APPLICATION NOTE

Measurement of isobaric c5 acylcarnitines in dried blood spots using QSight 225 MD UHPLC mass spectrometer.

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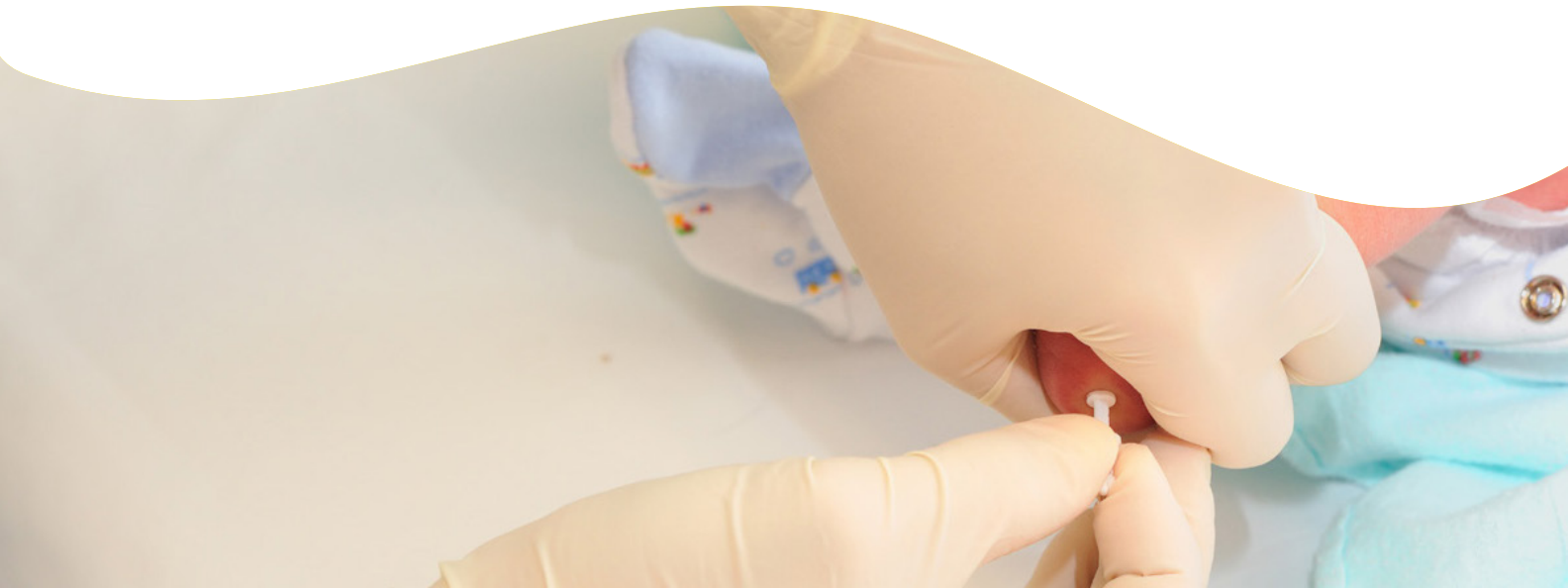
Introduction

Inborn errors of metabolism include a wide variety of conditions caused by mutations affecting the enzymatic activity. E.g., in isovaleryl-CoA dehydrogenase (IVD) deficiency, over 25 mutations in the IVD gene have been described, which typically result in phenotypic abnormalities due to the accumulation of isovaleric acid.[1,2]

Primary screening for IVD deficiencies is commonly based on the measurement of increased isovalerylcarnitine (C5) concentration in blood. By direct flow injection analysis tandem mass spectrometry (MS/MS) assay without liquid chromatography (LC) separation, this specific biomarker cannot be however distinguished from the three other C5 isobars valeryl-, pivaloyl-, and 2-methylbutyrylcarnitine. Especially, pivaloylcarnitine commonly causes false positive C5 screening results, because it can be present in blood due to use of antibiotics or nipple creams containing pivalic acid ester derivatives.[3,4]

Therefore, to allow more specific C5 marker measurement and to reduce false positive rates in the primary screening, we report here a gradient LC-MS/MS method using the Revvity QSight® 225 MD UHPLC mass spectrometer, which is capable to separate all four isobaric C5 acylcarnitines in dried blood spot (DBS).



