Figure 1. Mevalonic acid: metabolic pathway and analytical chemistry. (A) The mevalonate
pathway synthesises MVA from HMG-CoA through the rate-limiting enzyme 3-hydroxy-3-
methylglutaryl coenzyme A reductase. This enzyme reaction is inhibited by the statin class of drugs
in the treatment of hyperlipidemia; thereby serum and urinary MVA can be quantified to measure
drug efficacy. **(B)** MVA is converted to its less polar lactone at low pH for quantification by
LC–MS/MS. **(C)** Optimization of the pH-dependent conversion of MVA to mevalonolactone.
(D) Optimization of the rate of conversion of MVA to mevalonolactone at pH 2 and ambient
temperature or 60°C.
HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A; MVA: Mevalonic acid; MVL: Mevalonolactone.

plasma compared with man [23], with a range of
10.5–65.3 ng/ml in Sprague-Dawley rat plasma
[16,23], compared with 0.4–9.9 ng/ml in human
plasma [21,24–26]. The administration of HMG-
CoA reductase inhibitors to humans causes a
30–40% reduction of plasma MVA [27]. Pub-
lished data on urinary MVA excretion in non-
clinical species are lacking, although one report
observed a MVA clearance of 350 pmol/min in
rats [1]. Consequently, a robust analytical method
that can quantify MVA in urine for clinical and
nonclinical research is likely to require a wide
dynamic range.

MVA is a polar molecule and at low pH under-
goes spontaneous cyclisation to its lactone form
(mevalonolactone [MVL]; FIGURE 1B). More
recent quantification methods have favored the
use of LC–MS/MS for quantifying MVA in
human plasma [24,25,27], or human plasma and
urine [21]. To date there is no validated method
for MVA in the literature that can be used to

quantify the biomarker in rat and human urine.
Owing to its potential for high specificity and
sensitivity, LC–MS/MS is an attractive analyti-
cal platform for biomarker quantification, but a
frequent criticism is its perceived lack of inter-
laboratory reproducibility [28], hence it would be
advantageous to establish a LC–MS/MS based
MVA quantification method, which has been
validated for use in different laboratories.

The long-established concepts of drug bioana-
lysis and bioanalytical method validation do not
wholly apply to the quantification of endogenous
molecules such as MVA; as such, 'fit-for-purpose'
biomarker method development and validation
guidelines have been established recently [29,30],
which include routine assessment of dynamic
range, analytical precision and accuracy, relative
accuracy plus validation of calibration standards
made in surrogate matrices. Herein we detail the
first validation of a fit-for-purpose translational
MVA assay and its transfer between two separate

laboratories. We confirm the utility of this robust and validated assay for **translational medicine** by testing its biomarker potential in rats and humans, and demonstrating the diurnal variation of MVA excretion that is predicted by hepatic microsomal HMG-CoA reductase activity.

Materials & methods

■ Materials

(*R*)-MVA (purity ~96%) and MVL (purity ~97%) were obtained from Sigma-Aldrich (St Louis, MO, USA). 4,4,5,5-Tetradeutero-MVL (d_4-MVL, purity 97%) was obtained from CDN Isotopes (Pointe-Clare, Quebec, Canada). Methanol, ethyl acetate and formic acid were of analytical grade and were obtained from Fisher Scientific (Loughborough, UK). Urine for method development was obtained by spot collection from five healthy volunteers (two male, three female, mean age 23.2 ± 1.3 years) not subject to dietary or beverage constraints and from male Sprague-Dawley rats (150–250 g, pool of 12 donors) (Alderley Park, AstraZeneca). The urine was stored at -20°C. Urine for the rat biomarker investigation was collected from different animals via single housing in metabolic cages for 12 h between time points 10:00 and 22:00 (n = 8) or 22:00 and 10:00 h (n = 8), and stored at -80°C until analysis. The animals had free access to food and water throughout the 12 h periods. The phases of the illumination cycle in the animal accommodation were 08:00–20:00 (light) and 20:00–08:00 (dark). Protocols were undertaken in accordance with a license granted under the Animals (Scientific Procedures) Act 1986 and approved by the University of Liverpool Ethics Committee. Urine for the human biomarker investigation was obtained by spot collection in the morning (time points range 05:15–08:50) and evening (range 17:30–22:30) from 25 healthy children (mean age 9.10 years, range 0.9–16.7 years). Children were recruited from schools and a nursery with informed written consent obtained from carers or guardians on the behalf of the participating children. The study was conducted with the approval of the Liverpool East Research Ethics Committee. Urine samples were collected from each child by an age-appropriate method, normally a clean catch urine sample into a sterile container. Samples were centrifuged, aliquoted into smaller volumes, then frozen and stored at -80°C.

■ Optimization of the pH-dependent conversion of MVA to MVL

MVA (0.01, 0.1 and 0.5 µg/ml) was dissolved in water-methanol (9:1, v/v) that had been adjusted to various pH values between 2 and 6 by addition of formic acid. After 90 min at ambient temperature, aliquots (10 µl) were taken and analyzed by a LC–MS/MS method to identify the maximal peak area of MVL. The optimal pH (pH 2) was selected for further development. 0.1 µg/ml MVA was incubated at pH 2 and ambient temperature, 37 or 60°C with aliquots taken at 0, 15, 30, 60, 90, 120, and 180 min and analyzed by LC–MS/MS. MVL yield was also determined by overnight incubation at ambient temperature and pH 2. The optimal temperature (60°C) and incubation period (minimum of 15 min) for the extent of the reaction were chosen. Aliquots of MVL (0.1 µg/ml in water-methanol 9:1 v/v at pH 2) were incubated at 60°C for 120 min to establish analyte stability. The percentage of MVA converted to MVL was calculated from the concentration of MVL in MVA standards (4 and 6 µg/ml in distilled water) and in rat (n = 3) and human (n = 3) donors, that had been acidified (pH 2) and incubated at 60°C for a minimum of 15 min. Intra-day (n = 3 analyses per standard) and inter-day (n = 18, representing three analyses per standard, two standards and analyses on 3 days) variation of the conversion was also determined. The endogenous presence of MVL in human and rat urine was determined by analysis of 10 µl aliquots of unprocessed urine (from three rat and three human donors) using LC–MS/MS.

