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Short communication

Determination of mevalonic acid in human urine as mevalonic acid lactone by gas chromatography–mass spectrometry

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Abstract

Mevalonic acid in urine is converted to its lactone form by incubation overnight at acidic pH, extracted and analysed by GC–MS. The lower limit of quantitation of 7.5 ng/ml provides adequate sensitivity to measure changes in urinary excretion of MVA following administration of drugs which affect cholesterol synthesis. The assay is linear up to 300 ng/ml and has acceptable precision (<15%) and accuracy (<±20%) across the calibration range.

Keywords: Mevalonic acid; Mevalonic acid lactone

1. Introduction

Mevalonic acid (MVA) is a precursor of cholesterol and is synthesized by the enzyme 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMG-CoA). Assessment of urinary excretion of MVA can be used as a non-invasive surrogate marker to detect the inhibition of this enzyme by the class of drugs known as statins [1,2].

MVA is a poor ultraviolet chromophore, which precludes the use of HPLC analysis unless it is converted to a derivative such as the dehydrolactone [3]. Most published methods involve gas-chromatography–mass spectroscopy (GC–MS) of the lactone in either EI mode [1], CI mode [4], or as a derivative [2,5,6].

A number of published methods were evaluated. The method of Scopolla [6] which involves derivatisation and negative ion GC–MS was found to not have the required sensitivity using our equipment and was not amenable to high throughput. The method of Nozaki [1], involving conversion of MVA to the lactone (MVAL) overnight at pH 7.2, followed by extraction, silica clean-up, and GC–MS monitoring of ions at m/z 71 and 101, was too insensitive. A review of the literature [3,7] revealed that it was necessary to use a low pH to lactonise MVA.

Our approach was to develop a GC–MS method for MVAL in urine using solvent extraction and to monitor low-mass fragment ions. The assay needed to be sensitive enough to measure MVAL in urine from patients undergoing treatment to reduce choles-

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terol levels. It was necessary to use human urine containing endogenous analyte as a control matrix for standards and quality control (QC) samples.

2. Experimental

2.1. Chemicals

Mevalonic acid lactone (97%) was obtained from Sigma (Poole, Dorset, UK), deuterated mevalonic acid lactone (D_3MVAL, internal standard), (99%) from CDN Isotopes (Pointe-Claire, Quebec, Canada), and ^{14}C mevalonic-acid lactone from Amersham Life Sciences Ltd (Little Chalfont, Buckinghamshire, UK).

Phosphate buffer (1 M; pH 2): 136 g potassium orthophosphate in 500 ml deionised water adjusted to pH 2 with orthophosphoric acid and made up to 1 litre with deionised water.

Phosphate buffer (0.2 M; pH 7): 30.5 ml 0.2 M disodium hydrogen orthophosphate mixed with 19.5 ml 0.2 M sodium dihydrogen orthophosphate.

Solvents and other reagents were of analytical reagent grade.

2.2. Equipment

GC–MS was performed using a Hewlett-Packard 5890A gas chromatograph (Agilent Technologies, Cheadle Heath, Cheshire, UK) connected to a TRIO-1 quadrupole mass spectrometer (VG Masslab, Wythenshawe, Greater Manchester, UK) operated in the electron impact mode.

2.3. Samples/controls

Control urine for preparation of calibration curves was obtained from a donor who was undergoing treatment with a cholesterol-lowering drug, and who was known to excrete relatively low concentrations of MVA in urine. Urine samples for evaluation of assay specificity were obtained from volunteers who had participated in a clinical trial and taken a drug which would reduce the level of MVA in the urine.

2.4. Experimental procedure

Urine aliquots of 2 ml (sample or quality control only) were added to glass vials and 1 ml of a 0.2 μg/ml internal standard solution in 1 *M* phosphate buffer (pH 2) was added to each sample. The vials were vortex mixed and left to incubate at ambient temperature overnight.

Urine aliquots of 2 ml (standards only) were added to glass vials and 1 ml of a 0.2 μg/ml internal standard solution and the appropriate addition of MVAL, both in 1 *M* phosphate buffer (pH 2), were added to each sample. To minimise the concentrations of MVAL to enable a low intercept value on the standard curve, the standards were not left to incubate overnight but were prepared immediately before extraction.

Approximately 3 g of anhydrous granular sodium sulphate was added to each vial followed by 8 ml of ethyl acetate. The vials were mixed on a multi-vortexer for 10 min and the phases were allowed to separate at room temperature. Approximately 6 ml of each supernatant (ethyl acetate layer) was transferred to a clean glass vial containing approximately 3 g of anhydrous sodium sulphate and 5 ml of 0.1 *M* sodium phosphate buffer (pH 7) was then added. The vials were vortex mixed for approximately 10 min and the phases were allowed to separate. Approximately 3 ml of the ethyl acetate layer was transferred to a clean glass vial and reduced to dryness under a stream of nitrogen. The residues were re-dissolved in 100 μl of ethyl acetate and analysed by GC–MS.

