TECHNICAL NOTE 73759

Determination of inborn errors of metabolism

Quantification of amino acids and acylcarnitines in dried blood spots by FIA-MS/MS for clinical research

Author: Mariana Barcenas,
Thermo Fisher Scientific, Les Ulis, France

Keywords: Amino acids, acylcarnitines, dried blood spots, non-derivatized, mass spectrometry, TSQ Fortis MS

Goal

Implementation of a flow injection analysis-tandem mass spectrometry (FIA-MS/MS) method for simultaneous quantification of 13 amino acids and 13 acylcarnitines in dried blood spots

Introduction

Newborn screening (NBS) is a public health program provided by most of the countries around the world aimed at screening newborns for a list of serious genetic and metabolic disorders.[1-4] Determination of the amino acids and acylcarnitines profile is an integral part of the newborn screening programs. In the last decade, significant improvements in mass spectrometry (MS) technology enabled widespread use of MS/MS for newborn screening. MS can offer differentiation between true and false positive samples and also facilitate screening for untreatable disorders—all with a single injection.

In this report, an MS-based analytical method for determination of amino acids and acylcarnitines in dried blood spots (DBS) for inborn errors of metabolism (IEM) research is reported. FIA was performed on a Thermo Scientific™ Vanquish™ Flex Binary UHPLC system. Detection was performed on a Thermo Scientific™ TSQ Fortis™ triple-stage quadrupole mass spectrometer with heated electrospray ionization (HESI) by selected reaction monitoring (SRM). Reagents and controls were obtained from the MassChrom™ LC-MS/MS Complete Kit for Amino Acids and Acylcarnitines in Dried Blood Spots (Ref 57000) from Chromsystems Instruments & Chemicals GmbH (Gräfelfing, Germany).

Table 1. List of analytes

Amino acids	Amino acid internal standards	Acylcarnitines	Acylcarnitine internal standards
Alanine	d_4-Alanine	Carnitine (C0)	d_9-C0
Arginine	d_7-Arginine	Acetylcarnitine (C2)	d_3-C2
Aspartic acid	d_3-Aspartic acid	Propionylcarnitine (C3)	d_3-C3
Citrulline	d_2-Citrulline	Butyrylcarnitine (C4)	d_3-C4
Glutamic acid	d_5-Glutamic acid	Isovalerylcarnitine(C5)	d_9-C5
Glycine	$^{13}C_2$ ^{15}N-Glycine	Glutarylcarnitine (C5DC)	d_6-C5DC
Leucine/Isoleucine	d_3-Leucine	Hexanoylcarnitine (C6)	d_3-C6
Methionine	d_3-Methionine	Octanoylcarnitine (C8)	d_3-C8
Ornithine	d_6-Ornithine	Decanoylcarnitine (C10)	d_3-C10
Phenylalanine	d_5-Phenylalanine	Dodecanoylcarnitine (C12)	d_3-C12
Proline	d_7-Proline	Tetradecanoylcarnitine (C14)	d_3-C14
Tyrosine	d_4-Tyrosine	Hexadecanoylcarnitine (C16)	d_3-C16
Valine	d_8-Valine	Octadecanoylcarnitine (C18)	d_3-C18

Experimental

Target analytes

Target analytes and their respective internal standards are summarized in Table 1.

Sample preparation

Reagents from Chromsystems included an extraction reagent and two controls, as well as the internal standards.

Samples were prepared as described by Chromsystems using the following procedure:

1. The internal standards were reconstituted using the extraction reagent.
2. 3.2 mm punches were placed in a well plate, and 100 µL of extraction reagent (containing the internal standards) were added to each well.
3. The plate was shaken at 600 rpm for 20 minutes.
4. Extracted samples were transferred to a clean plate.

Liquid chromatography

Flow injection analysis was performed on a Vanquish Flex Binary UHPLC system, using an injection volume of 10 µL of extracted sample. The mobile phase was provided by Chromsystems. Details of the analytical method are reported in Table 2. Total runtime was 1.5 minutes.

Table 2. LC method description

Time (min)	Flow rate (mL/min)	A (%)
0.00	0.09	100
1.23	0.09	100
1.25	0.30	100
1.50	0.09	100

Mass spectrometry

Analytes and internal standards were detected by SRM on a TSQ Fortis triple-stage quadrupole mass spectrometer with heated electrospray ionization operated in positive ionization mode. A summary of the MS source conditions is reported in Table 3. SRM transitions with optimized collision energy and tube lens values are summarized in Table 4.

