Chapter 2

Quantification of Free Carnitine and Acylcarnitines in Plasma or Serum Using HPLC/MS/MS

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Abstract

Acylcarnitines are formed by esterification between fatty acids CoA or organic acids CoA molecules and carnitine. In various fatty acids oxidation defects and organic acidurias, there is increased concentration of corresponding acylcarnitines. Abnormalities in specific acylcarnitines are used in the diagnosis of fatty acids oxidation defects and organic acidurias. Most commonly used method for the assay of acylcarnitines is HPLC-tandem mass spectrometry (HPLC/MS/MS). A HPLC/MS/MS method is described for the quantification of number of acylcarnitines. The method involves butylation of carnitine/acylcarnitines using acidified butanol, HPLC flow injection, and measurement of acylcarnitines using precursor ion scan and multiple reactions monitoring (MRM).

Key words Fatty acid oxidation defects, Organic acidemia, Organic acidurias, HPLC, Mass spectrometry, Medium chain acyl-CoA dehydrogenase deficiency, Inborn error of metabolism, Inherited metabolic disorders

1 Introduction

Inborn metabolic disorders, although individually rare, are collectively quite common. It is estimated that inborn metabolic disorders have frequency of 1:500. A number of organic acidurias and most fatty acids oxidation defects can be diagnosed through the analysis of plasma acylcarnitine profile [1–5]. Organic acidurias are a diverse group of disorders commonly characterized by episodes of acidosis, vomiting, lethargy, coma, seizures, hypotonia, hypertonia, and developmental delay [6]. There are more than 12 fatty acids oxidation defects. Common clinical features of fatty acids oxidation defects include hypoglycemia, liver disease, cardiomyopathy, and sudden unexpected death [2].

HPLC-tandem mass spectrometry (HPLC/MS/MS) is the method of choice for the measurement of carnitine and acylcarnitines, although other methods such as gas chromatography–mass spectrometry and high-performance liquid chromatography have

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been described [1, 4, 7, 8]. HPLC/MS/MS method described here for the quantification of a number of acylcarnitines involves butylation of acylcarnitines using acidified butanol, HPLC flow injection, and measurement of acylcarnitines by precursor ion scan and multiple reactions monitoring (MRM). Deuterated internal standards are used for quantification of carnitine and acylcarnitines.

2 Materials

2.1 Samples

Collect 0.5 mL blood in red top (plain) or green top (heparin) tube. Centrifuge blood at 1200 × *g* for 7 min. Separate serum or plasma and refrigerate at 4 °C. Samples are stable for 2 months when refrigerated.

2.2 Solvents and Reagents

1. Formic Acid, 88 % ACS (Fisher).
2. UTAK blank human serum (UTAK Laboratories).
3. 3 N HCl in butanol (Regsil).
4. Mobile Phase (80:20 acetonitrile:water with 0.01 % formic acid): To a 1 L volumetric flask, add 800 mL acetonitrile, 114 μL 88 % formic acid, and then fill to the mark with deionized water. Stable for 1 year at room temperature.
5. Sample reconstitution solution (80:20 acetonitrile:water): To a 100 mL volumetric flask, add 80 mL acetonitrile and then fill to the mark with deionized water. Stable for 1 year at room temperature.

2.3 Internal Standards and Quality Controls

1. Internal standard mixture (NSK-B from Cambridge Isotope Laboratories): Dissolve in 200 mL of methanol. Concentrations of various acylcarnitines are listed in Table 1.
2. Quality controls:

Table 1
Concentrations of internal standards

Compound	Concentration (nmol/mL)
D9-L-Carnitine (C0)	0.760
D3-Acetycarnitine (C2)	0.190
D3-Propionylcanitine (C3)	0.038
D3-Butyrylcarnitine (C4)	0.038
D9-Isovalerylcarnitine (C5)	0.038
D3-Octanoylcarnitine (C8)	0.038
D9-Tetradecanoylcarnitine (C14)	0.038
D3-Hexadecanoylcarnitine (C16)	0.076

