Open Access

Original Article

Method Development and Validation of Methylmalonic Acid by Liquid Chromatography / Mass Spectrometry

Muhammad Hamid Awais, Muhammad Aamir, Sobia Irum Kirmani, Hafiza Monaza Batool*, Nisar Ahmed*, Sadiq Ali*

Department of Chemical Pathology, Armed Forces Institute of Pathology/National University of Medical Sciences (NUMS), Rawalpindi Pakistan,
*Department of Toxicology, Armed Forces Institute of Pathology/National University of Medical Sciences (NUMS), Rawalpindi Pakistan

ABSTRACT

Objective: To develop and validate an accurate and cost-effective method for analyzing methylmalonic acid (MMA) in serum on liquid chromatography/mass spectrometry.
Study Design: Cross sectional (validation) study.
Place and Duration of Study: Department of Chemical Pathology and Endocrinology, Armed Forces Institute of Pathology, Rawalpindi Pakistan, from Jul to Dec 2020.
Methodology: Method development was initiated to detect MMA using commercially available standards to achieve analyte signal. Liquid phase extraction was performed to extract the MMA from serum by using methanol, acetonitrile, and Formic Acid as an extracting solvent. Separation of the compound was achieved with Agilent SB-C18 column (4.6x150mm, 1.8 µm) using an isocratic elution having 20% mobile phase A (0.1% Formic Acid in water) and 80% mobile phase B (0.1% Formic Acid in Acetonitrile) at a flow rate of 0.7 mL/min.
Results: The AMR of MMA was 33-4227 nmol/L with LOD of 15 nmol/L. The lower limit of quantification was validated at 33 nmol/L. The calculated bias was -12.727. The within and between day imprecision at four concentrations levels were 0.7-7.5%. The method was found stable after the storage & freeze-thaw cycle. The integrity of the diluted sample was maintained for each dilution factor.
Conclusion: The presented method for MMA determination is accurate, cost-effective, specific, and has good clinical correlation. This method can be used in routine to accurately estimate MMA levels with good time management and less financial burden.

Keywords: Liquid Chromatography-Mass Spectrometry, Methylmalonic Acid, Validation.

How to Cite This Article: *Awais MH, Aamir M, Kirmani SI, Batool HM, Ahmed N, Ali S. Method Development and Validation of Methylmalonic Acid by Liquid Chromatography / Mass Spectrometry. Pak Armed Forces Med J 2025; 75(Suppl-5): S812-S816. DOI: https://doi.org/10.51253/pafmj.v75iSUPPL-5.7623*

INTRODUCTION

Levels of Methylmalonic acid (MMA) in serum are used as a marker for detecting methylmalonic academia and early indication of vitamin B12 deficiency. Methylmalonic academia is a rare inborn error of metabolism, presented in an autosomal recessive pattern, causing significant neurological symptoms in early neonatal life.[1] The prevalence of methylmalonic acidemia is 1/30,000 to 1/50,000 live births.[2] In healthy individuals, methylmalonyl-CoA is converted to Succinyl-CoA by an enzyme, methylmalonyl-CoA Mutase (MUT), which uses Cobalamin as a cofactor. Succinyl-CoA then enters the Krebs cycle. Deficient activity of MUT causes accumulation of methylmalonyl-CoA, and MMA is subsequently released.[3,4] Increased levels of MMA in plasma present as systemic acidemia in the initial days of neonatal life.[5] This leads to rapid deterioration of the clinical condition, leading to irreversible mental retardation or even death of the affected individuals if not diagnosed and treated promptly. Therefore, there is a need for an early diagnosis and treatment to prevent or minimize the progression of CNS dysfunctions. Vitamin B12 (Cobalamin) deficiency can lead to Megaloblastic anemia and irreversible neurological disorders.[6] It is an essential nutrient and plays a vital role in normal cellular functioning. Mild elevation of MMA (>400nmol/L) appears in the early stages of Vit B12 insufficiency,[12] even in the presence of normal Vit B12 levels (>156 pmol/L).[3] Thus, it makes MMA a sensitive early indicator of intracellular vitamin B12 deficiency, especially in patients with few or no abnormalities.[7]

