APPLICATION NOTE

Measurement of methylmalonic acid and 3-hydroxypropionic acid in dried blood spot using QSight MD mass spectrometer.

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Introduction

Elevated propionylcarnitine (C3) is commonly applied indicative marker for several metabolic disorders including propionic acidemia and methylmalonic acidemia. More specific biomarkers for these same two disorders are however methylmalonic acid (MMA) and 3-hydroxypropionic acid (3OHPA). An accumulation of these metabolites is caused by defected Methylmalonyl-CoA mutase or Propionyl-CoA carboxylase enzyme, respectively (Figure1).

These two biomarkers (MMA and 3OHPA) cannot be detected by methods using the direct flow injection analysis tandem mass spectrometry (MS/MS) assay without liquid chromatography (LC) separation. To improve this situation, we report here more selective method to measure MMA and 3OHPA concentrations in dried blot spot (DBS) samples by a rapid isocratic LC-MS/MS run.

Quick Facts:

- 2nd tier test method for monitoring and quantifying methylmalonic acid and 3-hydroxypropionic acid on dried blood spot
- Reduce or even eliminate false-positive results for propionylcarnitine (C3)
- $R^2 > 0.998$ for both compounds
- Monitoring for methylmalonic acidemia and propionic acidemia

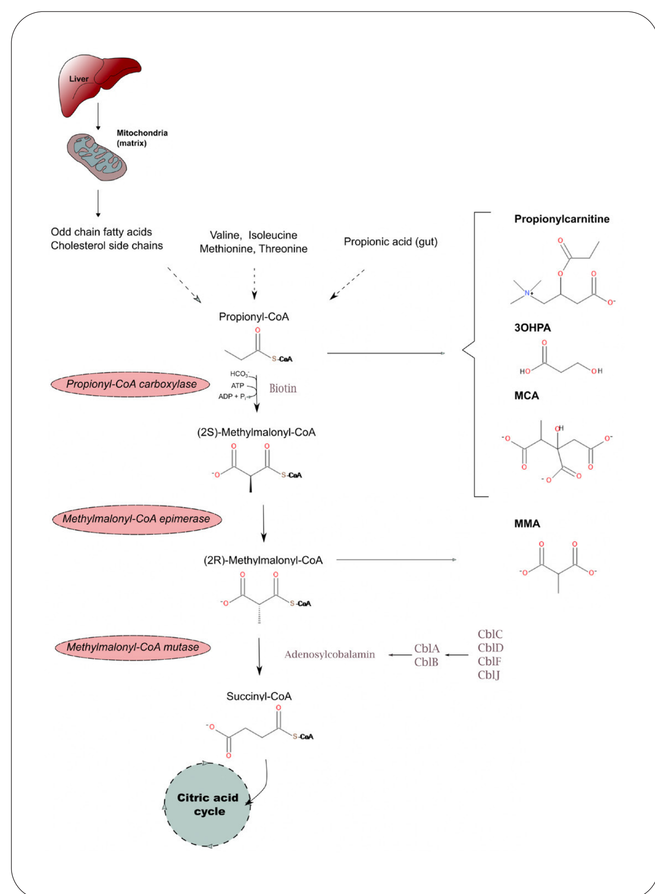

Figure 1: Biochemical pathway of propionate metabolism (simplified). 3OHPA: 3-hydroxypropionic acid; MMA: methylmalonic acid; MCA: methylcitric acid;

2. Methods

A 3.2 mm DBS disk was punched into the microplate well and incubated with 200 µL of extraction solution at 37oC for 20 minutes under stirring at 650-750 rpm. During the incubation the adhesive microplate cover was used to minimize evaporation. The extraction solution (50:50 acetonitrile: water with 0.05% formic acid) also contained the $^{2}H_3$-MMA internal standard (4 µM) for quantitation. After incubation the supernatant was transferred to a new microplate (or vial) and then 10 µL were injected in the LC-MS/MS system.The quantitative determination of both MMA and 3OHPA analytes was performed by isotope dilution method using the same isotopically labelled $^{2}H_3$-MMA internal standard (Cambridge Isotope Laboratories, Andover, MA, USA). This was because isotopically labelled form of 3OHPA is not commercially available.

2.1. Mass spectrometry conditions

The LC-MS/MS analysis was performed using the QSight® 210 MD mass spectrometer equipped with ESI source and operated in negative ion mode. Table 1 outlines the MS instrumental source parameter settings used during this method. The optimized MRM transition parameters for all the analytes are shown in Table 2.

