Clinical Application of Liquid Chromatography Tandem Mass Spectrometry Using Dried Blood Spot as a More Rapid Method for Determination of Methylmalonic Acid, Propionylcarnitine, and Total Homocysteine

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Abstract
Methylmalonic acidemia (MMA) should be diagnosed in early infancy and receive appropriate management promptly after the diagnosis to prevent severe complications leading to death. At present, a newborn screening (NBS) method using tandem mass spectrometry (MS/MS) identifies suspected patients with MMA by elevated propionylcarnitine. In addition, a liquid chromatography tandem mass spectrometry (LC/MS/MS) method using dried blood spot is effective to detect some metabolites as a second-tier test, and reduces the false-positive rate in NBS. However, these tests were only used in screening, and not applied as an examination for evaluating treatment. Herein, we describe a 57-day-old girl with MMA under treatment with cobalamin who had elevated urinary methylmalonic acid levels. We applied the LC/MS/MS method with a separation column to evaluate her cobalamin responsiveness, and discovered an insufficient cobalamin dose earlier than would have been possible using other methods. Based on the current data, this method seems to be applicable for the follow-up of the treatment of MMA patients. However, this should be confirmed with more experience with a larger number of cases and a wider spectrum of disorders.

Keywords
cobalamin responsiveness, liquid chromatography tandem mass spectrometry, methylmalonic acidemia, newborn screening.

Introduction
Methylmalonic acidemia (MMA) is a relatively common autosomal recessive inherited disorder caused by a deficiency of methylmalonyl-CoA mutase (MCM) or its cofactor adenosylcobalamin. Methylmalonyl-CoA apoenzyme deficiencies are subdivided into two subgroups, i.e., mut⁺ defect with some residual activity in the presence of high concentrations of adenosylcobalamin and mut⁻ defect with undetectable activity.[1] Patients with MMA display elevation of levels of propionylcarnitine (C3), methylmalonic acid, methylcitric acid, and 3-hydroxypropionic acid. These metabolites are derived from the propionate pathway, namely, isoleucine, valine, threonine, methionine, odd-chain fatty acids, and cholesterol metabolism.[2] Although the clinical presentation of patients with MMA is variable, the principal symptoms are vomiting, metabolic acidosis, hyperammonemia, and encephalopathy that could cause death. In addition, MMA patients with neonatal onset usually have a severe clinical course.[1,3–4] Even under appropriate metabolic management, patients with MMA can experience metabolic attacks and multisystemic disorders, including progressive renal disease,[3–4] cardiomyopathy,[5]

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neurodevelopmental disorders,[6–7] and pancytopenia.[8] These complications increase the morbidity and mortality rate in infancy and childhood.[1,4] To prevent this tragic progression, MMA should be diagnosed in early infancy and continue to receive appropriate management.

Recently, newborn screening (NBS) using tandem mass spectrometry (MS/MS) identifies suspected patients with MMA by elevated C3,[9–10] and effective new second-tier tests shorten the diagnostic process.[11–13] Although these tests improve the accuracy and speed of screening, they are not usually applied as an examination for evaluating treatment.

Herein, we describe a simple liquid chromatography tandem mass spectrometry (LC/MS/MS) method using dried blood spot (DBS) with a separation column, which can provide the acylcarnitine profile, methylmalonic acid level, and total homocysteine (tHcy) level in the same sample. The present article aimed to evaluate the effectiveness of this method for diagnosis and treatment of MMA.

Case Report

A 57-day-old Japanese girl with MMA was referred to our hospital because of the family’s recent relocation to Japan. She was the first child born to non-consanguineous Japanese parents, and had no significant family history. She was born at 38 weeks after an uneventful pregnancy in Dubai, where NBS identified elevated C3 (6.70 µmol/L; reference interval, < 3.60 µmol/L). At 12 days old, she had elevated C3 (9.17 µmol/L) and increased ratio with acetylcarnitine (C3/C2) (0.57; reference interval, < 0.25) on a secondary screening test. At 30 days old she was hospitalized to follow up the abnormal NBS results. A urinary organic acid analysis revealed a massive level of methylmalonic acid (8698 µmol/mmol creatinine; reference interval, < 11 µmol/mmol creatinine), without metabolic acidosis. Her ammonia level (62 µmol/L), cobalamin level (996 pg/mL), and serum homocysteine level (6.3 µmol/L) were normal. The acylcarnitine profile showed low free carnitine (11.7 µmol/L; reference interval, 27–49 µmol/L), and high acylcarnitine level (32.1 µmol/L; reference interval, 7–19 µmol/L). MMA was diagnosed and the patient was started on hydroxycobalamin injection 1 mg/day and oral L-carnitine 100 mg/kg/day. Urinary organic acid analyses, repeated at 4 and 10 days after initiation of treatment, showed decreased methylmalonic acid (4735 and 2823 µmol/mmol creatinine, respectively).

