

Development and Validation of a GC-FID Method for Diagnosis of Methylmalonic Acidemia

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Abstract

Background: Urinary organic acids are water-soluble intermediates and end products of the metabolism of amino acids, carbohydrates, lipids, and a number of other metabolic processes. In the hereditary diseases known as organic acidurias, an enzyme or co-factor defect in a metabolic pathway leads to the accumulation and increased excretion of one or more of these acidic metabolites. Gas chromatography is the most commonly-used technology to separate and identify these metabolites. In this report the analytical conditions for the determination of methylmalonic acid using a gas chromatography/flame ionization detector (GC-FID) are studied with the aim to establish a method to analyze organic acids in human urine.

Methods: Studies included the GC-FID method development, the conditions of the derivatization (trimethylsilylation) reaction, and the stability of the methylmalonic acid standard solution and trimethylsilyl derivatives during storage. Also, a systematic comparison between GC-FID and gas chromatography/mass spectrometry (GC-MS) was performed.

Results: The highest resolution and sensitivity were obtained at 60 °C with a 30 min reaction time. Standard solutions and derivatized samples were stable for 7 days at 4-8 °C. Relative standard deviations of within-day and day-to-day assay results were less than 5%. Methylmalonic acid was detected in thirty human urine samples by the proposed GC-FID, and the results were compared with gold standard technique GC-MS. The correlation coefficient between GC-MS and GC-FID was obtained with $R^2 = 0.997$.

Conclusions: The developed method was applied to the quantitative analysis of methylmalonic acid in urine from hospitalized children with methylmalonic acidemia. With this method we aim to support pediatric clinics in Iran and assist in clinical diagnostics.

Keywords: Gas chromatography/Flame Ionization Detector (GC-FID), Method development, Methylmalonic acidemia disorder, Urine organic acid analysis.

Introduction

Urinary organic acids are water-soluble intermediates and end products of the metabolism of amino acids, carbohydrates, lipids, and exogenous sources including food and food additives, drugs, and products of bacterial metabolism (1). In the hereditary diseases known as the organic acidurias, an enzyme

or co-factor defect in a metabolic pathway leads to the accumulation and increased excretion of one or more of these acidic metabolites (2). Depending on the site of the defect, only a few typical intermediates or a whole series of normal and abnormal metabolites may be involved. Thus, metabolic profiling of urinary

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organic acids has become an important tool for the detection and differential diagnosis of these inborn errors of metabolism (3). Because organic acids cover such a range of polarity and have different chemical properties related to the various functional groups present, for a procedure to be useful as a routine diagnostic method, one should be able to process several samples simultaneously with reasonable speed and accuracy (1). Gas chromatography (GC) is the most commonly-used technology to separate and identify more than 250 normal and pathological acidic metabolites detected in these diseases (4-7). For example, organic acid analysis via GC has helped to diagnose methylmalonic acidemia. In this disease, elevated excretion of methylmalonic acid was seen due to the impaired isomerization of L-methylmalonyl-CoA to succinyl-CoA during the oxidation of propionate. This reaction is catalyzed by the cobalamin-dependent mitochondrial enzyme L-methylmalonyl-CoA mutase (MCM, EC 5.4.99.2), using adenosylcobalamin (Adocbl) as a cofactor (8, 9).

The aim of this study was to determine the optimal conditions for the gas chromatography/flame ionization detector (GC-FID) analysis of methylmalonic acidemia using a simple and derivatization-free method to analyze methylmalonic acid. A GC-FID method was developed and optimized with respect to various parameters including oven temperature, injection conditions, and column clean-up. The stability of the analytes in their derivatized forms was also studied. The GC-FID method was validated in terms of accuracy, sensitivity, and repeatability for the analysis of organic acids in urine. The applicability of the GC-FID method to clinical samples was demonstrated.

Materials and Methods

Chemical and Reagents

In this study chemicals and reagents with highest purity available were purchased. Methylmalonic and pentadecanoic (PDA) acids were obtained from Sigma-Aldrich (Switzerland). N-Methyl-N-(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane from Sigma-Aldrich (Switzerland) was used as the derivatizing reagent. All solvents were GC Grade. Ethyl acetate was from Sigma-Aldrich (Germany), and anhydrous sodium sulfate was from Merck (Hohenbrunn, Germany).

Instrument

An Acme 6000 gas chromatography system (Younglin) equipped with a flame ionization detector was used for method development and validation. The column was a 5% diphenylpolysiloxane TRB-5 60 m x 0.32 mm capillary column with a film thickness of 0.5 μm .