GC–MS was performed using a TRIO-1 quadrupole mass spectrometer operated in the electron impact mode. The column was a fused-silica capillary (30 m×0.32 mm) coated with a 0.5-micron film of RTX5-MS (Thames Restek UK Ltd, Windsor, UK) and inserted directly into the source of the mass spectrometer. Helium was used as carrier gas at an inlet pressure of 0.3 bar and 2 μl splitless injections were performed into a 1.2-mm silanised glass liner. The injection port was maintained at 200°C and the source temperature was held at 200°C. The column temperature profile was 75°C for 0.5 min; 25°C/min to 150°C; 15°C/min to 200°C; 30°C/min to 275°C; and held at 275°C for 5 min. The ionisation voltage was 70 eV and selected ion monitoring was carried out for m/z 71 (quantitation ion), 58 (confirmatory

ion) and 74 (internal standard). Typical retention times for MVAL and D_3MVAL were 5.23 and 5.19 min, respectively. An example of a chromatogram is shown in Fig. 1.

Calibration was performed by regression analysis of the peak height ratio of m/z 71/74 against added concentration of MVAL. For each batch, the peak heights for m/z 71 were corrected for the contribution from non-deuterated mevalonic acid lactone present in the deuterated MVAL. Since the control urine used for calibration contained endogenous MVA, the calibration series was essentially a standard addition. The concentration of MVA in unknown samples was determined from the slope of the curve while the intercept allowed the background concentration of MVA in the control urine to be calculated. The calibration curve was linear over the range of 0 to 300 ng/ml MVAL. A curve using standards of 50 ng/ml and below was used for estimating lower concentrations of MVAL. Measured concentrations of MVAL were converted to concentrations of MVA by multiplying by the ratio of molecular mass.

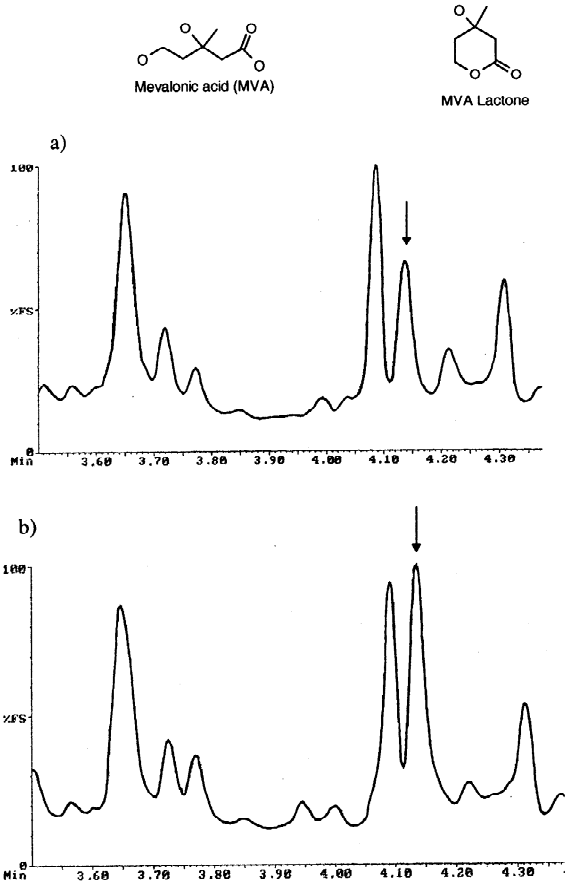

Fig. 1. Selected ion monitoring chromatograms for m/z 71 obtained from extracts of (a) control urine containing a low concentration of mevalonic acid (MVA), (b) the same urine spiked with 15 ng/ml mevalonic acid lactone (MVAL). The arrow shows the peak corresponding to MVAL.

3. Results

A number of columns were evaluated in the initial work including methyl silicone and RTX1701, but RTX5-MS was found to give the best peak shape and selectivity.

Initial time-course studies showed that a minimum of 6 h was required for complete lactonisation of MVA in urine at pH 2 and that once the lactone was formed, there was a tendency for rehydration under non-acidic conditions. In the procedure described, a pH 7 buffer wash was used to remove interfering impurities at the expense of significant losses of MVA lactone, which are consistent across the concentration range of the assay.

MVA lactone was adsorbed onto the GC column in the absence of urine extract matrix; therefore, it was necessary to prepare calibration standards and QC samples in urine. Additionally, because MVA is always present in urine, the achievable limit of quantitation is set by the background concentration of MVA in the control urine used to prepare the calibration curve, and the extent of lactonisation during sample preparation. To achieve the required limit of quantitation, calibration curves were prepared in urine known to contain low concentrations of MVA, which was exposed to acidic conditions for a minimum period of time. This resulted in a low intercept value on the standard curve.

