APPLICATION NOTE

Measurement of alloisoleucine and branched-chain amino acids in dried blood spot using QSight® 210 MD mass spectrometer.

Authors

Lorenzo Bacci
Roberto Bozic
Tero Lehtonen

Revvity, Inc.

Introduction

Maple syrup urine disease (MSUD) is an autosomal recessively inherited disease with a worldwide incidence of ca. 1:185000. It is caused by a defect of the branched chain alpha-keto acid dehydrogenase multienzyme complex (BCKDH). This results in a toxic accumulation of the branched chain amino acids (BCAAs): leucine (Leu), isoleucine (Ile) and valine (Val), and their corresponding alphaketo (i.e. 2-oxo) acid by-products in the blood, urine and body tissues (Figure 1).

MSUD is commonly based on the measurement of increased BCAA concentrations in blood as a sum of Leu, Ile and Val. The only pathognomonic marker for MSUD is D-alloisoleucine (Allo-Ile). By direct flow injection analysis tandem mass spectrometry (MS/MS) assay without liquid chromatography (LC) separation, this specific biomarker cannot be however distinguished from isobaric amino acids like Leu, Ile and 3-hydroxyproline (3-OH-Pro). To allow more specific marker measurement and to reduce false positive rates (also diet-related) in the MSUD screening, we report here a gradient LC-MS/MS method for quantifying Allo-Ile concentration in dried blood spot (DBS).

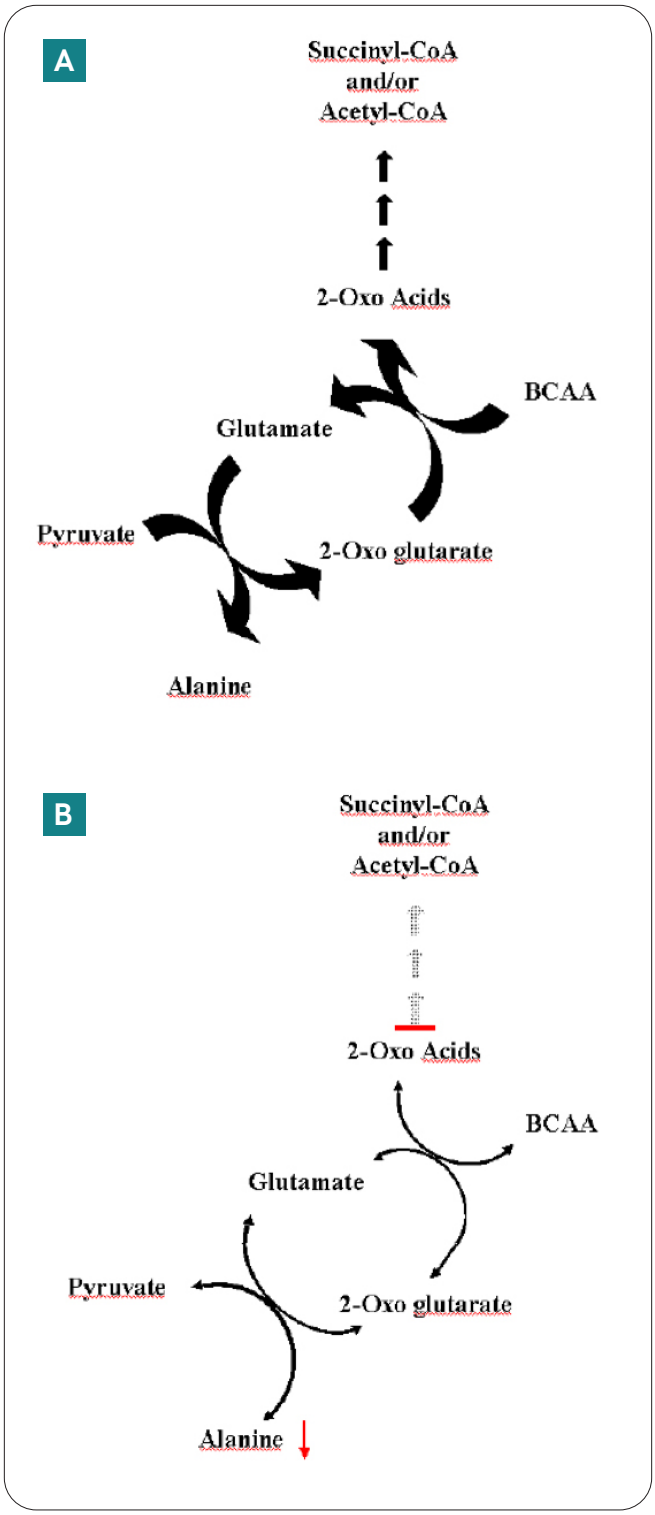

Figure 1: Alanine metabolism in healthy humans (a) and in patients with MSUD (b).

