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Validation and application of an assay for the determination of mevalonic acid in human plasma by liquid chromatography tandem mass spectrometry

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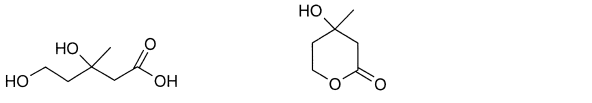
Abstract

The validation of a method for the determination of mevalonic acid (MVA; after conversion to the lactone, MVAL) in human plasma, using high-performance liquid chromatography with tandem mass spectrometry (HPLC–MS–MS), is reported. MVAL and deuterated internal standard were extracted from human plasma samples using automated solid-phase extraction. Analysis was conducted by column-switching, reversed-phase LC–MS–MS, using two hyper-cross-linked styrene–divinylbenzene copolymer sorbent reversed-phase columns. An assay range of 0.2–35 ng/ml and a lower limit of quantitation (LLOQ) of 0.2 ng/ml were achieved with acceptable accuracy and precision. MVA was stable in plasma under a variety of storage conditions.

Keywords: Mevalonic acid

1. Introduction

Mevalonic acid (MVA (see Fig. 1)) is synthesised by the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) [1] and is a precursor for cholesterol. Plasma mevalonate levels correlate with hepatic HMG-CoA reductase activity in rats [2] and humans [3] and are related to the rate of whole-body cholesterol synthesis [4]. Plasma MVA concentrations can therefore be used as a surrogate marker of HMG-CoA reductase inhibition by the statin class of drugs and thus as a measure of statin activity.



Mevalonic acid (MW 148) Mevalonic acid lactone (MW 130)

Fig. 1. Structures of MVA, MVAL.

Published methods were reviewed prior to development of the assay. The majority of published methods employ gas chromatography/mass spectrometry (GC–MS) detection. This may involve detection of the lactone [5,6] or a derivative [3,7,8].

Despite the precedence for GC–MS methods for the analysis of MVA our preference was for a high-performance liquid chromatography (HPLC)- based

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method with automation and high sample throughput. MVA is a poor UV chromophore, and therefore the use of an HPLC assay with ultra-violet detection was not considered feasible even if converted to a derivative such as the dehydrolactone [9].

A simple pH-dependent conversion of MVA to the lactone followed by automated extraction and HPLC–MS–MS detection appeared to offer advantages over existing methods. The methods of Del Puppo [10] and Scoppola [8], whilst effective, depended on an ion-exchange resin for the conversion of MVA to MVAL. The necessary sample preparation required after derivitisation, including filtration, solvent extraction and solid-phase extraction (SPE), would limit the application of automated sample preparation and would limit sample throughput. Similarly the method of Ishihama [6] also employed relatively lengthy sample preparation stages after derivitisation with Dowex 50W-X8. Whilst employing an acid-dependant conversion of MVA to MVAL, the sample preparation method employed by Yoshida [3] was also practically complex and time consuming.

Although the resolution capacity of HPLC is significantly lower than that for GC we expected that the tandem MS detection would enhance the selectivity of the detection system and allow the required sensitivity limits to be met.

A specific and sufficiently sensitive method for the determination of MVA in human plasma was needed to support clinical studies using statin drugs. Therefore, the objective of this study was to validate an analytical procedure for the determination of MVA in human plasma with a calibration range of 0.2–50 ng/ml. A lower limit of 0.2 ng/ml should allow detection of MVA following treatment with statin drugs whilst an upper limit of 50 ng/ml represents an approximate five-fold excess of levels found in normal healthy individuals.

The method involved conversion of mevalonic acid to mevalonolactone, SPE for sample preparation and HPLC with tandem mass-spectrometric detection using deuterated MVAL as an internal standard (I.S.). Due to endogenous MVA presence in human plasma and the need to achieve lower levels of quantification for this assay, it is necessary to use water in place of plasma for the preparation of all calibration standards and quality control (QC) samples at the LLOQ. The validity of using water in place of plasma was part of the investigation.