Table 2
Preparation of stock carnitine/acylcarnitine solutions

Compound	M.W.	Amount in vial (μg)	Reconstitute vial with this amount of methanol (mL)	Resulting concentration (nmol/mL)
C0	198	5000	2.52	10,000
C2	240	5000	2.1	10,000
C3	254	5000	1.97	10,000
C4	268	5000	1.86	10,000
C5	282	5000	1.77	10,000
C8	324	5000	1.54	10,000
C14	408	5000	1.22	10,000
C16	436	5000	1.15	10,000

Table 3
Preparation of primary combo carnitine/acylcarnitine solution

Compound	Stock solution (μL)	Resulting concentration (nmol/mL)
C0	800	4000
C2	400	2000
C3	20	100
C4	10	50
C5	10	50
C8	10	50
C14	10	50
C16	10	50

QS this combo carnitine/acylcarnitine solution to 2 mL using methanol (730 μL)

(a) Prepare stock of carnitine/acylcarnitine compounds (Cambridge Isotopes) according to Table 2. Stable for 2 years at −20 °C.

(b) Use stock carnitine/acylcarnitine compounds to prepare primary combo carnitine/acylcarnitine solution (Table 3). Stable for 2 years at −20 °C.

(c) Use primary combo carnitine/acylcarnitine solution to prepare working controls as shown in Table 4. Stable for 1 year at −20 °C.

Table 4
Preparation of working quality controls

	QC1	QC2	QC3	QC4
UTAK serum (μL)	200	995	980	950
Primary combo (μL)	0	5	20	50
0.9 % Saline (μL)	800	0	0	0
Resulting concentrations	Below normal limit	Approximately the upper limit of normal values	Approximately 4× upper limit of normal	Approximately >9× upper limit of normal

2.4 Analytical Equipment and Supplies

1. MS/MS 4000Q TRAP (AB Sciex).
2. Prominence HPLC (Shimadzu).
3. Dry block at 60 °C.
4. Sample evaporator, Turbovap (Zymark).
5. Fume Hood.

3 Methods

3.1 Stepwise Procedure

1. To a 1.5 mL microcentrifuge tube, add 20 μL sample or control.
2. Add 400 μL of working internal standard.
3. Close cap, vortex, and allow to stand for 5 min.
4. Centrifuge for 5 min at 12,000 × *g* for 10 min.
5. Transfer 300 μL of supernatant into a 13 × 100 mm tube.
6. Evaporate to dryness under a stream of nitrogen at 40 °C (*see* **Note 1**).
7. To the resulting residue, add 100 μL of 3 N HCl in butanol.
8. Cap tubes and incubate in dry block for 20 min at 60 °C.
9. Evaporate to dryness under a stream of nitrogen at 40 °C (*see* **Notes 2** and **3**).
10. Reconstitute with 900 μL of 80 % acetonitrile/water.
11. Transfer sample to autosampler vial.
12. Inject 50 μL onto HPLC/MS/MS.

3.2 Instrument Operating Conditions

See Tables 5, 6, 7, 8, and 9 for HPLC and mass-spectrometer conditions.

3.3 Data Analysis

1. Chemoview software (AB Sciex) is used for data analysis (*see* **Notes 4** and **5**).
2. Quantification of the various acylcarnitine species is based on the ratio of the analyte's response (intensity) to the response

Table 5
HPLC operating conditions

Time (min)[a]	Flow rate (mL/min)
0.15	0.30
0.16	0.03
3.50	0.03
3.51	1.00
4.00	1.00
4.01	0.03
5.00	0.30
6.00	Stop

[a]Will vary for different instruments depending on factors such as length/diameter of tubing and dead volume