When the patient returned to Japan and was admitted to our hospital, her height and weight were 54.4 cm (–1.0 SD) and 4.59 kg (–1.0 SD), respectively. She was developing normally with protein-restricted milk (92 kcal/100 mL, natural protein 0.5 g/100 mL). She did not present with any symptoms (hepatosplenomegaly, muscle weakness, or neurological abnormalities). Methylmalonic acid in urine was still high (0.432/10 µg HDA; reference interval, < 0.007/10 µg HDA; heptadecanoic acid [HDA] as an internal standard) in spite of drug treatment and dietary restriction. Laboratory data indicated normal levels of serum transaminases (AST 26 IU/L and ALT 20 IU/L) and ammonia (57 µmol/L), with no metabolic derangements such as hypoglycemia, hyperlipidemia, hyperuricemia, or hyperlactacidemia. Because of the high value of urinary methylmalonic acid with the previous dose of cobalamin, we increased the dose of cobalamin from 1 mg/day to 10 mg/day to assess cobalamin responsiveness accurately.

Materials and Methods

Written informed consent was obtained from the patient’s parents for all procedures and tests described herein, per the tenets of the Declaration of Helsinki, in addition to consent for publication of the patient’s data.

MS/MS analysis

The primary NBS assay that measures amino acids and acylcarnitines was carried out using the flow injection method (no separation column in the system; FI/MS/MS). The analysis was performed by a TQ Detector coupled with a 1525 µ binary HPLC pump and 2777C sample manager (Waters, Milford, MA, USA). LC/MS/MS analysis was performed by a Nexera HPLC system, coupled with an LCMS-8050 (Shimadzu Corporation, Kyoto, Japan) for the second-tier test, tHcy and methylmalonic acid analysis on DBS. Acylcarnitines and LC/MS/MS (assays of tHcy and methylmalonic acid) were analyzed separately, in three different assays from three separately punched DBSs from a single sample.

Electrospray ionization source was operated in both negative and positive mode for methylmalonic acid and the others, respectively. The LC separations were carried out on a SUPELCO Discovery HS F5-3 (150 × 2.1 mm, 3 µm) from Sigma-Aldrich Japan K.K. (Tokyo, Japan) and a Scherzo SS-C18 ion exchange multi-mode ODS column (150 × 3 mm, 3 µm) from Imtakt Corporation (Kyoto, Japan) for tHcy and methylmalonic acid, respectively. Sample preparation for the second-tier test was carried out using modification of the method by Turgeon et al.[14] LC conditions of tHcy and methylmalonic acid are shown in Supplementary Tables S1 and S2, respectively. The instrument was optimized automatically by an internal algorithm to monitor the transitions m/z 136.00 to m/z 90.1 and m/z 140.0 to m/z 94.10 for unlabeled and deuterium-labeled (d4) Hcy, and m/z 117.10 to m/z 73.15 and m/z 120.10 to m/z 76.20 for unlabeled and deuterium-labeled (d3) methylmalonic acid. D4-Hcy was converted from d8-homocystine after reduction by dithiothreitol added to the reaction solution. The results were acquired using LabSolutions Version 5.91 and LabSolutions Insight LCMS Version 3.10 (Shimadzu Corporation, Kyoto, Japan). Injection volume was 10 µL and 2 µL for acylcarnitines and LC/MS/MS, respectively.
Analysis of MCM activity

MCM activity was analyzed using a previously described method based on ultrahigh-performance LC/MS/MS.[15] MCM was obtained from 5x10^5 lymphocytes in Tris-sulfate buffer (pH 7.5) lysed by sonic disruption, and then adenosylcobalamin was added to the resultant lysate. After this solution was warmed at 37°C for a few minutes, it was spiked with methylmalonyl-CoA and incubated at 37°C for 15 min. The reaction was quenched with HClO_4, and acetyl-CoA (Internal Standard solution) was added. The solution was centrifuged, and the supernatant was analyzed by ultrahigh-performance LC/MS/MS to determine produced succinyl-CoA. MCM activity was evaluated using quantitation of succinyl-CoA produced.

Urinary organic acid analysis

The urine samples were spiked with 10 µg heptadecanoic acid (HDA) and 3-hydroxy myristic acid as internal standards, and treated with urease to remove interfering urea by a procedure extensively modified from that of Matsumoto and Kuhara.[16] The volume of urine including 20 µg of creatinine was used. Protein was precipitated with 900 µL of ethanol and removed by centrifugation. The deproteinized solution was then evaporated to dryness. Compounds of interest in the dried residue were converted to trimethylsilyl derivatives with 100 µL of N,O-Bis (trimethylsilyl) trifluoroacetamide with chlorotrimethylsilane and analyzed by gas chromatography/mass spectrometry.

