High sensitivity quantification of acylcarnitines using the SCIEX 7500 system

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Carnitine and acylcarnitines play an important role in the trafficking of fatty acids from the cytosol to the mitochondrial matrix with the additional responsibility of keeping the balance of CoA and acyl-CoA levels within the cell. Acylcarnitines are acetylated forms of L-carnitine produced from carnitine acyltranferases. These acyltransferases cover the entire range of fatty acid lengths, and depending on their chain length specificities, can be found in different tissue types.[1]

In many laboratories across the world, acylcarnitines and related molecules from a variety of sample types are quantified to investigate metabolic conditions, such as organic acidemias and fatty acid oxidation defects.[2] They have also been investigated as potential biomarkers for Type 2 Diabetes and other related conditions.[3] Whole blood from dried blood spots (DBS) is most commonly analyzed during screening, however, plasma, serum and urine are other common matrices used for analysis.

Carnitines and acylcarnitines vary in their hydrophobicity, ranging from small, polar carnitine to acylcarnitines with very long acyl chain lengths. This variability poses a unique challenge for chromatographic method development. To overcome this, chemical derivatization techniques are often needed to aid in the retention of these compounds on a reverse phase column.

The detection and quantification of acylcarnitine compounds can be challenging since many species occur as isomers and are found at very low concentrations in biological samples. Here, several acylcarnitine compounds were quantified in matrix, using diluted standards and the SCIEX 7500 system. Linearity, lower limits of quantification (LLOQ) and dynamic range were characterized and the sensitivity and reproducibility of the SCIEX 7500 system were evaluated. These experiments were performed without derivatization and with minimal sample preparation to quantify these low-level analytes in a biological matrix.

Key features of the SCIEX 7500 system for the quantification of acylcarnitines

- A high-throughput LC-MS method able to quantify acylcarnitine species
- A highly sensitive method that does not require derivatization of samples before analysis
- A robust method with high sensitivity and reproducibility and broad dynamic range (LDR)

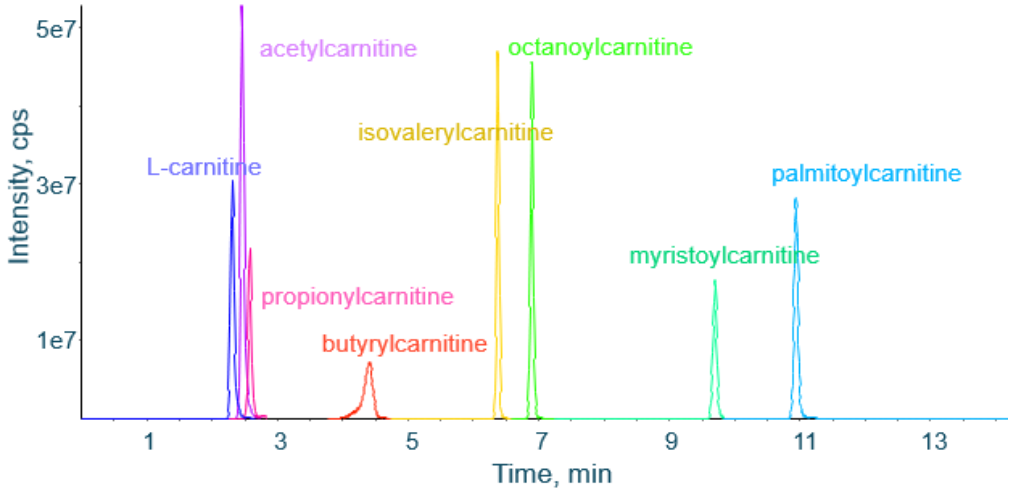

Figure 1. Extracted ion chromatogram (XIC) for 8 unlabeled acylcarnitine compounds.