Table 6
MS source parameters for both precursor ion scan and MRM

CUR	25
CAD	Medium
TEM	400
GS1	45
GS2	45
Ihe	ON
IS	4000
EP	10
CXP	3

Table 7
MS setting for MRM transitions

Compound	MRM transition	DP	CE	Internal standard	MRM transition	DP	CE
C0, Free carnitine	218.2 → 159	60	20	D9 C0	227.2 → 159	60	20
C2, Acetylcarnitine	260.2 → 141	51	21	D3 C2	263.2 → 141	58	21
C3, Propionylcarnitine	274.2 → 141	61	23	D3 C3	277.2 → 141	60	23
C4, Isobutyryl/butyrylcarnitine	288.2 → 141	60	24	D3 C4	291.2 → 141	60	24

Dwell time for all the compounds was 50 ms

Table 8
Transitions for acylcarnitines and internal standards

Compound	Precursor ion	Internal standard	Precursor ion
C5:1, Tiglylcarnitine	300.2 → 85.1	D9 C5	311.3 → 85.1
C5, Isovaleryl/2-methylbutyryl/Pivaloyl	302.2 → 85.1	D9 C5	311.3 → 85.1
C4-OH, 3-OH-butyrylcarnitine	304.2 → 85.1	D9 C5	311.3 → 85.1
C6, Hexanoylcarnitine	316.3 → 85.1	D9 C5	311.3 → 85.1
C5-OH, 3-OH-isovaleryl/2-methyl-3-OH-butyryl	318.2 → 85.1	D9 C5	311.3 → 85.1
C6-OH, 3-OH-hexanoylcarnitine	332.3 → 85.1	D3 C8	347.3 → 85.1
C8:1, Octenoylcarnitine	342.3 → 85.1	D3 C8	347.3 → 85.1
C:8, Octanoylcarnitine	344.3 → 85.1	D3 C8	347.3 → 85.1
C3-DC, Malonylcarnitine	360.3 → 85.1	D3 C8	347.3 → 85.1
C10:1, Decenoylcarnitine	370.3 → 85.1	D3 C8	347.3 → 85.1
C10, Decanoylcarnitine	372.3 → 85.1	D3 C8	347.3 → 85.1
C4-DC, Methylmalonylcarnitine	374.3 → 85.1	D3 C8	347.3 → 85.1
C5-DC, Glutarylcarnitine	388.3 → 85.1	D3 C8	347.3 → 85.1
C12:1, Dodecenoylcarnitine	398.3 → 85.1	D9 C14	437.4 → 85.1
C12, Dodecanoylcarnitine	400.3 → 85.1	D9 C14	437.4 → 85.1
C6-DC, 3-methyl-glutarylcarnitine	402.3 → 85.1	D9 C14	437.4 → 85.1
C12-OH, 3-OH-dodecanoylcarnitine	416.3 → 85.1	D9 C14	437.4 → 85.1
C14:2, Tetradecadienoylcarnitine	424.3 → 85.1	D9 C14	437.4 → 85.1
C14:1, Tetradecenoylcarnitine	426.4 → 85.1	D9 C14	437.4 → 85.1
C14, Tetradecanoylcarnitine	428.4 → 85.1	D9 C14	437.4 → 85.1
C14:1-OH, 3-OH-tetradecenoylcarnitine	442.4 → 85.1	D9 C14	437.4 → 85.1
C14-OH, 3-OH-tetradecanoylcarnitine	444.4 → 85.1	D9 C14	437.4 → 85.1
C16:1, Hexadecenoylcarnitine	454.4 → 85.1	D3 C16	459.4 → 85.1
C16, Hexadecanoylcarnitine	456.4 → 85.1	D3 C16	459.4 → 85.1
C16:1-OH, 3-OH-hexadecenoylcarnitine	470.4 → 85.1	D3 C16	459.4 → 85.1
C16-OH, 3-OH-hexadecanoylcarnitine	472.4 → 85.1	D3 C16	459.4 → 85.1
C18:2, Linoleylcarnitine	480.4 → 85.1	D3 C16	459.4 → 85.1
C18:1, Oleylcarnitine	482.4 → 85.1	D3 C16	459.4 → 85.1
C18, Stearoylcarnitine	484.4 → 85.1	D3 C16	459.4 → 85.1
C18:2-OH, 3-OH-linoleylcarnitine	496.4 → 85.1	D3 C16	459.4 → 85.1
C18:1-OH, 3-OH-oleylcarnitine	498.4 → 85.1	D3 C16	459.4 → 85.1
C18-OH, 3-OH-stearoylcarnitine	500.4 → 85.1	D3 C16	459.4 → 85.1