The recovery of MVAL from urine was determined by scintillation counting using ^{14}C-radiolabeled MVAL at 5 concentrations over the range of 0 to 500 ng/ml. The mean (SD) recovery was found to be 25.3% (6%), and was similar across the concentration range

Calibration curves were linear over the range of 0 to 300 ng/ml. Based on seven calibration runs, slope

(SD:SE)/intercept (SD:SE) values determined by non-weighted linear regression analysis were 0.0143(0.0045:0.0017)/0.0094(0.0355:0.0134) for an 11-point standard curve over the range of 0 to 300 ng/ml and 0.0141(0.0046:0.0017)/0.0186(0.0152:0.0058) for a 7-point standard curve over the range of 0 to 50 ng/ml MVAL with r^2 values >0.990.

Inter- and intra-assay accuracy and precision data over the range of 5 to 250 ng/ml MVAL were acceptable (Table 1), with accuracy always within $\pm 20\%$ and precision always less than 15%.

The lower limit of quantitation (LLOQ) was defined as the lowest concentration where accuracy was always $\pm 20\%$ and precision was $<15\%$. To calculate the LLOQ, it was necessary to take into account the background level of MVAL in control urine; therefore, the background level of MVAL in control urine was added to the spiked concentration and the sum converted into a concentration of MVA. The results of the experiments used to define the LLOQ are shown in Table 2. The background concentration of MVAL determined from the intercept value on three successive days ranged from 0.5 to 3 ng/ml, and the LLOQ was determined to be 7.5 ng/ml for MVA in urine.

Assay specificity was determined by analysing 10 urine samples from volunteers who had been dosed with a drug which reduces the MVA concentration in the urine. The peak area ratios of m/z 71 (quantitation ion) to m/z 58 (confirmatory ion) were evaluated using Dixon's outlier test [8]. This showed that these ratios all came from the same normal distribution demonstrating that there was no significant endogenous interference for the ions chosen.

MVAL was shown to be stable when stored as a dilution series (100, 10, 1 and 0.1 μg/ml) in 1 *M* pH 2 phosphate buffer for up to 8 days. The difference between freshly prepared and stored solutions was $<5\%$. MVAL was found to be less stable when stored as a concentrated solution (1 mg/ml) in methanol; a 14% reduction in concentration was measured after 8 days storage. This may have been due to rehydration to MVA under non-acidic conditions due to traces of water in methanol. MVAL was shown to be stable when stored in injection solvent (ethyl acetate) for 24 h. MVA was shown to be stable in urine which was stored at room temperature for 24 h and at -20°C or -80°C for 14 days. It was also stable to five freeze/thaw cycles whether the freeze temperature was -20°C or -80°C. Com-

Table 1
Accuracy and precision data for the determination of mevalonic acid lactone in urine

Concn. (ng/ml)	Assay number	Individual results (ng/ml)	Intra-assay mean (ng/ml)	Mean intra-assay precision (%) $n=7$	Mean intra-assay accuracy (%) $n=7$	Mean inter-assay precision (%) $n=3$[b]	Mean inter-assay accuracy (%) $n=3$[c]
5.0	1	5.4, 5.7, 5.3, 5.5, 5.4, 5.5, 5.8	5.5	3.5	110		
	2	4.9, 5.0, 4.8, 5.3, 5.3, 5.2, 6.3	5.3	9.7	105	9.8	102
	3	4.7, 4.4, 4.1, 3.9, 4.4, 4.6, 5.8	4.6	13.7	91.0		
35	1	31.9, 31.0, 30.8, 31.3, 32.2, 31.9, 32.1	31.6	1.8	90.3		
	2	33.9, 28.1, 27.0, 27.8, 28.0, 25.5, 29.5	28.5	9.3	81.6	5.2	86.3
	3	31.2, 28.0, 29.3, 30.6, 32.5, 32.2, 29.8	30.5	5.3	87.2		
80.0	1	72.3, 71,7, 70.4, 71.1, 71.3, 70.9, 73.5	71.6	1.4	89.5		
	2	70.4, 64.6, 70.4, 78.2, 64.1, 74.0, 66.6	69.8	7.4	87.2	1.7	87.8
	3	69.3, 71.0,70.5, 62.7, 69.8, 70.1, 72.2	69.4	4.5	86.7		
250[a]	1	255, 258, 260, 254, 255, 256, 260	257	1.0	103		
	2	244, 245, 240, 242, 239, 235, 227	239	2.6	95.5	3.7	100
	3	248, 243, 249, 257, 251, 252, 257	251	2.0	100		

[a] A measure of 1000 ng/ml diluted with three volumes of control urine and correction made for contribution from endogenous MVA.
[b] Based on the mean values for the three assays used for intra-assay performance.
[c] Based on the mean values for the three assays used for intra-assay performance.