2. Experimental

2.1. Chemicals

MVAL (99.3%) was obtained from Sigma–Aldrich (Poole, Dorset, UK) and deuterated MVAL, the I.S. from CDN Isotopes (Pointe-Claire, Quebec, Canada). Spiking solutions of MVAL and I.S. were prepared in methanol. Solvents and other agents were of analytical reagent grade.

2.2. Control plasma

Control human plasma (lithium heparin anticoagulant), for preparation of calibration curves, was obtained from human volunteers at Covance Laboratories Ltd., Harrogate, UK and was stored at −20 °C prior to use.

2.3. Experimental procedure

Calibration standards were spiked in water (surrogate control matrix) on the day of extraction. QC samples were prepared in bulk at the start of validation and were stored at −20 °C prior to analysis. A pool of plasma was analysed in the initial stages of validation to establish the mean concentration of endogenous MVA. Samples for the validation experiments were prepared by spiking stock solutions of MVAL in this pool of plasma, after which samples were stored at −70 °C prior to analysis.

2.4. Automated solid-phase extraction

Plasma samples were thawed in a water bath (25–35 °C) and then sonicated for 15 min. Plasma aliquots of 250 μl (sample or QC only) were added to glass tubes to which were also added I.S. (10 μl, 500 ng/ml), 0.1 N hydrochloric acid (500 μl) and water (750 μl); the tubes were then vortex mixed. Each sample solution (1510 μl) was individually transferred to an SPE cartridge (IST ENV 100 mg/3 ml) which had been primed with methanol (1 ml)

followed by 0.1 N hydrochloric acid (1 ml). The ENV cartridge contains a styrene–divinyl benzene material. The plasma samples were applied to the extraction cartridge shortly after the dilute hydrochloric acid had been added to the plasma. Each cartridge was washed with water (1 ml) followed by water:methanol (85:15 v/v), allowing the cartridges to dry completely. The analytes were eluted with 1 ml of methanol. The above extraction procedure was carried out by using a Gilson ASPEC (Middleton, WI, USA) automated SPE system. The resulting methanol extract solutions were evaporated to dryness under a stream of nitrogen. The residues were reconstituted in phosphate buffer (120 μl, pH 7) and submitted for analysis using LC–MS–MS.

2.5. LC–MS–MS

The HPLC system involved column switching on two hyper-cross-linked styrene–divinylbenzene copolymer sorbent reversed-phase columns: Spherclone 3 μm, 100 mm×3.2 mm (id) and Spherclone 3 μm, 150 mm×3.2 mm (I.D.) (Phenomenex UK Ltd., Cheshire, UK). The flow-rate was 0.5 ml/min, split approximately 4:1 into MS. The mobile phase comprised of: (a) 20 m*M* ammonium formate (pH 8); and (b) methanol, according to the gradient programme as shown in Table 1. The column switching was performed by the Alliance 2690 pump/autosampler (Waters, Watford, UK). In summary, samples were injected onto column A (Sphereclone column 3 μm 100 mm×3.2 mm) with ammonium formate carrier mobile phase. After 1 min, the mobile phase was changed to 90:10 ammonium formate:methanol and this maintained through column A for a further minute. At 3 min the mobile phase (maintained as 90:10 ammonium formate:methanol) route was switched to pass through columns A and B (Sphereclone column 3 μm 150 mm×3.2 mm) sequentially. At 10 min (after elution of MVAL), solvent was switched to 100% acetonitrile, through column A only, for one minute. The mobile phase was then switched to 100% ammonium formate for 8 min to re-equilibrate the column. The solvent was taken to waste at all times except for between 3 and 9 min where the mobile phase was taken into the mass spectrometer.