2. Method

A 3.2 mm DBS disk was punched into the microplate well and incubated with 150 µL of extraction solution at room temperature for 20 minutes under stirring at 650-750 rpm. During the incubation the adhesive microplate cover was used to minimize evaporation. The extraction solution (100% methanol) also contained the d_8-Val, d_3-Leu and d_{10}-Allo-Ile internal standards (5 µM) for quantitation. After incubation the supernatant was transferred to a new microplate, evaporated until dryness under gentle nitrogen flow and reconstituted with 50 µL of 0.1% tridecafluoroheptanoic acid (TDFHA) in water. The microplate was stirred (at 650-750 rpm) for 10 minutes before the analysis. Sample injection volume in the LC-MS/MS system was 10 µL and quantitative determination of Val, Leu and Allo-Ile analytes was performed by isotope dilution method using isotopically labelled d_8-Val, d_3-Leu and d_{10}-Allo-Ile internal standards (Cambridge Isotope Laboratories, Andover, MA, USA), respectively[1,2].

2.1. Mass spectrometry conditions

The LC-MS/MS analysis was performed using the QSight® 210 MD triple quadrupole mass spectrometer equipped with ESI source and operated in positive 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)	5850
HSID temp (°C)	190
Nebulizer gas setting	130
Drying gas setting	105
Source temp. (°C)	175
Dwell time (msec)	30

Table 2: Optimized MRM parameters

Compound	Precursor (m/z)	Fragment (m/z)	CCL 2	CC	EV
Leu/Ile/Allo-Ile	132.1	86.1	-27	-15	16
3-OH-Pro	132.1	86.1	-27	-15	16
Val	118.1	72.1	-24	-16	16
d8-Val (IS)	126.1	80.1	-24	-16	16
d3-Leu (IS)	135.2	89.2	-27	-15	16
d10-Allo-Ile (IS)	142.2	96.2	-27	-15	16

2.2. LC conditions

The LC separation was performed using a Supelco Discovery C18 column (50 mm x 2.1 mm, 5 µm) from Sigma Aldrich. The LC run time was ca. was 6 min with the following mobile phases and gradient conditions:

Mobile Phases: A H_2O with 0.1% TDFHA
B ACN with 0.1% TDFHA

Injection volume: 10 µL

Column temperature: RT

Gradient:

Time	Flow rate	%B
0	300	20
1	300	20
3	300	30
3.1	300	90
5	300	90
5.1	300	20
6	300	20

3. Results

When elevated Leu concentration is found in the NBS, the same blood sample is typically analyzed by LC-MS/MS to separate different Leu isomers: Ile, Allo-Ile and 3-OH-Pro. If the peak corresponding to Allo-Ile appears together with elevated Ile and Leu, the result supports the classical MSUD diagnosis.

The above reported LC-MS/MS method permits such a separation and quantification of Allo-Ile and other BCAAs from a single dried blood spot. The separation of isobaric compounds 3OH-Pro, Allo-Ile, Ile and Leu is demonstrated in Figure 2, with the retention times 0.85, 3.30, 3.46 and 3.70, respectively. Val concentration can be also monitored in this same assay, but it has not been shown in Figure 2 because only separation of isobaric compounds is highlighted.

As seen in the Figure 2 chromatograms A-C, the separated Allo-Ile peak is absent in healthy control sample (A), but clearly appears with true positive MSUD sample (B) in addition to elevated Ile and Leu. Also, the sample C, which was found presumptive MSUD positive in the initial MS/MS screen due to elevated Leu, the LC-MS/MS analysis confirmed this is actually a false-positive, because the initial abnormal result was due to elevated 3-OH-Pro, and not elevated Allo-Ile.