Preparation of Standard Solutions

A 10 g/l methylmalonic acid stock solution was prepared in absolute ethyl acetate. Three standard methylmalonic acid solutions of 10, 100, and 500 mM were prepared from this stock solution and used as controls to generate calibration curves. All standard solutions were stored in glass vials at -20 °C. Pentadecanoic acid was used as an internal standard; its 10 g/l stock solution was also prepared in absolute ethyl acetate (10).

Extraction, Derivatization, and Gas Chromatography

For the methylmalonic acid standard, 25 μl of PDA in ethyl acetate were aliquoted into a glass culture tube and the solvent was evaporated under a gentle nitrogen stream at 60 °C. The tube was then capped with a rubber serum stopper and 100 μl of trimethylsilyl (TMS) were injected through the stopper. The mixture was mixed on a vortexer and heated at 60 °C for 30 min in a hot water bath. The samples were kept at room temperature until analyzed. One μl of the prepared sample was taken through the stopper using a 10 μl Hamilton syringe and injected into the GC.

For the urine sample, a volume of urine corresponding to 250 μg of creatinine was placed in a screw-cap culture tube, diluted with de-ionized water to 2 ml, and the pH adjusted to 1.0 by addition of 6 mol/L HCl. The acidified sample was extracted successively with four 2-ml aliquots of ethyl acetate, with vigorous shaking. The organic layers were combined into a second tube, 25 μl of a 10 g/l PDA solution in ethyl acetate was added, and the combined extract was dried over 1 g of anhydrous Na_2SO_4 for 1 h. The sample was filtered with filter paper into a third tube and the Na_2SO_4 was washed 2 x with 1-ml portions of ethyl acetate. The extract was evaporated under a nitrogen stream at 60 °C. When the volume was about 0.5 ml, the solution was transferred to a 1 ml glass culture tube and the remaining solvent was

evaporated. The sample was then capped with a rubber serum stopper and 100 μ l of TMS was injected through the stopper. The sample was mixed on a vortexer and incubated at 60 $^{\circ}$ C for 30 min. The temperatures for the injectors and detectors were 250 and 300 $^{\circ}$ C, respectively. Helium was used as the carrier gas at a flow rate of 2 ml/min for the column. The oven was programmed to increase from 70 to 290 $^{\circ}$ C at a rate of 8 $^{\circ}$ C/min, with an initial isothermal delay of 0.5 min. One μ l of the prepared sample was taken through the stopper by a 10 μ l Hamilton syringe and injected into the GC (10).

Method Validation

Precision

Precision was evaluated by GC runs with the 10, 100, and 500 mM standard solutions under the optimal conditions three times in one day for the intra-day variation test and two times per day on three consecutive days for the inter-day variation test.

Repeatability

Three aliquots from one sample were injected at different times in one day to test repeatability. The relative standard deviations (RSDs) of the three assays were calculated to evaluate repeatability.

Recovery

The sample solution was spiked with 25 μ l of the standard solution to test sample recovery. The prepared samples were processed as described in Materials and Methods.

Statistical analysis

Data analyses, including calculation of standard deviation (SD) and linear regression, were conducted using Excel 2007 (Microsoft, USA). *P* values less than 0.05 were considered as statistically significant.

Results and Discussion

GC-FID Analysis Condition

Preliminary investigations aimed for adjustment and selection of the chromatographic conditions for the GC-FID analysis of TMS derivatives of urinary methylmalonic acid. We chose a column with wide applicability and not specifically for organic acids analysis. The effect of the rate of the oven temperature increase was determined to optimize chromatographic resolution and detection sensitivity. Different oven temperature programs and ramp rates were tested with a maximum oven temperature of 300 $^{\circ}$ C. Methylmalonic acid eluted at about 290 $^{\circ}$ C; therefore, this temperature was selected for the rest of the work as reported by Tanaka, et al (10). Next, the gradient temperature program in the GC oven and the injection were tested. The developed program provided separation of all analyte peaks within 30 min; about half the time reported in the literature (11). Fig. 1 shows a GC-FID analysis chromatogram obtained from the urine of a methylmalonic acidemia patient.