■ Extraction of MVL

Recovery of MVL from urine (50 µl) was achieved by LLE with ethyl acetate (1:4, v/v). The recoveries of MVL (0.4–6.0 µg/ml) and the IS, d_4-MVL (0.15 µg/ml), were determined by spiking MVL and IS (dissolved in acidified methanol 3% formic acid) into buffer (0.1 M potassium phosphate, adjusted to pH 2 with orthophosphoric acid) or into acidified rat (n = 3) or human (n = 3) urine . Samples were shaken vigorously for 10 min at ambient temperature and centrifuged at 8700 *g* for 10 min. Approximately 600 µl of the organic layer was taken and evaporated to dryness under oxygen-free nitrogen. The samples were reconstituted in 200 µl of water-methanol (9:1, v/v) containing 0.1% formic acid (pH 2.4) before injection of 10 µl of solution on to the HPLC column. The extracted compounds were assayed by comparison with standards (water-methanol

9:1, v/v, 0.1% formic acid) of the same concentration. For the urine extraction recovery, the endogenous concentration of MVL was factored into the calculation.

■ Calibration standards

To determine the sensitivity, range, precision and accuracy of the LC–MS/MS method, MVL standards (0.078–10 µg/ml in pH 2 buffer; n = 3) were spiked with IS (final concentration, 0.15 µg/ml), extracted with ethyl acetate, reconstituted in water-methanol-formic acid (9:1:0.1, v/v) and quantified by LC–MS/MS. Independently prepared QC MVL standards and a validation sample (VS, a small population sample, in this case of pooled human or rat urine, that is representative of the individual analytical samples) were prepared at the beginning of the validation, and analyzed alongside analytical samples and calibration standards to confirm batch acceptability and between-batch reproducibility of precision, accuracy and stability. Pooled human urine was prepared by mixing equal volumes of urine from five donors. The LOD was designated to be the concentration of extracted MVL with a peak height greater than three-times the background signal. The LLOQ and ULOQ were, respectively, the lowest and highest concentrations of extracted MVL with acceptable accuracy and precision. The acceptance criterion of precision was 15% coefficient of variation, or 20% for the low QC and LLOQ, while the acceptance criterion of accuracy was 85–115% or 80–120% for the low QC and LLOQ [29], which was determined by extraction of each of the nine calibration standards (three replicates) or three QC (six replicates) in three independent batches. A batch was deemed acceptable if more than four of the six QC replicates were within 85–115% of the set value, in accordance with **biomarker assay validation** guidelines [29]. LC–MS/MS carryover was determined by repeat injection of 10 µl of a 10 µg/ml MVL standard followed by injection and analysis of 10 µl of a blank reconstitution solution.

■ Assessment of matrix effects

Rat and human urine (50 µl aliquots from five donors) was acidified, extracted and reconstituted, and spiked with MVL and IS (final concentration 5 µg/ml) and serially diluted (water-methanol 9:1 v/v, 0.1% formic acid). The effect of dilution on the analyte/IS ratio was measured by the LC–MS/MS method. An ionization suppression/enhancement experiment

was conducted using the postcolumn infusion method. Due to the endogenous presence of the analyte in acidified urine, a solution of d_4-MVL (0.1 µg/ml) in water-methanol containing formic acid (9:1 v/v, 0.1%) was infused directly into the MS source at a constant rate of 10 µl/min (HPLC eluate entered the source at 280 µl/min) and 10 µl of a reconstituted ethyl acetate extract of an acidified human or rat urine sample (10 µl) was injected on to the HPLC column. The continuous MS/MS response to the d_4-MVL (m/z 135→75) was monitored.

■ Addition recovery

Human or rat urine (study samples) was spiked with a high concentration of MVA (in order to minimize the contributory effect of endogenous MVA) and the percentage recovery was used to evaluate relative accuracy. MVA in spiked (0.1, 0.5 or 4 µg/ml) and nonspiked human and rat urine (multiple donors) was converted to MVL, extracted, reconstituted and quantified by the LC–MS/MS method. The concentration of MVA in nonspiked urine was subtracted from the concentration determined in the aliquot of spiked urine and the percentage recovery was calculated.

■ Stability

Stability of MVL in the QC standards was determined by storage at room temperature, 4°C and at -20°C for 14 days. Freeze–thaw cycles of QC standards at -20°C and room temperature were also carried out. The stability of MVL in reconstituted extracts of urine samples at 4°C in the autosampler was also determined.

■ Validated method

Calibration standards were prepared on the day of analysis from 0.0156 to 10 µg/ml at pH 2 with IS (0.15 µg/ml). Aqueous QC standards (0.045, 2 and 6 µg/ml in laboratory 1 and 0.1, 4 and 9 µg/ml in laboratory 2, at pH 2) and a pooled rat urine or human urine VS were prepared at the start of the validation process and stored at -20°C. Urine or MVL calibration standards (50 µl) were incubated with 148 µl of buffer (0.1 M potassium phosphate, pH 2) at 60°C for 25 min. After cooling to ambient temperature, samples and standards were spiked with 2 µl of d_4-MVL solution (15 µg/ml acidified aqueous stock, final concentration 0.15 µg/ml). The analytes were extracted with 800 µl of ethyl acetate: the samples were shaken vigorously for a minimum of 10 min at ambient

temperature before centrifuging at 8700 *g* for 10 min. Approximately 600 µl of the organic layer were removed, evaporated to dry residue under oxygen-free nitrogen and reconstituted in 200 µl of water-methanol (9:1, v/v) containing 0.1% formic acid before injection of 10 µl of solution on to the HPLC column.

Chromatographic separation was achieved on an Acquity UPLC® (Waters, Millford, MA, USA) (laboratory 1) or Dionex UltiMate® 3000 LC system (Fisher Scientific, Loughborough, UK) (laboratory 2) with a Synergi™ 2.5-µm Hydro RP column (100 × 2.0 mm; Phenomenex, Macclesfield, UK). The LC method employed solvent A (water-0.1% formic acid) and solvent B (methanol-0.1% formic acid) (TABLE 1). The LC–MS/MS analysis time was 5 min per sample. A MRM method was optimized on an AB Sciex 4000 QTRAP® instrument (Foster City, CA, USA) in positive-ion mode using automatic and manual tuning with aqueous standards of MVL. The precursor ion for MVL was m/z 131, and the transition m/z 131→69.4 was used for the quantification of MVL with reporter transitions at m/z 131→113 and m/z 131→71. The reporter transitions and retention time of MVL conferred specificity. The IS d_4-MVL was monitored at m/z 135→75 in laboratory 1 and m/z 135→73 in laboratory 2. The following conditions were used for LC–MS/MS acquisition: curtain gas setting, 10.0; collision gas, 4.0; IonSpray voltage, 5500 V; temperature, 450°C; ion source gas 1, 90 in laboratory 1 (20 in laboratory 2); ion source gas 2, 20; interface heater, on; dwell time, 150 ms; declustering potential, 50 V; Q1 and Q3 resolution, high. Individual transition parameters are given in TABLE 2.