Table 9
MS setting for precursor ion scans

Polarity	Positive
Scan mode	Profile
Resolution Q1	Unit
Resolution Q3	Unit
Setting time	5.0 ms
MR pause	5.0 ms
MCA	No
Step size	0.10 Da
Center/width	No
Scanning range	210–550
Scan time	6 s
DP range	45–65
CE range	30–55

(intensity) of an appropriate isotopically labeled internal standard using the following calculations:

$$\text{Unknown conc}(\text{nM}) = \frac{\text{Intensity Analyte} \times \text{IS conc}(\text{nM}) \times \text{Dilution Factor}(21)}{\text{Intensity IS}}$$

3. Intensities used for quantification of C0, C2, C3, and C4 are obtained through MRM mode (Table 7). Intensities for all other acylcarnitine quantifications are obtained through precursor ion mode (Tables 8 and 9).
4. While analyzing data, it is important to evaluate interferences (*see* **Notes 6–8**).
5. Typical total ion chromatogram is shown in Fig. 1.
6. Typical precursor ion spectrum is shown in Fig. 2.

4 Notes

1. Drying time is ~5 min. May vary with nitrogen flow rate and type of equipment.
2. Drying time is ~10 min. May vary with nitrogen flow rate and type of equipment.
3. Make sure that extract is completely dry.
4. External calibration is not used in this assay. Chemoview software is used only for quantification. Chromatographic review

