Fast and reliable method for the analysis of methylmalonic acid from human plasma

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Keywords: Methylmalonic acid (MMA), succinic acid (SA), SOLAµ WAX, Acclaim Surfactant Plus, LC-MS/MS, SPE, mixed-mode ion-exchange, micro-elution, vitamin B12 deficiency, cobalamin deficiency

Goal
To describe an accurate and precise high-throughput analytical technique for the analysis of methylmalonic acid (MMA) utilizing mixed-mode ion-exchange micro-scale solid phase extraction (SPE), followed by liquid chromatography coupled with triple quadrupole mass spectrometry (LC-MS/MS). This technique further separates MMA from the known endogenous interference succinic acid (SA), a compound that has the same molecular weight as MMA and demonstrates a similar fragmentation pattern, which can cause issues with selectivity in mass spectrometry (MS) detection. Deuterated MMA (MMA-d3) was used as an internal standard.

Introduction
Typical analysis for MMA is performed by gas chromatography-mass spectrometry (GC-MS) with lengthy chromatography and time consuming derivitization steps. By utilizing Thermo Scientific™ SOLAµ™ WAX solid phase extraction (SPE) plates, a fast, reproducible, and reliable method can be created to accurately measure levels of MMA from plasma samples. High levels of recovery for MMA can be achieved with minimal matrix effects in a high-throughput workflow.

SOLAµ products provide reproducibility, robustness, and ease-of-use at low elution volumes by utilizing the revolutionary SOLA SPE technology. This removes the need for frits delivering a robust, reproducible format, which ensures highly consistent results at low elution volumes.

SOLAµ products deliver:
- Lower sample failures due to high reproducibility at low elution volumes
- Increased sensitivity due to lower elution volumes
- The ability to process samples that are limited in volume
- Improved stability of biomolecules by reduction of adsorption and solvation issues

Measuring MMA presents issues to the bioanalyst due to the high endogenous levels; obtaining blank matrices for matched standards can present a problem. Phosphate buffer saline (PBS) is regularly used as a surrogate matrix because of its low cost and availability; however, it is often not a close match for the sample requiring analysis. Treated or stripped plasma can be used as a surrogate matrix, but in order to remove trace levels of any particular compound, the level of processing required can be so high that the surrogate is no longer a close matrix match.

Mixed-mode ion-exchange SPE can be employed as a technique to achieve high levels of recovery and low levels of matrix effects. Provided monitoring of matrix to a surrogate is carried out on a batch-by-batch basis; samples can be extracted alongside calibration standards prepared in a surrogate matrix with confidence in the accuracy of the data.
The Thermo Scientific™ Acclaim™ Surfactant Plus column, based on novel mixed-mode chromatography technology and advanced surface chemistry, provides both reversed-phase and anion-exchange mechanisms. The result of which is a specific elution order of cationic, nonionic, amphoteric, and anionic surfactants and, therefore, a highly selective stationary phase.

MMA analysis can also suffer from interference by succinic acid (Figure 2) due to the similarity in structure, molecular weight and fragmentation pattern observed by MS/MS. Excellent liquid chromatographic separation can be achieved in just 3.5 minutes using an Acclaim Surfactant Plus analytical column prior to detection by a Thermo Scientific™ TSQ Vantage™ MS/MS.

No derivitization steps are used prior to injection onto the chromatographic system, so 96 samples can be processed and ready for analysis within 1 to 2 hours.

**Experimental**

**Consumables**
- SOLAµ WAX plate (P/N 60209-005)
- Acclaim Surfactant Plus 3 µm, 150 x 2.1 mm id (P/N 078951)
- Acclaim Surfactant Plus 5 µm, 3.0 x 10 mm drop-in guard (P/N 078959)
- 96-well square well microplate (P/N 60180-P212)
- Thermo Scientific™ Webseal™ mat (P/N 60180-M120)
- Fisher Scientific™ LC-MS grade water (P/N 10095164)
- Fisher Scientific LC-MS grade methanol (P/N 10636545)
- Fisher Scientific LC-MS grade acetonitrile (P/N 10055454)
- Fisher Scientific analytical grade formic acid (P/N 10063427)
- Phosphate buffer saline (PBS) (P/N 10151570)