Method evaluation

The robustness, reliability, and reproducibility of the method were evaluated in terms of intra- and inter-assay precision and accuracy for all analytes.

Table 3. MS settings

Source type	Heated electrospray inization (HESI)
Vaporizer temperature	150 °C
Capillary temperature	300 °C
Spray voltage (positive mode)	3500 V
Sheath gas	15 AU
Sweep gas	0 AU
Auxiliary gas	5 AU
Data acquisition mode	Selected-reaction monitoring (SRM)
Source fragmentation	10 V
Collision gas pressure	1.5 mTorr
Cycle time	0.800 s
Q1 mass resolution (FWMH)	0.7
Q3 mass resolution (FWMH)	0.7

Table 4. SRM transitions, collision energies, and tube lens values

Analyte	Precursor ion	Product ion	Internal standard	Precursor ion	Product ion	Collision energy (V)	Tube lens (V)
Alanine	90.038	44.071	d_4-Alanine	94.075	48.125	12	84
Arginine	175.088	70.054	d_7-Arginine	182.162	77.179	24	85
Aspartic acid	134.038	116.125	d_3-Aspartic acid	137.175	119.196	8	83
Citrulline	176.162	113.125	d_2-Citrulline	178.162	115.125	17	60
Glutamic acid	148.088	130.054	d_5-Glutamic acid	153.138	135.125	10	75
Glycine	76.088	30.179	$^{13}C_2{}^{15}N$-Glycine	79.088	32.196	13	130
Leucine	132.125	86.125	d_3-Leucine	135.138	89.125	10	83
Methionine	150.050	132.982	d_3-Methionine	153.088	136.125	10	84
Ornithine	133.038	70.054	d_6-Ornithine	139.162	76.196	18	68
Phenylalanine	166.112	120.125	d_5-Phenylalanine	171.162	125.125	14	82
Proline	116.112	70.125	d_7-Proline	123.175	77.196	16	89
Tyrosine	182.162	136.125	d_4-Tyrosine	186.162	140.125	14	88
Valine	118.125	72.125	d_8-Valine	126.162	80.250	11	84
C0	162.125	85.054	d_9-C0	171.162	85.054	21	77
C2	204.112	85.071	d_3-C2	207.125	85.071	20	64
C3	218.125	85.054	d_3-C3	221.162	85.054	20	71
C4	232.162	85.071	d_3-C4	235.212	85.054	20	77
C5	246.162	85.054	d_9-C5	255.162	85.054	21	78
C5DC	276.175	85.071	d_6-C5DC	282.175	85.054	24	90
C6	260.212	85.054	d_3-C6	263.175	85.125	22	94
C8	288.212	85.125	d_3-C8	291.212	85.125	23	99
C10	316.212	85.125	d_3-C10	319.212	85.125	24	104
C12	344.212	85.125	d_3-C12	347.250	85.125	25	99
C14	372.300	85.054	d_3-C14	375.300	85.125	25	110
C16	400.350	85.125	d_3-C16	403.350	85.125	27	116
C18	428.350	85.125	d_3-C18	431.400	85.054	28	118

Intra-assay precision for each day was evaluated in terms of percentage coefficient of variation (%CV) using the controls at two different concentration levels in replicates of five (n=5). Inter-assay precision was evaluated as the %CV on the full set of samples (control samples at two levels in replicates of five prepared and analyzed on three different days, n=15). Analytical accuracy was evaluated in terms of percentage bias between nominal and average calculated concentrations using quality control samples at two different levels provided by Chromsystems (0192 and 0193 batches #1519 and #2518).

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 4.1 software. Quantification of the analytes is done by comparison with the corresponding isotopically labeled internal standard, using the formula Conc = A Area/IS Area × IS conc.

Results and discussion

Representative chromatograms for phenylalanine, acetylcarnitine, and the corresponding internal standards are reported in Figure 1.