Table 2
Data used to define lower limit of quantitation (LLOQ) for mevalonic acid in human urine during three assay days

Assay day number	Spiked concentration of MVAL (ng/ml)	Calculated background level of MVAL (ng/ml)	Total MVAL (spike+ background) (ng/ml)	Total as MVA equivalent[a] (ng/ml)	Mean total MVA (LLOQ) value (ng/ml)
1	5.0	3.0	8.0	9.1	
2	5.0	1.3	6.3	7.2	7.5
3	5.0	0.5	5.5	6.3	

[a] Mevalonic acid concentration=Mevalonic acid lactone concentration×148/130, where 148 and 130 are the molecular masss of mevalonic acid and mevalonic acid lactone, respectively.

pared with analyses of fresh urine, differences of <5% were found in the analyses of urine after 14 days storage and five freeze/thaw cycles at both temperatures.

An example of an application of the method is a clinical study (4522IL/0004) comparing the effects of administering rosuvastatin to healthy volunteers at different times of the day (am and pm). The 24-h urine collections were made prior to, and after 14 days of administration of a single daily administration of 10 mg rosuvastatin. Pre-dose urinary concentrations of MVA were between 34–323 ng/ml and after 14 doses within the range of 17 to 142 ng/ml. The urinary excretion of MVA was reduced by approximately 30% for both times of dosing. The mean urinary excretion was decreased from 200 μg/day to 134 μg/day by am dosing and from 240 μg/day to 133 μg/day by pm dosing. The clinical results show that the assay is suitable to cover the range of concentrations of urinary MVA encountered in therapeutic dosing of statin drugs.

4. Discussion

The method described is capable of throughput in the order of at least 30 samples per-day and although it involves an overnight incubation step this is not rate limiting, because it merely involves a simple thawing and buffer addition at the end of the working day before the main analysis on the next day. The method is technically simple to perform since the use of a deuterated internal standard removes the need for accurate volumetric transfers and it uses inexpensive disposable tubes and transfer pipettes. It is considered that the use of GC–MS in the EI mode is a more rugged technique that CI. Previous methods have not been validated in urine down to low concentrations and the method provides a clear strategy for accomplishing low level measurements by appropriate control of lactonisation in a control urine matrix known to contain a low concentration of MVA.

A review of the literature reveals a variety of conditions during which lactonisation of MVA is claimed to occur. At one extreme Nozaki [1] reported a method which involves lactonisation in 1 *M* pH 7.2 buffer overnight. In our experience this would not adequately convert MVA to its lactone. Another approach which has been used is lactonisation in the presence of an ion-exchange resin [4,9], followed by solid-phase extraction clean-up, but a simpler liquid incubation with acid was preferred for a urine matrix. If the conditions are strongly acidic, MVAL is further dehydrated to the dehydrolactone [3]. Our time course experiments were carried out by incubating urine with pH 2 buffer and measuring the yield of MVAL. An incubation time of 6 h was needed before a plateau concentration was reached, and no further change (which could have indicated conversion to the dehydrolactone) occurred up to 16 h. An overnight incubation was chosen as a convenient procedure to ensure consistency of lactonisation. It is essential to prepare calibration curves in the control urine matrix rather than pH 2 buffer. The matrix has a carrier effect during chromatography and in its absence MVAL is almost totally adsorbed onto the column at low concentrations. This finding is in contrast to Ishihama [4] who reported that calibration curves for a GC–MS assay for MVAL in urine were prepared in water. The assay transferred well between different GC columns of the spe-

cification shown, but it was found that the peak shape for MVAL deteriorated after 200–300 injections necessitating replacing the column. Neither removing the front portion of the column nor washing the column would regenerate satisfactory peak shape. Replacing the column is considered to be an acceptable consumable cost given the overall simplicity of this assay. The procedure is amenable to high sample throughput since there is no derivatisation, other than conversion of MVA to its lactone.

The assay procedure shows a relatively low overall recovery of MVAL from urine. During method development it was found that when MVAL was extracted from pH 2 buffered urine and the concentrated extract was analysed by GC–MS, the peak shape for MVAL deteriorated rapidly when successive samples were injected. This may have been due to an adverse effect of residual acidic buffer on the column. This problem was resolved by washing the acidic buffer extract with pH 7 buffer. This step enabled good chromatography of MVAL for long periods, and an additional benefit was a reduction in endogenous compounds which eluted close to MVAL. An adverse consequence of this additional stage was substantial losses of MVAL, most probably due to rehydration of the MVAL to MVA at pH 7. Despite the low recovery, it is still possible to achieve the required sensitivity, accuracy, and precision, because standards are extracted from urine alongside unknown samples in the presence of deuterated MVAL as internal standard.

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