Quantitative determination of MMA helps clinicians identify and monitor inherited metabolic disorders (IMDs) like methylmalonic academia and cobalamin deficiency.[7] Various analytical techniques like electrophoresis, GC/MS, and LC-MS/MS have

Correspondence: Dr Muhammad Hamid Awais, Department of Chemical Pathology, Armed Forces Institute of Pathology, Rawalpindi Pakistan
Received: 15 Nov 2024; revision received: 26 Jan 2022; accepted: 15 Mar 2022

been used to determine MMA in biological fluids. Mass spectrometry-based methods have been widely tested, among which GC/MS has been routinely applied to quantitate MMA. However, due to labor-intensive sample preparation steps such as solid-phase extraction, derivatization, evaporation, and ultra-filtration,[8,9] GC/MS has been replaced by LC-MS/MS with less time-consuming sample preparation and better sensitivity.[10]

As the commercially available prefabricated kits for estimation of MMA on LC-MS/MS are too expensive and the cost has to be borne by the patients, most cases remain undiagnosed in developing countries due to cost restraints. So, there is a need to introduce a more efficient, cost-effective, yet highly accurate LC-MS/MS method for early detection and monitoring of MMA. In the present study, we have developed and validated an in-house method on LC-MS/MS with simple sample preparation protocols, shorter run time, and cutting the price to less than half. The primary aim is to benefit the poor population.

METHODOLOGY

The cross sectional (Validation study) was conducted at Department of Chemical Pathology and Endocrinology, Armed Forces Institute of Pathology, Rawalpindi Pakistan, from July to December 2020 after taking prior approval of the Institution Ethical Review Board (FC-CHP19-16/READ-IRB/21/145).

Inclusion Criteria: For patient samples following criteria was used: adults aged 18–70 years, and the two Groups were: Healthy controls with normal vitamin B12 levels and Patients with confirmed or suspected vitamin B12 deficiency.

Exclusion Criteria: Following were the exclusion criteria**:** degraded samples: hemolyzed, lipemic, or icteric samples, Patients with renal failure as elevated MMA may result from kidney dysfunction.**,** recent Vitamin B12 supplementation: within the last 4 weeks.

Methylmalonic acid (MMA) standard and deuterated MMA (MMA-d3) as internal standards were purchased from CERILLIENT. All the calibrators and controls were purchased from UTAK-USA. Formic acid, Acetonitrile & Methanol were purchased from Merck. The Millipore water purification system was used to prepare fresh Ultra-pure water. Commercially available blank serum (UTAK-USA) was used.

For sample preparation, 100µl of calibrators, controls, and patient serum were aliquoted in a sample preparation vial followed by the addition of 300µl of extracting solution (Methanol: Acetonitrile: Formic Acid in 2:2:1) and 100 µl of d3-methylmalonic acid as internal standard. The mixture was mixed for 30 seconds on a vortex mixer & subsequently centrifuged at 11500 RPM for five minutes. Centrifuged supernatant was then transferred to a sample vial through a 0.2µm filter and analyzed by LC-MS/MS.

The Chromatographic separation was carried out on the Agilent SB-C18 column (4.6x150mm, 1.8 µm). The column temperature was kept at 45°C, and the injector volume was 5 µL. A 0.7 mL/min flow rate was used for linear elution using a mobile phase containing 20% mobile phase A (0.1% Formic Acid in water) and 80% mobile phase B (0.1% Formic Acid in acetonitrile). A 0.2m membrane filter was used to filter the mobile phase components, followed by an ultrasonic bath to degas them. Quantification and detection were conducted using a triple quadruple quadrupole LC-MS/MS 6460 equipped with an electrospray ionization source and jet stream technology with Mass hunter software. The analysis was performed using a negative ionization mode. The detector was operated using multiple reaction monitoring (MRM). For desolvation and collision, nitrogen gas was used. Tables I and II summarize MS parameters and acquisition data, respectively.

