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Application News

LCMS™-8050 High-Performance Liquid Chromatograph Mass Spectrometer

Identification of C5-Acylcarnitines Using Flow Injection Analysis-Tandem Mass Spectrometry

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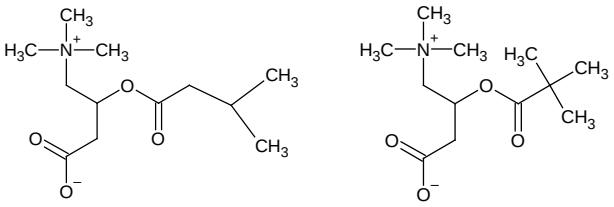
User Benefits

- C5-acylcarnitines (isovalerylcarnitine and pivaloylcarnitine) can be distinguished without special reagents or instruments by a few modifications of the analytical method in conventional flow injection analysis-tandem mass spectrometry.

■ Introduction

In newborn screening, amino acids and acylcarnitines in a dried blood spot (DBS) are analyzed by flow injection analysis-tandem mass spectrometry (FIA-TMS) to find amino acid, organic acid, and fatty acid metabolic disorders. FIA-TMS provides excellent throughput but is normally unable to distinguish isomers because a column is not used. For example, isovalerylcarnitine (i-C5), which is a marker metabolite of isovaleric acidemia (IVA), is isobaric with pivaloylcarnitine (p-C5), 2-methylbutyrylcartinine, and n-valerylcartinine. p-C5 can be present in a DBS due to newborn or maternal use of pivalate-containing antibiotics, but i-C5 and p-C5 cannot be distinguished by FIA-TMS. There are many reports of false positives derived from p-C5. An analytical method to distinguish i-C5 and p-C5 is needed to reduce false positives of IVA. This Application News describes a technique (patent pending) to distinguish i-C5 and p-C5 using a reference ion.



Isovalerylcarnitine
(marker for isovaleric acidemia)

Pivaloylcarnitine
(antibiotic-derived metabolite)

Fig. 1 C5-acylcarnitine Isomers

■ Analytical Conditions

The analytical instruments used were a Nexera™ UHPLC system and the LCMS-8050 (Fig. 2). The LCMS-8050 is a high-sensitivity and high-speed triple quadrupole mass spectrometer capable of simultaneous analysis of trace compounds. Table 1 shows the analytical conditions used for HPLC and MS. The injection volume is 1 µL and the analysis time is 1 minute in Shimadzu's method for FIA-TMS. This reduces mass spectrometer contamination and allows high throughput analysis of many samples.

Table 1 Analytical Conditions

[HPLC Conditions] (Nexera × 3)	
Mobile Phases:	NeoBase Kit
Flowrate:	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 Volume:	1 µL
[MS Conditions] (LCMS-8050)	
Ionization:	ESI (Positive mode)
Mode:	MRM (*m/z* 246.2 > 85.0, 187.1)
Nebulizing Gas Flow:	3.0 L/min
Drying Gas Flow:	10.0 L/min
DL Temp.:	250 °C
Block Heater Temp.:	400 °C

■ Samples

A DBS (sample A) with high levels of C5-acylcarnitine derived from an IVA patient and a DBS (sample B) with high levels of C5-acylcarnitine derived from pivalate-containing antibiotics were analyzed. The i-C5 and p-C5 in these samples were also analyzed by LC/MS/MS with a column. In sample A, p-C5 was not detected and only i-C5 was detected, while in sample B i-C5 was not detected and only p-C5 was detected.

Fig. 2 LCMS™-8050

■ Pretreatment

DBS samples were prepared in accordance with the standardized protocol of a non-derivatized method using the NeoBase kit (PerkinElmer). A single 3 mm DBS punch was placed in each well of a 96-well assay plate. 100 µL of the extraction solution containing an internal standard of acylcarnitines and amino acids was added to each well. The plate was shaken at 700 rpm at 45 °C for 45 minutes. After centrifugation for 5 minutes, the supernatant was analyzed.