■ Application to study samples

MVA was quantified in urine acquired from male Sprague-Dawley rats in 12 h collections (time range 10:00–22:00 or 22:00–10:00) and urine from 25 healthy children obtained by spot collection in the morning (time range 05:15–08:50) and evening (time range 17:30–22:30).

■ Calculation & normalization

Quantification of MVA in urine was achieved by linear regression, plotting the nominal MVL concentration against the analyte-IS peak area ratio. The calculated concentrations were adjusted according to the within-batch estimate of the conversion of MVA to MVL. The simplest calibration model was selected for quantifying the concentration of analyte in standards and urine in accordance with the guidelines for 'fit-for-purpose' biomarker method validation [29].

The MVA concentration was normalized to the urinary creatinine concentration (UCr) as determined by the Jaffe-based assay: urine (25 µl of urine diluted one in 25) was incubated with 125 µl of alkaline picrate solution (0.14 M NaOH, 10 mM Na_2HPO_4, 90 mM sodium borate, 10 mM sodium dodecyl sulphate, 25% v/v picric acid, 2.7% v/v DMSO) for 2 min at room temperature before addition of 5 µl of acid solution (10:2:88 v/v acetic acid:concentrated H_2SO_4:distilled water) and further incubation at room temperature for 10 min with shaking. Plates were read at 490 nm and creatinine was estimated using creatinine calibration standards. Data are reported as nanograms (or micrograms) of MVA per milligram of creatinine (ng/mg UCr).

Results

■ pH-dependent conversion of MVA to MVL

The acid-catalyzed formation of MVL from MVA was optimized by incubating aqueous standards at a range of pH values (FIGURE 1C). Below pH 3 the amounts of MVA and MVL were observed to decrease and increase, respectively. Based on

Table 1. HPLC conditions for the analysis of extracted urine samples, standards and QCs.

Time (min)	%A	%B	Flow rate (ml/min)
Initial	95	5	0.30
2.00	95	5	0.30
2.01	90	10	0.30
2.70	90	10	0.30
2.80	95	5	0.30
5.00	95	5	0.30

Phenomenex Synergi™ Hydro RP column (2.5 µm, 100 A, 100 × 2.00 mm) linked to an Acquity UPLC® or Dionex Ultimate® 3000 and the 4000 QTRAP®. The mobile phase is at pH 2.4.

Table 2. MS parameters for each transition in the MRM method for laboratory 1 and laboratory 2.

Analyte	Q1 mass (*m/z*)	Laboratory 1			Laboratory 2		
		Q3 mass (*m/z*)	Collision energy (eV)	Collision exit potential (V)	Q3 mass (*m/z*)	Collision energy (eV)	Collision exit potential (V)
Mevalonolactone	130.956	113.1	13	8	113.1	13	8
Mevalonolactone	130.956	71.0	20	12	71	20	12
Mevalonolactone	130.956	69.4	15	10	69.4	20	20
IS	134.945	75.0	20	12	73.4	15	5

these results, the production of MVL at pH 2 was examined over 180 min at ambient temperature, 37 and 60°C. No significant difference was observed between the rate of MVL production at ambient and physiological temperature; incubations at 60°C for 25 min had a greater rate of MVL production (Figure 1D). The percentage converted to MVL overnight at ambient temperature was not significantly different from the yield of MVL after 25 min at 60°C: 70.8 ± 3.9% (n = 6) versus 67.5 ± 1.3% (n = 6), respectively; therefore an overnight incubation offered no advantage. The percentage conversion of MVA to MVL in aqueous standards under the optimal conditions was 69.5 ± 4.4% (day 1; n = 6), 66.0 ± 3.9% (day 2; n = 6) and 67.5 ± 1.3% (day 3; n = 6). In three rat urine donors the percent conversion of MVA to MVL was 73.8 ± 6.5% and in three human donors 70.6 ± 2.7%; aqueous standards in this experiment gave an equivalent conversion of 71.1 ± 2.5%. Data are given as mean ± SD. Inter-day variation of 3.6% was observed and, as a result, aqueous MVA standards were converted at the same time as study samples to determine percentage conversion for each batch. In the analysis of 10 µl of unprocessed rat (n = 3) and human (n = 3) urine by the LC–MS/MS method, MVL was not detected.

■ **Extraction recovery**
The analyte and IS were not extracted by ethyl acetate to the same extent, with 72.7 ± 2.1% (n = 6) of the analyte recovered compared with 34.4 ± 3.5% IS in laboratory 1. In laboratory 2 it was observed that extraction recovery of the analyte and IS was lower at 33.7 ± 1.0% (n = 6) and 17.5 ± 1.7% (n = 5), respectively, but the analyte-IS peak area relationship was still reproducible, linear and accurately quantifiable by LC–MS/MS. Increasing the extraction time to 30 min in laboratory 2 gave equivalent recoveries of analyte (73.6 ± 6.3%) and IS (38.5 ± 4.3%) as observed in laboratory 1. The extraction recovery of the analyte and IS was assessed in human (n = 3) and rat (n = 3)

donors. The mean extraction recovery of 0.4, 1.0 and 6.0 µg/ml MVL from three rat urine donors was 64.0 ± 8.1%, 62.5 ± 4.8% and 54.3 ± 5.0%, respectively, and the mean recovery of 0.15 µg/ml IS was 27.9 ± 3.3%. The mean extraction recovery of 0.4, 1.0 and 6.0 µg/ml MVL from three human donors was 72.8 ± 16.1%, 62.1 ± 4.4% and 64.7 ± 8.7%, and the mean recovery of 0.15 µg/ml IS was 31.5 ± 3.3%. The peak area ratio of analyte to IS corrects for the variability in the extraction recovery between samples, enabling the accurate quantification of MVL. The peak areas of MVL and the IS in calibration standards and samples of equivalent concentration were similar. As an example the mean peak area of MVL in one rat study sample was determined to be $2.5 \times 10^6 \pm 5.2 \times 10^4$ and, using the analyte/IS peak area ratio, was calculated to be 5.9 µg/ml MVL. The closest standard, a QC at 6 µg/ml, had a similar MVL peak area of $2.8 \times 10^6 \pm 2.7 \times 10^5$. The IS peak area for the rat urine sample was $1.2 \times 10^5 \pm 3.2 \times 10^3$ and for the QC was $1.3 \times 10^5 \pm 7.0 \times 10^3$, giving ratios of 20.8 and 21.5, respectively. The extracted IS peak areas varied by 7.9% across the rat urine study and by 16.0% across the human urine study.

