APPLICATIONS

A Selective Method for Quantitation of Underivatized Methylmalonic Acid (MMA) in Plasma

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Outside of the lab, Laura enjoys spoiling her dog Maggie and subjecting her husband to novel methods of torture, such as endless playlists of sad songs and long walks on the beach to catch Pokémon.

Overview

- Fast, reproducible method for underivatized MMA
- Resolution of isobaric species
- Clean extract from plasma

Introduction

Methylmalonic acid (MMA) is a small dicarboxylic acid. This hydrophilic molecule can present chromatographic challenges both in achieving adequate retention under reversed phase conditions as well as resolution from the isomeric/isobaric species such as succinic acid, especially at low analyte concentrations. To combat these challenges, many published LC/MS/MS methods require a sample derivatization step. In this technical note, we present a fast, reproducible LC/MS/MS method to analyze underivatized MMA by utilizing a unique Luna® Omega 1.6 µm PS C18. The method runtime is 5 minutes including column re-equilibration. For sample preparation procedure we used Strata®-X-AW solid phase extraction to produce a clean sample from plasma. Analyte detection was performed using negative mode electrospray ionization of a triple quadrupole MS.

Materials

Methylmalonic acid and methyl-D3-malonic acid standards were purchased from Cerilliant (Round Rock, TX). Succinic acid, formic acid, acetic acid, ammonium hydroxide, and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile and methanol were purchased from Honeywell (Morris Plains, NJ). Purified water was obtained using a Sartorius® arium® comfort II filtration system (Göttingen, Germany). Egg albumin was sourced from eggs purchased from a local grocery store (Torrance, CA). Quality controls in serum were purchased from UTAK (Valencia, CA). Pooled human K_2EDTA plasma was purchased from BioreclamationIVT (Westbury, NY).

Experimental Conditions

The calibration curve was prepared using a matrix of 2% egg albumin in 50 mM ammonium acetate, pH 7, since the plasma obtained for this project was not MMA-free. Seven points across a linear range of 50 nmol/L to 5000 nmol/L were tested. The internal standard was prepared in 50:50 water/acetonitrile with MMA-D3 at 50 µmol/L.

Analyte recovery was tested at two concentrations using pooled plasma spiked with MMA at 250 nmol/L and 750 nmol/L above the endogenous MMA level. Percent recovery for each concentration level was calculated as the mean area ratio of pre-extraction spiked samples divided by the mean area ratio of post-extraction spiked samples.

A separate set of plasma samples was spiked with succinic acid at 1.5 µg/mL above the endogenous level to test chromatographic resolution of a higher concentration of succinic acid from MMA. All studies were performed with N=3 replicates.

Sample Pretreatment

Combine 0.5 mL of 1% aqueous acetic acid and 50 µL of internal standard with 100 µL blank, standard, or sample.

SPE Method

Cartridge	Strata-X-AW 30 mg/1 mL
Part Number	8B-S038-TAK
Condition	1 mL of methanol
Equilibrate	1 mL of 1 % acetic acid in water
Load	Pretreated sample (see above)
Wash	0.5 mL of methanol/water (50:50)
Dry	5 to 10 minutes at max vacuum (or positive pressure)
Elute	2 x 0.6 mL 2 % ammonium hydroxide in methanol
Dry Down Extract	Evaporate solvent to dryness @ 45-50 °C under a gentle stream of nitrogen
Reconstitute	200 µL of mobile phase A (0.1 % formic acid in water)

LC Conditions

Analytical Column: Luna Omega 1.6 µm PS C18
Dimensions: 50 x 2.1 mm
Part No.: 00B-4752-AN
Gurad: SecurityGuard™ ULTRA Luna Omega PS C18 Cartridges: AF0-8497
Mobile Phase: A: 0.1% Formic acid in Water
B: 0.1% Formic acid in Acetonitrile

Gradient:

Time (min)	B (%)
0.01	2
2	90
3	90
3.01	2
5	2

Flow Rate: 0.4 mL/min
Injection Volume: 5 µL
Temperature: 40°C

MS/MS Conditions

Detector: SCIEX 4000 QTRAP®
Mode: Negative Ionization Mode
Scan Type: MRM
Curtain Gas (CUR): 10.0 psi
Collision Gas (CAD): Medium
IonSpray Voltage (IS): -4500 V
Temperature (TEM): 600 °C
Ion Source Gas 1 (Gas1): 50 psi
Ion Source Gas 2 (Gas2): 50 psi
Interface Heater (ihe): On