Retesting of these tier 2 samples often becomes a bottleneck, as the samples must be re-prepared and then subjected to subsequent analytical tests, which adds time, complexity and room for human error to the process. Here, an automated workflow using QSight 225 MD mass spectrometer, coupled with Revvity Specimen Gate™ Laboratory software, is also possible, where samples that are positive for tier 1 marker are automatically retested via tier 2 methodologies, with minimal need for human intervention and without the need for further sample preparation.

Experimental

Chemicals and materials

Perfluoroheptanoic acid (PFHA, Sigma-Aldrich) was used as an ion pairing mobile phase additive.2 Isovaleryl-L-carnitine, pivaloyl-L-carnitine, 2-methylbutyryl-L-carnitine and valeryl-Lcarnitine hydrochlorides were purchased from Amsterdam University Medical Centers, Netherlands. 2H9-labeled isovaleryl-L-carnitine used as internal standard (IS) was purchased from CDN Isotopes, Canada.

Sample Preparation

A 3.2 mm DBS disk was punched into the microplate well and incubated with 125 µL of extraction solution at 45 °C for 30 minutes under shaking at 650 - 750 rpm. During the incubation an adhesive microplate cover was used to minimize evaporation. The extraction solution (78:22 methanol:water with 3 mM oxalic acid) also contained the $^{2}H_{9}$-isovalerylcarnitine internal standard (1.57 µM) for signal normalization and quantitation. After incubation, 100 µL of supernatant was transferred to a new microplate, and then 3 µL were injected in the LC-MS/MS system. Quantitative determination of C5 isobars was performed using 6 level blood calibrators for each analyte with final concentrations of 0.2, 0.3, 1, 2, 5 and 10 µM.

Here, an automated workflow using the Revvity QSight 225 MD mass spectrometer, coupled with Revvity Specimen Gate Laboratory software, is also possible. Worklists for flagged positives tier 1 samples are automatically generated via Revvity Specimen Gate Laboratory software, and users can resample from the same exact sample that was tested via tier 1 without the need for further sample preparation.

Mass spectrometry conditions

The LC-MS/MS analysis was performed using the QSight 225 MD 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)	4500
HSID™ Temp (°C)	300
Nebulizer Gas Setting	250
Drying Gas Setting	175
Source Temp (°C)	350
Dwell time (ms)	20

Table 2: Optimized MRM parameters.

Compound	Precursor (m/z)	Fragment (m/z)	CCL2	CE	EV
$^{2}H_{9}$-Isovaleryl-L-carnitine (IS)	255.2	85.0	-45	-27	30
Isovaleryl-L-carnitine	246.2	85.0	-45	-27	30
2-Methylbutyryl-L-carnitine	246.2	85.0	-45	-27	30
Valeryl-L-carnitine	246.2	85.0	-45	-27	30
Pivaloyl-L-carnitine	246.2	85.0	-45	-27	30

LC conditions

The LC separation was performed using a Brownlee SPP C18 column (50 x 2.10 mm, 2.7 µm, part number: N9308402), SPP C18 guard column (2.1 x 5 mm, part number: N9308513), and the corresponding guard column holder (part number: N9308534). The LC run time was 5.2 min with the following conditions and mobile phase gradient as listed in Table 3.

Table 3: LC gradient.

Mobile phase A:	10% acetonitrile and 0.1% perfluoroheptanoic acid (PFHA) in water		
Mobile phase B:	0.1% PFHA in acetonitrile Injection volume: 3 µL Column temperature: 30 °C Flow rate: 0.450 mL/min		
	Time	**A (%)**	**B (%)**
	0.01	88	12
	0.20	88	12
	4.00	81	19
	4.10	5	95
	4.50	5	95
	4.60	88	12
	5.20	88	12

Results and discussion

As shown by chromatograms in Figure 1, the above described LC-MS/MS method allowed to resolve the isovaleryl-Lcarnitine from pivaloyl-L-carnitine and other two C5 isobars: methylbutyryl-L-carnitine and valeryl-L-carnitine. The retention times were 4.00, 3.63, 3.75 and 4.22 min, respectively.