Mutation analysis

Genomic DNA was extracted from the peripheral blood leukocytes of the patient. A mutation analysis of MMUT, PCCA, PCCB, ABCD4, HCF1, LMBRD1, MMAA, MMAB, MMACHC, and MMADHC was performed by next-generation sequencing (Nextseq Sequencing System, Illumina, San Diego, CA, USA) at the Kazusa DNA Research Institute. Common genetic variations were identified using the following public databases: Exome Aggregation Consortium (ExAC), NCBI ClinVar, Human Genetic Variation Database (HGVD), and Human Gene Mutation Database (HGMD).

In silico analysis

Phylogenetic information was obtained via Vertebrate Multiz Alignment & Conservation in the UCSC Genome Browser (https://genome.ucsc.edu/). We used PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and PROVEAN and SIFT (http://provean.jcvi.org/index.php) predictive algorithms to evaluate the pathogenicity of the identified sequence variants.

Results

The results indicated that C3, C3/C2, and methylmalonic acid levels were decreased on DBS, although tHcy was stable at the normal value. A urinary organic acid analysis also revealed a decrease in methylmalonic acid. Plasma amino acid analysis could not detect homocysteine. These examinations were performed simultaneously 14 days after increasing the dose of cobalamin (Table 1). The LC/MS/MS method took 3-5 days until results were given to physicians, whereas the urinary organic acid analysis took 2-3 weeks.

The amounts of produced succinyl-CoA in the patient were low (mean 0.35 pmol/min/5.0x10^5; 0.332 and 0.372 pmol/min/5.0x10^5) compared with those in control samples (mean 42.67 pmol/min/5.0x10^5; 40.97, 42.08, and 44.97 pmol/min/5.0x10^5). These data revealed that MCM activity in our patient was 0.82% of the control.

Next-generation sequencing revealed heterozygous missense mutations (c.1292T>C [p.Met431Thr, located in exon 6] and c.1808G>A [p.Arg603Lys, located in exon 10]) in a responsible gene, MMUT, of the patient. The former mutation was novel, whereas the latter was previously reported as mut^MMA.

Table 1. Laboratory data for the case before and after cobalamin increase

| Age (months) | 1    | 2    | 3    | 7    | 13   | 18   | Cut-off value |
|--------------|------|------|------|------|------|------|---------------|
| Natural protein (g/kg/day) | –    | 0.8  | 0.8  | 0.9  | 1.6  | 1.8  |               |
| Cobalamin (mg/day) | 0    | 1    | 10   | 10.5 | 10.5 | 10.5 |               |
| MS/MS in DBS (µmol/L) | 9.17 | 6.79 | 2.52 | 5.45 | 7.36 | 6.05 | < 3.60        |
| LC/MS/MS | C3/C2 | 0.57 | 0.37 | 0.08 | 0.20 | 0.28 | 0.28 | < 0.25        |
| MMA | –    | 11.46| 1.02 | –    | –    | –    | < 3.00        |
| tHcy | –    | 2.37 | 2.64 | –    | –    | –    | < 7.00        |
| Urinary organic acid analysis (/10 µg HDA) | MMA | –    | 0.432| 0.043| 0.295| 0.434| 0.373| < 0.007       |
| Plasma amino acid analysis (nmol/mL) | tHcy | 6.3  | –    | ND   | –    | –    | –    | > 15.0        |

F/MS/MS, flow injection tandem mass spectrometry; LC/MS/MS, liquid chromatography tandem mass spectrometry; DBS, dried blood spot; MMA, methylmalonic acid; tHcy, total homocysteine; ND, not detected
According to ClinVar, ExAC, HGVD, and HGMD, p.Met431Thr was not listed. Both Met431 and Arg603 are conserved throughout species. Upon PolyPhen-2, PROVEAN, and SIFT analyses, p.Met431Thr was predicted as “probably damaging”, “deleterious”, and “damaging”. However, PolyPhen-2, PROVEAN and SIFT analyses predicted p.Arg603Lys to be "benign", "neutral", and “tolerated” respectively.

At present, the patient is 18 months old and developing normally with no symptoms, metabolic acidosis, or hyperammonemia under high-dose cobalamin (10.5 mg/day), oral L-carnitine (50 mg/kg/day), and mild dietary restriction (natural protein 1.8 g/kg/day).

Discussion

The patient’s disease was diagnosed as MMA and she was started on treatment with a combination of cobalamin and carnitine, but the value of her urinary methylmalonic acid remained high because of the insufficient dose of cobalamin in Dubai. Using the LC/MS/MS method and DBS with the separation column, we could quickly determine cobalamin responsiveness in this case and relieve dietary restrictions by increasing the dose of cobalamin. Although the values of C3 and urinary methylmalonic acid increased after alleviation of protein restriction, these data at 18 months (natural protein 1.8 g/kg/day) were lower than before increasing the dose of cobalamin (natural protein 0.8 g/kg/day). In addition, our patient’s growth and development are good, and she has had no clinical symptoms. Therefore, we believe that reintensification of protein restriction is not necessary under high-dose cobalamin.