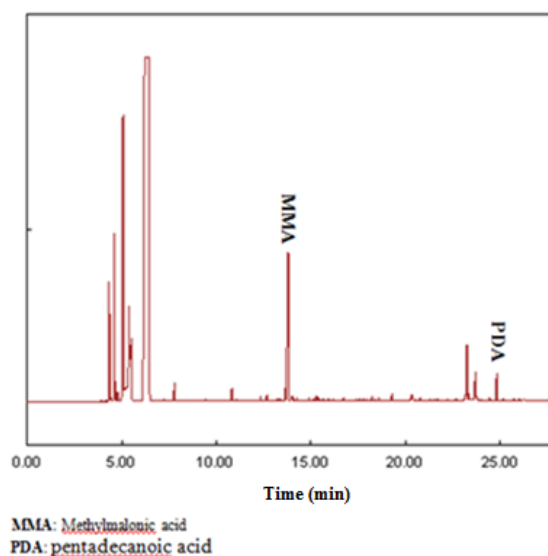


Fig. 1. A typical chromatogram of urinary organic acids in urine from a methylmalonic acidemia patient.

Stability Studies

Stability tests of the standard and urine samples showed that TMS derivatives methylmalonic acid remained stable during storage at 4-8 °C at least for 7 days. Thus, it is advised that standard solution and urine samples of organic acids should not be kept in

the refrigerator for more than 7 days. Also, the internal standard (PDA) remained stable for 7 days when stored at 4-8 °C. Table 1 shows the stability of TMS derivative of methylmalonic acid stored at 4-8 °C at 2, 4, and 7 days.

Table 1. Stability of the TMS derivative of methylmalonic acid stored at 4-8 °C at 2, 4, and 7 days

No	Methylmalonic acid	Time (day)		
		2 Mean	4 Mean	7 Mean
1	Standard (100mM)	1.3	1.25	1.22
2	Urine of a methylmalonic acid patient	4.3	4.1	3.9

*Mean values were calculated based on the methylmalonic acid to PDA peak area ratio.

Method Validation

Calibration curves showed linear regression for methylmalonic standards with the following equation: $Y = 48.67X + 1198$ ($R^2 = 0.995$, $P < 0.01$). The precision of the intra- (three times per day) and inter-day (twice a day for three consecutive days) data was indicated by

RSDs, which were less than 5.42% for methylmalonic acid at the three concentrations tested (Table 2). These results suggest that the TMS derivative of methylmalonic acid is stable. The accuracy was determined by back calculation of precision samples at the three concentrations against a calibration curve prepared each day.

Table 2. Precision of the intra- and inter-day measurements

Concentration (mM)	Intra-day ^a			Inter-day ^b		
	Peak areas Mean (SD)	RSD%	Precision %	Peak areas Mean (SD)	RSD%	Precision %
Low (10 mM)	1146.55 (9.288)	0.8	99.3	1175.7 (16.879)	1.4	86
Medium (100 mM)	5796.24 (75.64)	1.3	92	3877.5 (52.877)	1.36	89
High (500 mM)	26346.55 (1307.14)	4.96	99	27013 (1464.9)	5.42	96

The concentration of methylmalonic acid in a normal urine sample was 5 mmol/mmol creatinine. This sample was selected for recovery analysis. After recovery the 100 mM methylmalonic acid standard solution was added, after which the average concentration of methylmalonic acid was 105.25 mmol/mmol creatinine. The average recovery was 99.1% (SD 1.25%) with RSDs of 1.2% (n = 4). Thus, this chromatographic system is suitable for quantitative determination of urinary methylmalonic acid. Fig. 2 shows the urine chromatogram before and after recovery.

Comparison of GC-FID with GC-MS

The proposed GC-FID was applied to evaluate the concentration of methylmalonic acid in human urine samples. The results obtained using the proposed GC-FID methods in the determination of concentration of methylmalonic acid in thirty clinical urine samples were compared with those obtained by the gold standard GC-MS method. As can be seen in Fig. 3, the correlation coefficient between GC-FID and GC-MS was 0.997. This correlation indicates influence the detection precision and sensitivity of the GC-FID, when GC-MS are not available.

Analysis of abnormal urines for methylmalonic acidemia (Application to clinical sample)

Diagnosis of methylmalonic acidemia is usually easy; up to 20 mg/mg of creatinine is readily detectable (Fig. 3). In cases in which a large methylmalonic acid peak is not detected in urine, but clinical symptoms

indicate methylmalonic acidemia, vitamin B12 (cobalamin) is administered. In B12-responsive methylmalonic acidemia, the urinary methylmalonic acid concentration may be low (12). Therefore, the GC-FID method was applied to analyze organic acids in urine samples obtained from hospitalized children.