■ Identification Using a Reference Ion

In the multiple reaction monitoring (MRM) mode, compounds can be distinguished by differences in reference ion ratio (Fig. 3, peak intensity of reference ion (b)/peak intensity of quantitative ion (a) × 100). *m/z* 246.2>85.0 and *m/z* 246.2>187.1 were used as the quantitative ion and the reference ion of C5-acylcarnitine, respectively. Fig. 3 shows MRM chromatograms of standard solutions of i-C5 and p-C5. The reference ion ratio of i-C5 was lower than that of p-C5, and the reference ion ratio of i-C5 and p-C5 was 20.44% and 28.21%, respectively. To distinguish i-C5 and p-C5, similarities of reference ion ratio of i-C5 and p-C5 were described as i-C5 score and p-C5 score (scored from 0 to 100 where 100 is a perfect match) by the following equation:

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

The reference ion ratio of sample A and B was 20.48 and 28.54, respectively. The i-C5 score was higher than the p-C5 score in sample A and the p-C5 score was higher than the i-C5 score in sample B (Table 2). Based on the above results, the C5-acylcarnitine in sample A was estimated to be i-C5 and the C5-acylcarnitine in sample B was estimated to be p-C5. The estimations using i-C5 and p-C5 scores were consistent with the identification results using LC/MS/MS with a column.

Table 2 i-C5 and p-C5 Scores in DBS Analyses

DBS	C5 Conc. (µmol/L)	i-C5 Score	p-C5 Score
A	5.32	98.0	72.6
B	3.72	58.8	97.7

■ Summary

This article shows that i-C5 and p-C5 can be distinguished by FIA-TMS using a reference ion.

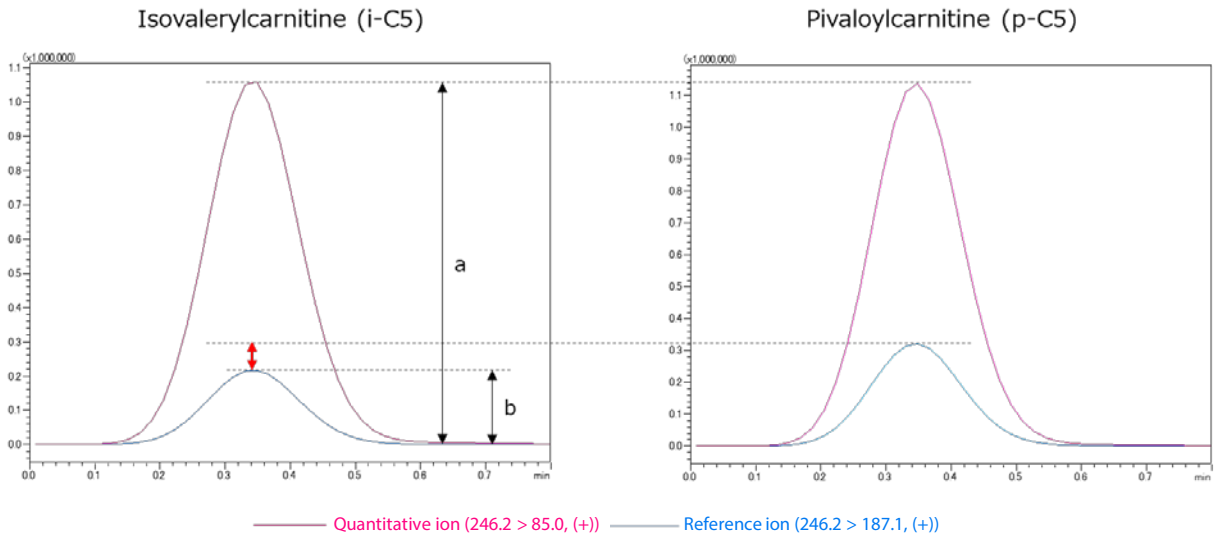


Fig. 3 MRM Chromatograms of Isovalerylcarnitine and Pivaloylcarnitine

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