The Quattro 1 (Micromass, Manchester, UK) tandem mass spectrometer was operated using electrospray ionisation in the positive ion mode; the ions monitored were: 148>131 Da (MVAL) and 155>138 Da (deuterated MVAL). A second mass transition of 131>71 Da (MVAL) was also used for confirmation purposes during the assay development stage. Quantitation was carried out using the 148>131 Da transition due to its greater sensitivity.

Major LC–MS–MS operating parameters were:

- nebuliser gas flow, 15 l/h
- bath gas flow, 300 l/h
- collision gas (Argon) pressure, 2.0×10^{4} MBar
- collision energy, 148>131 (MVAL) (20 eV); 155>138 (deuterated MVAL) (20 eV)
- cone voltage, 10 V
- capillary voltage, 3.25 kV
- dwell time, 0.2 s
- inter-channel delay, 0.02 s
- analysis time, 15.3 min

Concentrations of MVAL in calibration standards and study samples were determined using least-

Table 1
LC–MS–MS gradient programme

Time Minutes	% Solvent A	% Solvent B	Column	Waste/MS
0	100	0	A	Waste
1.0	100	0	A	Waste
2.0	90	10	A	Waste
3.0	90	10	A+B	MS
9.0	90	10	A+B	MS
10.0	0	100	A	Waste
11.0	0	100	A	Waste
12.0	100	0	A	Waste

squares linear regression with the reciprocal of the concentration ($1/x$) as weighting to improve accuracy at low levels.

2.6. Validation

To assay MVA, it was necessary to convert it to MVAL under acidic conditions. This conversion was necessary to produce a more lipophilic compound which could be extracted from plasma and chromatographed by reversed-phase HPLC. As MVA is an endogenous compound it was not possible to prepare standards and QCs in control plasma; standards and the LLOQ-QC were prepared in water. To verify this approach, standard curves were prepared in water and plasma, and the calibration slopes were compared.

Specificity was evaluated on the basis of the parent product ion transition from six individual spiked samples and six individual plasma blanks (endogenous levels) together with the processed surrogate blanks. Results were compared to those obtained from the second mass transition to assess the selectivity of the detection.

Due to MVA being endogenous to plasma, assessment of recovery was made using the deuterated I.S. instead of MVA itself. Recovery was assessed at two levels. Replicates of control plasma (containing a fixed concentration of MVA) were extracted both containing (test) and omitting (reference) the I.S. Post-extraction, I.S. was added to the reference set. Recovery of MVA was assessed at each concentration level by comparison of the mean peak ratios (analyte to I.S.) for I.S. recovery samples and reference samples. Recovery was defined as the reciprocal ratio of the peak area ratios in the I.S. recovery samples to that obtained for the corresponding reference samples.

The intra- and inter-assay accuracy and imprecision was determined from the coefficient of variation of the observed concentrations at each level tested. Accuracy was determined by comparing mean observed and theoretical concentration at a range of levels. Intra-assay accuracy and imprecision were assessed on six replicates at six levels within one batch. Inter-assay accuracy and imprecision were assessed on six replicates at four levels performed on four separate occasions.

2.7. Stability

The chemical stability of MVA was assessed in: (a) control human plasma after storage at room temperature for 24 h; (b) in human plasma exposed to three freeze–thaw cycles; and (c) in plasma extracts stored refrigerated for 48 h. In each case, six replicates at the stability levels 8.47, 18.47 and 25.47 ng/ml were analysed. In addition, long-term (16 months) stability was assessed in human plasma after storage at -20 and $-70\,^{\circ}C$.

3. Results

The $M+NH_4^+$ (m/z 148) ion was the predominant Q1 ion and the product ion m/z 131 (loss of NH_3) the most abundant and thus the most sensitive. The equivalent transition (155→138) was monitored for the deuterated (D_7)internal standard. These Q1 and fragment ions, with loss of NH_3 produced the most sensitive detection for MVAL and its deuterated internal standard.

