Quantitative Analysis of Methylmalonic Acid in Plasma Using a Q Exactive Focus High-Resolution Mass Spectrometer for Clinical Research

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Key Words
Q Exactive Focus, methylmalonic acid, MMA, clinical research

Goal
To evaluate the performance of the Thermo Scientific™ Q Exactive™ Focus benchtop quadrupole-Orbitrap mass spectrometer as a quantitative platform for analysis of methylmalonic acid in plasma.

Application Benefits
- Wide quantitative analytical range from 25 to 100,000 nM
- Sensitive and selective analytical method
- Highly confident analyte identification with accurate mass and ion ratio
- Robust method without matrix effects
- Implemented on versatile analytical platform that can be used by the research laboratory for other analytical applications

Introduction
Clinical research laboratories perform analysis of methylmalonic acid (MMA) using LC-MS because of that technique's sensitivity and selectivity. Usually, the quantitative method is developed on a triple quadrupole mass spectrometer. Here we evaluated a method implemented on a Q Exactive Focus ultra-high resolution quadrupole-Orbitrap mass spectrometer for the best selectivity.

Methods
Calibration Standards and QC samples
Because it is impractical to obtain analyte-free plasma matrix, calibration standards in the range of 25 to 100,000 nM were prepared in aqueous 2% acetonitrile diluent. Pooled plasma containing MMA at a concentration of 87.5 nM was used as Level 0 Quality Control. Level 1 (260 nM) and Level 2 (583 nM) Quality Controls were purchased from RECIPE™ Chemicals and Instruments GmbH™ (ClinChek™ control kit P/N MS5082).

Sample Preparation
Samples were mixed with the internal standard (IS) D_3-MMA, processed by protein precipitation and evaporation followed by derivatization to produce dibutyl-esters of MMA and SA. Parameters of the derivatization reaction were optimized to obtain the required reaction efficacy in approximately 20 minutes during the derivatization reagent drying time.

Liquid Chromatography
Succinic acid (SA), which is a structural isomer of MMA present in plasma, can interfere with MMA analysis and must be separated chromatographically. A 2.8-minute chromatographic elution through a Thermo Scientific™ Accucore™ C8 column (2.6 µm, 50 x 2.1 mm, P/N 17226-052130) at room temperature was performed using a Thermo Scientific™ UltiMate™ 3000RS liquid chromatography pump with OAS autosampler. Mobile phases consisted of 0.1% formic acid in water and 0.1% formic acid in methanol (Fisher Chemical Optima™ grade) for phases A and B, respectively. Using this chromatographic method, MMA was separated from succinic acid.

Mass Spectrometry
Compounds were detected on a Q Exactive Focus benchtop quadrupole-Orbitrap mass spectrometer equipped with an Ion Max™ source and an atmospheric-pressure chemical ionization (APCI) probe. Data were acquired in parallel-reaction monitoring (PRM) mode. In this mode, a single precursor ion was selected in the quadrupole with an isolation width of 2.0 *m/z* and fragmented in the HCD cell using optimized, compound-specific collision energy. The resulting MS/MS product ion spectrum was detected in the Thermo Scientific™ Orbitrap™ analyzer at a resolution of 70,000 (FWHM at *m/z* 200).

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Method Evaluation

The limits of quantitation (LOQ) and linearity ranges were evaluated by collecting calibration curve data. Method precision and accuracy were evaluated by running quadruplicate replicates of calibrators and QCs on three different days. Method accuracy was assessed by calculating %recovery for QC samples and for 10 donor plasma samples spiked with 100 nM of MMA. Matrix effects were evaluated by comparing internal standard peak areas in 10 different donor plasma samples to internal standard signal in neat solution.

Data Analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ software. For each analyte, the most abundant fragment from the MS/MS spectrum was selected as the quantifying ion and another specific fragment was selected as a qualifying ion. The resulting chromatograms were extracted and reconstructed with a mass accuracy of 5 ppm for quantification and confirmation with ion ratio calculations. The average ion ratio calculated for analyte confirmation was about 15% and individual ratios were within 30% (relative) of averages calculated from the calibrators.

Figure 1a and Figure 1b show representative MS/MS spectra for MMA and D_3-MMA, internal standard (IS), with quantifying and qualifying ions specified.

