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Reducing the False Positive of Isovaleric Acidemia in Newborn Screening using Flow Injection Analysis-Tandem Mass Spectrometry

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1. Overview

In this study, we investigated a flow injection analysis-tandem mass spectrometry (FIA-TMS) method to distinguish isovalerylcarnitine (i-C5) and pivaloylcarnitine (p-C5). The reference ion ratio of p-C5 was higher than that of i-C5. Similarities of the reference ion ratio of i-C5 and p-C5 (i-C5 score and p-C5 score) were used to distinguish i-C5 and p-C5 in dried blood spots (DBS). In DBS samples with high level of C5-acylcarnitine, it was possible to correctly distinguish i-C5 or p-C5 using i-C5 score and p-C5 score.

2. Introduction

Newborn screening (NBS) is an important public health program for improving children's health in most developed and developing countries. It has provided early diagnosis and early treatment of congenital diseases, and has been prevented babies from becoming handicapped or even dying. FIA-TMS has been applied to NBS testing. Isovaleric acidemia (IVA) is one of the organic acidemias, and is a target disease for NBS. Although i-C5 is an index of IVA, i-C5 cannot be distinguished from p-C5 originating from the use of pivalate-conjugated antibiotics by FIA-TMS. Therefore, there are many reports of the false positive derived from p-C5. In addition to p-C5, 2-methylbutyrylcarnitine (m-C5) and n-valerylcarnitine (n-C5) exist as isomers of C5-acylcarnitine.
Several reports have reported the usefulness of secondary testing using column separation by LC-MS/MS, but a simpler method is preferred in terms of cost and time for operation.
In this study, we developed a new FIA-TMS method for a first-tier test to distinguish i-C5 and p-C5 in DBS. Therefore, we investigated the methods to distinguish the isomers of C5-acylcarnitine using the specific product ion or the reference ion ratio under the conventional FIA-TMS condition. To confirm the effectiveness of this new FIA-TMS method in a first-tier test, DBS samples with high level of C5-acylcarnitine were analyzed.

3. Materials and Methods

3-1. Sample.

11 DBS samples (case1-11) with high level of C5-acylcarnitine derived from pivalate-conjugated antibiotics and 4 DBS samples (case12-15) with high level of C5-acylcarnitine derived from IVA patients were analyzed.

3-2. Sample Pretreatment

DBS samples were prepared in accordance with standardized protocols of non-derivatized method using NeoBase kit (Perkin Elmer). In brief, a single 3 mm DBS punch was placed in each well of 96-well assay plate. 100 µL of the extraction solution containing internal standard of acylcarnitines and amino acids was added to each well. The plate was shaken at 700 rpm at 45°C for 45 min and the supernatant was transferred to another plate for analyses.

3-3. Analytical Conditions

Nexera™ MP system coupled with a LCMS™-8050 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan) was used for FIA-TMS. Detailed analytical conditions to distinguish i-C5 and p-C5 are shown below.

UHPLC (Nexera MP system)

Mobile phase: NeoBase Kit
Flow rate: 0.1 mL/min (0 min)→0.05 mL/min (0.1 min)
→0.1 mL/min (0.65 min)→0.5 mL/min (0.66-1 min)
Injection vol.: 1 µL

MS (LCMS-8050)

Ionization: ESI (Positive mode)
Mode: MRM (*m/z* 246.2>85.0, 187.1)
DL temp.: 250°C HB temp.: 400°C
Nebulizing gas: 3.0 L/min Drying gas: 10 L/min

Figure 1 Nexera MP + LCMS-8050

4. Result

4-1. Reference Ion Ratio

In the MRM mode, the reference ion for qualitative analysis is used in addition to the quantitative ion for quantification. The reference ion ratio (ratio of peak intensity of reference ion to peak intensity of quantitative ion) is effective for identifying compounds. So, we investigated the reference ion ratio of i-C5, p-C5, m-C5 and n-C5. As product ions of C5-acylcarnitine, *m/z* 187.1, 85.0, 57.1, 41.1 and 29.1 were generated. From the viewpoint of peak intensity, the highest m/z 246.2>85.0 (CE: -23 V) and the second highest m/z 246.2>187.1 (CE: -15 V) were used as the quantitative ion and the reference ion, respectively. Other product ions (m/z 57.1, 41.1 and 29.1) were also examined as the reference ion, but they were not effective in terms of peak intensity and reproducibility. Figure 2 shows MRM chromatograms of i-C5, p-C5, m-C5 and n-C5. As results of continuous analyses (n=6), the reference ion ratio of i-C5, p-C5, m-C5 and n-C5 was 19.97±0.61%, 27.25±0.56%, 22.89±0.43% and 18.07±0.77%, respectively.