Methods

Sample preparation: Labeled and unlabeled standards were purchased from Cambridge Isotopes (p/n: NSK-B-1, NSK-B-US-1). The unlabeled standards were serially diluted from stock solutions to concentrations ranging from 1:50 to 1:1,000,000. Labeled standards were spiked in at a 1:100 dilution across all samples.

Ten µL of Wistar rat plasma was extracted using 490 µL of 80:20 methanol:water and then vortexed. The samples were spun for 10 minutes at 15000 rpm. An insert vial was loaded with 150 µL of supernatant and 1 µL was injected. Triplicate injections were analyzed.

Chromatography: An ExionLC system with a Phenomenex Kinetex column (C18, 2.6 µm, 150x4.6 mm; p/n 00F-4462-E0) was used to perform the separation. The total run time was 15.5 min. A 1.0 µL injection was used. Table 1 shows the chromatographic gradient used.

Table 1. Chromatographic gradient.

Time (min)	Flow (µL/min)	B Conc (%)	B Curve
3.0	*600*	*15.0*	*0*
3.5	*600*	*75.0*	*0*
11.0	600	*99.0*	*0*
12.5	*600*	*99.0*	*0*
13.50	*600*	*15.0*	*0*

Mobile phase A: 99:1 water:methanol with 0.1% Formic Acid
Mobile phase B: 1:99 water:methanol with 0.1% Formic Acid

Mass spectrometry: Samples were analyzed on the triple quadrupole version of the SCIEX 7500 system using the Scheduled MRM algorithm in the positive ion mode. Table 2 lists the source conditions for analysis.

Table 2. Source conditions for the SCIEX 7500 system.

Parameter	Value
CUR	*45*
CAD	*10 (medium)*
Temp	*475*
IS	*1400*
GS1	*45*
GS2	*70*

Data processing: All data were acquired and processed using SCIEX OS software 2.1.0.

Quantification results

Figure 1 shows extracted ion chromatograms for 8 unlabeled acylcarnitine compounds in diluted 1:50 neat solution. Compounds ranging in size from L-carnitine toand short-chain acetylcarnitine, to medium-chain octanoylcarnitine, to long-chain palmitoylcarnitine in neat solution at the 1:50 dilution, to highlight the optimized separation.

Next, the standards were diluted into extracted rat plasma across a broad concentration range to determine the sensitivity of the method. Lower limits of quantification (LLOQs) were calculated for all 8 analytes. Table 3 lists all LLOQs for each standard in the assay. The MRM signals observed at the lowest concentration analyzed for 4 of the analytes are shown in Figure 2. These are integrated peaks for the 1:1,000,000 dilution of the unlabeled L-carnitine, acetylcarnitine (short-chain), octanoylcarnitine (medium-chain) and myristoylcarnitine (long-chain) compounds. While the 1:1,000,000 dilution was the final point in the calibration curve, the response from some analytes in the assay suggest that quantification at even lower concentrations might be possible.

In Figure 3, 5 analytes that exhibited 4.5 orders of linear dynamic range (LDR) are shown. The LDR value for each analyte is shown in Table 3. These values were calculated for each analyte based on 3 replicates at each dilution, ranging from 1:50 to 1:1,000,000, of the stock solutions of the commercially available standards.

