 for MVA and m/z 154 → 59 for D7-MVA, with a dwell time of 200 ms. The autosampler cooler was maintained at 10°C. Analyst software (version 1.4; Applied Biosystems) was used for data registration and calibration.

Sample collection

After validation, to study the effect of rosuvastatin on plasma MVA levels in rat (n = 5), the method was used to quantify plasma MVA concentrations after 10 mg/kg oral administration. Blood samples were collected in tubes containing lithium heparin before drug administration and 0.5, 1, 1.5, 6, 16, and 24 h thereafter. The samples were centrifuged at 2,500 *g* for 10 min. The separated plasma was stored at −70°C until analysis.

Sample preparation

Samples were thawed in water. Plasma aliquots of 500 μl were added to a glass tubes to which were also added IS (100 μl, 200 ng/ml), 0.1 N HCl (1 ml), and water (0.5 ml); the tubes were than vortex-mixed. The sample solution was allowed to equilibrate for 30 min to convert MVA to MVAL. Each sample solution was individually transferred to a solid-phase extraction cartridge (IST ENV+; 100 mg/3 ml) that had been preconditioned with methanol (1 ml) followed by 0.1 N HCl (1 ml). Each cartridge was washed with 0.1 N HCl (1 ml) followed by water (1 ml) and 15% methanol in water. The cartridges were allowed to dry. The analytes were eluted with 3 × 0.5 ml of methanol. The resulting methanol extract solutions were evaporated to dryness under a stream of nitrogen at 15 p.s.i. and 40°C bath temperature for 15 min. The residues were reconstituted in 0.2% ammonium hydroxide solution (100 μl) to convert MVAL to MVA. Aliquots of 10 μl were injected into the LC-MS/MS apparatus for analysis.

Standard curves

The calibration curve (CC) standards were prepared in water by adding known amounts of MVA. Lower limit of quantification (LLOQ) QC and low-quality control (LQC) samples were obtained by spiking MVA in water; the final concentrations were 0.5 and 1.9 ng/ml, respectively. Middle-quality control (MQC) and high-quality control (HQC) samples were obtained by spiking in plasma with concentrations of 21.9 and 41.3 ng/ml, respectively. The bulk-spiked CC and QC samples were stored at −70°C. The endogenous MVA level obtained in plasma (12.2 ng/ml) was added to spiked plasma samples to obtain corrected concentrations for MQC and HQC samples. All calibration curves consisted of one blank sample and eight calibration points in the concentration range of 0.5–50 ng/ml. The concentrations were corrected for potency and amount weighed. The resulting peak area ratios were plotted against the concentrations.

Validation

Specificity. The approach of using water for the preparation of CC and lower QC standards has been verified and reported previously (11, 12). A specificity exercise was performed for both water and plasma. Individual blank plasma samples, LLOQ QC samples, and water (blank) (n = 6) were prepared according to the sample preparation procedure described above and screened for interference.

Recovery. The recovery exercise was performed at all QC levels by comparing the response (area) of processed QC samples

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with those of directly injected QC samples. The dilutions were made in 0.2% ammonium hydroxide solution to keep conditions the same.

Matrix effect. To study the matrix effect, blank plasma samples were processed and spiked later to obtain MQC and HQC concentrations. The response (area) was compared with directly injected samples at MQC and HQC levels.

Inter-assay and intra-assay imprecision and accuracy. Inter-assay and intra-assay imprecision and accuracy were evaluated by spiking known amounts of MVA and IS in plasma (n = 5). Four different concentrations were used, and samples were prepared according to the procedure mentioned above. Intra-assay imprecision and accuracy were assessed within one batch, whereas inter-assay imprecision and accuracy were assessed on three separate occasions.

Dilution integrity and partial volume analysis. MQC and HQC samples were diluted two and four times with water for dilution integrity. Partial volume analysis was performed at one-half and one-fourth the processing volumes at MQC and HQC levels. The samples were processed according to the procedure mentioned above in five replicates.

Stability. The stability of MVA was studied in human plasma and water at room temperature (bench top) for 6 h and in an autoinjector for 20 h. The bulk-spiked plasma and water samples stored at −70°C underwent three freeze-thaw cycles. Stock solution stability studies were performed at room temperature for 4 h and in refrigeration for 22 days. In addition, a long-term (28 days) stability study was done in human plasma stored at −70°C. The stability of D7-MVA was assessed in an autoinjector for 20 h. Stock solution stability studies were performed at room temperature for 4 h and in refrigeration for 112 days. Stock solution stability studies were carried out at the MQC level, and working IS was prepared from fresh and refrigerated stock solutions. The drug and IS response ratios of stored and fresh stocks were compared. In other stability studies, five replicates of LQC and HQC were analyzed.