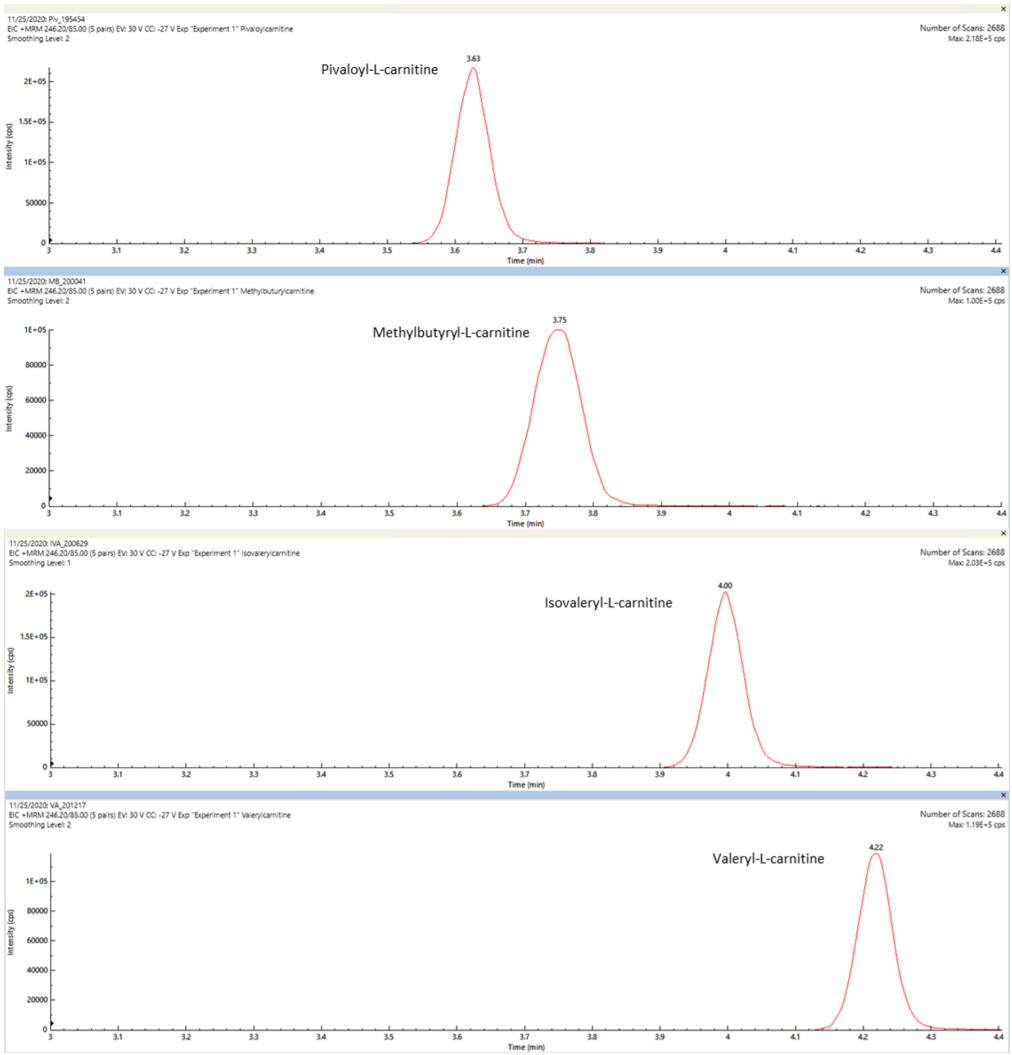


Figure 1: Chromatograms of four individual C5 isobars pivaloyl-L-carnitine, methylbutyryl-L-carnitine, isovaleryl-L-carnitine and valeryl-L-carnitine.

The LC-MS/MS method characteristics were demonstrated by using non-enriched endogenous blood and several enriched concentrations of each C5 isobar alone and in combined mixture of all four C5 isobars. The tested linearity ranges were: 0.2-10 µM covering the expected normal (C5 < 0.5 µM) and abnormal (C5 typically 0.6-12 µM) concentration ranges in IVD deficiencies. As shown in Table 4 and Figure 2, good overall linearity results (coefficient of determination R^2>0.99), acceptable precision and reasonable recovery (%) ranges were obtained for all four C5 isobars.

Table 4: QC metrics of 6 extractions of the C5 isobars in DBS with three enrichment levels: Level 1 0.25 µM; Level 2 1.5 µM; and Level 3 8.0 µM

Analyte	Level 1		Level 2		Level 3	
	CV %	Accuracy %	CV %	Accuracy %	CV %	Accuracy %
Pivaloylcarnitine	3.9	107	4.1	101	1.5	99
Methylbutyrylcarnitine	11.7	112	5.0	96	7.3	103
Isovalerylcarnitine	2.1	115	3.3	100	4.9	97
Valerylcarnitine	4.2	108	3.5	98	7.1	98