The method was validated using US Food and Drug Administration (FDA) guidelines.[11-13] The assay was validated for Linearity (calibration curve) with recovery, Limit of detection (LOD), a Lower limit of quantification (LLOQ), Analytical measurement range (AMR), Sensitivity, Precision, Accuracy, Selectivity, Carryover, Stability, and Dilution effects.

RESULTS

The assay's linearity was evaluated using the calibration curves generated by using eight calibrators of various concentrations spanning the analytical range of MMA. Three independent replicates calculated each calibration level. The calibration curve was created by running eight working calibrators in triplicate with different concentrations and selecting the best fit among them. Throughout the analytical measurement range (AMR) of 33 - 4227 nmol/L, there was a linear relationship between the concentration of analytes and their peak areas (concentration detected)

(Figure-1). Table-III shows linearity and correlation coefficients of ≥0.998 for calibration curves.

Table-I: MS Parameters for MMA Analysis in Serum

Parameters	
Scan Type	MRM
Polarity	Negative
Gas Temperature	300°C
Gas Flow	10 L/min
Sheath Gas Temp	400°C
Sheath Gas Flow rate	10 L/min
Nebulizer pressure	55 Psi
Capillary Voltage	1750 V

The limitation of detection (LOD) was considered minimum concentration, providing a peak height three times the baseline noise, 15 nmol/L. At the same time, the lower limit of quantification (LLOQ) was determined by analyzing the lowest concentration that can be quantitatively determined with acceptable accuracy & precision, which also defined the assay's sensitivity. LLOQ of 33 nmol/L was calculated with a bias of -12.7% and precision of 7% (<20%). Accuracy & precision were determined using four QC levels (LLOQ, Low, Mid, High) spanning AMR of MMA. Each Level was extracted & run in five replicates on three different occasions. Accuracy was assessed by percent bias, calculated as a difference between the average and true concentrations divided by the true. Percent recovery of each compound during four different concentrations at which it was measured satisfied the acceptance criteria (15%). For precision, within & between day data was evaluated by calculating mean, SD & % CV at four concentration levels for MMA, which were less than 15%.

MMA's selective detection capability was demonstrated during the validation of the test. Testing blanks determined the selectivity. Blank serum was spiked from different sources after following the sample extraction protocols. The chromatograms were assessed through MRM and their retention times.

Table-II: Acquisition Parameters of MMA in Serum on Triple Quadrupole LCMS.

Compound	ISD	Precursor Ion	Product Ion	Dwell	Fragmentor	Collision Energy
MMA	-	116.9	73	50	62	12
MMA	-	116.9	55	50	62	24
MMAd3	Yes	119.9	76	50	66	12
MMAd3	Yes	119.9	58	50	66	24

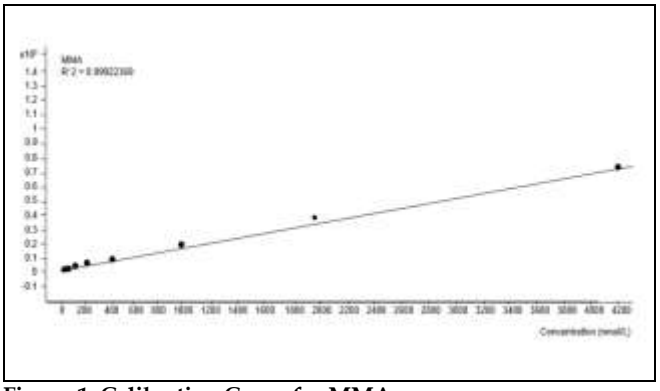

Figure-1: Calibration Curve for MMA

Analyte retention time and internal standard retention time were not distorted in the blank chromatogram. Finally, MMA chromatograms were adequate, especially at LLOQ (Figure 2). A post-run of one minute was given in our method to minimize the interferences of carryover. In this assay, there was no carryover since the response in blank was less than ±20% of the LLOQ. The stability of the extracted sample was checked at the short-term and long-term storage and after the freeze-thaw cycle. To check the sample's integrity after dilution, we developed dilution protocols for the patient's serum samples with the highest MMA >4227 nmol/L concentration.

