Poster Note 64367

Simple Sample Preparation for Measuring Methylmalonic Acid in Blood Serum by LC-APCI-MS/MS

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Overview

Purpose: Measure methylmalonic acid (MMA) in blood serum for research purposes only by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with atmospheric pressure chemical ionization (APCI) using a simple protocol for sample preparation that would produce the most reliable results. The method must be capable of running simultaneously with others on a multi-channel LC system coupled to an APCI-MS/MS system.

Methods: Specimens were subjected to protein precipitation by mixing in acetonitrile containing an internal standard (IS). After centrifugation, a portion of each supernatant was dried and derivatized to form butyl-MMA and butyl-MMA-IS. These analytes were then separated from butyl-succinic acid by reversed-phase chromatography and selectively detected and quantified by MS/MS using APCI.

Results: Our five-minute LC-MS/MS method repeatedly achieved a quantitative range of 50 to 1,000 nM with a throughput of 12 samples per hour on a single channel of a multi-channel LC system.

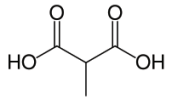
Introduction

MMA is an intermediate metabolite bound to coeznzyme A (CoA) formed as isoleucine, methionine and valine as well as odd-chain fatty acids are metabolized. These catabolic pathways are completed as methylmalonyl CoA is converted to succinyl CoA by a mutase enzyme that requires the cofactor Vitamin B12 (Cobalmin). Blood and urine levels of MMA increase if this enzyme is missing or defective or is not able to work if Vitamin B12 is lacking. Succinic acid (SA), which is normally present in much higher concentrations, interferes with the analysis of MMA by LC-MS methods. SA and MMA are isomers having identical molecular weight (Figure 1) and must be separated before detection and quantification.

Consequently, many protocols for sample preparation involve laborious and expensive solid-phase or liquid extraction followed by ion-exchange chromatography coupled to electro-spray ionization (ESI) of a mass spectrometer. Typically, negative-ion detection is not as sensitive as positive-ion detection and ESI is prone to ion suppression by matrix interferences. Derivatization methods to form butyl esters of MMA and other organic acids have been used with both LC-MS or GC-MS analysis. We developed a simple approach to sample preparation similar to that of Turgeon, et al (1), which was used to measure MMA, homocysteine and methylcitric acid in dried blood spots.

FIGURE 1. Chemical structures and characteristics.

Methylmalonic Acid (MMA)



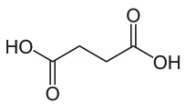
$C_4H_6O_4$

MW = 118.09

pK_{a1} = 3.0

pK_{a2} = 5.7

Succinic Acid (SA)



$C_4H_6O_4$

MW = 118.09

pK_{a1} = 4.2

pK_{a2} = 5.6

Methods

Consumables

HPLC-grade solvents, reagents, and most other consumables were from Thermo Fisher Scientific. Methylmalonic acid and methyl-D_3-malonic acid standards were from Cerilliant (Round Rock, TX). Calibrators were made by mixing these standards with a diluent of 1% bovine serum albumin in phosphate-buffered saline. MMA controls were from UTAK (Valencia, CA).

Sample Preparation

Protein precipitation was achieved by mixing 100 µL of each calibrator, control and specimen with 200 µL of acetonitrile containing the MMA-D3 internal standard (IS). After centrifugation, 100 uL of each supernatant was dried in a glass tube by a stream of nitrogen at 40°C for 15 minutes. The residue in each tube was reacted with 200 µL of butylation reagent (10% acetyl chloride in 1-butanol) at 65°C for 15 minutes. The reagent was then evaporated by a stream of nitrogen at 40°C for 15 minutes. The remaining residue was dissolved with 100 µL of 50% methanol in water. The mixture was transferred to a micro vial which was positioned in the autosampler for 20 µL injections.

Liquid Chromatography

The multi-channel LC system was a Thermo Scientific™ Transcend™ LX4 equipped with binary-solvent pumps and a dual-arm autosampler configuration. A Thermo Scientific™ Accucore™ C8, 2.6 µm, 50 x 2.1 mm HPLC column was heated to 50ºC by a Thermo Scientific™ Hot Pocket™ column heater. The mobile phase conditions are described in Figure 2.

FIGURE 2. Liquid chromatography conditions.

		Step	Start	Sec	Flow	Grad	%A	%B	Comments
Column 1	Accucore C8, 2.6um, 50x2.1mm	1	0.00	10	0.50	Step	45.0	55.0	Load sample
Loading Pump	Binary	2	0.17	30	0.50	Ramp	35.0	65.0	Separate analytes
A	Water + 0.1% Formic Acid	3	0.67	90	0.50	Ramp	30.0	70.0	Elute analytes
B	Methanol + 0.1% Formic Acid	4	2.17	60	0.50	Step	-	100.0	Wash column
		5	3.17	110	0.50	Step	45.0	55.0	Equilibrate column

Start data 1.1 min **Data window: 1.0 min** **Total run time: 5.0 min**

Mass Spectrometry System Control & Data Analysis

Thermo Scientific™ TraceFinder™ with Aria™ MX software was used to control the Transcend II LX4 and Endura MS/MS systems, submit batches to desired channels as well as for analyzing data and reporting results.

The Thermo Scientific™ TSQ Endura™ triple-quadrupole mass spectrometer was used with APCI. Ion source and MS/MS conditions are described in Figure 3. The method utilized a unique “electro-clean” step.