The retention time for MVAL and deuterated MVAL was approximately 8.3 min. The apparent retention time appeared marginally longer for deuterated MVAL than for the unlabelled compound. This may be attributed to the data sampling mechanism of the MS detector, and may also be explained if the deuterium atoms replacing hydrogen atoms make the compound marginally more lipophilic.

The parent product ion transition from the six individual spiked matrix samples was consistent (the peaks occurred at the same retention time) with endogenous peaks from the six individual blank matrix samples. Therefore, the method was considered specific and selective for the determination of MVAL in human plasma.

The second mass transition of 131>71Da (MVAL), used for confirmation purposes, produced very similar results (data not shown) to those obtained using the 148>131Da transition. Although not providing definitive proof of selectivity, this result strongly implies that the detection was specific for MVAL.

Within the method development stage of the assay (employing isocratic HPLC, without column-switching) it became obvious that there was a lot of

interference from plasma components on the detection of MVAL. This was not unexpected as the compound was of low molecular mass and the mass transition used in the detection was a non-specific loss. It was clear that minor modifications to the SPE step of the assay were unlikely to overcome this and therefore the move to column-switching was made. Although this came at a price in terms of analysis time it was considered an acceptable price to derive a reliable method.

Example chromatograms for MVAL samples prepared in: (a) water; (b) human plasma; and (c) control plasma samples (endogenous background) are illustrated in Fig. 2.

The mean recovery on extraction of I.S. from plasma was 51.2% (45.6 and 56.7% at concentrations of 10 and 17 ng/ml, respectively). Whilst the extraction efficiency for MVA (assessed using the I.S.), was moderate at 51.2%, because the assay achieved the desired sensitivity, this was not considered to be a significant problem.

The mean endogenous level of MVA was calculated at 8.47 ng/ml (range 6.85–9.96 ng/ml) and this figure was used as the LOQC (low QC). The nominal concentrations for the MeQC (medium QC), HiQC (high QC), ULOQQC (upper limit of quantification QC) and DiQC (dilution QC) samples were therefore corrected from the spiked concentrations 10, 17, 20 and 40 ng/ml to the total concentrations 18.47, 25.47, 28.47 and 48.47 ng/ml, respectively.

The slopes of the calibration curves (Table 2) were shown to be essentially the same in water and plasma (mean slope for six individual plasma curves ranged was 0.048895 (S.E. 0.00121) compared with a slope of 0.04491 for the curve prepared with water), with mean coefficient of determination for the six plasma curves of 0.99395 (S.E. 0.00093). The intercept was found to pass acceptably close to zero (0.05176 for water versus 0.279087 ± 0.043283 (S.E.) for plasma). The similarity in the slopes between water and plasma indicated that water was a suitable surrogate matrix. Example chromatograms of samples prepared in water and control plasma are shown in Fig. 2.

Eleven calibration standards (0.2, 0.4, 1, 2, 5, 10, 15, 20, 25, 30, 35 ng/ml) were employed over the concentration range of 0.2–35 ng/ml. The coefficients of determination of the calibration standards were all ≥ 0.98603 (range 0.98603–0.99722). The characteristics of the calibration line are presented in Table 3. The inter-assay imprecision for the calibration standards across the concentration range of 1–35 ng/ml was $\leq 16.3\%$.

The intra-assay imprecision observed in the test samples ranged from 4.1 to 14.7%, while intra-assay accuracy ranged from 95.0 to 114.2% (Table 4). The inter-assay imprecision ranged from 12.8 to 15.7% and accuracy from 88.9 to 99.0% (Table 5). Both intra- and inter-assay inaccuracy and imprecision were considered acceptable as they were $< \pm 20\%$ and $< 20\%$, respectively, at all concentrations.