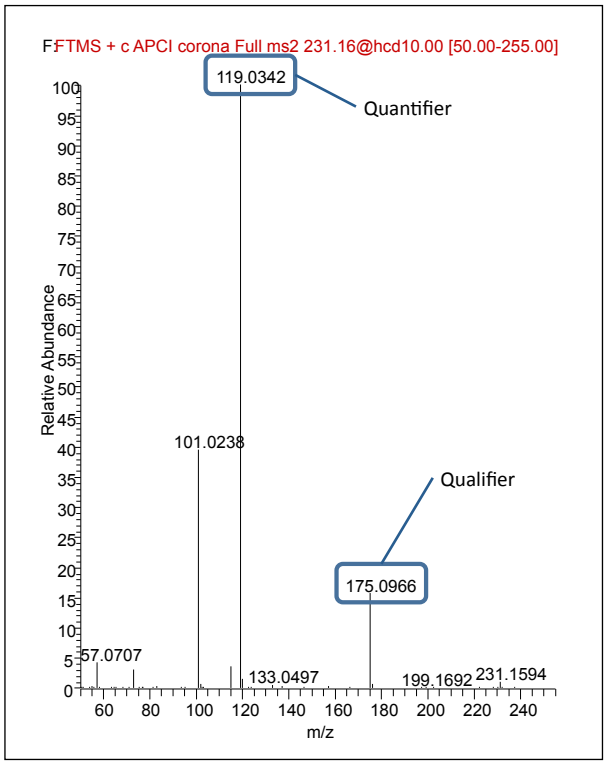

Figure 1a. MS/MS spectra for MMA with specified quantifying and qualifying ions.

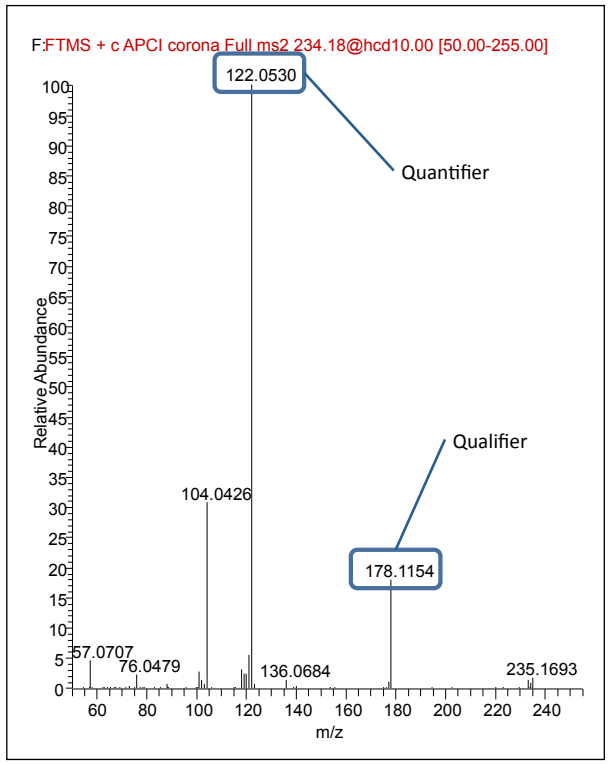

Figure 1b. MS/MS spectra for d_3-MMA with specified quantifying and qualifying ions.

Results and Discussion

Limits of quantitation (LOQs) were defined as the lowest concentrations that had back-calculated values within 20% and ion ratio within the specified range. Using these criteria, the limit of quantitation was 25 nM. The upper calibration range was 100,000 nM. Figure 2 presents stick mode chromatograms of quantifying and qualifying ions from the lowest calibration standard reconstructed with mass accuracy of 5 ppm. More than 20 scans across the peak were collected.