Table 1: MS conditions

ESI voltage (V)	- 4800
HSID™ temp (°C)	250
Nebulizer gas setting	250
Drying gas setting	100
Source temp. (°C)	200
Dwell time (ms)	30

Table 2: Optimized MRM and compound dependent parameters

Compound	Precursor (m/z)	Fragment (m/z)	CCL 2	CC	EV
3OHPA	89.0	58.9	20	14	-6
Lactic acid (LA)	89.0	43.0	17	17	-20
MMA	116.9	73.1	23	13	-7
$^{2}H_{3}$-MMA (IS)	119.9	76.1	23	13	-7
Succinic acid (SA)	117.0	73.0	30	17	-7

2.2. LC conditions

The LC separation was performed using a Phenomenex Gemini C6-phenyl column (150 X 2.00 mm, 3 µm) and precolumn cartridge (4 X 2.00 mm). The LC was run with an isocratic flow with a run time of 4 min under the following conditions:

Mobile Phases: A water with 0.05% formic acid
B acetonitrile with 0.05% formic acid

Isocratic flow ratio: 50% B

Flow rate: 200 µL/min

Injection volume: 10 µL

Column temperature: RT

3. Results

As shown in Figure 2, the above described LC conditions permitted to resolve the MMA and 3OHPA analytes from two commonly seen and disturbing isobaric endogenous compounds: succinic acid (SA) and lactic acid (LA), respectively. The retention times of LA, 3OHP, SA and MMA were 1.61, 1.92, 2.07 and 2.68, respectively.

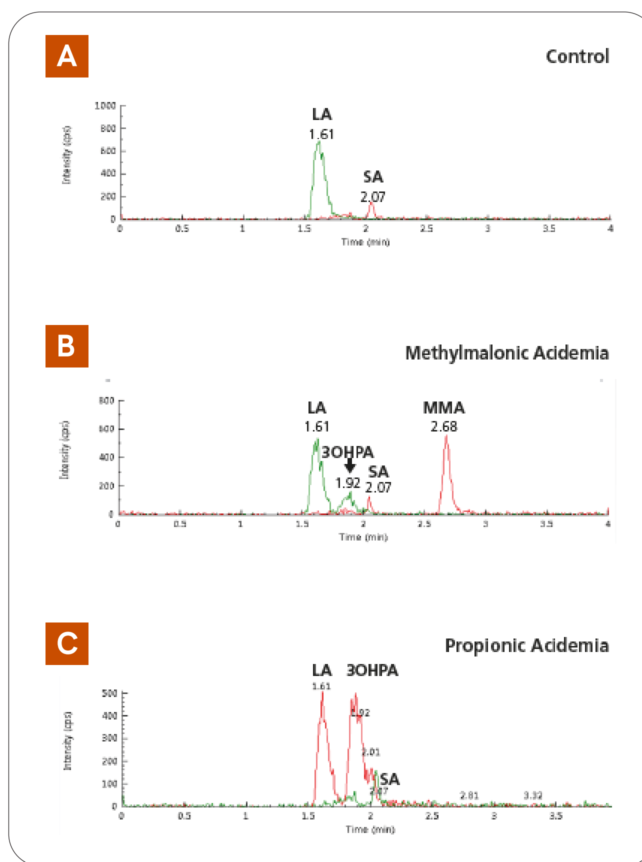

Figure 2: Extracted ion chromatograms of control (A), Methylmalonic Acidemia (B) and Propionic Acidemia (C) samples.

The LC-MS/MS method linearity was demonstrated by using several enriched concentrations of DBS calibrators. The determined linearity ranges were: 2.5-100 µM for MMA and 10- 400 µM for 3OHPA. As shown in Table 3 and Figure 3, good overall linearity (coefficient of determination R^2) results and reasonable recovery (%) ranges were also obtained for both MMA and 3OHPA analytes.

Table 2: Linearity and recovery results in DBS.

Analyte	Linearity (R^2)	Recovery (%)
MMA	0.9987	78-110
3OHPA	0.9989	84-116

Figure 3: Calibration curves in DBS.

4. Conclusions

The results obtained confirm the applicability of LC-MS/MS method for measuring and monitoring the MMA and 3OHPA biomarker concentrations in DBS samples. This reported method can be potentially used for the 2nd tier testing of propionic acidemia and methylmalonic acidemia to reduce commonly seen false-positive rates for C3 marker only.

References

1. La Marca G et al. Rapid 2nd-Tier Test for Measurement of 3-OH-Propionic and Methylmalonic Acids on Dried Blood Spots: Reducing the False-Positive Rate for Propionylcarnitine during Expanded Newborn Screening by Liquid Chromatography–Tandem Mass Spectrometry. Clin Chem 2007; 53: 1364-1369.

2. Monostori P et al. Simultaneous determination of 3-hydroxypropionic acid, methylmalonic acid and methylcitric acid in dried blood spots: Second-tier LC-MS/MS assay for newborn screening of propionic acidemia, methylmalonic acidemias and combined remethylation disorders.. PLoS ONE 12(9): e0184897. https://doi.org/10.1371/journal.pone.0184897

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