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Clinical research

A reliable and simple FIA-MS/MS method for the quantitation of amino acids and acylcarnitines in dried blood spots

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Keywords

Vanquish MD HPLC, TSQ Quantis MD MS, TraceFinder LDT software, screening of newborns, ClinSpot kit, inborn errors of metabolism, dried blood spots, non-derivatized amino acid and acylcarnitine kit, flow injection analysis-tandem MS

Goal

Develop and verify a flow injection analysis-tandem MS (FIA-MS/MS) method to quantitate 12 amino acids and 13 acylcarnitines in dried blood spots to be implemented in laboratories performing primary screening for inherited metabolic disorders as part of a recommended screening program for newborns.

Introduction

The screening of newborns is a government-sponsored public health program aiming to detect severe inherited genetic disorders. An early-stage diagnosis of these disorders, sometimes before any symptoms appear, can lead to immediate medical intervention and treatment, therefore minimizing the unnecessary suffering of the patient and the family. In the U.S., the Recommended Uniform Screening Panel (RUSP) is a list of disorders that the Advisory Committee on Heritable Disorders in Newborns and Children of the U.S. Department of Health and Human Services (HHS) has selected based on the criteria that the disorders 1) have selective and sensitive detection methods, 2) have health outcomes that are well understood, 3) have an effective treatment, and 4) could affect the future reproductive decisions of the family.[1,2] Following HHS's recommendation, each state integrates the RUSP into its universal programs for the screening of newborns. Most states screen for the majority of disorders on the RUSP, while some states screen for additional disorders. Newer conditions are nominated to the Advisory Committee and can be adopted into future RUSP updates.

Out of the 60 disorders listed in the RUSP (as of July 2018), 34 are readily detected by tandem-MS, among which abnormal levels of some amino acids and acylcarnitines are indicative of 33 metabolic disorders in organic acid, fatty acid oxidation, and amino acid disorders.[3] Measuring amino acids and acylcarnitines by flow injection analysis-tandem MS (FIA-MS/MS) is the predominant methodology in primary screening of metabolic

disorders in these programs.[3] In this report, a FIA-MS/MS method was developed on a Thermo Scientific™ Vanquish™ MD HPLC and a Thermo Scientific™ TSQ Quantis™ MD MS operated in the selected reaction monitoring (SRM) mode. Quality control dried blood spot samples, reagents, and consumables were obtained from ClinSpot™ LC-MS/MS Complete Kits, Amino Acids and Acylcarnitines in Dried Blood Spots (DBS) – non-derivatized (Ref MS10200, RECIPE Chemicals + Instruments Gmbh, Germany). The developed method verifies the detection of 12 amino acids and 13 acylcarnitines from DBS samples and meets the need for semi-quantitative determination of amino acids and acylcarnitine for the screening of newborns.

Experimental

Sample preparation

The ClinMass™ Internal Standard (IS, Ref MS10012A) was reconstituted using Reagent A (Ref MS10021) following the instruction of the ClinSpot LC-MS/MS Complete Kits, Amino Acids and Acylcarnitines in Dried Blood Spots (DBS) – non-derivatized (Ref MS10200). This solution was used to extract analytes from ClinChek™ – Control Dried Blood Spot (DBS) (Ref MS10182), which came with two control levels (Levels I and II). A 3.2 mm disc was punched from the DBS control cards and placed in a 96/370 µL well plate (Ref MS10040). To extract the analytes, 100 µL of the reconstituted IS solution was added to the well plate, which was sealed by the PE/PP protective sheet (Ref MS10042) and left on an Eppendorf™ ThermoMixer™ mixing device with agitation at 700 rpm at room temperature. After 30 min, the extract was transferred to another 96/370 µL well plate and sealed by the PE/PP protective sheet for LC-MS analysis. For the inter-day and intra-day precision measurements, each control level was prepared five times over three days.

Liquid chromatography

A Vanquish MD HPLC was used to deliver the extracted sample to the MS via isocratic flow injection analysis. The LC-MS system was conditioned with the provided mobile phase (Ref MS10010) for at least 10 minutes prior to sample injection. Each of the five replicate samples were injected twice at an injection volume of 3 µL for a total of 30 injections over three days. The LC conditions are specified in Table 1.