■ **Calibration range, accuracy & LOQ**
Several weighted regression models (1/x, 1/x², 1/x³) were tested to improve the residuals in the calibration model [31], see **Supplementary Table 1**. The best model was linear and forced through the origin, with acceptable precision and accuracy across the analytical range with $R^2 > 0.99$ without the need to weight the data (Figure 2A). The LOD of extracted MVL was 0.0078 µg/ml. The LLOQ was 0.0156 µg/ml in laboratory 1 and 0.0313 µg/ml in laboratory 2; the ULOQ was 10 µg/ml. Independently prepared QC standards also reached acceptable precision and accuracy in both laboratory 1 and laboratory 2; individual data are shown in **Tables 3 & 4**. Carryover of the analyte to subsequent blank injections on the LC–MS/MS was not detected.
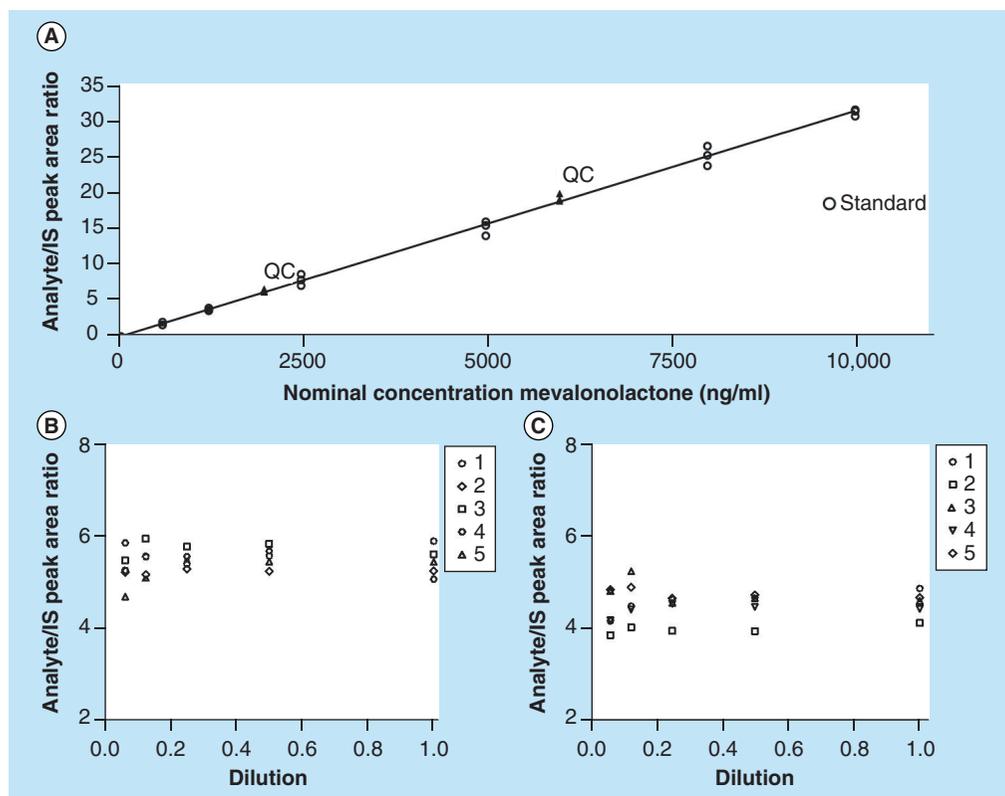
Figure 2. Validation of the surrogate matrix for the mevalonolactone calibration and QC standards. (A) The linear relationship between nominal concentration and the analyte to IS ratio. Independently prepared QC standards were within ±15% of the nominal concentration; data shown are mean values of three batches (n = 3 for each batch) from laboratory 1. **(B)** Dilution linearity of mevalonolactone extracted from rat urine (n = 5). **(C)** Dilution linearity of mevalonolactone extracted from human urine (n = 5). Dilution linearity of the analyte/IS peak area ratio confirms the parallelism of the surrogate matrix.

■ **Parallelism of rat urine to surrogate matrix**

To validate the utility of a surrogate matrix for the calibration standards, rat (n = 5) and human (n = 5) urine that had been acidified, extracted and reconstituted was serially diluted, and the analyte and IS response was determined by LC–MS/MS **(FIGURE 2B & C)**. The observed analyte response remained within 20% of what was expected in both rat and human urine up to a dilution of one in 128. The observed IS response only remained within 20% of the expected response in both rat and human urine up to a dilution of one in 16, as further dilution reduced the IS response below the LOD. These data suggest that the rat and human urine matrix has no significant effect on the MS detection of MVL and the IS.

■ **Matrix effects**

Matrix effects were further investigated by postcolumn infusion of d_4-MVL and injection of 10 µl of a reconstituted ethyl acetate extract of acidified human or rat urine. The MS/MS response of the IS was monitored throughout the elution and there was no significant suppression or enhancement of the signal for d_4-MVL at or around the retention time of the analyte **(FIGURE 3A & B)**.

■ **Relative accuracy**

Rat and human urine spiked (0.1, 0.5 or 4 µg/ml MVA) and nonspiked was extracted and assayed to determine relative accuracy. The mean concentration of MVL in the nonspiked urine was subtracted from that of the spiked urine. In laboratory 1, the recovery of a 4 µg/ml spike in rat urine was 105.7 ± 9.9%. In laboratory 2, the recovery of the 0.1, 0.5 or 0.4 µg/ml spike in rat urine was 96.0 ± 7.6%, 111.0 ± 6.9% and 105.9 ± 2.3%, respectively. Recovery of the 0.1, 0.5 or 0.4 µg/ml spike in human urine was assessed in laboratory 2 and was calculated to be 96.0 ± 4.8%, 118.2 ± 3.5 and 107.3 ± 0.4%, respectively. Data are mean ± SD. Full recovery

Table 3. Laboratory 1 intra-day validation of the extraction, reconstitution and LC–MS/MS analysis of mevalonolactone calibration and QC standards across three batches. Accuracy is within 85–115% and precision is within 15% CV.