MRM Transitions

ID	Q1 Mass (Da)	Q3 Mass (Da)	Time (msec)	DP (volts)	EP (volts)	CE (volts)	CXP (volts)
MMA 1	117.0	72.9	100	-32	-10	-13	-15
MMA 2	117.0	54.9	100	-32	-10	-34	-15
MMA-D3	120.1	75.9	100	-36	-10	-13	-15

Results and Discussion

In this method, succinic acid was well-resolved chromatographically from MMA, including the samples spiked with succinic acid at roughly 10-fold (1.5 µg/mL) above the endogenous level of the MMA in pooled plasma (**Figure 1**). Calibration curves for extracted samples covered a range from 50 nmol/L to 5000 nmol/L with $r^2 = 0.999$ (**Figure 3**). Signal-to-noise for the lowest calibrator was >25 with accuracy ≥90% (**Figure 2** and **Table 1**). Precision and accuracy met our criteria, with %CV ≤15% and accuracy within ±15% for all replicates (**Tables 1, 2**). Analyte recovery was demonstrated at two concentrations with percent recovery of 114% for ~700 nmol/L and 102% for ~1160 nmol/L, respectively (**Table 3**). Precision for all plasma samples, including the ~385 nmol/L unspiked plasma sample, were within the acceptable range at ±10% (**Table 3**).

We were able to analyze MMA and avoid a derivatization step by using a mixed-mode UHPLC column that contains both a positively charged ligand for retention of acidic compounds and a C18 ligand for added selectivity. MMA was more than adequately retained and well-resolved from succinic acid on a Luna Omega 1.6 µm PS C18, 50 x 2.1 mm column. Through the efficiency gained by using a 1.6 µm particle, a fast 5-minute runtime was achieved, including column re-equilibration.

A SPE procedure was developed to ensure a good degree of sample cleanliness to accommodate the small 1.6 µm particle size. By selecting SPE as the sample preparation technique, we were able to use a small sample volume of only 100 µL of plasma. The weak anion exchange mechanism of the sorbent selectively retained methylmalonic acid and produced good analyte recovery.

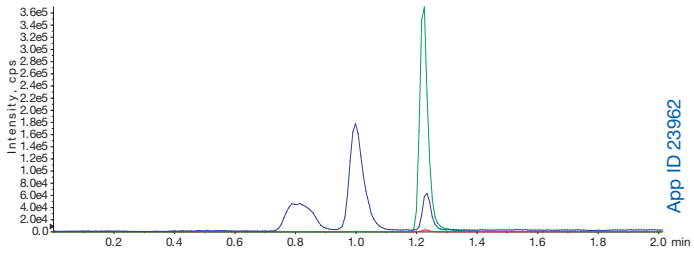

Figure 1. Representative chromatogram of an extracted sample. Pooled human plasma was spiked with standards to 1.5 µg/mL of succinic acid and 750 nmol/L of methylmalonic acid above the endogenous concentrations and processed by solid phase extraction. Peaks in order of elution: plasma interference (0.81 min), succinic acid (1.00 min), methyl-D3-malonic acid (1.20 min), and methylmalonic acid (1.23 min).

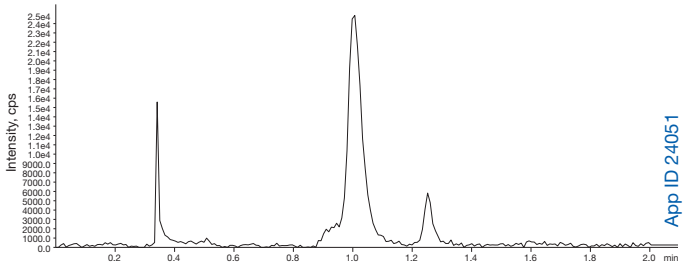

Figure 2. Representative chromatogram of the lowest calibrator (Cal. 1), 50 nmol/L MMA, extracted. Peaks in order of elution: interference (0.34 min), succinic acid (1.00 min), and MMA (1.25 min).

Table 1. A seven point calibration curve from 50 nmol/L to 5000 nmol/L was evaluated.

Sample Name	Concentration (nmol/L)	Average Calculated Concentration N=3 (nmol/L)	Accuracy (%)	% CV	Analyte Signal-to-Noise
Cal. 1	50	47	93.9	7.59	32
Cal. 2	100	101	101	13.8	61
Cal. 3	250	262	105	7.45	124
Cal. 4	500	495	99.1	3.97	221
Cal. 5	1000	1025	103	4.17	443
Cal. 6	2500	2467	98.7	2.41	1035
Cal. 7	5000	5003	100	2.82	1907

Table 2. Two levels of quality controls (200 nmol/L and 1000 nmol/L) were evaluated.