Table 1. Vanquish MD HPLC conditions

Time (min)	Flow rate (mL/min)	Mobile phase (Ref MS10010) (%)
0.00	0.1	100
0.35	0.1	100
0.36	0.5	100
0.75	0.5	100
0.76	0.1	100
1.00	0.1	100

Mass spectrometry

The analyte quantification was achieved using a TSQ Quantis MD mass spectrometer equipped with a Thermo Scientific™ OptaMax™ NG ion source with a heated electrospray ionization probe operated in positive mode. The MS source parameters and SRM properties are listed in Table 2. SRM transitions, optimized collision energies, and RF lens settings for the compounds are shown in Table 3.

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ LDT software (ver 1.0). The analyte (A) concentrations were estimated based on the corresponding isotopically labeled internal standard (IS) using the equation:
Conc (A) = Area (A)/Area (IS) × Conc (IS)

Table 2. TSQ Quantis MD MS settings

MS source parameters	
Ion source type	HESI (OptaMax NG ion source)
HESI probe position	Center - 1.0 - L/M (x - y - z)
Spray voltage	+3,200 V
Sheath gas (Arb)	50
Aux gas (Arb)	5
Sweep gas (Arb)	0
Ion transfer tube temp (°C)	325
Vaporizer temp (°C)	100
SRM properties	
Dwell time (ms)	10
Q1 resolution (FWHM)	0.7
Q3 resolution (FWHM)	1.2
CID gas (mTorr)	1.5
Source fragmentation	5

Table 3. SRM transitions, collision energies, and RF lens for the analytes and their internal standards

Analytes	Precursor (*m/z*)	Product (*m/z*)	IS	Precursor (*m/z*)	Product (*m/z*)	CE (V)	RF lens (V)
Alanine (Ala)	90.1	44.1	$^{13}C_3$,^{15}N-Ala	94.1	47.1	13	76
Arginine (Arg)	175.1	70.1	$^{13}C_6$-Arg	181.1	74.1	25	112
Citrulline (Cit)	176.1	113.1	$^{2}H_7$-Cit	183.1	120.1	17	78
Glutamate (Glu)	148.1	84.1	$^{13}C_5$-Glu	153.1	88.1	18	77
Glycine (Gly)	76.1	30.1	^{13}C,^{15}N-Gly	78.1	32.2	13	51
Leucine (Leu)	132.1	86.1	$^{2}H_3$-Leu	135.1	89.1	11	72
Methionine (Met)	150.2	104.2	$^{2}H_3$-Met	153.2	107.2	12	77
Ornithine (Orn)	133.2	70.2	$^{2}H_6$-Orn	139.2	76.2	19	65
Phenylalanine (Phe)	166.1	120.1	$^{13}C_6$-Phe	172.1	126.1	14	84
Proline (Pro)	116.2	70.2	$^{13}C_5$-Pro	121.1	74.2	16	78
Tyrosine (Tyr)	182.1	136.1	$^{13}C_6$-Tyr	188.1	142.1	15	87
Valine (Val)	118.1	72.2	$^{2}H_8$-Val	126.1	80.2	13	97
Carnitine (C0)	162.1	85.0	$^{2}H_9$-C0	171.1	85.0	22	119
Acetylcarnitine (C2)	204.1	85.0	$^{2}H_3$-C2	207.1	85.0	21	116
Propionylcarnitine (C3)	218.1	85.0	$^{2}H_3$-C3	221.1	85.0	21	125
Butyrylcarnintine (C4)	232.2	85.0	$^{2}H_3$-C4	235.2	85.0	21	125
Isovalerylcarnitine (C5)	246.2	85.0	$^{2}H_9$-C5	255.2	85.0	23	137
Glutarylcarnitine (C5DC)	276.2	85.0	$^{2}H_9$-C5DC	285.2	85.0	25	129
Hexanoylcarnitine (C6)	260.2	85.0	$^{2}H_3$-C6	263.2	85.0	23	141
Octanoylcarnitine (C8)	288.2	85.0	$^{2}H_3$-C8	291.2	85.0	24	156
Decanoylcarnitine (C10)	316.2	85.0	$^{2}H_3$-C10	319.2	85.0	25	168
Dodecanoylcarnitine (C12)	344.2	85.0	$^{2}H_3$-C12	347.2	85.0	26	181
Tetradecanoylcarnitine (C14)	372.2	85.0	$^{2}H_3$-C14	375.2	85.0	26	206
Hexadecanoylcarnitine (C16)	400.3	85.0	$^{2}H_3$-C16	403.3	85.0	28	213
Octadecanoylcarnitine (C18)	428.3	85.0	$^{2}H_3$-C18	431.3	85.0	29	233

