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Analysis of Methylmalonic Acid in Serum Using the Xevo TQ-S micro for Clinical Research

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APPLICATION BENEFITS

- Analytical selectivity of the chromatographic method provides separation of isomeric species
- LC-MS/MS enables high sample throughput using multi-well plate
- Excellent agreement to an independent LC-MS/MS method

WATERS SOLUTIONS

Ostro™ Phospholipid Removal Plate

ACQUITY™ UPLC™ 1.7 µm CSH™ C_{18} Column

ACQUITY UPLC I-Class System with Fixed Loop (FL)

Xevo™ TQ-S micro

MassLynx™ Software

TargetLynx™ Application Manager

KEYWORDS

MMA, protein precipitation, phospholipid removal, LC-MS/MS, Xevo TQ-S micro

INTRODUCTION

Vitamin B12 plays an integral role in critical biological functions, such as DNA synthesis, formation of erythrocytes, and maintenance of the myelin sheath in the nervous system. In its role as a co-factor, vitamin B12 aids the methyl-malonyl-CoA mutase catalyzed conversion of methylmalonyl-CoA to succinyl-CoA, with methylmalonic acid (MMA) being an important by-product in this pathway. MMA concentrations are elevated when there is insufficient vitamin B12 to help catalyze this reaction, meaning that MMA is a candidate biomarker for clinical research investigations into the function of vitamin B12. LC-MS/MS is an ideal platform to perform this analysis. Chromatographic separation is key in separating MMA from isobaric and isomeric species, such as succinic acid, that can cause a significant concentration bias. To help facilitate adoption of this methodology, a simplified workflow is necessary to enable a high throughput of samples.

Here we describe a clinical research method utilizing Ostro phospholipid removal plate technology for the extraction of MMA from serum. Chromatographic separation was performed on an ACQUITY UPLC I-Class System using an ACQUITY UPLC 1.7 µm CSH C_{18} Column followed by detection on a Waters™ Xevo TQ-S micro Tandem Quadrupole Mass Spectrometer to enable quantification of very low physiological concentrations of MMA (Figure 1).

Figure 1. The Waters ACQUITY UPLC I-Class System and Xevo TQ-S micro Mass Spectrometer.

EXPERIMENTAL

LC conditions

System:	ACQUITY UPLC I-Class (FL) with Column Heater (CH)
Needle:	20 µL
Column:	ACQUITY UPLC CSH C_{18} 2.1 × 100 mm, 1.7 µm (p/n: 186005297)
Pre-column:	In-line filter (p/n: 205000343)
Mobile phase A:	Water with 0.2% formic acid
Mobile phase B:	Acetonitrile with 0.2% formic acid
Weak wash solvent:	Mobile phase A, 900 µL
Strong wash solvent:	Mobile phase B, 600 µL
Column temp.:	40 °C
Injection volume:	10 µL
Flow rate:	See table 1
Gradient:	See table 1
Run time:	3.3 minutes

MS conditions

System:	Xevo TQ-S micro
Resolution:	MS1 (0.75 FWHM) and MS2 (0.75 FWHM)
Acquisition mode:	Multiple Reaction Monitoring (MRM) (see Table 2 for details)
Polarity:	ESI-
Capillary:	0.50 kV
Source temp.:	150 °C
Desolvation temp.:	600 °C
Inter-scan delay:	Automatic
Inter-channel delay:	Automatic

Data management

MassLynx Software v4.2 with TargetLynx Application Manager

Sample preparation

Methylmalonic acid certified reference solutions and methylmalonic acid-$^{13}C_4$ stable labelled internal standard were purchased from Sigma Aldrich (Poole, UK).

Calibrators were prepared in 1% (w/v) bovine serum albumin (BSA) in phosphate buffered saline and quality controls (QCs) were prepared in pooled human plasma (BioIVT, UK). Methylmalonic acid calibrators were prepared over the range of 21–1270 nmol/L. QC concentrations were 152, 364, and 982 nmol/L. Additional MMA QCs (Recipe, Germany) in reconstituted lyophilized serum with concentrations at 264 and 598 nmol/L were also used to evaluate performance of the method. *Note: To convert SI units to conventional mass units divide by 8.468 for methylmalonic acid (nmol/L to ng/mL).*

Sample extraction

Samples were centrifuged at 3000 g for five minutes prior to extraction. 100 µL of sample was added directly to an Ostro phospholipid removal plate (p/n: 186005518). 25 µL of internal standard solution (83 nmol/L methylmalonic acid-$^{13}C_4$) and 400 µL 1% formic acid in acetonitrile were added, mixing vigorously after each reagent addition. Using a vacuum, samples were eluted into a 2-mL collection plate (p/n: 186002482) and then evaporated to dryness using nitrogen at 50 °C. Samples were reconstituted in 60 µL 1% formic acid in water, mixed vigorously, and centrifuged for two minutes at 3000 g prior to injection.