Sample Name	Target Value (nmol/L)	Calculated Concentration (nmol/L)	%CV	Published Range (nmol/L)*
Low QC - 1	200	205	5.44	170-230
Low QC - 1	200	205		
Low QC - 3	200	212		
High QC - 1	1000	1260	4.82	870-1170
High QC - 2	1000	1180		
High QC - 3	1000	1140		

*Manufacturer's recommended ranges

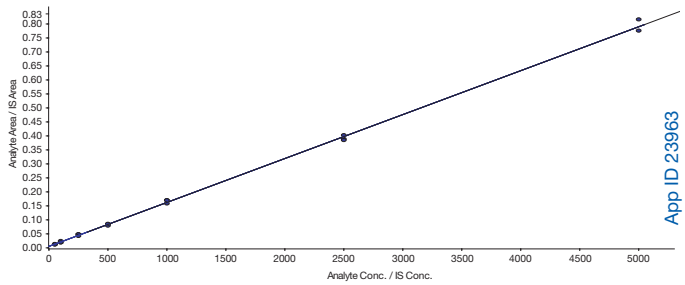

Figure 3. Calibration curve plot using a linear regression with 1/x weighting factor. $r^2 = 0.999$

Table 3. Analyte recovery was tested at two concentrations of spiked plasma.

Sample Name	Spike (nmol/L)	Average Calculated Concentration N=3 (nmol/L)	% CV	% Recovery
Prespiked 250 nmol/L in plasma	250	696	9.44	114
Prespiked 750 nmol/L in plasma	750	1157	2.00	102
Extracted unspiked plasma	0	385	3.01	N/A

Conclusion

This work here demonstrates a selective SPE procedure and LC/MS/MS method for quantitation of MMA in plasma. The mixed-mode stationary phase of Luna® Omega PS C18 was successfully able to chromatographically resolve underivatized MMA from succinic acid and a plasma interference peak with better than baseline resolution. The UHPLC method has a 5-minute runtime (including column re-equilibration). A method utilizing Strata® X-AW weak anion exchange SPE sorbent was developed to produce a clean extract from plasma. The reproducible method demonstrated good analyte recovery for MMA.

References

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APPLICATIONS

Ordering Information

Luna® Omega PS C18

1.6 µm Minibore Columns (mm)

Phases	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	SecurityGuard™ ULTRA Cartridges‡ 3/pk
PS C18	00A-4752-AN	00B-4752-AN	00D-4752-AN	00F-4752-AN	AJ0-9508 for 2.1 mm ID

‡SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

5 µm Minibore Columns (mm)

Phases	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	SecurityGuard Cartridge(mm) 4 x 2.0* 10/pk
PS C18	00A-4753-AN	00B-4753-AN	00D-4753-AN	00F-4753-AN	AJ0-7605 for 2.0-3.0 mm ID

5 µm MidBore™ Columns (mm)

Phases	50 x 3.0	100 x 3.0	150 x 3.0	SecurityGuard Cartridge(mm) 4 x 2.0* 10/pk
PS C18	00B-4753-Y0	00D-4753-Y0	00F-4753-Y0	AJ0-7605 for 2.0-3.0 mm ID

5 µm Analytical Columns (mm)

Phases	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	SecurityGuard Cartridge(mm) 4 x 3.0* 10/pk
PS C18	00B-4753-E0	00D-4753-E0	00F-4753-E0	00G-4753-E0	AJ0-7606 for 3.2-8.0 mm ID

SecurityGuard Cartridges require holder, Part No.: KJ0-4282

Strata®-X-AW

Format Tube	Sorbent Mass	Part Number	Unit
	30 mg	8B-S038-TAK**	1 mL (100/box)
	30 mg	8B-S038-TBJ	3 mL (50/box)
	60 mg	8B-S038-UBJ	3 mL (50/box)
	100 mg	8B-S038-EBJ	3 mL (50/box)
	100 mg	8B-S038-ECH	6 mL (30/box)
	200 mg	8B-S038-FBJ	3 mL (50/box)
	200 mg	8B-S038-FCH	6 mL (30/box)
	500 mg	8B-S038-HBJ	3 mL (50/box)
	500 mg	8B-S038-HCH	6 mL (30/box)
Giga™ Tube			
	500 mg	8B-S038-HDG	12 mL (20/box)
	1 g	8B-S038-JDG	12 mL (20/box)
	1 g	8B-S038-JEG	20 mL (20/box)
	5 g	8B-S038-LFF	60 mL (16/box)
96-Well Plate			
	10 mg	8E-S038-AGB	2 Plates/Box
	30 mg	8E-S038-TGB	2 Plates/Box
	60 mg	8E-S038-UGB	2 Plates/Box
96-Well Microelution Plate			
	2 mg	8M-S038-4GA	ea

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