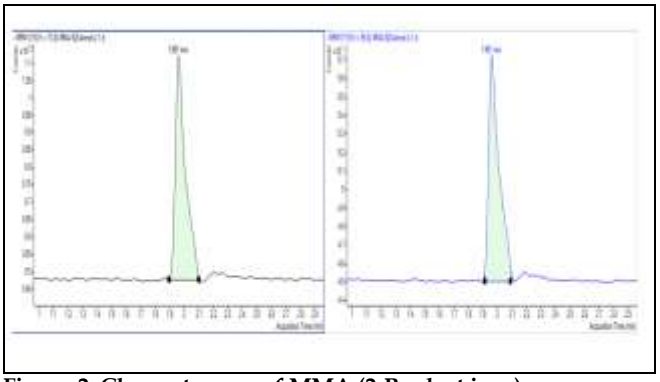

Figure-2: Chromatogram of MMA (2-Product ions)

Table-III: Accuracy & Precision of MMA

Sample	Accuracy			Precision			
				Within day		Between days	
	Nominal Value	Mean (nmol/L)	Bias %	Mean±SD (nmol/L)	CV(%)	Mean±SD (nmol/L)	CV(%)
LLOQ	33	28.8	-12.7	28.8±1.9	6.6	29.3±2.2	7.5
Low	256	250	-2.3	256±6.6	2.7	256±4.8	1.9
Middle	1056	1007	-4.6	1007±10	1.0	1040±25.3	2.4
High	4227	4242	0.4	4242±44.8	1.1	4246±30.2	0.7

The linear regression data for MMA has shown LOD of 15 nmol/L, LOQ of 33 nmol/L, AMR 33 – 4227 nmol/L and r2 0.998.

DISCUSSION

This study has developed a simple and sensitive method on LC-MS/MS for quantitation of serum MMA. With this new method, we have achieved our goal of making it cost-effective and accurate. Our method used a much-reduced sample volume (100(L) compared to the previous GC/MS procedure having a sample volume of 275µL.[14-17] Our extraction protocol was less laborious, less time-consuming, and was found to be highly specific with greater sensitivity for MMA. Previously reported GC/MS methods required Silyl derivatization of MMA for its determination, which was time-consuming and throughput was limited to 36 samples/run.[18] These concerns have been addressed in our newly developed method, with run time reduced to 2.4 minutes, and sample throughput has also been increased to 100 samples/run. Many analytical columns were tested, and SB-C18 was selected to get excellent resolution with a sharp, well-defined peak.

Our method used in-house extracting solvents and mobile phases, making it highly cost-effective. The LOD (15 nmol/L) of our newly developed LC-MS/MS method was improved compared to other LC/MS methods with a LOD of 22.1 described by Ekaterina M. Mineva.[19] Previously, no study has been done in Pakistan concerning method development and validation of MMA but relying on costly prefabricated columns and mobile phases. The cost per test was too high, which had to be borne by the patients. This is the first study that focused on the accuracy of method development and its cost and simplicity.

CONCLUSION

In summary, we have developed and validated an accurate and economical method on LC-MS/MS to analyze MMA in serum with important features of low imprecision, good sensitivity, low sample volume, high throughput, and high accuracy.

Conflict of Interest: None.

Funding Source: None.

Authors' Contribution

Following authors have made substantial contributions to the manuscript as under:

MHA & MA: Data acquisition, data analysis, critical review, approval of the final version to be published.

SIK & HMB: Study design, data interpretation, drafting the manuscript, critical review, approval of the final version to be published.