QC	Nominal concentration (ng/ml)	Batch 1				Batch 2				Batch 3			
		Calculated concentration (ng/ml)	Accuracy (%)	Precision (%CV)	n	Calculated concentration (ng/ml)	Accuracy (%)	Precision (%CV)	n	Calculated concentration (ng/ml)	Accuracy (%)	Precision (%CV)	n
Low	45	44.0	97.8	5.3	6	45.2	100.4	3.2	6	41.1	91.4	2.7	6
Mid	2000	1991.0	99.5	2.1	6	2048.1	102.4	4.9	5	1855.7	92.8	3.4	6
High	6000	5881.7	98.0	4.2	6	6199.3	103.3	6.7	5	5427.7	90.5	4.1	6
Standard													
1	15.6	16.6	106.0	8.3	3	16.2	103.9	5.6	3	16.3	104.4	3.8	2
2	31.3	31.0	99.3	2.5	3	33.9	108.5	4.1	3	29.6	94.8	1.3	2
3	62.5	65.8	105.3	10.8	3	62.3	99.7	5.2	3	58.5	93.6	4.3	3
4	625	610.5	97.7	1.6	3	644.9	103.2	10.5	3	629.4	100.7	4.1	3
5	1250	1221.4	97.7	1.9	3	1255.5	100.4	5.2	3	1130.2	90.4	0.9	3
6	2500	2475.9	99.0	3.1	3	2589.0	103.6	9.8	3	2338.4	93.5	0.6	3
7	5000	5416.7	108.3	5.1	3	4930.5	98.6	6.6	3	4535.7	90.7	4.2	3
8	8000	7914.6	98.9	2.7	3	8149.9	101.9	5.4	3	7830.4	97.9	6.4	3
9	10000	10129.3	101.3	5.2	3	10093.6	100.9	1.5	3	10489.1	104.9	7.8	3

of the spiked analyte from multiple donors suggests that this method can be used for the accurate quantification of MVA in both human and rat urine.

■ Reproducibility

Reproducibility of the full extraction and analysis method was determined by quantifying MVA in rat and human urine (VS) divided into three batches stored at -20°C. MVA was quantified on three separate occasions from each of these batches (n = 3 per analysis) over 20 days (**Figure 4A & B**). Within-batch variation was similar between laboratories, with 2.01–4.58% coefficient of variation in laboratory 1 and 1.45–5.25% in laboratory 2; while between-batch variation was 6.15% in laboratory 1 and 6.35% in laboratory 2.

■ Stability

MVL QC standards were stable at room temperature, 4°C and at -20°C across 14 days, and at 60°C for 120 min (**Table 5**). Freeze–thawing did not affect the integrity of aqueous standards.

■ Biomarker application

MVA was quantified in the urine collected from male Sprague-Dawley rats during two 12 h collections, time ranges 10:00–22:00 and 22:00–10:00 (**Figure 4C**). The excretion of MVA was significantly greater (unpaired t-test; $p < 0.0001$) in urine collected during the time range 22:00–10:00 (6.2–13.0 µg/mg UCr; mean 9.7 ± 2.3 µg/mg UCr) compared with the time range 10:00–22:00 (1.6–5.2 µg/mg UCr; mean 3.4 ± 1.3 µg/mg UCr). When expressed as total MVA excretion by factoring in urine volume, the diurnal differences remained and urine collected in time range 22:00–10:00 represented a mean excretion of 110.0 ± 27.8 µg MVA/12 h (81.7–153.2 µg) compared with urine collected in time range 10:00–22:00 with a mean excretion of 21.5 ± 6.6 µg MVA/12 h (12.8–33.1 µg).

MVA was also quantified in paired morning and evening urines obtained by spot collection from healthy children (n = 25). The excretion of MVA in evening spot collections had a median of 292.4 ng/mg UCr (n = 25, inter quartile range 245.4 ng/mg UCr), whereas the median concentration of MVA in morning spot collections was 427.0 ng/mg UCr (n = 25, inter quartile range 230.1 ng/mg UCr). The paired and mean data are represented in **Figure 4D & E**; in 15 out of 25 pairs, morning collections had significantly greater concentrations of MVA than evening

collections (p < 0.05; t-test, paired, two-sided) where the morning excretion was, on average, 105% greater; however, in four individuals the concentration of MVA was significantly greater in the evening collection (p < 0.005; t-test, paired, two-sided). The remaining six morning and evening pairs had numerical differences but were not statistically significant.

Discussion

Translational studies of the mevalonate pathway are hindered by the lack of a simple and validated, cross-species quantification method for the urinary biomarker of HMG-CoA reductase activity, MVA. The only published LC–MS/MS assay of urinary MVA has a short dynamic range of 20–1000 ng/ml validated in human urine; in addition the protocol requires an initial sample volume of 125 µl [21]. We have developed, validated and applied an assay for MVA by LC–MS/MS that can be used for the analysis of rat and human urine, and can be transferred between laboratories. With a substantial dynamic range (0.0156–10 µg/ml), the assay is sufficient to determine normal biological and perturbed concentrations of MVA in both rat and human urine. Administration of statins is known to reduce the production of MVA by 30–40% [6,32,33]. Given that the physiological urinary concentration of MVA in humans ranges between 116 and 1227 ng/ml, the validated dynamic range of this assay will comfortably address perturbed MVA biosynthesis. The assay also benefits from requiring only 50 µl of urine, making the assay applicable where sample volumes are limited (e.g., rodent studies), and a short analytical run-time of 5 min.