**Sample handling equipment**

**SPE hardware**
- Thermo Scientific™ HyperSep™ 96 vacuum manifold (P/N 60103-351)
- Vacuum pump, European version (P/N 60104-241)
- Vacuum pump, North American version (P/N 60104-243)

**Compounds and matrix**

**Compounds**
- Methylmalonic acid (MMA)

**Internal standards**
- d₃-methylmalonic acid (MMA-d₃)

**Matrix**
- Human plasma and phosphate buffer solution

**Calibration and quality control (QC) preparation**

Working solutions of 5 µL of MMA prepared in methanol were added to 95 µL of PBS to give concentrations in the range of 15 to 1200 ng/mL. QC samples were prepared in PBS and control human plasma with lithium heparin anti-coagulant at 25, 200, and 1000 ng/mL levels.

**Endogenous level investigation samples**

Blank plasma was spiked with internal standard to measure endogenous levels of MMA.

**Sample pre-treatment**

First, 10 µL of 500 ng/mL methanol stock solution of internal standard (MMA-d₃) was added to each PBS or plasma calibration and QC sample. Then, 10 µL of methanol was added to each blank PBS or plasma sample. Finally, 300 µL of 15 mM ammonium acetate (pH 4 with formic acid) was added to each PBS or plasma sample and vortexed.
Sample preparation

<table>
<thead>
<tr>
<th>SPE plate type</th>
<th>SOLAμ WAX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condition</td>
<td>Apply 200 µL of methanol</td>
</tr>
<tr>
<td>Equilibrate</td>
<td>Apply 200 µL 15 mM ammonium acetate (pH 4 with formic acid)</td>
</tr>
<tr>
<td>Load</td>
<td>Load the entire pretreated sample</td>
</tr>
<tr>
<td>Wash 1</td>
<td>Apply 500 µL of 15 mM ammonium acetate (pH 4 with formic acid)</td>
</tr>
<tr>
<td>Wash 2</td>
<td>Apply 200 µL of methanol</td>
</tr>
<tr>
<td>Elution</td>
<td>2 × 50 µL of 5% ammonia solution in water</td>
</tr>
</tbody>
</table>

During development it was found that the extracts could either be directly injected onto the chromatographic system or blown down under nitrogen at 50 °C and reconstituted in 50 µL of the starting mobile phase conditions for a more concentrated extract.

Separation conditions

<table>
<thead>
<tr>
<th>Instrumentation</th>
<th>Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Acclaim Surfactant Plus 3 µm, 150 × 2.1 mm</td>
</tr>
<tr>
<td>Guard column</td>
<td>Thermo Scientific™ Hypersil GOLD™ 5 µm, 10 × 4 mm drop-in guard</td>
</tr>
<tr>
<td>Mobile phase A</td>
<td>15 mM ammonium acetate pH 4 (with formic acid)</td>
</tr>
<tr>
<td>Mobile phase B</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Gradient</td>
<td>See Table 1</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.450 mL/min</td>
</tr>
<tr>
<td>Column temperature</td>
<td>30 °C</td>
</tr>
<tr>
<td>Injection details</td>
<td>15 µL</td>
</tr>
<tr>
<td>Injection wash</td>
<td>Mobile phase A solvent 1</td>
</tr>
<tr>
<td>Injection wash</td>
<td>30:30:30:10 methanol/IPA/water/acetonitrile solvent 2</td>
</tr>
</tbody>
</table>

Table 1. LC gradient conditions

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>3.5</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>3.6</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>4.0</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>4.1</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>5.5</td>
<td>80</td>
<td>20</td>
</tr>
</tbody>
</table>