Method conditions

Table 1. Gradient table for the separation of MMA. Operating backpressure at the initial conditions was approximately 9000 psi.

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.45	99	1	Initial
0.3	0.45	99	1	6
1.3	0.45	80	20	6
1.5	0.45	5	95	11
2.0	0.45	99	1	11

Table 2. MRM parameters for methylmalonic acid and its stable isotope labelled internal standard. Dwell times were set to automatic with 20 points across the peak over four seconds. Qualifier ion conditions are in parentheses. MS scan window was 1.0–1.8 minutes with the time period outside this diverted to waste.

Compound	MRM	Cone (V)	Collision (V)
Methylmalonic acid	117.1>73.1 (55.1)	15	8 (20)
Methylmalonic acid -$^{13}C_4$	121.1>76.1	15	8

RESULTS

No significant interferences (recovery within ±15% bias) were observed at the retention time of methylmalonic acid, when succinic acid, homocysteine, and homocystine were examined. Chromatographic separation of MMA and succinic acid is demonstrated in Figure 2. No significant interferences (recovery within ±15% bias) were observed when other endogenous compounds were examined (albumin, bilirubin, uric acid, intralipid, triglycerides, and cholesterol).

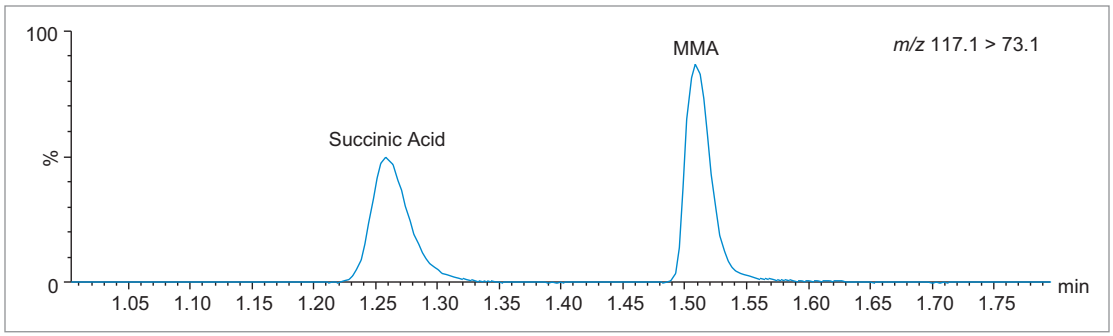

Figure 2. Chromatographic separation of MMA and succinic acid using the ACQUITY UPLC CSH C_{18} Column.

No significant system carryover (<20% of the lowest calibrator) was observed from high concentration samples into subsequent blank injections. A 1:5 dilution was successfully employed on high concentration samples at 4234 nmol/L, providing a mean accuracy of 103% for methylmalonic acid with an RSD of 3%.

Analytical sensitivity investigations were performed using methylmalonic acid spiked into stripped serum over three occasions across and below the calibration range (n=30 at each concentration). The method would allow for precise quantification (<20% RSD) at 21 nmol/L for methylmalonic acid. The S/N (PtP) was >10 at 21 nmol/L.

Total precision of MMA was determined in pooled plasma and Recipe serum controls by extracting and quantifying five replicates of the QC material per day over five separate days (n=25). Repeatability was assessed by analyzing five replicates at each QC level. Low, mid, and high concentrations in pooled plasma were 152, 364, and 982 nmol/L (Table 3), and level 1 and 2 concentrations in Recipe controls were 264 and 598 nmol/L (Table 4).

Table 3. Total precision and repeatability for the analysis of methylmalonic acid at three concentrations in pooled plasma.

	Total QC precision			QC repeatability		
Compound	**Low**	**Mid**	**High**	**Low**	**Mid**	**High**
Methylmalonic acid	4.4%	6.1%	5.8%	4.4%	3.7%	4.6%

Table 4. Total precision and repeatability for the analysis of methylmalonic acid at two concentrations in reconstituted lyophilized serum Recipe controls.

	Total QC precision		QC repeatability	
Compound	**Level 1**	**Level 2**	**Level 1**	**Level 2**
Methylmalonic acid	6.3%	5.5%	5.5%	4.8%

The method was shown to be linear for methylmalonic acid (6.8–1524 nmol/L) when different ratios of high and low concentration pools of the analytes were combined and analyzed. In addition, calibration lines were linear with coefficient of determinations (r^2) >0.995 for all analyses.