RESULTS

A high-performance liquid chromatographic mass spectrometric method for the estimation of MVA in human plasma has been developed and validated according to the principles of Good Laboratory Practices. The plasma was validated over a concentration range of 0.5–48.5 ng/ml. Sample cleanup was accomplished by solid-phase extraction using C-18, ENV+ cartridges. The reconstituted samples were analyzed by LC-MS/MS using a HyPurity Advance (4.6 × 50 mm) column. The retention times of MVA and D7-MVA were between 0.8 and 0.9 min,

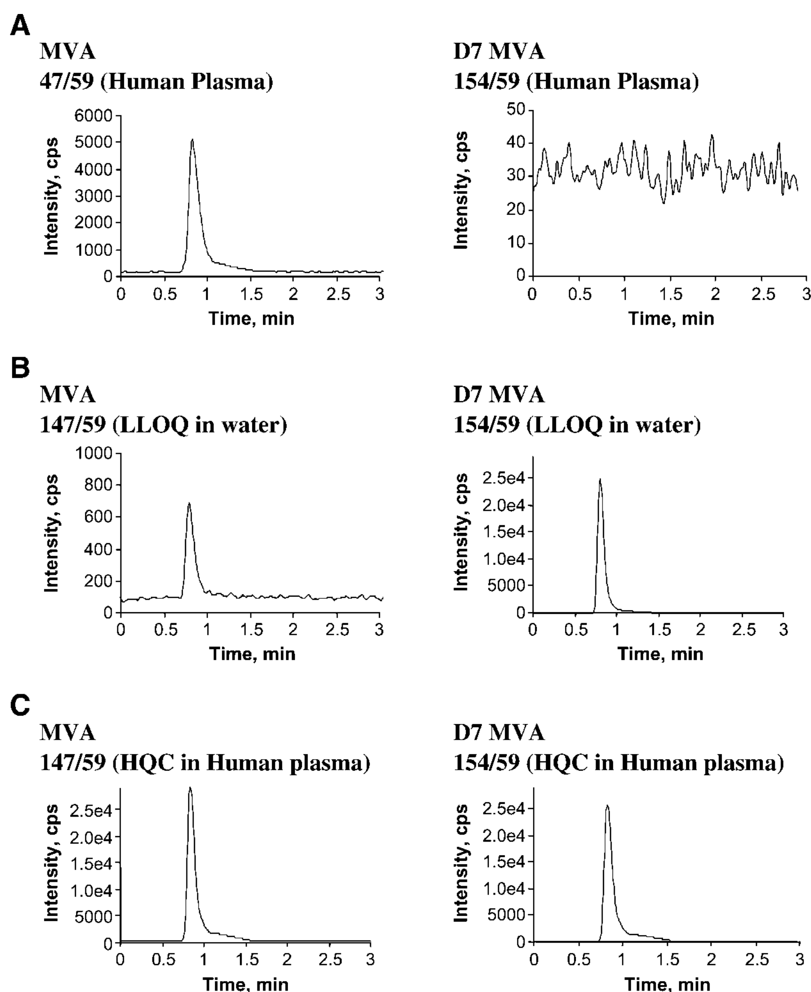

Fig. 2. Representative chromatograms. A: Plasma extract (endogenous background) without internal standard. B: Mevalonic acid (MVA) sample prepared in water. C: Spiked human plasma. D7 MVA, deuterated mevalonic acid; HQC, high-quality control; LLOQ, lower limit of quantification.

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with a total run time of 3 min. Representative chromatograms of MVA in blank plasma, LLOQ QC, and HQC samples are shown in **Fig. 2**.

The lower limit of quantitation was 0.5 ng/ml for MVA. The between-run precision and accuracy for MVA at 0.5 ng/ml were 6% and 105%, respectively. The linearity of the method was determined by a weighted least-squares regression analysis of an eight point standard curve. The calibration lines were shown to be linear from 0.5 to 48.5 ng/ml. Best-fit calibration lines of the ratio of MVA to IS peak area versus the concentration of calibration standards were determined by least-squares regression analysis with weighting factors of $1/x^2$. The r^2 values were consistently >0.99 during the course of validation.