MS conditions

<table>
<thead>
<tr>
<th>Instrumentation</th>
<th>Thermo Scientific™ TSQ Quantiva™ triple quadrupole mass spectrometer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionization source</td>
<td>Heated electrospray ionization (HESI)</td>
</tr>
<tr>
<td>Polarity</td>
<td>Negative</td>
</tr>
<tr>
<td>Scan time</td>
<td>0.02 s</td>
</tr>
<tr>
<td>Q1 (FWHM)</td>
<td>0.75</td>
</tr>
<tr>
<td>Q3 (FWHM)</td>
<td>0.75</td>
</tr>
<tr>
<td>Compound transition details</td>
<td>See Table 2</td>
</tr>
</tbody>
</table>

Table 2. Compound transition details

<table>
<thead>
<tr>
<th>Compound</th>
<th>Methylmalonic acid</th>
<th>d₃-Methylmalonic acid</th>
<th>Succinic acid</th>
<th>Succinic acid (confirmatory ion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor (m/z)</td>
<td>117.0</td>
<td>120.1</td>
<td>117.1</td>
<td>117.1</td>
</tr>
<tr>
<td>Products (m/z)</td>
<td>73.1</td>
<td>76.1</td>
<td>73.1</td>
<td>99.1</td>
</tr>
<tr>
<td>Collision energy (V)</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>S-lens (V)</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>45</td>
</tr>
</tbody>
</table>

Data processing

Thermo Scientific™ LCQUAN™ quantitative software was used for data processing.
Results and discussion

Due to the high levels of MMA present in control human plasma normally used in preparation of calibration and quality control samples, a surrogate matrix of phosphate buffer solution was used. Human plasma was used to spike additional QC samples in order to confirm that accuracy, precision, and matrix effects are still in acceptable limits when compared to PBS samples. Excellent levels of accuracy and precision were observed for MMA using the SOLAμ WAX SPE plate. High levels of recovery were also seen, as well as low levels of matrix effects, on both PBS and plasma samples giving a high degree of confidence in the analytical results.

Chromatography

Excellent separation of MMA and SA was achieved using the Acclaim Surfactant Plus analytical column as shown in Figure 3.

![Figure 3. Separation of succinic acid (SA) at 1.71 min RT and methylmalonic acid (MMA) at 2.37 min RT](image)

Linearity

Extracted MMA standards from PBS gave a linear calibration curve over the dynamic range of 15 to 1200 ng/mL with an $R^2$ of 0.9953 (Figure 4 and Table 3). A linear $1/x^2$ weighting was applied to the calibration curve.

![Figure 4. MMA linearity over a dynamic range of 15 to 1200 ng/mL](image)
Table 3. Accuracy data for calibration standards of MMA from PBS over a range of 15 to 1200 ng/mL, n=2

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Nominal Concentration (ng/mL)</th>
<th>Average Calculated Concentration (ng/mL)</th>
<th>% Bias from nominal</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>15</td>
<td>14.8</td>
<td>-1.31%</td>
</tr>
<tr>
<td>S2</td>
<td>25</td>
<td>25.0</td>
<td>0.00%</td>
</tr>
<tr>
<td>S3</td>
<td>50</td>
<td>49.8</td>
<td>-0.46%</td>
</tr>
<tr>
<td>S4</td>
<td>100</td>
<td>111</td>
<td>10.9%</td>
</tr>
<tr>
<td>S5</td>
<td>200</td>
<td>201</td>
<td>0.510%</td>
</tr>
<tr>
<td>S6</td>
<td>400</td>
<td>386</td>
<td>-3.55%</td>
</tr>
<tr>
<td>S7</td>
<td>600</td>
<td>593</td>
<td>-1.18%</td>
</tr>
<tr>
<td>S8</td>
<td>800</td>
<td>798</td>
<td>-0.29%</td>
</tr>
<tr>
<td>S9</td>
<td>1000</td>
<td>980</td>
<td>-1.98%</td>
</tr>
<tr>
<td>S10</td>
<td>1200</td>
<td>1170</td>
<td>-2.65%</td>
</tr>
</tbody>
</table>

R² value: 0.9951

Accuracy and precision
QC samples were run in replicates of six at concentrations of 25, 200, and 1000 ng/mL. The precision and accuracy of the QC extracted from PBS is presented in Table 4. The mean endogenous levels of MMA in human plasma is shown in Table 5. The precision and accuracy of the QC extracted from plasma is presented in Table 6.