NA & SA: Conception, data acquisition, drafting the manuscript, approval of the final version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

REFERENCES

1. Tecleab A, Schofield R, Ramanathan L, Carlow DC. A simple and sensitive method for quantitative measurement of methylmalonic acid by turbulent flow chromatography and tandem mass spectrometry. Journal of chromatography & separation techniques 2016; 7(5).
2. Magera MJ, Helgeson JK, Matern D, Rinaldo P. Methylmalonic acid measured in plasma and urine by stable-isotope dilution and electrospray tandem mass spectrometry. Clinical chemistry 2000; 46(11): 1804-1810.
3. Herrmann W, Obeid R. Ursachen und frühzeitige Diagnostik von Vitamin-B12-Mangel. Dtsch Arztebl 2008; 105(40): 680-685.
4. Klee GG. Cobalamin and folate evaluation: measurement of methylmalonic acid and homocysteine vs vitamin B12 and folate. Clinical chemistry 2000; 46(8): 1277-1283.
5. Plessl T, Bürer C, Lutz S, Yue WW, Baumgartner MR, Froese DS. Protein destabilization and loss of protein-protein interaction are fundamental mechanisms in cblA-type methylmalonic aciduria. Human mutation 2017; 38(8): 988-1001.
6. Green R, Allen LH, Bjørke-Monsen A-L, Brito A, Guéant J-L, Miller JW, et al. Vitamin B 12 deficiency. Nature reviews Disease primers 2017; 3(1): 1-20.
7. Sloan JL, Carrillo N, Adams D, Venditti CP. Disorders of intracellular cobalamin metabolism. GeneReviews®[Internet] 2018.
8. Pandor A, Eastham J, Beverley C, Chilcott J, Paisley S. Clinical effectiveness and cost-effectiveness of neonatal screening for inborn errors of metabolism using tandem mass spectrometry: a systematic review. NIHR Health Technology Assessment programme: Executive Summaries 2004.
9. Ozben T. Expanded newborn screening and confirmatory follow-up testing for inborn errors of metabolism detected by tandem mass spectrometry. Clinical Chemistry and Laboratory Medicine (CCLM) 2013; 51(1): 157-176.
10. Cox E, White A. Methyl-malonic acid excretion: an index of vitamin-B12 deficiency. Lancet 1962; 2: 853-856.
11. Fenton W, Gravel R, Rosenblatt D. Disorders of Propionate and Methylmalonate Metabolism in The Metabolic and Molecular Bases for Inhertited Disease. Edited by: Scriver CR, Beaudet AL, Sly WS, Valle D. McGraw-Hill, Inc., New York; 2001.
12. Kushnir MM, Nelson GJ, Frank EL, Rockwood AL. High-throughput analysis of methylmalonic acid in serum, plasma, and urine by LC-MS/MS. Method for analyzing isomers without chromatographic separation. Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Springer 2016: 159-173.
13. Mak J, Wong BT, Kwan T, Le A, Cowan TM. Rapid underivatized method for quantitative methylmalonic acid by liquid chromatography–tandem mass spectrometry. The Journal of Applied Laboratory Medicine 2018; 3(3): 408-417.

14. Carvalho VM, Kok F. Determination of serum methylmalonic acid by alkylative extraction and liquid chromatography coupled to tandem mass spectrometry. Analytical biochemistry 2008; 381(1): 67-73.
15. Schmedes A, Brandslund I. Analysis of methylmalonic acid in plasma by liquid chromatography–tandem mass spectrometry. Clinical chemistry 2006; 52(4): 754-757.
16. FDA U. Guidance for industry, bioanalytical method validation (2018). 2001.
17. Pfeiffer C, Gunter E, editors. Automated assay for methylmalonic acid (MMA) in plasma. Clinical chemistry; 1999: Amer Assoc Clinical Chemistry.
18. Straczek J, Felden F, Dousset B, Gueant JL, Belleville F. Quantification of methylmalonic acid in serum measured by capillary gas chromatography—mass spectrometry as tert.-butyldimethylsilyl derivatives. Journal of Chromatography B: Biomedical Sciences and Applications 1993; 620(1): 1-7.
19. Mineva EM, Zhang M, Rabinowitz DJ, Phinney KW, Pfeiffer CM. An LC-MS/MS method for serum methylmalonic acid suitable for monitoring vitamin B12 status in population surveys. Analytical and bioanalytical chemistry 2015; 407(11): 2955-2964.

.....