Results and discussion

The most common primary screening method for the 33 RUSP recommended metabolic disorders is via the semi-quantitation of some amino acids and acylcarnitines by flow-injection analysis-tandem MS (FIA-MS/MS). Utilizing the Vanquish MD HPLC and TSQ Quantis MD MS, we verified the reliable detection of 12 amino acids and 13 acylcarnitines using the ClinSpot LC-MS/MS Complete Kits, Amino Acids and Acylcarnitines in Dried Blood Spots (DBS) (non-derivatized). The quantification was achieved by comparing the SRM area ratio of the analyte with the corresponding internal standards.

Figure 1 shows results for one analyte, proline, to demonstrate TraceFinder LDT software data processing results. The measured amount, %Diff (comparing to the theoretical amount), %RSD (of the calculated amount), %CV (of the area), etc. are shown in the Sample Results table window. TraceFinder LDT software uses the caution flag to expedite the data review process. Samples outside the predefined acceptance criteria are flagged. The analyte and IS chromatograms from one raw file are shown in the Compound Details Plot, and the chromatograms of the same analyte from different raw files are shown in Compound-centric Plot.

The Trueness of Measurement (accuracy) results are listed in Table 4, and the inter- and intra-day precision measurements are specified in Table 5. All the measured values were within the target range listed by the ClinChek – Control Dried Blood Spot (DBS) (Ref MS10182) specification sheet, except Orn, which was slightly lower. This is due to the fact that the original target ranges were obtained and optimized from other vendors of mass spectrometers, which would exhibit different ionization efficiencies for the same compound. After consulting with RECIPE, all the measured levels were deemed acceptable because the %RSD of all compounds was below 12%, indicating the method was robust and reproducible.