Matrix effect investigations for methylmalonic were performed using individual donor samples (n=6). The matrix factor calculated is shown in Table 5. Normalized matrix factor calculations, based on the analyte:internal standard response ratio, demonstrated that the internal standards compensated for any matrix effects observed.

Table 5. Mean (range) matrix factor and %RSD based on both peak area and analyte:internal standard response ratio.

Compound	Mean matrix factor (range) peak area	RSD	Mean matrix factor (range) response ratio	RSD
Methylmalonic acid	1.00 (0.68–1.50)	21.9%	0.96 (0.89–1.04)	4.2%

Stability of extracted MMA samples onboard the autosampler at 8 °C was demonstrated for 67 hours, with mean QC concentrations within 2% of the previously calculated values.

A method comparison to an independent LC-MS/MS method for the analysis of 94 methylmalonic acid serum samples was performed. Statistical analysis using Deming regression demonstrated no statistically significant proportional (p = 0.1950) or constant bias (p = 0.1911) with a fit of y = 1.05x–11.77 (Figure 3A). Bland-Altman agreement for methylmalonic acid demonstrated a mean method bias of -0.8%, demonstrating excellent agreement with the independent LC-MS/MS method for methylmalonic acid (Figure 3B).

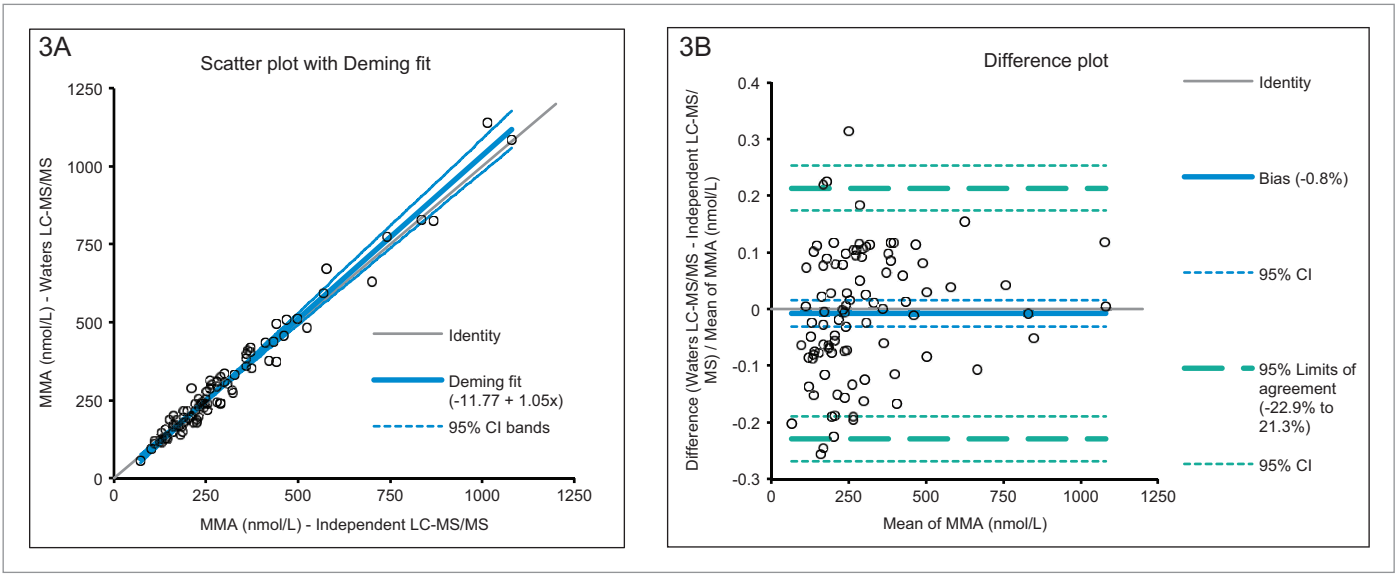

Figure 3A. Deming regression comparing the Waters LC-MS/MS method to the independent LC-MS/MS method for methylmalonic acid in serum. 3B. Bland-Altman agreement comparing the Waters LC-MS/MS method to the independent LC-MS/MS method for methylmalonic acid in serum.

CONCLUSIONS

An analytically sensitive and selective clinical research method has been developed for the analysis of methylmalonic acid using the Xevo TQ-S micro.

The Xevo TQ-S micro enables the analysis of physiologically low levels down to 21 nmol/L of MMA, while only using 100-µL sample volume. Excellent levels of precision across the calibration range have been demonstrated. Comparison to an independent LC-MS/MS method demonstrated excellent agreement in serum samples. In-well protein precipitation using Ostro plates facilitated a simple sample preparation workflow for high throughput analysis of MMA.

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