Separation and Quantitation of Total Plasma Homocysteine and Methylmalonic Acid by LC-MS/MS Analysis

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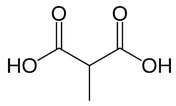
Introduction

Homocysteine is an endogenous sulfur containing amino acid that is formed during the metabolism of methionine to cysteine. Homocysteine is irreversibly catabolized by transsulfuration to cysteine or remethylated to methionine with the aid of B-vitamins. Genetic mutation or deficiencies of the essential B vitamins can tend to lead to different metabolic disorders including hyperhomocysteinemia and Homocystinuria, also known as Cystathionine Beta-Synthase.

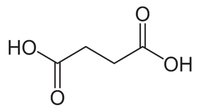
In up to 99% of plasma samples, homocysteine is present in the oxidized and bound forms in which it is bound via a sulfur-sulfur bridge with itself (homocystine). For this reason, sample preparation tends to be different for homocysteine compared to other amino acids and usually requires derivatization. Therefore, plasma sample reduction by sulfhydryl reagents, such as dithiothreitol is performed to convert all the oxidized and bound forms of homocystine (dimer) to homocysteine (monomer) in order to quantify total plasma homocysteine levels. Total plasma homocysteine normally ranges from 5 to 15 micromol/L with differences related to age, gender and lifestyle. Elevated total plasma homocysteine levels above 15 micromol/L can lead doctors to suspect the disorders mentioned above.

Methylmalonic Acid (MMA) is a small, water-soluble organic acid that is necessary for the metabolism and energy production by breaking down proteins. It can be used as a biomarker for some disorders involving vitamin B12 such as methylmalonic acidemia and megoblastic anemia. MMA also has a naturally occurring isomer, succinic acid. These two must be chromatographically separated for accurate quantitation of MMA and correct diagnosis.

Methylmalonic Acid



Succinic Acid



Simultaneous measurement of methionine, homocysteine, and methylmalonic acid helps in the differential diagnosis of cobalamin disorders without the need for additional amino acid testing. Therefore, a rapid 4-minute method was developed that showed good chromatographic separation of these metabolites was achieved using a Raptor Polar X 50 x 2.1 mm, 2.7 µm and a Polar X guard column with a shorter sample preparation with the use of DTT.

Methods

Table 1: Analytical Conditions

Analytical Column:	Raptor Polar X 2.7 µm, 50 x 2.1 mm (Restek Cat #9311A12)	
Guard Column:	Raptor Polar X Guard Column Cartridge (Restek Cat #9311A0252)	
Mobile Phase A:	0.5% Formic Acid in Water	
Mobile Phase B:	Acetonitrile	
Flow:	0.6 mL/min	
Gradient:	Time (min)	%B
	1.00	55
	3.00	10
	3.01	85
	4.00	STOP
Column Temp.:	40 degrees C	
Injection Volume:	5 µL	

Table 2: Analyte Transitions

Analyte	RT	Q1 Transition	Q3 Transition	Polarity
L-Methionine	0.75	150.0	104.1	+
DL-Homocysteine-D4	0.79	140.1	94.1	+
L-Homocysteine	0.81	136.2	90.1	+
L-Cysteine	1.00	122.1	76.0	+
L-Homocystine	1.29	268.9	136.0	+
Succinic Acid 1	1.32	117.0	72.9	-
Succinic Acid 2	1.32	117.0	55.1	-
Cystathionine 1	1.45	221.3	119.8	-
Cystathionine 2	1.45	221.3	113.9	-
Methylmalonic Acid-D3 1	2.91	120.1	58.0	-
Methylmalonic Acid-D3 2	2.91	120.1	75.9	-
Methylmalonic Acid 1	2.92	117.0	55.1	-
Methylmalonic Acid 2	2.92	117.0	72.9	-

Sample Preparation

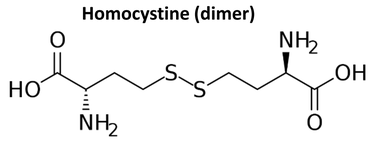
Plasma Samples: 100 µL of plasma was added to a micro centrifuge tube along with 5 µL IS (5 ng/mL, DL-homocysteine-d4 and MMA-D3), followed by 20 µL of 0.5M DTT. Next, the sample was vortexed for 10 seconds. The samples were then incubated at room temperature in darkness for 30 minutes. After the 30 minutes, 300 µL Methanol was added. The sample was vortexed for 10 seconds and then centrifuged for 10 minutes at 4000 rpm. The supernatant was pipetted into a vial containing a vial insert, and then injected for LC-MS/MS analysis.

Control Samples: Phosphate buffered saline (PBS) was used in place of the plasma for control samples because it did not have endogenous levels of the metabolites, and it shared a similar salt concentration to plasma. PBS was diluted to a 1:9 concentration with HPLC water to obtain 10x PBS solvent. This solvent was used to prepare calibration and QC standards. A 50 µg/mL and 5 µg/mL homocysteine (monomer) and MMA stock solutions were prepared in PBS. 100 µl PBS standards were prepared at the range of 25 - 5000 ng/mL and mixed with 5 µL of internal standard (5 µg/mL, DL-homocysteine-d4 and MMA-D3). The rest of the sample preparation was the same as above.