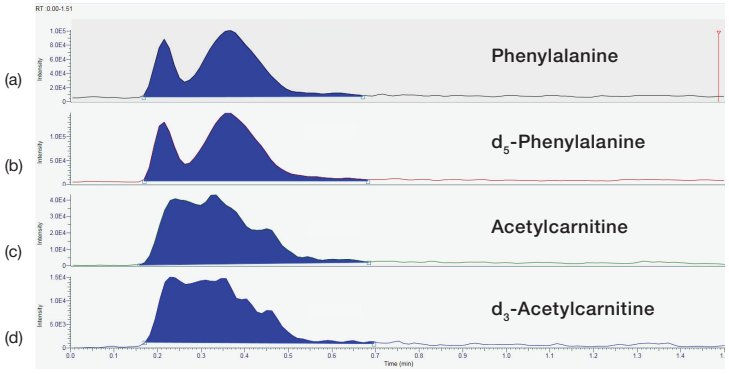

Figure 1. FIA-MS/MS profiles for (a) phenylalanine, (b) d_5-phenylalanine, (c) acetylcarnitine, and (d) d_3-acetylcarnitine

The reported method showed good reproducibility, with the maximum intra- and inter-assay precision below 9% and 12.5%, respectively, for all analytes. C5DC showed slightly higher values (14.9% and 17.7% for intra- and inter-assay precision, respectively). Results for intra- and inter-assay precision are reported in Tables 5 and Table 6, respectively.

Analytical accuracy for all analytes was always within the acceptance range provided by the supplier, with values between -12.8% and 11.8% (Table 7).

Table 5. Analytical intra-assay precision for controls 0192 and 0193 batches #1519 and #2518

Analyte	Control I			Control II		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
	CV (%)	CV (%)	CV (%)	CV (%)	CV (%)	CV (%)
Alanine	5.4	3.5	5.3	4.7	5.3	5.5
Arginine	6.2	4.3	3.4	2.5	3.0	4.4
Aspartic acid	7.8	6.0	5.4	3.7	4.7	5.3
Citrulline	7.8	4.5	6.3	5.3	5.4	5.5
Glutamic acid	5.5	4.0	4.2	4.4	5.0	5.3
Glycine	8.3	7.8	8.5	6.7	6.3	6.8
Leucine	5.5	2.2	5.7	2.2	5.0	5.1
Methionine	8.0	6.5	7.9	6.1	4.8	6.1
Ornithine	5.4	5.9	5.5	2.4	3.9	3.9
Phenylalanine	5.8	2.6	5.9	2.6	4.3	4.6
Proline	5.2	2.5	4.9	3.5	4.3	4.7
Tyrosine	5.0	3.6	4.6	2.7	3.0	4.3
Valine	6.0	2.5	6.0	2.8	5.3	5.1
C0	5.6	3.0	4.1	3.6	4.9	5.5
C2	6.1	2.7	6.7	3.0	4.4	5.2
C3	6.6	4.5	5.9	3.1	5.5	5.4
C4	7.6	5.5	5.4	4.6	5.0	6.6
C5	7.0	6.8	7.3	3.9	6.1	5.6
C5DC	14.9	12.8	12.3	9.4	10.6	11.9
C6	8.7	5.6	8.5	5.1	6.3	6.0
C8	7.4	5.8	8.7	3.6	4.6	6.2
C10	8.2	5.0	8.1	6.2	5.3	5.8
C12	5.2	4.1	7.8	3.3	5.6	6.9
C14	6.3	3.3	7.5	3.0	5.1	6.2
C16	6.3	2.8	6.1	2.9	5.9	6.1
C18	6.2	3.6	6.5	3.5	4.2	5.4

Table 6. Analytical inter-assay precision results for controls 0192 and 0193 batches #1519 and #2518

Analyte	Control I CV (%)	Control II CV (%)
Alanine	8.1	5.7
Arginine	7.6	11.2
Aspartic acid	7.1	6.6
Citrulline	7.5	6.0
Glutamic acid	5.6	6.0
Glycine	8.7	7.7
Leucine	8.7	4.7
Methionine	12.1	5.9
Ornithine	5.9	8.3
Phenylalanine	8.3	4.6
Proline	7.5	5.4
Tyrosine	6.4	4.5
Valine	10.5	5.2
C0	8.5	4.8
C2	8.0	4.8
C3	8.9	4.9
C4	7.9	5.5
C5	9.6	5.7
C5DC	17.7	13.2
C6	10.6	5.6
C8	9.9	5.1
C10	12.3	5.4
C12	11.3	5.6
C14	11.0	5.5
C16	9.9	5.8
C18	7.6	6.1