The limit of quantification (LOQ) was set at 0.2 ng/ml, as this was the lowest concentration at which acceptable accuracy ($> 80\%$) and imprecision ($< \pm 20\%$) were obtained. However, in some runs, the sensitivity of the mass spectrometer was insufficient to quantify values below 1 ng/ml and in these instances the LOQ was corrected to 1 ng/ml. In these instances, samples producing results below 1 ng/ml were reanalysed in subsequent batches until positive results were obtained for all samples. Thus, in practice, despite a higher LofQ in some runs, only values below 0.2 ng/ml were reported as not quantifiable.

Mean MVA concentrations at baseline and in stability samples, after storage under a variety of test conditions are summarised in Table 6. Results for all storage conditions differed by less than 6.7% from baseline. MVA was therefore shown to be chemically stable in human plasma after storage at room temperature for 24 h, after three freeze–thaw cycles, and as refrigerated extracts for 48 h. In addition, plasma extracts were stable when refrigerated for up to 48 h. Stability of MVA in human plasma following frozen storage at -20 and $-70\,^{\circ}C$ was also confirmed over a period of 15 months. Although stability was assessed over 16 months it was evident with time that the baseline (time zero) results were anomalous and therefore the results obtained at 1 month were used as the reference. The reason that the baseline values were considered anomalous was that higher results were obtained at all subsequent stability assessment times. The baseline samples put down for the long term frozen storage were not the same ones used to assess the shorter term storage and freeze/thaw assessments and thus, these latter assessments