This method builds upon published methods, incorporating acid-catalyzed conversion of MVA to its lactone form MVL [21,24,25], extraction of the lactone and the IS with ethyl acetate [25], and analysis by positive-ion LC–MS/MS [24,25], but it is validated to a large dynamic range to encompass MVA quantification in human and rat urine, has an optimized MVA→MVL conversion step, is without matrix effects and has been demonstrated to transfer between analytical laboratories. During the inter-laboratory validation we observed an increase in the LLOQ from 15.6 ng/ml in laboratory 1 to 31.3 ng/ml in laboratory 2, which could be explained by the extraction recovery in laboratory 2; which was approximately 50% lower than in laboratory 1. Different shaker equipment was used in the two laboratories, which may account for

Table 4. Laboratory 2 intra-day validation of the extraction, reconstitution and LC–MS/MS analysis of MVL calibration and QC standards across three batches. Accuracy is within 85–115%, precision is within 15% CV and within 20% CV for the LLOQ.

| QC	Nominal concentration (ng/ml)	Batch 1				Batch 2				Batch 3			
		Mean calculated concentration (ng/ml)	Accuracy (%)	Precision (%)	n	Mean calculated concentration (ng/ml)	Accuracy (%)	Precision (%)	n	Mean calculated concentration (ng/ml)	Accuracy (%)	Precision (%)	n
Low	100	112.0	112.0	8.2	6	111.6	111.6	10.9	6	108.8	108.8	6.4	6
Mid	4000	4129.0	103.2	3.7	6	3805.5	95.1	1.0	6	3899.2	97.5	3.1	6
High	9000	9649.6	107.2	3.0	6	8935.3	99.3	1.9	6	9040.6	100.5	1.3	6
Standard													
1	31.3	37.6	120.3	7.0	2	33.0	105.7	4.9	3	36.7	117.6	1.5	2
2	62.5	68.9	110.3	8.9	3	59.1	94.6	12.5	3	67.9	108.6	4.8	3
3	625	642.8	102.9	2.8	3	642.8	102.9	2.8	3	643.6	103.0	3.4	3
4	1250	1224.1	97.9	1.3	3	1251.5	100.1	0.6	3	1274.1	101.9	4.0	3
5	2500	2513.5	100.5	2.5	3	2513.3	100.5	3.5	3	2519.2	100.8	1.1	3
6	5000	5055.5	101.1	2.6	3	5039.5	100.8	2.6	3	4993.8	99.9	2.1	3
7	8000	8146.7	101.8	1.4	3	7812.4	97.7	0.8	3	7937.8	99.2	2.0	3
8	10000	9898.3	99.0	1.3	3	10166.8	101.7	0.7	3	10068.2	100.7	0.2	3
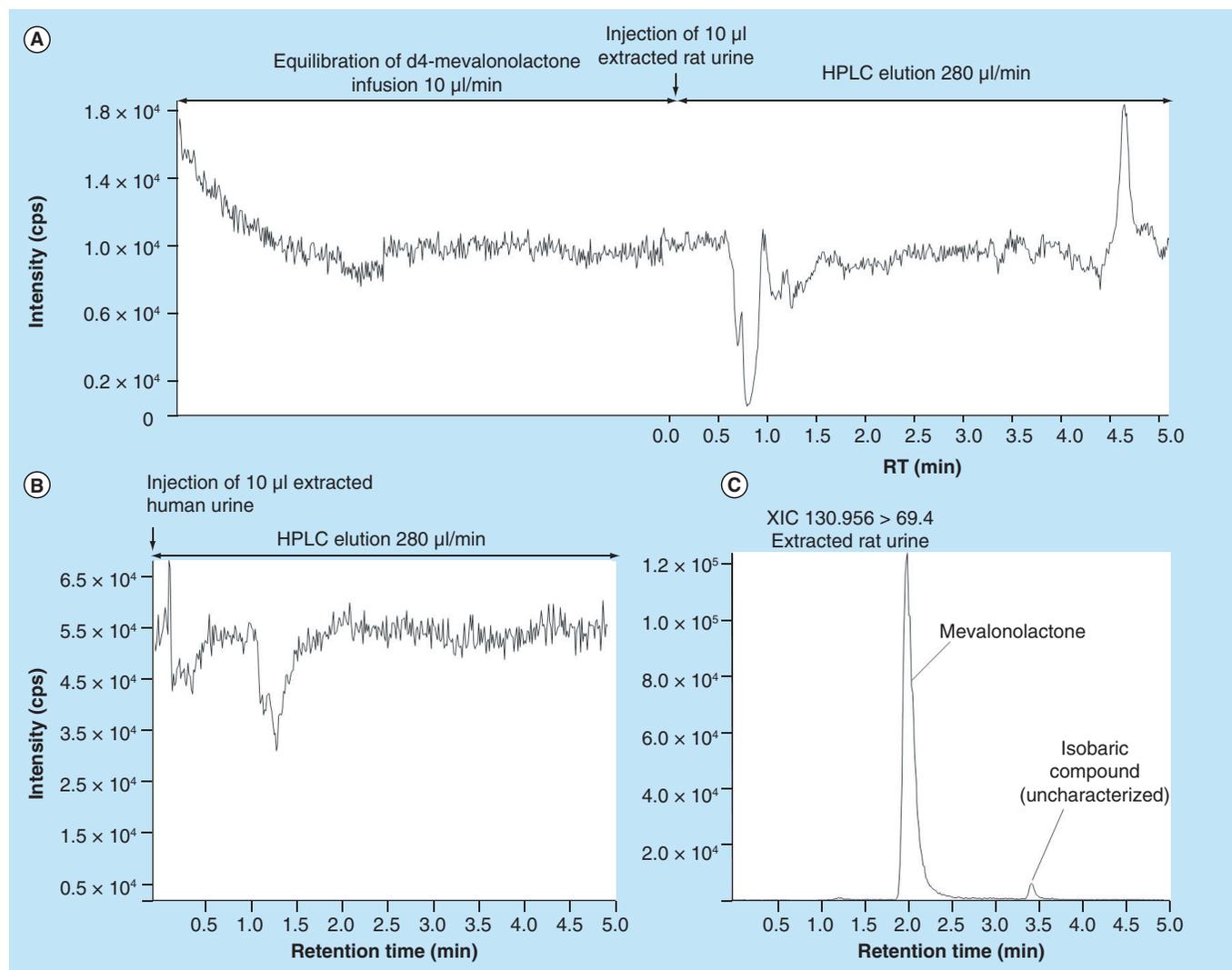
Figure 3. Mevalonolactone assay: assessment of matrix effects and assay selectivity. LC–MS/MS chromatograms showing the absence of ionisation suppression/enhancement of the IS signal (*m/z* 135→75 for d$_4$-mevalonolactone; retention time, 2.05 min) by co-eluting materials extracted from **(A)** the acidified rat and **(B)** human urine matrix with ethyl acetate. **(C)** The extracted ion chromatogram of a reconstituted ethyl acetate extract (10 µl injection) of acidified rat urine (50 µl) showing mevalonolactone derived from endogenous mevalonic acid calculated to be at a concentration of 3.6 ± 0.06 µg/ml and a minor, unidentified, noninterfering compound eluting at 3.5 min.

the disparity, however, increasing the extraction period to 30 min in laboratory 2 gave equivalent recoveries to laboratory 1. Therefore, when transferring this method to other laboratories and using different processing equipment, the extraction process must be re-optimized in order to retain sufficient sensitivity.