Table 7. Accuracy for controls 0192 and 0193 batches #1519 and #2518

Analyte	Control I (µmol/L)	Calculated conc. (µmol/L)	Bias (%)	Control I range (µmol/L)	Outcome	Control II (µmol/L)	Calculated conc. (µmol/L)	Bias (%)	Control II range (µmol/L)	Outcome
Alanine	358	373	4.2	164–552	Normal	736	645	-12.3	323–1149	Normal
Arginine	98.0	90.4	-7.7	36.0–160	Normal	225	196	-12.8	115–335	Normal
Aspartic acid	144	134	-6.7	93.0–195	Normal	261	286	9.7	173-350	Normal
Citrulline	67.0	74.3	11	47.0–87.0	Normal	300	273	-8.9	221–379	Normal
Glutamic acid	444	402	-9.5	286–603	Normal	730	794	8.7	502–958	Normal
Glycine	218	221	1.2	158–278	Normal	649	611	-5.8	456–842	Normal
Leucine	302	275	-8.8	166–438	Normal	504	544	8.0	335–673	Normal
Methionine	60.0	55.4	-7.7	19.0–101	Normal	191	206	7.8	76.0–306	Normal
Ornithine	199	179	-10	117–281	Normal	547	488	-10.8	343–751	Normal
Phenylalanine	149	158	5.8	95.0–203	Normal	436	456	4.6	269–603	Normal
Proline	311	304	-2.3	229–393	Normal	774	846	9.3	475–1074	Normal
Tyrosine	160	153	-4.7	108–212	Normal	556	515	-7.4	381–731	Normal
Valine	239	225	-5.7	150–328	Normal	424	435	2.7	278–570	Normal
C0	51.8	48.5	-6.4	28.9–74.7	Normal	101	112	10.9	63.0–139	Normal
C2	23.2	25.9	11.8	15.9–30.5	Normal	60.1	63.4	5.4	37.2–83.0	Normal
C3	5.03	5.00	-0.7	2.91–7.15	Normal	13.0	12.4	-5.0	8.44–17.6	Normal
C4	0.940	0.860	-8.0	0.440–1.44	Normal	3.29	3.60	9.1	1.92–4.66	Normal
C5	0.540	0.560	4.6	0.280–0.800	Normal	2.17	2.23	2.8	1.22–3.12	Normal
C5DC	0.550	0.570	3.2	0.130–0.970	Normal	2.60	2.48	-4.6	1.20–4.00	Normal
C6	0.460	0.500	9.0	0.270–0.650	Normal	2.12	2.26	6.8	1.35–2.89	Normal
C8	0.510	0.480	-5.9	0.270–0.750	Normal	2.17	2.22	2.4	1.22–3.12	Normal
C10	0.450	0.460	2.5	0.260–0.630	Normal	1.96	2.06	5.1	1.08–2.84	Normal
C12	0.450	0.440	-3.3	0.200–0.700	Normal	2.09	2.06	-1.7	1.37–2.81	Normal
C14	0.460	0.450	-2.9	0.240–0.680	Normal	2.09	2.02	-3.5	1.24–2.94	Normal
C16	4.60	4.42	-4.0	2.79–6.41	Normal	13.2	12.3	-6.6	8.08–18.3	Normal
C18	2.61	2.43	-7.0	1.45–3.77	Normal	8.28	8.09	-2.3	4.47–12.1	Normal

Conclusion

The MassChrom LC-MS/MS Complete Kit for Amino Acids and Acylcarnitines in DBS from Chromsystems was developed by an FIA-MS/MS workflow and implemented on a Vanquish Flex Binary UHPLC system coupled to a TSQ Fortis triple-stage quadrupole mass spectrometer. SRM data acquisition was used to provide high selectivity and sensitivity. The method proved to be robust and reliable, and met the required sensitivity requirements typically demanded by clinical research laboratories.

References

1. Lemonde, H. *Pediatr. Child Health*, **2014**, *25*, 103–107.
2. Marca, G.J. *Pharm. Biomed. Anal.*, **2014**, *101*, 174–182.
3. Lund, A.M. *Mol. Genet. Metab.*, **2012**, *107*, 281–293.
4. Sandlers, Y. *Transl. Res.*, **2017**, *189*, 65–75.

Find out more at **thermofisher.com/clinicalresearch**