The imprecision of the assay was measured by the percentage coefficient of variation over the concentration range of LLOQ QC, LQC, MQC, and HQC samples during the course of validation. The accuracy of the assay was defined as the absolute value of the ratio of the calculated mean values of the QC samples to their respective nominal values, expressed as percentages. Within-batch precision ranged from 1% to 18%, and within-batch accuracy ranged from 97% to 116%. Intra-assay precision ranged from 1% to 17%, and intra-assay accuracy ranged from 98% to 109%. Inter-assay precision ranged from 3% to 12%, and inter-assay accuracy ranged from 99% to 108%.

The room temperature stock stability at 4 h was 101% for MVA and 111% for IS. The refrigerated stock solution stability on the 22nd day for MVA was 105%, and that for IS on the 112th day was 100%. The autoinjector stability results demonstrate that MVA and IS are stable for 20 h. The mean stability ranged from 97% to 102% for MVA and from 98% to 102% for IS. The mean stability of MVA in human plasma ranged from 97% to 102% and 92% to 98% for one and three freeze-thaw cycles, respectively. During bench-top stability analysis, MVA was found to be stable up to 4 h, and the mean stability ranged from 94% to 102%. MVA was found to be stable for up to 28 days of storage (plasma) below −50°C, and the mean stability ranged from 100% to 107% (**Table 1**). The absolute recovery of MVA and IS was calculated for replicate spiked QC samples (MQC and HQC). Results indicate overall recoveries of 21% for MVA and 21% for IS. The percentage matrix effect was 46% for analyte and 73% for IS.

The results demonstrate acceptable dilution integrity for two and four times. Within-batch precision and accuracy for two times dilution were 5–8% and 103–104%, respectively, whereas within-batch precision and accuracy for four times dilution were 6–7% and 100–107%, respectively. These results demonstrate acceptable partial volume analysis for one-half volume (250 µl) and one-fourth volume (125 µl) analysis (**Table 2**). Within-batch imprecision and accuracy for one-half volume were 4–8% and 104–105%, respectively, whereas at one-fourth volume, within-batch imprecision and accuracy were 6–9% and 102–103%, respectively (Table 2).

The validated method was successfully applied to measure MVA in rat plasma. One-fourth volume (125 µl) of plasma was used and read against CC in water. The QC samples were run to cross-validate the method. Plasma concentrations decreased significantly after oral rosuvastatin (10 mg/kg) administration (**Fig. 3**). Because the reduction was 30–40%, assay sensitivity (0.5 ng/ml) was sufficient.

TABLE 1. Stability of MVA and D7 MVA (LQC samples in water and HQC samples in plasma)

Storage Condition	Mean MVA Concentration: Baseline QC (CV %)	Mean MVA Concentration: After Storage (CV %)	Difference from Baseline
	ng/ml		%
MVA			
Three freeze-thaw cycles[a]			
LQC	2 (12)	2 (4)	−10
HQC	40 (2)	41 (2)	1
Bench top (room temperature) (6 h)[a]			
LQC	2 (12)	2 (7)	−8
HQC	40 (2)	40 (2)	0.5
Autoinjector, refrigerated extracted sample (20 h)[a]			
LQC	2 (12)	2 (11)	1
HQC	40 (2)	41 (1)	2
Long term, <−50°C (28 days)			
LQC	Read against fresh calibration curve	2 (12)	7
HQC		42 (7)	−0
D7 MVA (internal standard)			
Autoinjector, refrigerated extracted sample (20 h)			
LQC	13 (9)	13 (8)	−1
HQC	1 (2)	1 (1)	−3

CV, coefficient of variation; D7 MVA, deuterated mevalonic acid; HQC, high-quality control; LQC, low-quality control; MVA, mevalonic acid; QC, quality control.

[a] Done on the same day with same baseline samples (n = 5).

DISCUSSION

In this study, a reverse-phase HPLC method with mass spectrometric detection using D7-MVA as an IS was developed. Various combinations of organic and aqueous phases were tried, and better chromatography with lower baseline was achieved using ammonium formate buffer (10 mM, pH 8.0) and acetonitrile (70:30, v/v) as the mobile phase. Response was observed in the range of 0.2–0.8 ml/min flow rate and was optimized to 0.8 ml/min.