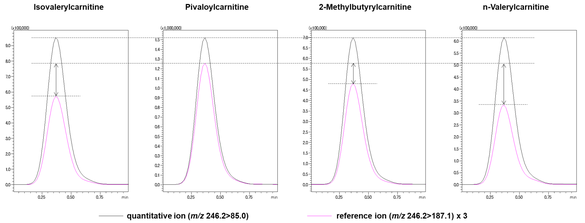

Figure 2 MRM chromatograms of C5-acylcarnitine isomers

There was no so big difference in the reference ion ratio among i-C5, m-C5 and n-C5, but the reference ion ratio of p-C5 was higher than that of other isomers. It was suggested that p-C5 can be distinguished from other isomers by the reference ion ratio. In DBS samples with high level of C5-acylcarnitine, the amount of m-C5 and n-C5 is lower than that of i-C5 and p-C5. The identification of m-C5 and n-C5 is not needed in a first-tier test. In a first-tier test, the identification of i-C5 and p-C5 is important. So, we considered the identification of i-C5 and p-C5 using similarities of reference ion ratio of i-C5 and p-C5. Similarities of reference ion ratio of i-C5 and p-C5 are described as i-C5 score and p-C5 score by the following equation:

$$\text{i-C5 score} = 100 - |R_{DBS} - R_{i\text{-}C5}| / R_{i\text{-}C5} \times 100$$
$$\text{p-C5 score} = 100 - |R_{DBS} - R_{p\text{-}C5}| / R_{p\text{-}C5} \times 100$$

where R_{DBS} is the reference ion ratio when DBS sample is analyzed, $R_{i\text{-}C5}$ is the reference ion ratio when the standard solution of i-C5 is analyzed and $R_{p\text{-}C5}$ is the reference ion ratio when the standard solution of p-C5 is analyzed.

4-2. Identification of i-C5 and p-C5 in DBS

To investigate the effectiveness of i-C5 score and p-C5 score for identification of i-C5 and p-C5 in DBS, 11 of DBS samples (case1-11) with high level of C5-acylcarnitine derived from pivalate-containing antibiotics and 4 of DBS samples (case12-15) with high level of C5-acylcarnitine derived from IVA patients were analyzed by LC-MS/MS (n=4) and FIA-TMS (n=8). LC-MS/MS was conducted to investigate the percentage of i-C5, p-C5, m-C5, and n-C5 in these DBS samples. The percentage of i-C5, p-C5, m-C5 and n-C5, the concentration of C5-acylcarnitine, i-C5 score, and p-C5 score are shown in Table1.

In analyses of 11 of DBS samples that were derived from pivalate-containing antibiotics, p-C5 score was higher than i-C5 score. The C5-acylcarnitine in these samples was correctly estimated as being from p-C5. In analyses of 4 of DBS samples that were derived from IVA patients, i-C5 score was higher than p-C5 score. The C5-acylcarnitine in these samples was correctly estimated as being from i-C5. The correlation coefficients between the percentage of i-C5 in DBS and i-C5 score and between the percentage of p-C5 in DBS and p-C5 score were 0.93 and 0.85, respectively, indicating a strong correlation.

Table 1 Analysis results of 15 of DBS samples

Case	% of total C5 isomers				C5 Conc. (µmol/L)	i-C5 Score	p-C5 Score
	i-C5	p-C5	m-C5	n-C5			
1	12.2	79.7	8.1	0	0.66	61.4±4.9	**97.3±2.1**
2	9.6	90.4	0	0	0.96	35.5±4.8	**80.8±4.8**
3	0	100.	0	0	1.31	56.7±5.0	**95.9±3.3**
4	0	100.	0	0	1.33	59.4±3.5	**97.4±1.6**
5	0	100.	0	0	1.37	48.7±3.0	**90.4±2.2**
6	0	100	0	0	1.64	55.1±1.2	**95.0±0.9**
7	0	100	0	0	1.66	55.2±1.6	**95.1±1.2**
8	0	100	0	0	1.90	55.5±3.7	**95.3±2.7**
9	0	100	0	0	3.72	58.8±1.6	**97.7±1.2**
10	0	100	0	0	3.83	42.3±1.8	**85.7±1.3**
11	0	100	0	0	10.98	60.3±1.5	**98.7±1.0**
12	100	0	0	0	2.57	**91.0±1.8**	79.0±1.3
13	100	0	0	0	2.66	**93.3±3.5**	77.3±2.6
14	100	0	0	0	5.32	**98.0±1.6**	72.6±1.9
15	99.9	0.1	0	0	13.61	**98.9±1.0**	72.8±1.1

5. Conclusions

- ✓ **We have developed a new FIA-TMS method, which allows to distinguish i-C5 and p-C5 using the difference of reference ion ratio.**
- ✓ **Our method is expected to reduce the false positive rate in cases of increased C5-acylcarnitine without extra cost and time.**
- ✓ **Since our method requires only minor modifications to existing analytical methods, it will not be difficult to implement in many laboratories.**