The IS was chosen to correct for between-sample inconsistencies. Ideally, the IS should behave in the same manner as the analyte throughout the analytical method. Our data have shown that the extraction recovery of the d$_4$-MVL was approximately 50% lower than MVL and had a fractionally earlier elution time (2.05 min) relative to the analyte (2.10 min) in the polar

reversed-phase HPLC method, which could suggest that d$_4$-MVL is more polar than MVL. Similar observations of reversed-phase HPLC resolution and different extraction recoveries between a low molecular weight analyte and its d$_4$-IS have been reported [34,35]. Despite this, the analyte-IS relationship was consistent and linear, as shown by the validation. Therefore, these differences in behavior between analyte and IS are not limiting for the quantification of MVA, and d$_4$-MVL, which was also used successfully in another published method [25], is a suitable IS.

The use of MS/MS to assay MVL derived from MVA in urine encountered a significant selectivity challenge. In extracts of rat urine,
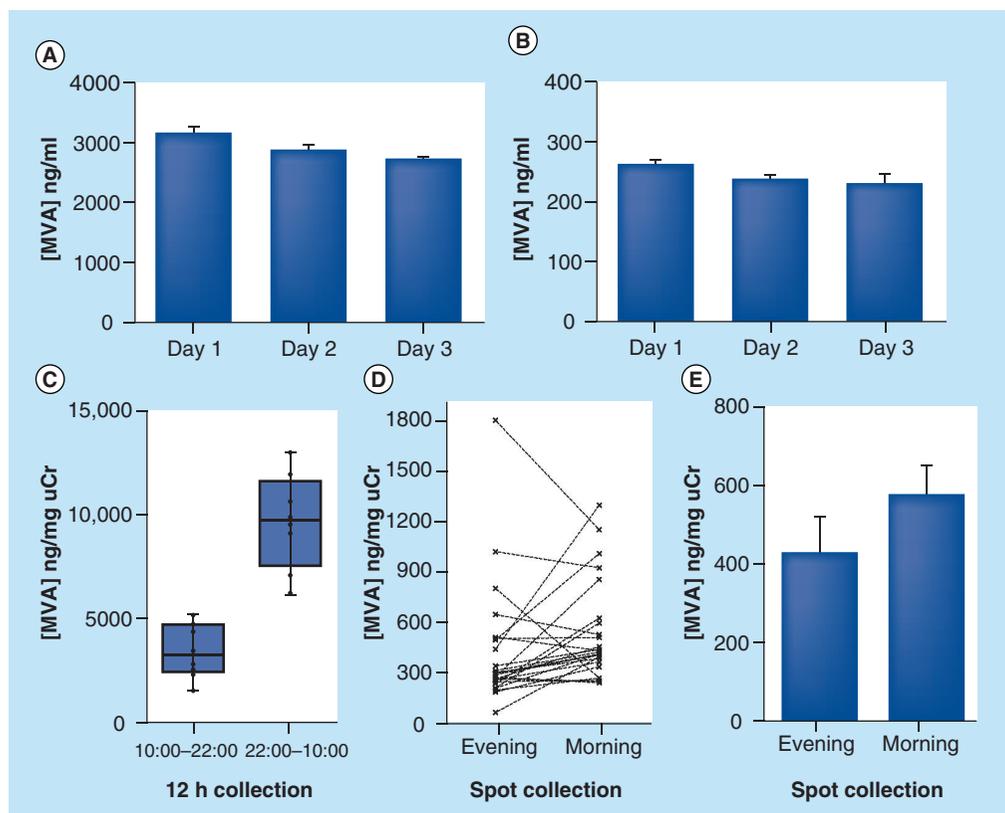
Figure 4. Reproducibility of mevalonic acid determination on 3 separate days. For **(A)** a rat validation sample and **(B)** a human validation sample. **(C)** Concentration of urinary MVA determined in 12 h collections from male Sprague-Dawley rats displayed as mean, upper and lower quartiles with the range (n = 8) and mean of each individual (n = 3). **(D)** Concentration of MVA in urine obtained by spot collection from healthy human children in the evening and the following morning; data are plotted as the mean concentration (n = 3) determined for each morning and evening pair. **(E)** Mean concentration of urinary MVA in the statistically significant morning and evening spot collections from healthy human children (n = 19) with standard error.
MVA: Mevalonic acid.

single-reaction monitoring with *m/z* 131→69.4 detected an isobaric compound that differed from MVL in retention time (**FIGURE 3C**). This compound was left uncharacterized as its presence did not interfere with the quantification of the analyte. However, we used a combination of chromatographic retention time and MRM reporter transitions to confer enhanced specificity on our assay. MVA is a very polar compound and was found to be incompatible with a number of widely used reversed-phase columns. Screening of several column chemistries identified a reversed-phase polar end-capped column that retained MVA and MVL satisfactorily.

Table 5. Stability of aqueous mevalonolactone standards at the LLOQ and QC levels assessed after three cycles of freeze–thawing and at room temperature, 4 and -20°C for 14 days. Data are presented as the mean percentage recovery ± SD (n = 3).

Storage conditions	LLOQ		Low QC		Mid QC		High QC	
	Mean recovery (%)	SD (%)	Mean recovery (%)	SD (%)	Mean recovery (%)	SD (%)	Mean recovery (%)	SD (%)
-20° freeze–thaw	100.5	1.0	100.1	1.4	100.9	1.7	101.7	2.5
Room temperature 14 days	96.8	1.2	95.2	1.5	98.7	0.8	96.7	1.7
4°C 14 days	98.0	2.8	96.9	3.4	96.5	2.5	96.1	0.2
-20°C 14 days	96.0	2.3	95.0	1.7	96.1	1.4	94.4	0.2

The high endogenous concentrations of MVA in rat and human urine negate the use of 'blank' matrix for calibration standards. Calibration standards of MVL were prepared in pH 2 buffer, extracted and reconstituted in the same manner as urine. The use of a surrogate matrix was validated by demonstrating that the human and rat urine matrix did not affect the percentage of MVA converted to MVL, the extraction recovery of MVL and the IS, and the MS response of the analyte and IS, a phenomenon termed '**parallelism**'. By analyzing the effect of matrix compared with aqueous standards at each stage of the assay, we found that the urine matrix had no appreciable bearing on the processing, detection and quantification of MVA.