There is a few report on the evaluation of high doses cobalamin for MMA patients. Recent case series (3 cbl C patients, 1 cbl A patient, and 1 cbl IX patient) showed that higher cobalamin dose produced biochemical responses such as lower range plasma tHcy and higher methionine values as well as low urinary methylmalonic acid.[20] The mechanism of cobalamin responsiveness may be explained by distinct mutations in the MMUT gene, same as the paper of tetrahydrobiopterin responsive phenylalanine hydroxylase deficiency.[21] In our patient, two mutations, c.1292T>C and c.1808G>A, were identified in a compound heterozygous state, in a potentially recessive pattern of inheritance. The novel mutation, c.1292T>C (p.Met431Thr) is responsible for the cobalamin responsiveness described in this case. Therefore, on the basis of the above findings, our patient’s disease was diagnosed as mut- MMA.

Although screening using MS/MS reveals suspected patients with MMA by elevated C3,[9–10] the diagnostic process after a primary screening is typically complex.[2] To distinguish between MMA and propionic acidemia, elevated levels of methylmalonic acid, 3-hydroxypropionic acid, and methylcitric acid should be detected by urinary organic acid analysis. Isolated elevation of urinary methylmalonic acid without hyperhomocysteinemia and low cobalamin level is caused by MMA, including deficiency of MCM, Cbl A, Cbl B, and Cbl D. Cobalamin responsiveness should also be assessed in every MMA patient by repeating a urinary organic acid analysis or acylcarnitine profile analysis. Traditionally, this process takes time and often causes treatment delay. In addition, NBS using MS/MS creates a high number of false-positive cases, leading to frequent recalls, family anxiety, long time to diagnosis, and unnecessary medical cost.[12,23] Decreased gestational age and increased body weight are associated with increased C3, which enhances the false-positive rate.[24]

Recently, to simplify the second-tier testing, and to reduce the false-positive rate in NBS, effective methods have been reported. For example, LC/MS/MS revealed the simultaneous determination of tHcy, methionine, methylmalonic acid, and methylicitric acid in plasma or serum,[13] and another paper reported that LC/MS/MS detected methylmalonic acid, 3-hydroxypropionic acid, and methylcitric acid in the DBS as in primary screening.[12] DNA analysis using next-generation sequencing is also beneficial.[11] Although these second-tier tests decrease time to diagnosis, and the emotional and financial burdens of unneeded medical precautions, they are currently only used as assays in NBS, not as examinations for evaluating treatment. Wang et al reported good correlation between DBS methylmalonic acid concentration by LC/MS/MS and urinary methylmalonic acid concentration by gas chromatography mass spectrometry. In addition, they mentioned the promise of LC/MS/MS for the follow-up of children with MMA.[25] However, they did not use LC/MS/MS in clinical practice for patients with MMA.

Patients with MMA may suffer from metabolic strokes and hyperammonemia during the course of treatment, even if adequate at the time of diagnosis. Movement disorders, spastic quadraparesis, or paraparesis are potential sequela of metabolic strokes.[4] A history of hyperammonemia was related to lower measures for full-scale IQ.[7] Therefore, before triggering a metabolic attack, it is important to evaluate whether the current management method is valid.

The LC/MS/MS method, particularly using labeled molecules as internal standards, is characterized by increased accuracy...
and precision. Persichilli et al. showed that several LC/MS/MS methods have been reported for tHcy determination showing superior analytical quality and high throughput, some of which are also applicable to large routines.[26]

Herein, we described a simple and accurate LC/MS/MS method with a separation column, which can provide the acylcarnitine profile, methylmalonic acid level, and tHcy level in the DBSs in the same sample. This method revealed cobalamin responsiveness of the patient with MMA earlier than would have been possible using other methods. Based on the current data, this method seems to be applicable for the follow-up of the treatment of MMA patients. Furthermore, this rapid determination in DBS allows for better second-tier tests that would allow screening of combined MMA/homocystinuria conditions (cbl C, D, F, J), and evaluates cobalamin responsiveness in the follow-up of positive newborn screen cases for MMA. However, this should be confirmed with more experience with a larger number of cases and a wider spectrum of disorders.

Conclusion

This article described a simple, rapid, and accurate LC/MS/MS method, which can be applied not only to screen for MMA, but also to develop therapeutic strategy.

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Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Supplementary Material

The following online material is available for this article:
Table S1 - LC conditions for analysis of tHcy
Table S2 - LC conditions for analysis of methylmalonic acid

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