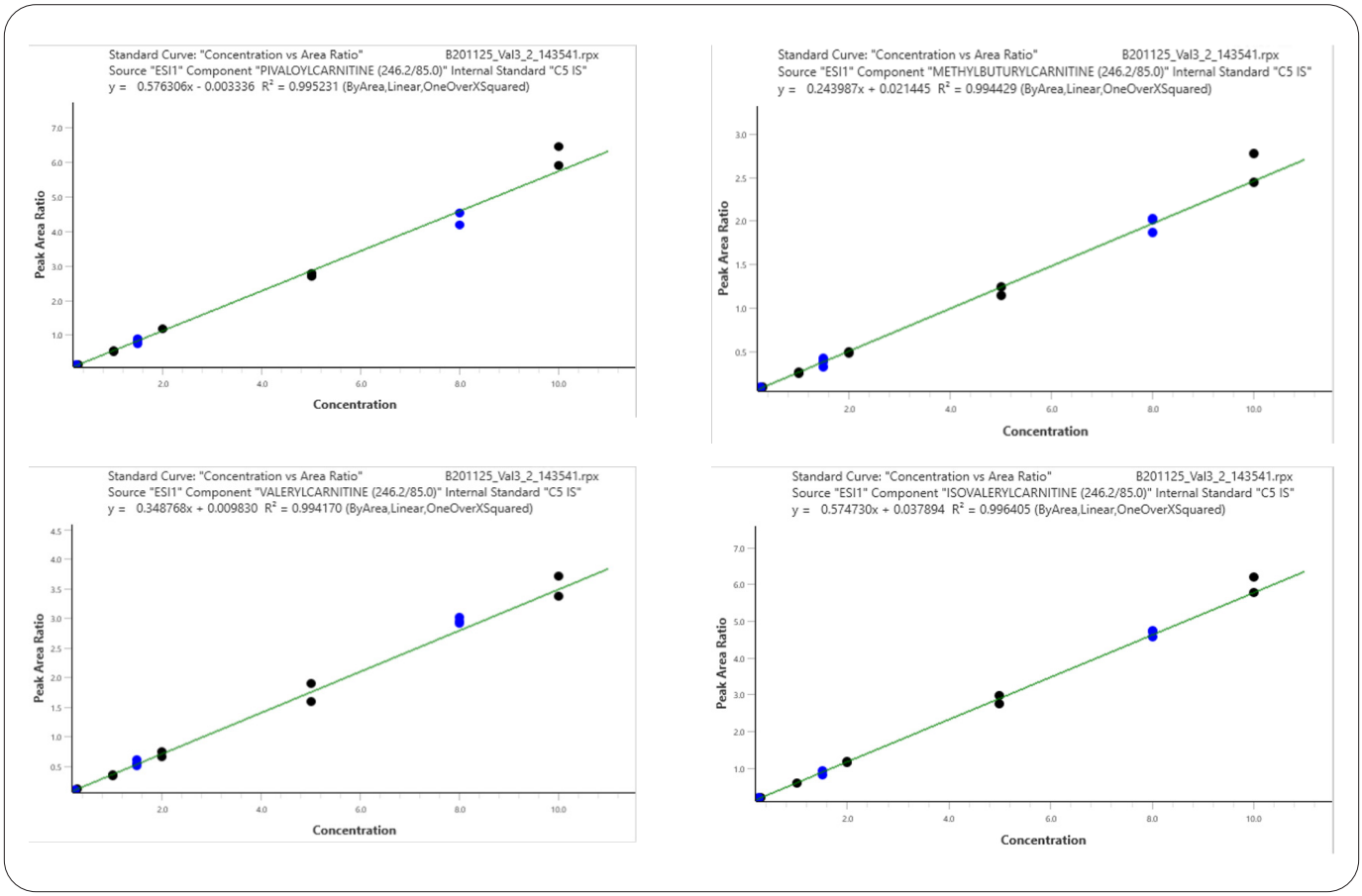


Figure 2: Calibration curves of four C5 isobars (6 level calibrators are reported as black dots, instead the blue dots are the QCs enriched levels mentioned in Table 4).

Alternatively, also a direct isotope dilution quantitation without an external calibration curve was tested. For this, a compound specific relative response factor was first determined for each analyte based on six different DBS enrichment levels, and then studied with three measured control levels over two test plates on two separate days. As summarized in Table 5, also this approach gave reasonable accuracy and precision results.

Table 5: QC metrics of 9 extractions of the C5 isobars in DBS with three enrichment levels: Level 1 0.25 µM; Level 2 1.5 µM; and Level 3 8.0 µM).