FIGURE 3. Mass spectrometry conditions.

Polarity switch in “Electro-Clean” step desorbs contamination from corona discharge needle.

Results

Quantitation Range Achieved

As shown in Figure 4, the desired analytical range from 50 to 1,000 nM (5.9 to 118 ng/mL) was consistently achieved using the quantifying MS/MS transition of 231 > 119 for butyl-MMA. This transition is much more specific for butyl-MMA than for butyl-SA, which is usually present in much higher concentrations. Unfortunately, the only other transition that could be used to confirm peak identity by ion ratios is 231 > 101, which gives a strong signal for butyl-SA. Consequently, specimens with MMA concentrations less than 150 nM often fail ion ratio conformation, as shown in Figure 5. Fortunately, it is necessary to confirm MMA concentrations in blood serum above 300 nM in most research applications.

FIGURE 4. Typical quantitation results.

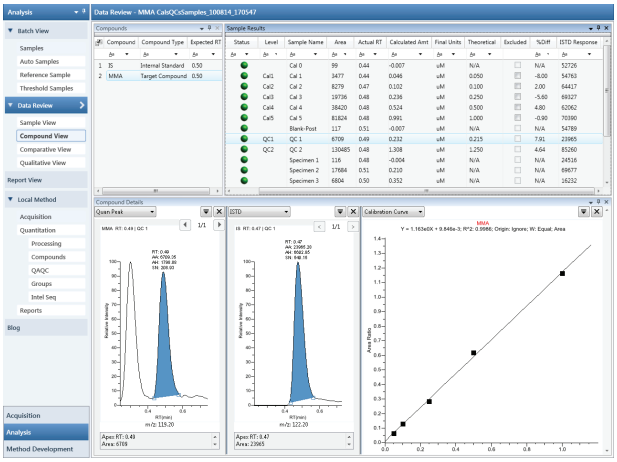
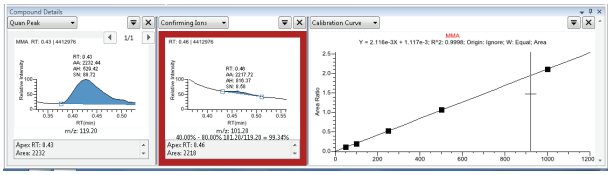


FIGURE 5. Ion ratios at low MMA concentrations.



Interferences and Accuracy

Internal standard peak areas among blood serum specimens varied by as much as five-fold due to matrix components that interfered with the butylation reaction. We are investigating ways to minimize these interferences. However, the calculated amounts of MMA concentrations in the QCs and specimens agreed with those from a reference laboratory within +/- 15%.

Repeatability

For QCs I and II, inter-batch and intra-batch precisions were typically less than 7% CV, as shown in Tables 1a and 1b, respectively.

TABLE1. Inter- and intra-batch precisions.

a

	MMA Intra-Batch Precision	
Injection	QC1 (nmol/L)	QC2 (nmol/L)
1	320	1020
2	330	980
3	300	1030
4	330	970
5	300	1060
6	290	1050
7	290	1040
8	330	1050
9	350	960
10	300	1020
11	320	1040
12	320	1030
13	330	940
14	310	1040
15	290	980
16	350	1050
17	330	1030
18	320	1020
19	340	1020
20	320	1000
Mean	318.5	1016.5
STD	18.72	33.76
CV (%)	5.9	3.3

b

		MMA Inter-Batch Precision	
Day	Injection	QC1 (nmole/L)	QC1 (nmole/L)
9/30/2014	1	260	970
	2	270	960
	3	260	910
	4	270	910
	5	270	920
10/1/2014	1	280	1020
	2	270	980
	3	270	1020
	4	270	980
	5	290	1060
10/2/2014	1	290	970
	2	250	890
	3	270	980
	4	260	1060
	5	260	1010
10/3/2014	1	270	1120
	2	300	1060
	3	310	1110
	4	290	1070
	5	270	1070
	Mean	274	1003.5
	SD	15.01	67.69
	CV (%)	5.48	6.75

Throughput and Multi-channeling with Other APCI - MS/MS Methods

MMA batches submitted to one or two channels had throughputs of 12 or 23 injections per hour, respectively. If demand for MMA is much less than for 25-OH-VitDs, one channel can be used while the other three are used for the VitDs. Thus, 8 injections from MMA and 36 injections of 25-OH-VitDs are completed in one hour.

Conclusions

- Butylation-derivatization of “crashed” blood serum provided reliable measurements of MMA concentrations using LC-MS/MS with APCI.
- An analytical range from 50 to 1,000 nM was achieved.
- In some specimens, ion-ratio confirmations of butyl-MMA peaks may fail at concentrations below 150 nM due to interference by much larger butyl-SA peaks.
- This method can be used simultaneously with other LC-APCI-MS/MS research methods, such as 25-OH-Vitamin Ds and steroids, on a multi-channel UHPLC system.

References

1. C.T. Turgeon, M.J. Magera, C.D. Cuthbert, P.R. Loken, D.K. Gavrilov, S. Tortorelli, K.M. Raymond, D. Oglesbee, P. Rinaldo and D. Matern, Determination of total homocysteine, methylmalonic acid and 2-methylcitric acid in dried blood spots by tandem mass spectrometry, Clinical Chemistry 2000; 56:1686-1695.

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