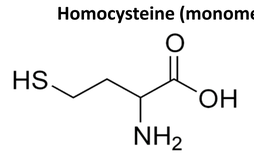
Results and Discussion

Optimization of DTT: Dithiothreitol, also referred to as DTT or Cleland's Reagents, is a reducing agent that forms a stable six-membered ring with an internal disulfide bond that is resistant to oxidation. A time study was performed, where 20 µL of 0.5M DTT was added to 100 µL plasma samples. The samples were incubated at 10 minutes, 20, 30, 40, 50 and 60 minutes, precipitated with 300 µL methanol, centrifuged for 10 minutes and analyzed by LC-MS/MS. It was found that 30 minutes incubation time was enough to obtain the maximum recovery of homocysteine and further incubation will only make the sample preparation more time consuming.

Homocystine (dimer)



Homocysteine (monomer)



Dithiothreitol (DTT)

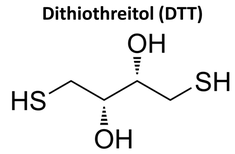


Figure 1: Recovery of Homocysteine Based on Incubation Time of DTT

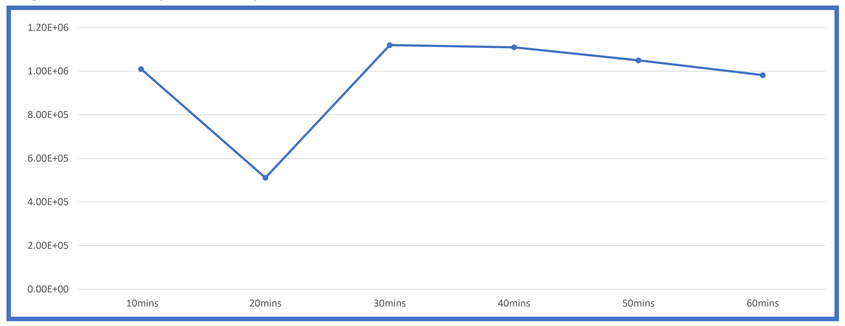


Figure 2: Homocystine (Dimer) and Homocysteine (Monomer) without the Use of DTT in Sample Preparation

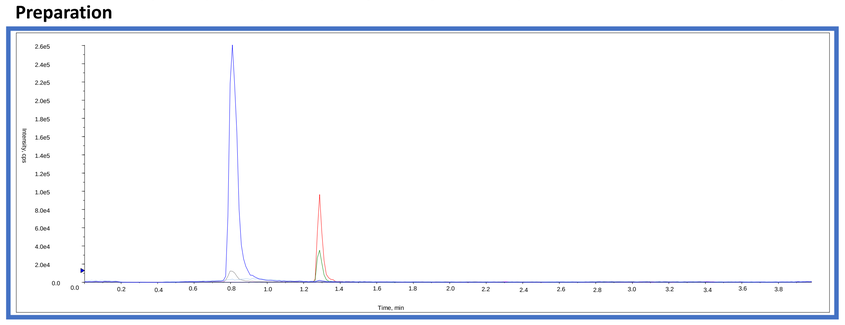
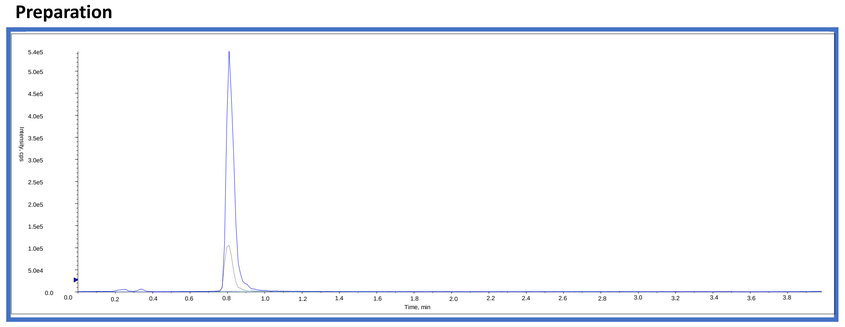
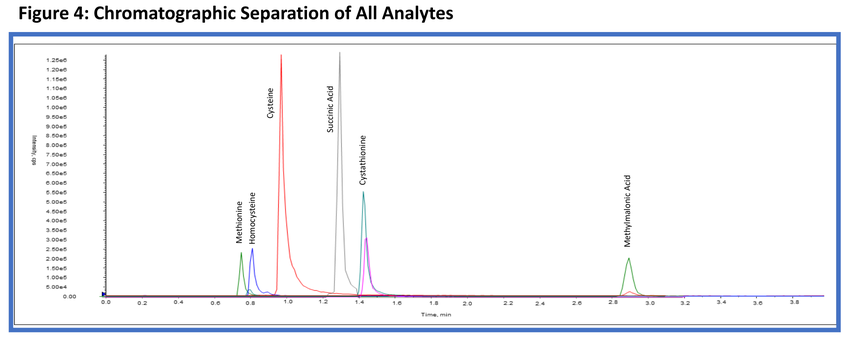


Figure 3: Homocystine (Dimer) and Homocysteine (Monomer) with the Use of DTT in Sample Preparation



Chromatographic Performance: Good separation and quantitation of the metabolites was achieved in 4 minutes. Good baseline separation of methionine and homocysteine allowed easy peak identification and quantitation. Reproducible chromatographic performance (retention, peak shape, and sensitivity) was observed upon continuous 500 injections demonstrating good method robustness. Reproducible retention times were also observed over the testing of multiple lots of the PolarX columns. Carryover was not observed.