Analyte	Level 1		Level 2		Level 3	
	CV %	Accuracy %	CV %	Accuracy %	CV %	Accuracy %
Pivaloylcarnitine	9.4	99	8.5	88	6.8	97
Methylbutyrylcarnitine	10.4	127	9.5	86	13.5	93
Isovalerylcarnitine	4.3	114	6.6	84	4.2	92
Valerylcarnitine	7.0	102	11.2	91	4.2	107

When elevated C5 concentration is found in the primary screening, the same blood sample is typically analyzed by LC-MS/MS to separate different C5 isobars and confirm if the seen elevation is caused by increased isovaleryl-L-carnitine indicating IVD deficiency or pivaloyl-L-carnitine potentially deriving from antibiotics use. As seen in Figure 3 chromatograms A-B, the separated isovaleryl-L-carnitine peak is absent or negligible in healthy control sample (A), but clearly appears with contrived positive IVD deficiency sample (B). Also, in the sample C chromatogram representing newborn sample after known antibiotic treatment (C), the pivaloyl-L-carnitine peak is clearly dominating but no isovaleryl-L-carnitine peak is seen.

Conclusions

The results obtained confirm the applicability of the LC-MS/MS method using QSight 225 MD mass spectrometer for measuring and monitoring the isobaric C5 biomarker concentrations in DBS samples. This reported method can be potentially used as a second-tier test to reduce commonly seen false-positive rates due to the antibiotic derived C5 interference caused by pivaloylcarnitine.

Moreover, the Revvity QSight 225 MD mass spectrometer, coupled with Revvity Specimen Gate® Laboratory software, offers attractive solutions for newborn screening labs, and others who want to automate retesting of samples.

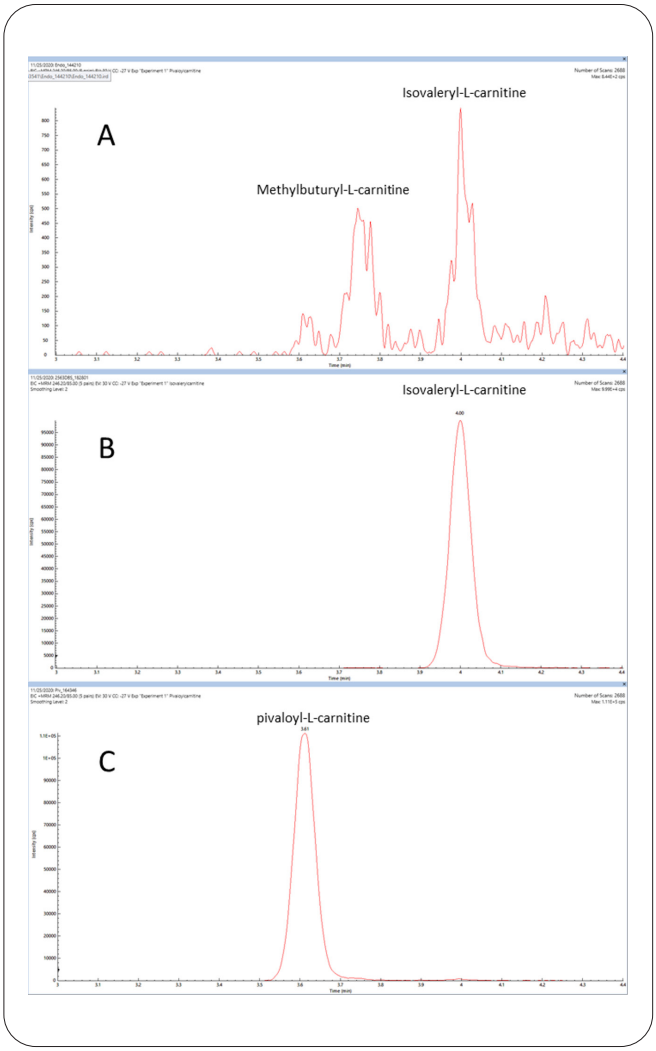


Figure 3: Extracted ion chromatograms of healthy adult DBS sample (A), contrived positive IVD deficiency DBS sample (0.8 µM) (B), and DBS sample enriched with 0.8 µM Pivaloylcarnitine (C).

References

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3. Abdenur, J.E. et al. (1998): Diagnosis of isovaleric acidaemia by tandem mass spectrometry: False positive result due to pivaloylcarnitine in a newborn screening programme. J. Inher. Metab. Dis. 21, 624–630.

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