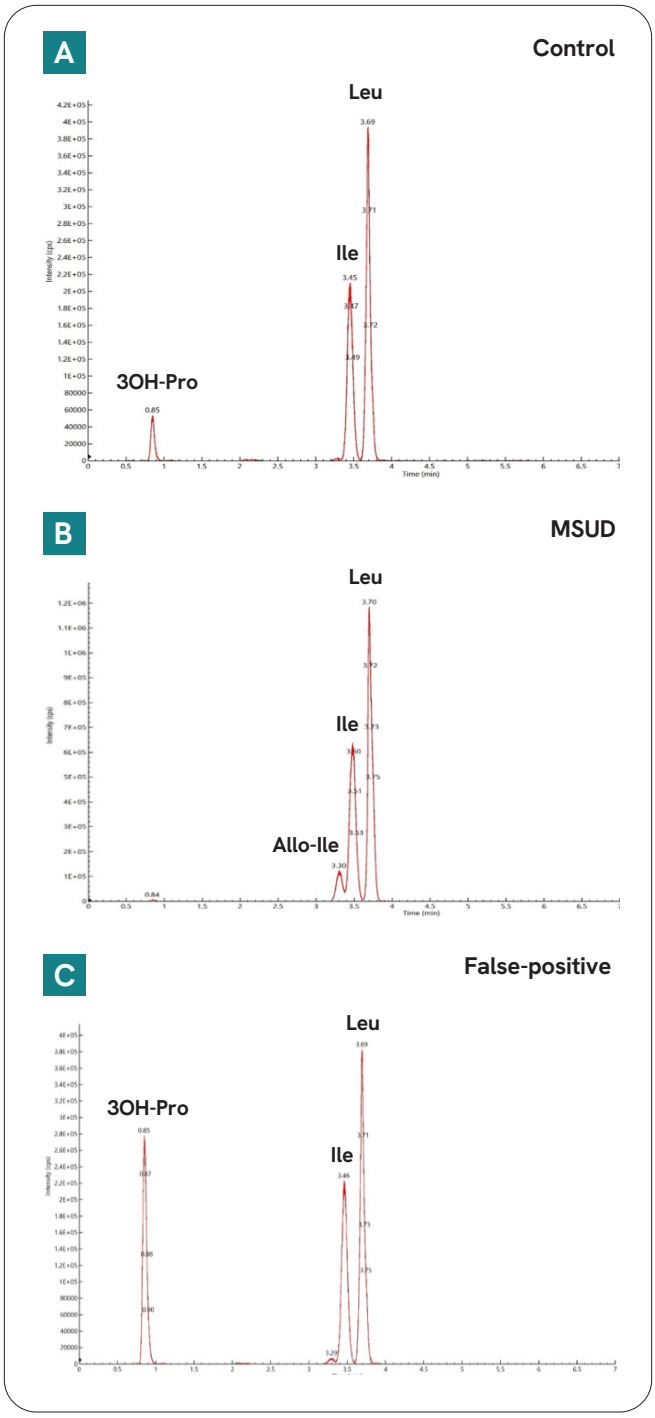

Figure 2: Extracted ion chromatograms of healthy control (A), MSUD positive (B) and false-positive samples (C).

4. Conclusions

The results obtained confirm the applicability of LC-MS/MS method for measuring and monitoring the BCAAs and alloisoleucine biomarker concentrations in DBS samples.

References

1. Zoppa M. et al, Method for the quantification of underivatized amino acids on dry blood spots from newborn screening by HPLC–ESI–MS/MS. J. Chromatogr. B 831 (2006) 267-273.

2. Oglesbee D. et al, Second-Tier Test for Quantification of Alloisoleucine and Branched-Chain Amino Acids in Dried Blood Spot to Improve Newborn Screening for Maple Syrup Urine Disease (MSUD). Clin. Chem. 54:3 (2008) 542-549.

3. Fingerhut R. et al, A Rapid and Sensitive UPLC-MS/MS-Method for the Separation and Quantification of Branched-Chain Amino Acids from Dried Blood Samples of Patients with Maple Syrup Urine Disease (MSUD). Int. J. Neonatal Screen 2 (2016) 2.