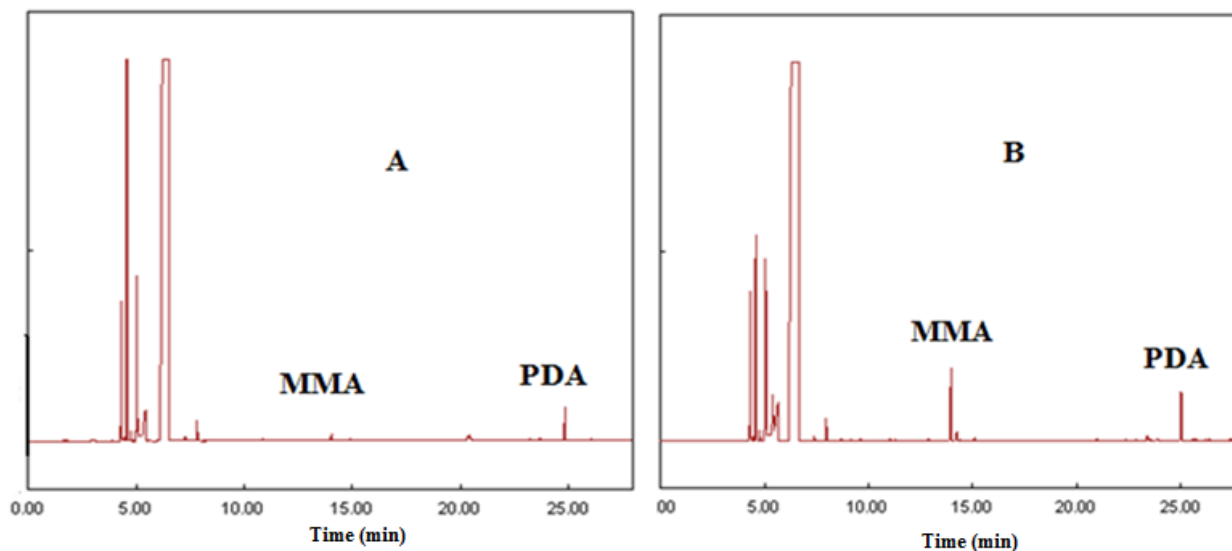


Fig. 2. The chromatogram of a normal urine sample before (A) and after (B) recovery.