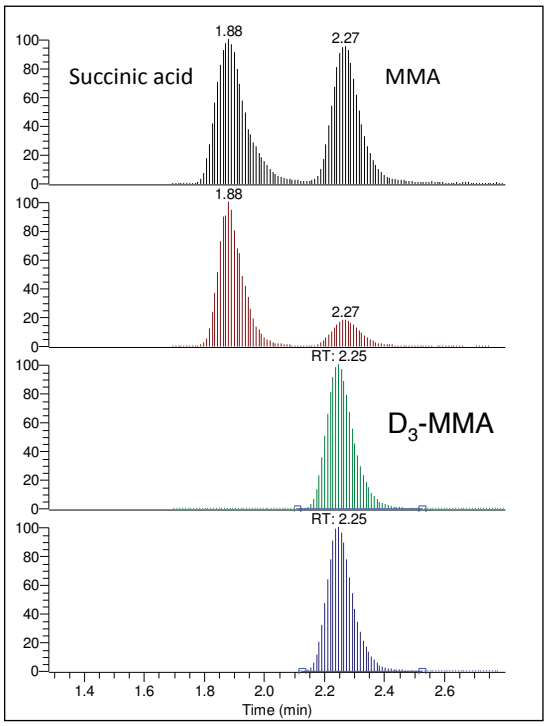

Figure 2. Stick mode chromatogram of the lowest calibration standard (25 nM) reconstructed with mass accuracy of 5 ppm.

Figure 3 shows a representative calibration curve, along with quantifying and qualifying ion chromatograms for the lowest calibration standard. Calibration standards' precision was better than 2.8%, and accuracy was within ±7%.

Method accuracy calculated as % recovery in spiked QCs and donor plasma samples ranged from 91.8% to 103%. This shows that the use of the surrogate matrix for the calibration curve will produce accurate results for samples in plasma matrix.

Intra-assay precision was better than 1.5% (Table 1), and inter-assay precision was better than 1.4% (Table 2). Figure 4 presents chromatograms of quantifying and qualifying ions in plasma QC samples.

Table 1. Intra-assay precision and accuracy.

	QC Level 0	QC Level 1	QC Level 2
Concentration (nM)	87.5	260	583
Precision (%RSD)	<5.1	<1.0	<0.84
Accuracy (%recovery)	99.6–101	91.8–92.9	95.9–96.4

Table 2. Intra-assay precision and accuracy.

	QC Level 0	QC Level 1	QC Level 2
Concentration (nM)	87.5	260	583
Precision (%RSD)	1.4	0.88	0.72
Accuracy (%recovery)	100	92.3	96.1