Matrix effects are commonly encountered in electrospray ionization when a matrix component that co-elutes with the analyte(s), but may not be detected in the MRM channel, attenuates or enhances the MS signal, and consequently compromises accuracy. Jemal *et al.* experienced extensive suppressive matrix effects when they used negative-ion detection of MVA regenerated from MVL to quantify MVA in human urine [21]. We exploited the $[M+H]^+$ precursor ion generated abundantly by an eluent containing formic acid instead of the $[M+NH_4]^+$ precursor generated by ammonium formate eluents [24,25]. We did not encounter suppression or enhancement of the positive-ion d_4-MVL signal in the postcolumn infusion experiment and the addition recovery experiment gave near 100% recovery from rat and human urine.

The stability of MVA in various matrices, including urine, has been widely cited in the literature. In human plasma there was <6.7% change from baseline over 16 months under various storage conditions [24]. Similarly, in water, urine and plasma there was <8.2% change from baseline for at least 7 weeks [21]. We have included interim stability data from storage and freeze–thaw experiments for our QC standards composed of MVL. In addition, we have safeguarded against potential analyte instability by analysis of a urine validation sample in each batch. However, additional long-term studies should be carried out to confirm previous reports of analyte stability.

To demonstrate the utility of the validated assay MVA was quantified in rat urine collected during the time range 10:00–22:00 or 22:00–10:00 and in paired morning-evening spot collections from healthy children. The production of MVA, represented by the plasma concentration and urinary excretion, is subject to diurnal variation in humans [14,15,32] and the hepatic microsomal activity of HMG-CoA reductase in rats displays a marked diurnal variation [14,16]. However, early studies were unable to demonstrate a statistically significant diurnal fluctuation of plasma MVA in rats [2]. The present assay successfully distinguished between two 12 h rat urine collections, with 22:00–10:00 collections containing more than threefold the amount of MVA than 10:00–22:00 collections. These data correspond well to published data on the diurnal activity of hepatic HMG-CoA reductase [36]. The discrepancy between the present data and the lack of significance found in the flux of plasma MVA by Kopito *et al.* is likely to do with study design rather than the analytical method; in comparison to the present study, rats were allowed access to food between time points 09:00 and 12:00 and plasma mevalonate was measured across nine time points with different groups of animals at each time point (n = 5–6 per group) [2]. Given the interindividual variation displayed between the animals in the present study, it is more likely that significant diurnal fluctuations would have been seen if the concentration of plasma MVA over time had been compared in samples obtained from matched animals.

Our pilot biomarker investigation also quantified MVA in urine obtained by paired morning and evening spot collection from healthy children with a mean age of 9.10 years. In 60% of individuals the concentration of MVA was greater in the morning spot collection and these increases ranged from 27 to 469%. In four individuals a significantly lower urinary MVA content was observed in the morning compared with the evening collection. These data conform with published urinary MVA data where a study of healthy volunteers observed increases of 6 to 122% in MVA excretion in eight of 11 volunteers in 12 h urine collected between time points 19:00 and 07:00 compared with 07:00 and 19:00; in the remaining three volunteers this trend was reversed [37]. Correspondingly, plasma MVA was observed to vary fivefold in a 40 year old male with a maximum at 07:00 and a minimum at 22:00 [32].

Notably, normalization of the MVA concentration to UCr concentration or urine volume increased the differences between MVA excretion observed in the timed collections of the rat study. UCr normalization was especially important to account for inconsistencies in

urine output between the spot collections from the healthy children, as prolonged timed collections were not practical. However, there is some disagreement over the value of creatinine normalization for urine biomarkers in nonsteady state diseases such as acute kidney injury [38,39], and consideration of the changes in creatinine clearance must be made when selecting a method of normalization for urinary MVA as a biomarker.

Conclusion

The MVA quantification method presented here has been validated according to fit-for-purpose guidelines for biomarkers. The method displays precision (±15%CV) and accuracy (85–115%), and circumvents the bioanalytical difficulties of MVA as an analyte – its endogenous presence in biofluids and polarity. This method is suitable for quantification of endogenous MVA in rat and human urine, and can be successfully transferred between laboratories in support of studies of mevalonate pathway flux as a biomarker of disease, pharmacological efficacy and response to cellular injury. In a prototype biomarker study, urinary excretion of MVA by male rats and healthy human children was shown to undergo the diurnal variation predicted by the known diurnal variation of hepatic HMG-CoA reductase activity.

Future perspective

With the increasing importance of established and novel biomarkers in the realm of disease and therapeutics, the field of bioanalysis has had to appreciate the difference between quantifying drugs and their metabolites and **endogenous biomarkers**. Yet just as in drug quantification, consistency between laboratories in the execution and validation of methods is important to the reliability of data, especially if such data are to have a clinical impact. For HMG-CoA reductase and MVA, their pivotal position in cholesterol and isoprenoid biosynthesis makes them an interesting target for future disease research. As a potential biomarker of multiple end-points, the development of a reliable MVA quantification method will only enhance translational research into this limitless area. It is hoped that regulatory authorities will formulate guidelines for bioanalysts that will clarify and compliment the study of endogenous biomarkers and help progress a biomarker from basic research to clinical application.

Executive summary

Background

- Mevalonic acid is produced by the rate-limiting enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase and is a key metabolic intermediate of the mevalonate pathway.
- The mevalonate pathway and therefore 3-hydroxy-3-methylglutaryl coenzyme A reductase are important mediators of sterol and nonsterol biosynthesis, and therefore play an important role in multiple health and disease states.
- The study of mevalonic acid, and any biomarker, would benefit from an accurate, precise, site-transferable and translational method of quantification.
- To this end, a LC–MS/MS quantification assay has been developed and validated according to fit-for-purpose biomarker guidelines.

Results

- The assay is accurate and precise over a biologically relevant dynamic range allowing quantification of mevalonic acid in human and rat urine with a small 50 µl sample volume.
- The method has been successfully transferred between laboratories with different instruments with minimal optimization required.

Application

- Mevalonic acid demonstrated diurnal urinary excretion patterns in male Sprague-Dawley rats and healthy human children, thus illustrating the utility of the method.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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