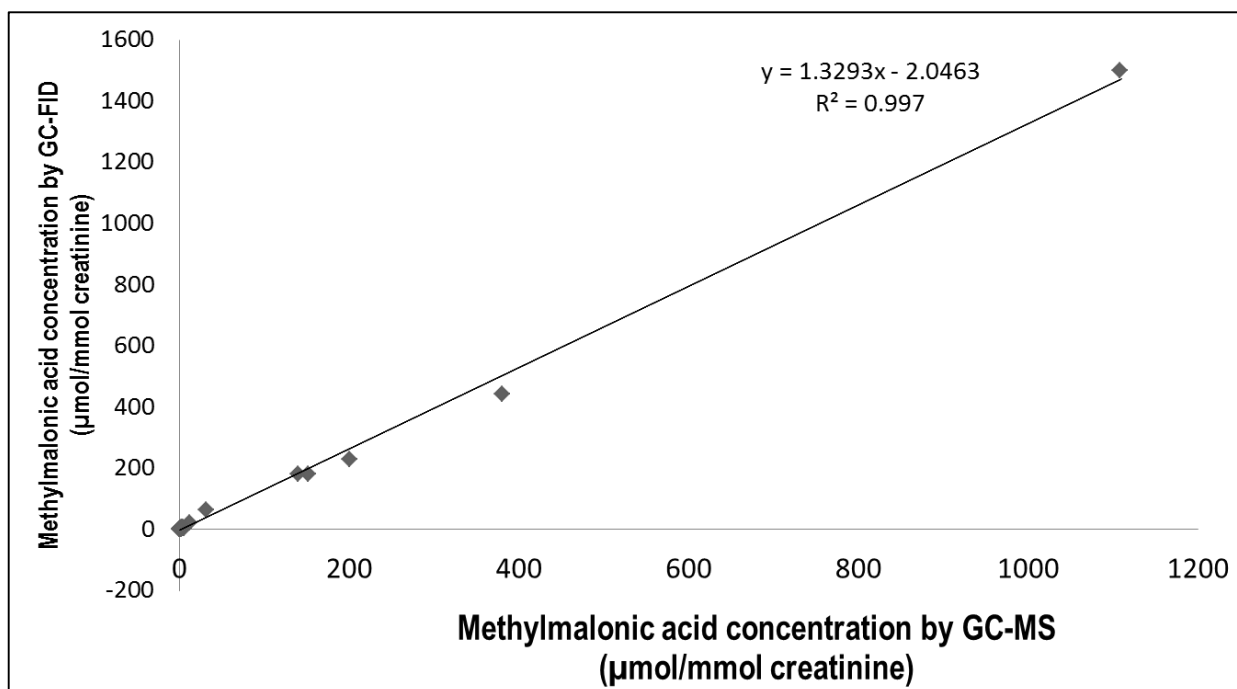


Fig. 3. Evaluation of methylmalonic acid in human urine samples with GC-FID and GC-MS.

Conclusion

In the present paper we report the development, optimization, and validation of a GC-FID method for the quantitative determination of methylmalonic acid in urine. In our study we focused on the optimization of the derivatization of this organic acid by TMS and the stability of TMS derivatives. Also, the construction and systemically comparison of GC-FID with GC-MS were performed for detection of methylmalonic acid and a good correlation was obtained. All of these indicated that in the clinical diagnosis, the GC-FID for detecting of methylmalonic acid was a convenient, economical, and time-saving method for screening, prognosis and monitoring of methylmalonic acidemia

References

1. Verhaghe BJ, Llever MF, DeLeenheer AP. Solid-Phase Extraction with Strong Anion-Exchange Columns for Selective Isolation and Concentration of Unnary Organic Acids; *Clin chem.* 1988 Jun; 34 (6): 1077- 83
2. Chalmers RA, Healy MJR, Lawson AM, Watts RWE. Urinary organic acids in man. II. Effects of individual variation and diet on the urinary excretion of acidic metabolites. *Clin chem.* 1976 May; 22 (8): 1288-91
3. Holland JF, Leary JJ, Sweeley CC. Advanced instrumentation and strategies for metabolic profiling [Review]. *J Chromatogr.* 1986 Jun; 379:3-26.
4. Liebich HM. Analysis of acidic metabolites by capillary column GC and GC/MS [Review]. *J High Reaolut Chromatogr.* 1983 Dec; 6 (12): 640-50.
5. Niwa T. Metabolic profiling with gas chromatography- mass spectrometry and its application to clinical medicine [Review]. *J Chromatogr.* 1986 Jun; 379:313-45.
6. Tuchman M, Bowers LD, Fregien KD, Crippin PJ, Krivit W. Capillary gas chromatographic separation of urinary organic acids. Retention indices of 101 urinary acids on a 5% phenylmethyl silicone capillary column. *J Chromatogr Sci* 1984 May; 22 (5):198-202.
7. Christou C, Gika HG, Raikos N, Theodoridis G. GC-MS analysis of organic acids in human urine in clinical settings: a study of derivatization and other analytical parameters. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2014 Aug; 964:195-201.
8. Keyfi F, Sankian M, Moghaddassian M, Rolfs A, Varasteh AR. Molecular, biochemical, and structural analysis of a novel mutation in patients with methylmalonyl-CoA mutase deficiency. *Gene.* 2016 Jan; 576: 208-13.
9. Van Gosen L. Organic acidemias: a methylmalonic and propionic focus. *J Pediatr Nurs.* 2008 Jun; 23 (3):225-33.
10. Tanaka K, West-Dull A, Hine DJ, Lynn TB, Lowe T. Gas-Chromatographic Method of Analysis for Urinary Organic Acids. II. Description of the Procedure, and Its Application to Diagnosis of Patients with Organic Acidurias. *Clin chem.* 1980 Dec; 26 (13): 1847- 53
11. Kuhara T. Diagnosis and monitoring of inborn errors of metabolism using urease-pretreatment of urine, isotope dilution, and gas chromatography-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2002 Dec; 781 (1-2):497-517.
12. Rosenberg LE, and Scriver CR. Disorders of amino acid metabolism. In: *Metabolic Control and Disease*, Bondy PK, Rosenberg LE, Saunders WB. 8th ed., 1980, pp 583-776.

disorder when GC-MS are not available. We are now improving and expanding the GC-FID method to diagnose methylmalonic acidemia, aiming to support pediatric clinics in Iran and assist in clinical diagnostics.

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