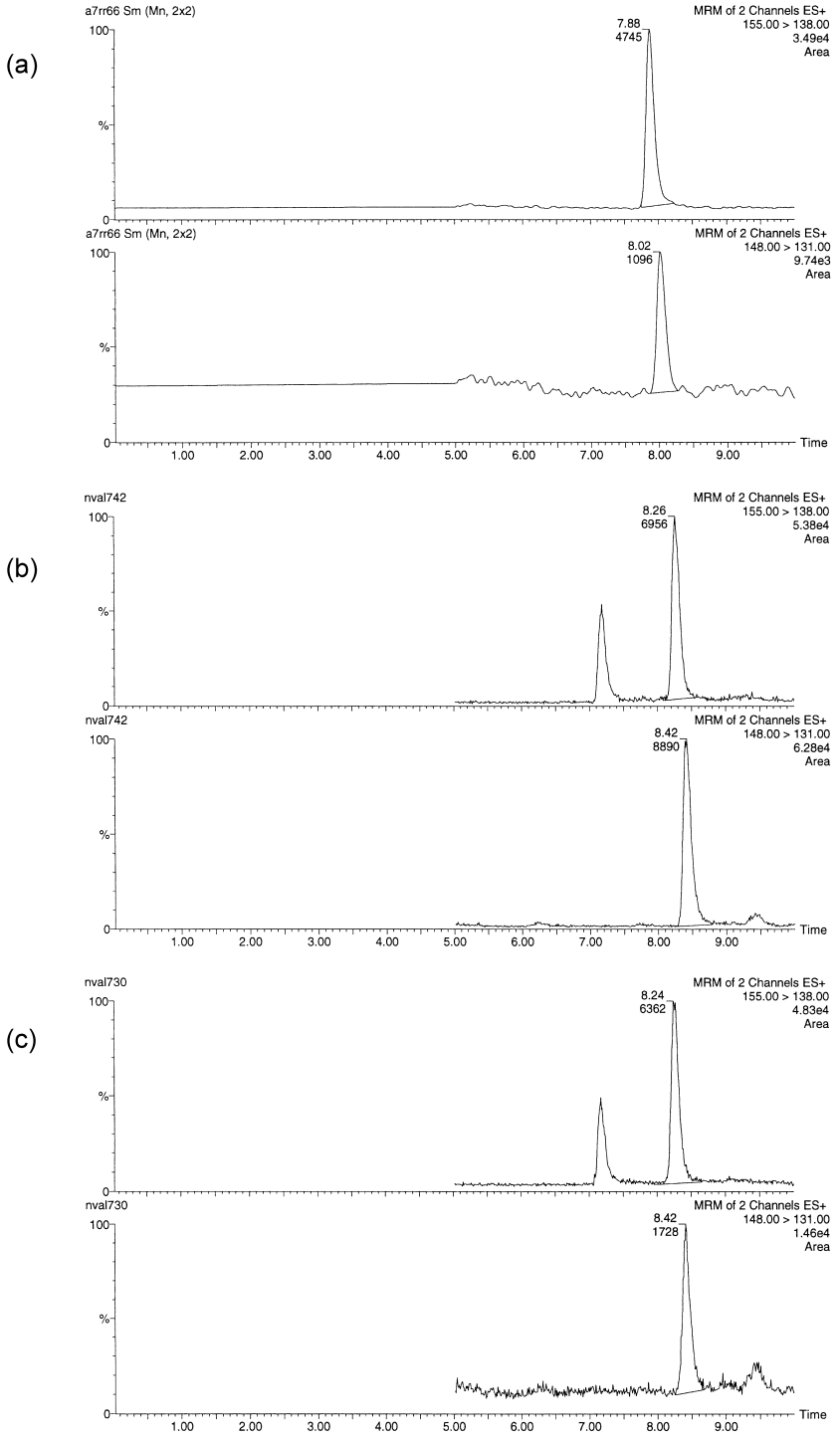

Fig. 2. Specimen chromatograms of: MVAL samples prepared in: (a) water; (b) spiked human plasma; and (c) control plasma extract (endogenous background).

Table 2
Characteristics of calibration regression line data prepared in the plasma from six different individuals and in water

Run	Slope	Intercept	Coefficient of determination (r^2)
1 (plasma)	0.05394	0.25461	0.99490
2 (plasma)	0.05063	0.47272	0.99671
3 (plasma)	0.04888	0.22163	0.99303
4 (plasma)	0.04625	0.32366	0.99000
5 (plasma)	0.04684	0.18351	0.99408
6 (plasma)	0.04683	0.21839	0.99498
Mean (plasma)	0.04889	0.27909	0.99498
S.E. (plasma)	0.00121	0.04328	0.00093
7 (water)	0.04491	0.05176	0.99049

Table 3
Characteristics of calibration regression line data obtained during the validation procedure

Run	Slope	Intercept	Coefficient of determination (r^2)
1	0.04485	0.02504	0.98603
2	0.04491	0.05176	0.99049
3	0.04512	0.03896	0.99722
4	0.04650	0.02203	0.98973
5	0.04243	0.05372	0.99684
Mean	0.04476	0.00066	0.03830
S.E.	0.00656	0.99206	0.00217

Table 5
Inter-assay accuracy and precision

QC concentration (ng/ml)	8.47	18.47	25.47	48.47
Accuracy	88.9	91.5	89.9	99.0
Imprecision	15.7	14.2	15.1	12.8
N	24	24	23	24

are not impacted by the anomalous baseline results. The concentrations determined at 16 months were less than 6% different to those obtained at 1 month at each concentration and under each storage temperature.

The successful application of the assay to the measurement of MVA in human plasma is provided by the data derived from a clinical study (4522/IL0004) to investigate the pharmacodynamics of rosuvastatin (Crestor™), following repeated morning or evening dosing to healthy volunteers (Fig. 3) [11]. Rosuvastatin is a new HMG-CoA reductase inhibitor which has recently completed phase III clinical development. The two predose profiles illustrate the diurnal rhythm of plasma MVA which reflects the known diurnal pattern of cholesterol synthesis. Plasma MVA concentrations are at their lowest at approximately midday, climbing to a peak at around midnight. Both morning and evening dosing with rosuvastatin produced marked reductions in plasma MVA concentrations. This reduction in MVA concentrations across the dosing interval is consistent with the inhibition of HMGCoA reductase by rosuvastatin. The pattern of concentrations was similar with morning dosing to the un-dosed pattern, but with lower concentrations. With evening dosing, the pattern of concentrations as well as the absolute levels were modified. The observed reductions in plasma MVA translated into marked reductions in LDL-cholesterol which were essentially the same with morning and evening dosing [11]. Fig. 3 also illustrates that the 0.2 ng/ml LOQ achieved with the