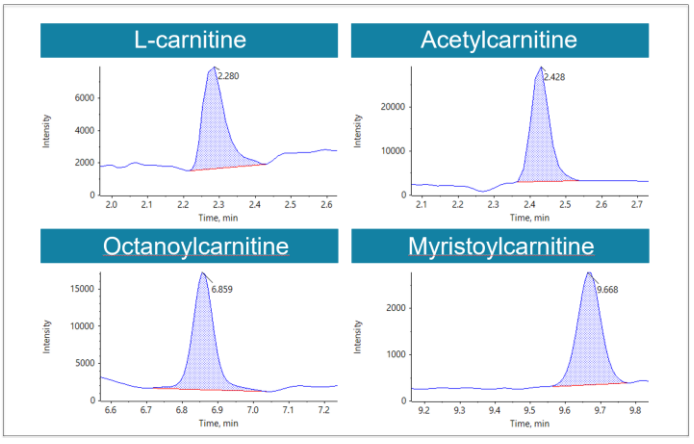

Figure 2. MRM chromatograms for the 1:1,000,000 dilution point in the concentration curve. Examples of the signals obtained at the lowest concentration measured. Signal was generated at the following concentrations: 152 pM L-carnitine, 38 pM acetylcarnitine, 7.6 pM octanoylcarnitine and 7.6 pM myristoylcarnitine.

Figure 3. Linear dynamic range for several acylcarnitine compounds. Selected compounds exhibited 4.5 orders of linear dynamic range. Note that wider LDR might be possible for some compounds, as detection at lower concentrations is possible based on observed signals.

To assess reproducibility and robustness, 3 replicates of carnitine and acylcarnitines at their respective LLOQ concentrations in matrix were analyzed. A summary of the accuracy and %CV results is shown in Table 3. The reproducibility observed at the LLOQ was <6% across all the analytes, indicating strong reproducibility. The LDR observed for some analytes was 4.5 orders.

Table 3. Calibration curve statistics. The observed LLOQ, accuracy and %CV at the LLOQ and LDR of the calibration for acylcarnitine compounds in matrix is reported.

Name	LLOQ* (pM)	Accuracy	%CV	LDR
L-carnitine	*152*	*106.51*	*5.88*	*4.5*
acetylcarnitine	*38*	*107.13*	*5.67*	*4.5*
propionylcarnitine	*15.2*	*113.43*	*3.81*	*4*
butyrylcarnitine	*76*	*119.45*	*0.94*	*3.5*
isovalerylcarnitine	*15.2*	*92.98*	*0.44*	*4*
octanoylcarnitine	*7.6*	*119.31*	*2.2*	*4.5*
myristoylcarnitine	*14.13*	*91.46*	*3.87*	*4.5*
palmitoylcarnitine	*15.2*	*99.8*	*4.45*	*4.5*

**calculated concentration from calibration curve*

Next, the endogenous levels of carnitine and acylcarnitines in 10 µL of extracted Wistar rat plasma were assessed. In brief, this plasma was diluted 1:20 utilizing a simple sample preparation, without derivatization or SPE extraction was employed, and 1 µL of plasma was injected on the column. Table 4 illustrates the sensitivity of the SCIEX 7500 system using this minimal extraction protocol. The concentrations of each acylcarnitine compound range from low µM to high nM and provide excellent reproducibility in a biological matrix.

Table 4. Concentration and %CV for endogenous acylcarnitine compounds in extracted Wistar rat plasma.

Name	Concentration (µM)	%CV
L-carnitine	*0.8085*	*2.11*
acetylcarnitine	*0.7183*	*4.61*
propionylcarnitine	*0.0956*	*5.91*
butyrylcarnitine	*0.0879*	*8.89*
isovalerylcarnitine	*0.0067*	*1.52*
octanoylcarnitine	*0.0007*	*2.12*
myristoylcarnitine	*0.0033*	*2.44*
palmitoylcarnitine	*0.0131*	*6.32*

Conclusions

- The SCIEX 7500 system can quantify acylcarnitine species using LC-separation, yielding a high-throughput, robust and reproducible method that does not require derivatization of samples before analysis
- The SCIEX 7500 system provides unparalleled sensitivity and could be utilized to quantify other low-level acylcarnitines that are present in biological matrices without the need for extensive sample preparation or derivatization
- Since the SCIEX 7500 provides greater depths of sensitivity using plasma, researchers could further dilute the sample or require less sample for extraction, allowing consideration of alternative sample types for analysis.

References

1. Meierhofer D. (2019) Acylcarnitine profiling by low-resolution LC-MS. *PLoS ONE*, **14(8)**, 1-11.

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