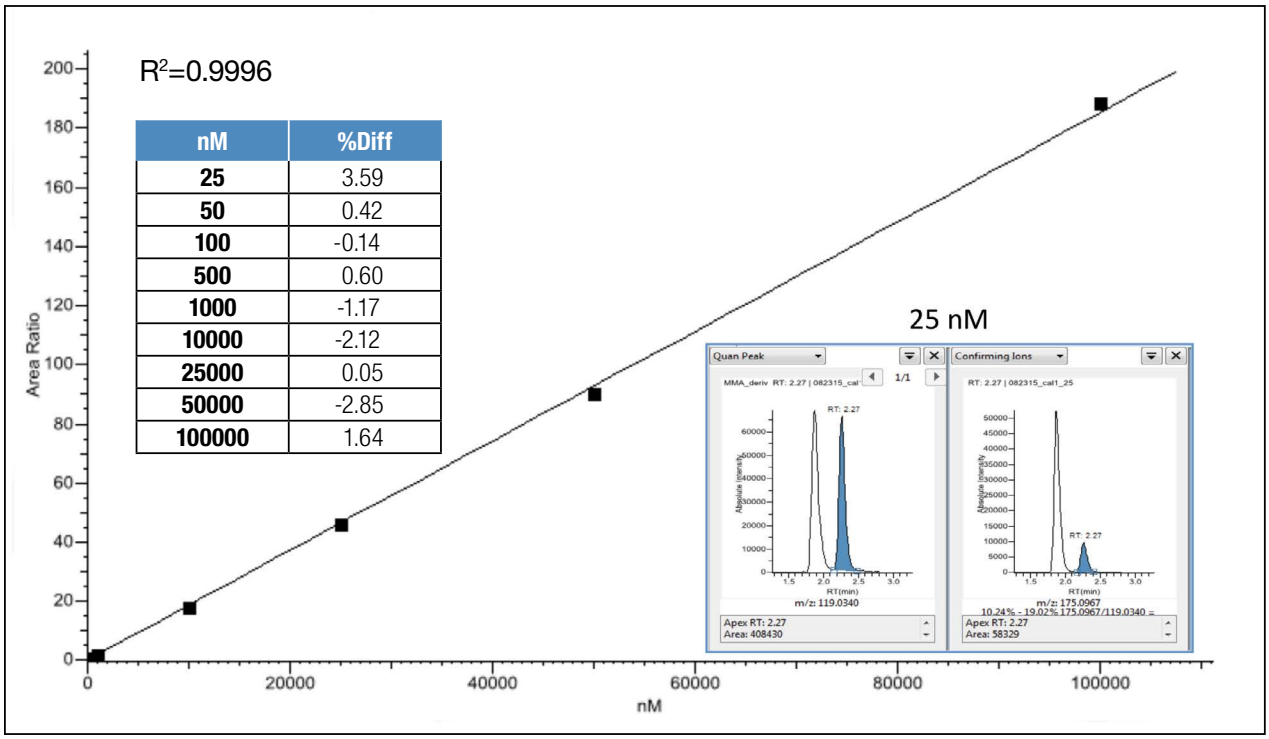

Figure 3. Representative calibration curve and the chromatogram for the lowest calibration standard (25 nM).

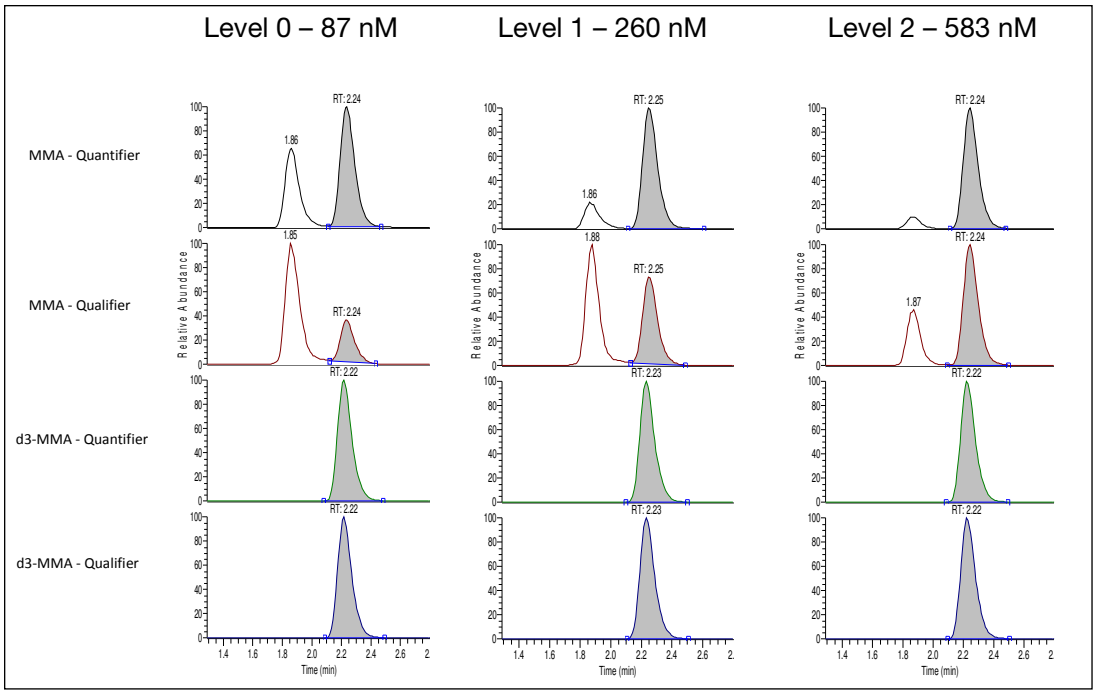

Figure 4. Chromatogram of quantifying and qualifying ions in QC samples reconstructed with mass accuracy of 5 ppm.

Matrix effects were not observed. Internal standard recovery in ten donor samples, calculated as the ratio between internal standard peak area in plasma and internal standard peak area in neat solution, was within method analytical error and ranged from 95.4% to 102%.

Conclusion

We demonstrated a sensitive method with a wide dynamic range using simple and economical sample preparation for the analysis of MMA in plasma samples using a high-resolution mass spectrometer. The results show that the Q Exactive Focus Orbitrap mass spectrometer, which is used extensively for screening in forensic toxicology laboratories, can be also used for quantitative analysis in clinical research laboratories. With the short run time and data acquisition window, this analytical method can be put into use on a 4-channel Thermo Scientific™ Transcend™ II LC system to provide data for 50 samples per hour.

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