Table 4
Intra-assay accuracy and precision

QC concentration (ng/ml)	0.2	8.47	18.47	25.47	28.47	48.47
Accuracy	95.0	100	103.1	106.8	100.6	114.2
Imprecision	14.7	13.0	10.7	5.7	4.1	11.3
N	6	6	6	6	6	6

Table 6
Stability of MVA in plasma

Storage conditions QC level (ng/ml)	Mean MVA concentration (ng/ml)		Difference from baseline (%)
	Baseline QC (C.V.%)	After storage (C.V.%)	
Room temperature (24 h)			
8.47	6.30 (10.4)	6.71 (10.3)	6.6
18.47	15.15 (6.5)	15.58 (9.3)	2.9
25.47	22.40 (12.5)	21.91 (9.6)	−2.2
Three freeze–thaw cycles			
8.47	7.18 (12.7)	7.13 (10.5)	−0.7
18.47	17.14 (13.2)	16.84 (7.6)	−1.8
25.47	22.98 (5.6)	22.95 (5.6)	−0.1
Refrigeration of extracted samples (48 h)			
8.47	8.18 (7.8)	7.63 (5.8)	−6.7
18.47	16.28 (15.9)	15.55 (10.3)	−4.5
25.47	22.40 (12.5)	21.25 (6.9)	−5.1
−20 °C (15 months)			
6.79	6.79 (4.4)[a]	6.53 (12.5)	−1.15
26.56	26.56 (6.8)[a]	25.16 (6.0)	1.91
−70 °C (15 months)			
6.98	6.98 (14.4)[a]	6.90 (6.0)	−1.15
24.63	24.63 (4.8)[a]	25.1 10.3)	1.91

[a] 1 month stability samples used as baseline.

assay was sufficient for measuring normal endogenous plasma MVA levels as well as reduced levels produced by statin treatment.

4. Discussion

The main challenges in developing and validating a method for determining MVA in human plasma are that MVA is a polar, low molecular mass (MW 148), endogenous compound which circulates at trace levels.

The current study used SPE for sample preparation and HPLC reversed-phase column switching with tandem mass-spectrometric detection using deuterated MVA as an I.S. The method has a high degree of in-built selectivity. The use of a styrene–divinyl benzene SPE phase and a C_{18} reversed-phase HPLC column allows two separation chemistries to be used to separate endogenous materials in plasma from the target analyte. The reversed-phase column switching aids selective elimination of co-extractants so that they are excluded from the MS source, therefore making the MS–MS detection more robust and reliable. The assay was initially intended to use conventional isocratic HPLC but there was too much interference from co-extractants to establish a reliable method. Whilst the column-switching made the assay more complex than initially desired (though no more complex from a manual procedures point of view) the assay still allowed the necessary sample throughput and the assay proved robust and reliable in practice.

The results indicate that the method described is

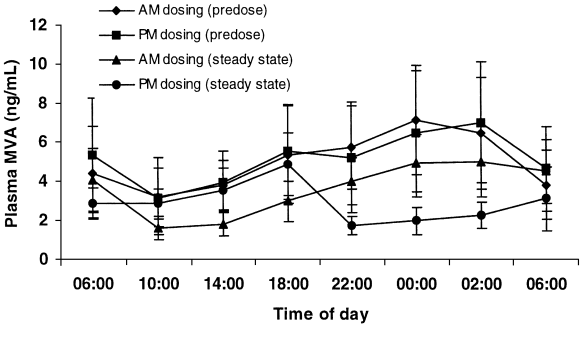

Fig. 3. Gmean plasma concentrations of MVA before and after 14 daily doses of rosuvastatin in the morning or evening.