Table 4. Accuracy data for QC samples of MMA from PBS, n=6

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Nominal Concentration (ng/mL)</th>
<th>Average Calculated Concentration (ng/mL)</th>
<th>% Bias from nominal</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low QC</td>
<td>25</td>
<td>24.3</td>
<td>-2.66%</td>
<td>9.18%</td>
</tr>
<tr>
<td>Mid QC</td>
<td>200</td>
<td>178</td>
<td>-11.0%</td>
<td>4.54%</td>
</tr>
<tr>
<td>High QC</td>
<td>1000</td>
<td>897</td>
<td>-10.3%</td>
<td>3.44%</td>
</tr>
</tbody>
</table>

Table 5. Mean endogenous levels of MMA in human plasma, n=6

<table>
<thead>
<tr>
<th>Blank Plasma with Internal Standard</th>
<th>Mean Measured Endogenous Levels from Plasma (ng/mL)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=6</td>
<td>12.2</td>
<td>12</td>
</tr>
</tbody>
</table>

Table 6. Accuracy data for QC samples of MMA from plasma, n=6

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Nominal Concentration (ng/mL)</th>
<th>Measured MMA Concentration from Blank Plasma (ng/mL)</th>
<th>Adjusted Nominal Concentration (ng/mL)</th>
<th>Calculated Concentration</th>
<th>% Bias from Adjusted Nominal</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low QC</td>
<td>25</td>
<td>12.2</td>
<td>37.2</td>
<td>39.2</td>
<td>5.20%</td>
<td>3.7</td>
</tr>
<tr>
<td>Mid QC</td>
<td>200</td>
<td>12.2</td>
<td>212</td>
<td>233</td>
<td>9.8%</td>
<td>4.9</td>
</tr>
<tr>
<td>High QC</td>
<td>1000</td>
<td>12.2</td>
<td>1012</td>
<td>1014</td>
<td>0.153%</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Recovery and matrix effects
Recovery and matrix effects from both matrices are presented in Table 7.

Table 7. Recovery and matrix effects data for QC samples of MMA from PBS and plasma, n=6

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Recovery from PBS Samples</th>
<th>Matrix Effects</th>
<th>Recovery from Plasma Samples</th>
<th>Matrix Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low QC</td>
<td>91</td>
<td>-8</td>
<td>81</td>
<td>-10</td>
</tr>
<tr>
<td>Mid QC</td>
<td>92</td>
<td>-4</td>
<td>105</td>
<td>-12</td>
</tr>
<tr>
<td>High QC</td>
<td>96</td>
<td>-10</td>
<td>81</td>
<td>-22</td>
</tr>
</tbody>
</table>

\[ \text{Recovery} = \frac{\text{Average Response of Sample}}{\text{Average Response of Overspike}} \times 100 \]

\[ \text{Absolute % Matrix Suppression} = 100 - \frac{\text{Average Response of Overspike}}{\text{Average Response of Solution Standard}} \times 100 \]
Conclusion

- The use of SOLAµ WAX mixed-mode ion-exchange SPE allows for fast, reliable extraction of MMA from human plasma.

- The cleanliness of the final extraction allows the use of a surrogate matrix in preparation of calibration standards enabling analysis of normal and abnormal levels of MMA.

- Excellent levels of accuracy and precision were observed on all compounds using the SOLAµ WAX SPE plate. High levels of recovery as well as low levels of matrix effects were also seen on both PBS and plasma samples, giving a high degree of confidence in the analytical results.

- Known interference from succinic acid can easily be avoided via chromatographic separation within 3 minutes by using an Acclaim Surfactant Plus analytical column.

Useful links

AppsLab Library
The eWorkflow and the Chromeleon Backup (cmbx) file can be downloaded at AppsLab Library:
appslab.thermoscientific.com