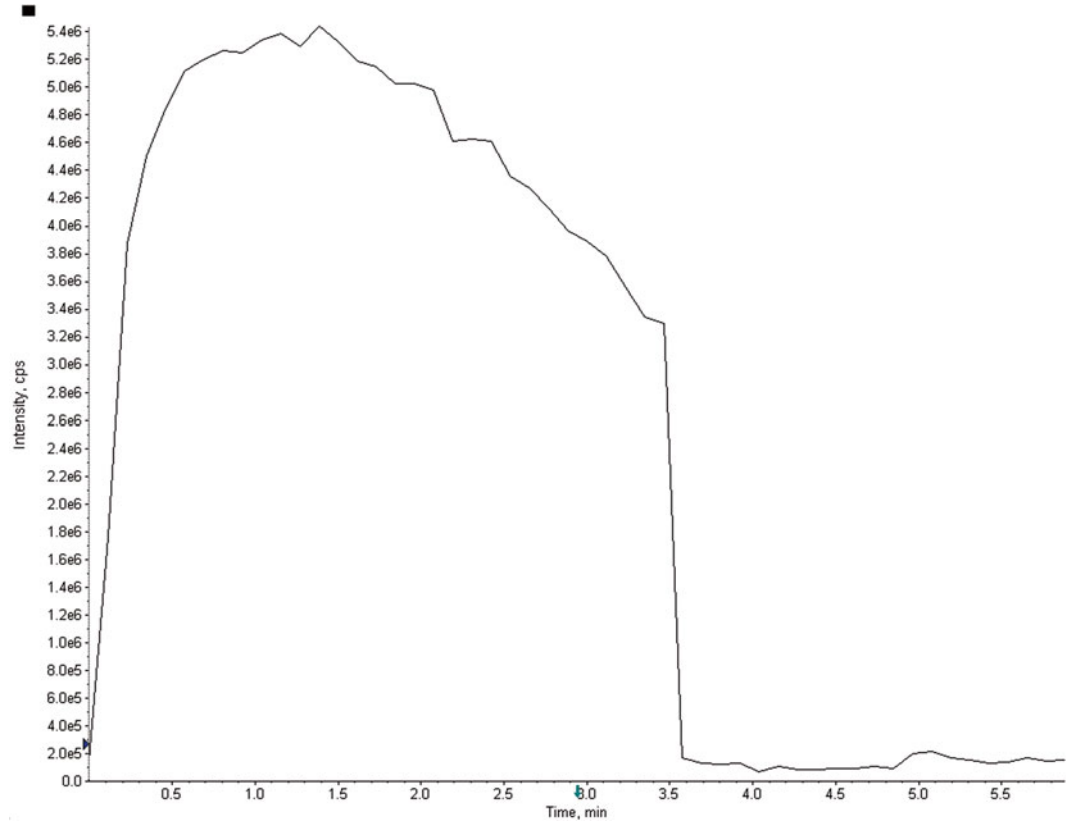

Fig. 1 Total ion chromatogram for carnitine/acylcarnitines

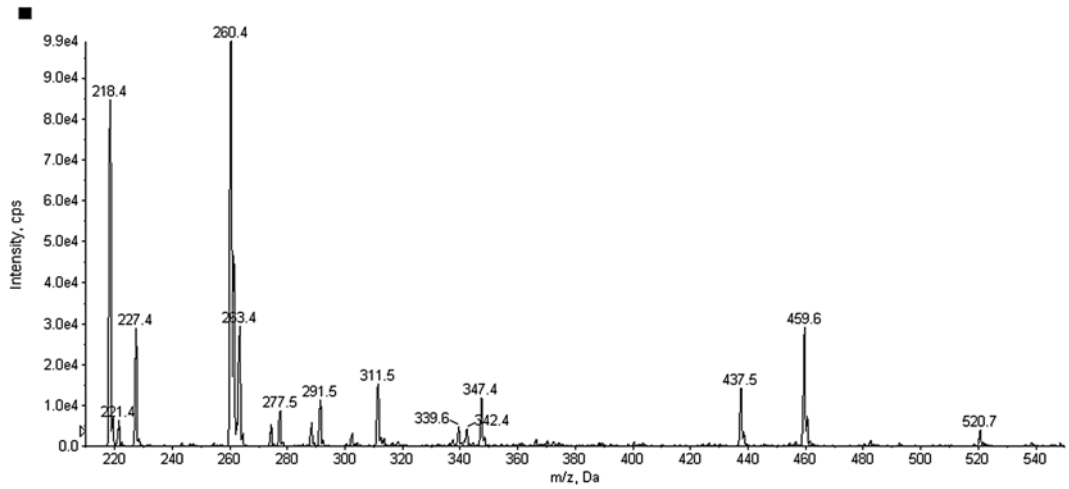

Fig. 2 Typical precursor ion spectrum from a healthy individual

is performed on the Analyst Software (AB Sciex). Ion suppression is also monitored in Analyst Software (AB Sciex) by comparing highest intensity of the total ion chromatogram (TIC) to the lowest intensity of the TIC. The highest intensity should be at least ten times greater than the lowest intensity.

5. Carryover is monitored by running the internal standard preparation after the most concentrated control (QC4). This injection of the internal standard should have C0 and C2 values of less than 1.0 nmol/mL. All other acylcarnitines should be less than 0.1 nmol/mL.
6. In precursor ion scan, glutamate interferes with C2 quantification due to $260 \rightarrow 85$ transition. This interference is avoided by using C2 MRM of $260 \rightarrow 141$.
7. Cefotaxime interferes with C16:1-OH due to $470 \rightarrow 85$ transition.
8. Isotope of formiminoglutamate (FIGLU) with m/z of 288 interferes with C4. This is avoided by using MRM $288 \rightarrow 141$ for C4.

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