Figure 4: Chromatographic Separation of All Analytes



Linearity: Using $1/x^2$ weighted linear regression, the analytes showed acceptable linearity with r^2 values of 0.995 or greater, and % deviations (from nominal concentrations) were <15%, in all accuracy and precision experiments.

Table 3: Linearity Results over 3 Days of Testing for Homocysteine and MMA

	Day 1	Day 2	Day 3	Average
Homocysteine	0.9921	0.9988	0.9952	0.9954
MMA	0.9964	0.9964	0.9992	0.9973

Accuracy and Precision: Precision and accuracy analyses were performed over the course of multiple days. The method accuracy was demonstrated with recovery of within 15% of the nominal concentrations for all mid and high QC levels and 15% for LLOQ (Table 4). The quantitative range for homocysteine was 50-5000 ng/mL, and the range for methylmalonic acid was 25 – 5000 ng/mL.

Table 4: Inter-Day Accuracy and Precision of Quality Control Samples in PBS

	Homocysteine			MMA		
	Avg Conc. (ng/mL)	Avg. Accuracy (%)	% RSD	Avg Conc. (ng/mL)	Avg. Accuracy (%)	% RSD
QC 25 ng/mL	NA			24.2	95.2	11.0
QC 50 ng/mL	47.6	94.7	13.0	NA		
QC 150 ng/mL	153	103	5.80	158	105	10.9
QC 600 ng/mL	598	99.6	2.90	583	97.4	5.40
QC 1500 ng/mL	1540	103	5.30	1483	99.1	5.50
QC 3000 ng/mL	3183	106	2.80	3170	106	10.5

* NA indicates that the concentration was not applicable for the specific analyte.

Quantitative Repeatability: Three different lots of plasma from BIOIVT were tested on their own and then with a known spiked amount of a calibrator in order to quantify to levels of homocysteine in each of the lots. One sample from each of the different plasma lots were also prepped and injected multiple times during the same day to show intra day repeatability (Table 5). Multiple samples were prepped from the same lot and were tested over the course of multiple days to assess day to day repeatability (Table 6).

Table 5: Intra-Day Homocysteine Repeatability for Standard Addition Samples

Plasma Lot	Expected	Average	% Difference	%RSD
HMN527618	1008.3	907.2	10.0	3.80
HMN527619	1055.4	946.6	10.3	12.0
HMN488920	1000	1142	14.2	5.50

Table 6: Intra-Day MMA Repeatability for Standard Addition Samples

Plasma Lot	Expected	Average	% Difference	%RSD
HMN527618	2506	2606	4.0	7.5
HMN527619	2666	2902	8.9	3.2
HMN488920	3620	3320	8.3	1.4

Table 7: Inter-Day Homocysteine Repeatability for Standard Addition Samples

Plasma Lot	Expected	Average	% Difference	%RSD
HMN527618	1013.2	1086.6	7.2	3.2
HMN527619	1049.6	1060.3	1.0	7.4

Table 8: Inter-Day Homocysteine Repeatability for Standard Addition Samples

Plasma Lot	Expected	Average	% Difference	%RSD
HMN527618	2531	2566	1.4	7.3
HMN527619	2716	2792	2.8	3.4

Conclusion

- A quick and easy sample preparation approach using DTT
- Rapid 4-minute LC-MS/MS method for the separation and quantitation of homocysteine and methylmalonic acid for the differential diagnosis of cobalamin disorders.
- Separation of additional analytes including methionine, cysteine, succinic acid, and cystathionine using a Raptor Polar X 50 x 2.1 mm, 2.7 µm column Raptor Polar X EXP Guard Column Cartridge
- Separation of methylmalonic acid from its main isobaric interference - succinic acid

References

Jiang, Yi, et al. "Simultaneous determination of plasma total homocysteine and methionine by liquid chromatography-tandem mass spectrometry." Clinica Chimica Acta, vol 464, 2017, pp. 93-97.

Persichilli, Silvia, et al. "A Simplified Method for the Determination of Total Homocysteine in Plasma by Electrospray Tandem Mass Spectrometry." Journal of Separation Science, vol. 33, no. 20, 2010, pp. 3119–3124., https://doi.org/10.1002/jssc.201000399.

Weaving, G, et al. "Simultaneous quantitation of homocysteine, cysteine and methionine in plasma and urine by liquid chromatography-tandem mass spectrometry." Ann Clin Biochem, vol 43, 2006, pp. 474-480.