The extraction of MVA is highly pH-specific (11, 12). The sample was equilibrated with 0.1 M HCl to convert MVA to MVAL. The equilibration time was optimized to 30 min after evaluating response at 15, 30, 45, and 60 min. Both liquid-liquid and solid-phase extraction procedures were assessed initially for the extraction of MVA from plasma. Better sample cleanup and reproducibility were obtained using

TABLE 2. Dilution integrity and partial volume analysis

QC Sample	Dilution Integrity: MQC	HQC	MQC	HQC	Partial Volume Analysis: MQC	HQC	MQC	HQC
Dilution/volume	2	4	2	4	2	4	2	4
Imprecision	8	6	5	7	8	9	4	6
Accuracy	104	100	104	107	104	102	105	103
Number	5	5	5	5	5	5	5	5

MQC, middle-quality control.

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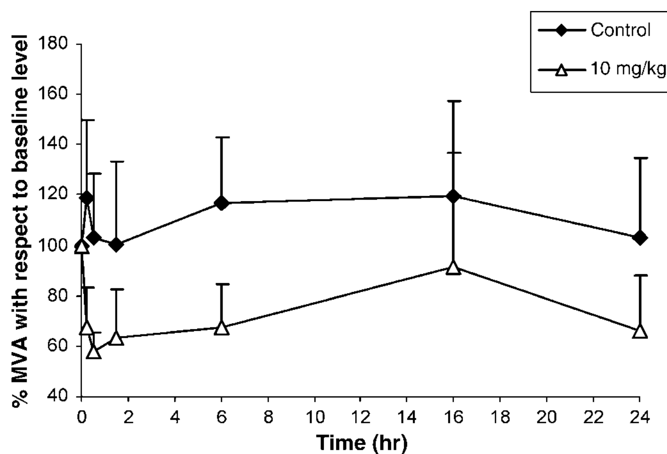

Fig. 3. Plasma MVA levels in rat (n = 5) after a single oral dose of control vehicle or rosuvastatin (10 mg/kg). Error bars represent SD.

polystyrene-divinylbenzene cartridges. The method described is sensitive, selective, precise, and accurate for the determination of MVA in human plasma at very low concentrations (<1 ng/ml) over a concentration range extending up to 50 ng/ml. The method has been validated for a maximum batch size of 103 samples (with a total run time of 6.5 h). The use of water as a surrogate matrix allows the assay to be used down to the required lower limit of 0.5 ng/ml, which would not be possible if standards were prepared in plasma containing endogenous MVA.

In previously reported methods, recovery was determined by comparing processed plasma concentrations against spiked processed blank plasma samples (11, 12). This approach to determine recovery does not capture the effect of any constant endogenous substance that may reduce the response. Hence, the calculated recovery will not be the actual recovery. During validation, recovery was calculated by comparing processed plasma QC sample concentrations against the same aqueous concentrations for MVA and D7-MVA prepared in reconstitution solution (0.2% ammonium hydroxide).

The matrix effect was determined by comparing spiked processed blank plasma QC samples against the same aqueous concentrations for MVA and D7-MVA. A significant matrix effect was observed. The low recovery is attributable to the matrix effect. During method development, six different plasma samples were spiked to add 10 ng/ml to endogenous MVA. The response observed in spiked plasma was increased proportionately compared with that in blank plasma, and similar results were seen with QC samples. It can be concluded that the presence of some constant endogenous substance other than MVA or any reagent effect during sample processing can contribute to the matrix effect. Hence, this method can be used to obtain accurate plasma MVA concentrations.

The method was successfully applied to estimate rat plasma MVA levels after a single oral dose of rosuvastatin (10 mg/kg). Although the rat is not an adequate model for studying lipid metabolism, it has been used extensively to study the mevalonate pathway (2, 8, 16). No diurnal rhythm was observed, unlike in humans, and the normal range of plasma MVA in rats was found to be 20–40 ng/ml, as reported previously (16). A significant decrease was observed in rat plasma MVA levels after rosuvastatin administration.

In conclusion, a simple, sufficiently sensitive, and reproducible method was developed for the quantification of MVA in plasma, and the developed method has been validated. The stability studies demonstrated that MVA was stable during normal assay procedures and in long-term frozen storage conditions (below −50°C). This should allow clinical samples to be stored and analyzed efficiently. The matrix effect and recovery should be differentiated to improve the sensitivity of the method and to capture the effect of any endogenous interference. The use of MVA as biomarker needs to be explored further.

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