suitable for the determination of MVA in human plasma from sub-ng/ml levels (LLOQ of 0.2 ng/ml) over a concentration range extending to 35 ng/ml. The method has been validated for a maximum batch size of 86 samples (with a total run time of 30 h) which allows a high throughput with a method that involves practically simple and non-labour-intensive procedures. Whilst the chromatographic run time for the current method was longer than that used for the methods of Del Puppo and Scopolla, the savings in time and effort over these relatively laborious methods (which include an overnight derivatisation) represents a significant improvement over these methods.

The use of water standards and LLOQ QCs allows the assay to be used down to the required lower limit of 0.2 ng/ml, which would not be possible if standards were prepared in plasma containing endogenous MVA. This approach was supported by the similarity of curves produced in plasma and water; the outcome is probably the result of using a deuterated analogue as the I.S.

In studies such as this, instability leading to concentration changes of $>\pm 20\%$ is likely to compromise the integrity of the data. The current assay was associated with changes from baseline of no more than $\pm 6.7\%$ for all storage conditions. Thus, the stability studies demonstrated that MVA was chemically stable during normal assay procedures and in long-term frozen storage. This should allow clinical study samples to be stored and analysed efficiently.

5. Conclusion

A method using SPE followed by column-switching HPLC–MS–MS was validated to assay plasma concentrations of MVA over the range 0.2–35 ng/ml. The assay was successfully applied to the analysis of plasma samples for MVA following the dosing of the HMG-CoA reductase inhibitor rosuvastatin.

References

[1] T.S. Parker, D.J. McNamara, C.D. Brown, R. Kolb, E.H. Ahrens, A.W. Alberts, J. Tobert, J. Chen, P.J. De Schepper, J. Clin. Invest. 74 (1984) 795.
[2] G. Popják, G. Boehm, T.S. Parker, J. Edmond, P.A. Edwards, A.M. Fogelman, J. Lipid. Res. 20 (1979) 716.
[3] T. Yoshida, A. Honda, N. Tanaka, Y. Matsuzaki, B. He, T. Osuga, N. Kobayashi, K. Ozawa, H. Miyazaki, J. Chromatogr. 613 (1993) 185.
[4] T.S. Parker, D.J. McNamara, C. Brown, O. Garrigan, R. Kolb, H. Batwin, E.H. Ahrens, Proc. Natl. Acad. Sci. USA 79 (1982) 3037.
[5] S. Nozaki, T. Nakagawa, A. Nakata, S. Yamashita, K. Kameda-Takemura, T. Nakamura, Y. Keno, K. Tokunaga, Y. Matsuzawa, Eur. J. Clin. Pharmacol. 49 (1996) 361.
[6] Y. Ishihama, N. Mano, Y. Oda, H. Hiyoshi, H. Ikuta, Y. Yoshida, Rapid. Commun. Mass. Spectrom. 8 (1994) 377.
[7] S. Siavoshian, C. Simoneau, P. Maugeais, L. Marks, L. Rodary, J. Gardette, K. Krempf, Clin. Chim. Acta 243 (1995) 129.
[8] A. Scoppola, V.M.G. Maher, G.R. Thompson, N.B. Rendell, G.W. Taylor, J. Lipid Res. 32 (1991) 1057.
[9] K. Gondoh, S. Itoh, Y. Tsukada, Rinsho Kagaku 24 (1998) 89.
[10] M. Del Puppo, G. Cighetti, M. Galli Kienle, L. De Angelis, Biomed. Environ. Mass. Spectrom. 18 (1989) 174.
[11] P.D. Martin, P.D. Mitchell, D.W. Schneck, J. Clin. Pharmacol. 40 (2000) 1056.