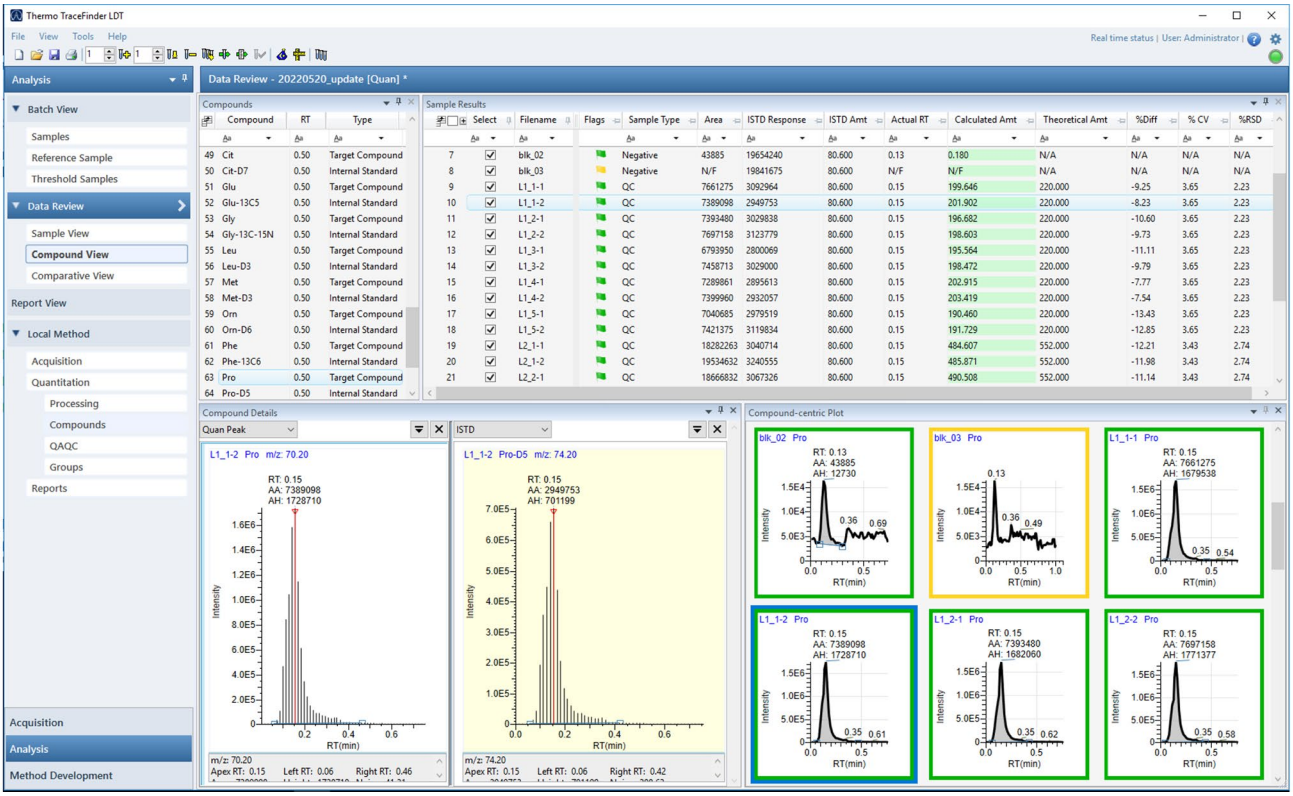

Figure 1. The TraceFinder processing results of Pro shown in the Compound View

Table 4. Trueness of measurement (acceptance criteria: %CV < 35; measured values within the control value range)

Analytes	Control Level I (ranges) (μM)	Measured values (μM)	%CV	Acceptance	Control Level II (ranges) (μM)	Measured values (μM)	%CV	Acceptance
Ala	481.0 (312.7 - 649.4)	425.1	7.4	Pass	1053.0 (737.1 - 1368.9)	965.9	4.2	Pass
Arg	8.3 (2.9 - 13.7)	3.7	3.9	Pass	19.2 (8.6 - 29.8)	9.6	2.7	Pass
Cit	24.5 (13.5 - 35.5)	18.5	4.5	Pass	179.0 (89.5 - 268.5)	175.7	2.6	Pass
Glu	579.0 (376.4 - 781.7)	477.1	3.8	Pass	737.0 (515.9 - 958.1)	615.8	2.4	Pass
Gly	459.0 (275.4 - 642.6)	361.0	5.9	Pass	1110.0 (666.0 – 1554.0)	937.8	4.1	Pass
Leu	248.0 (161.2 - 334.8)	201.8	6.1	Pass	690.0 (448.5 - 931.5)	580.6	6.4	Pass
Met	20.7 (12.4 – 29.0)	15.6	7.1	Pass	370.0 (240.5 - 499.5)	305.7	2.9	Pass
Orn	158.0 (55.3 - 260.7)	65.7	4.2	Pass	570.0 (313.5 - 826.5)	269.2*	5.5	Pass
Phe	94.1 (65.9 - 122.3)	76.2	6.0	Pass	755.0 (490.8 - 1019.3)	637.4	2.6	Pass
Pro	220.0 (154.0 – 286.0)	196.1	6.2	Pass	552.0 (358.8 - 745.2)	505.5	2.7	Pass
Tyr	75.2 (45.1 - 105.3)	65.8	4.7	Pass	558.0 (362.7 - 753.3)	515.3	3.0	Pass
Val	153.0 (84.2 - 221.9)	117.7	6.2	Pass	427.0 (256.2 - 597.8)	340.4	6.1	Pass
C0	39.1 (17.6 - 60.6)	29.6	5.1	Pass	196.0 (98.0 – 294.0)	176.8	3.9	Pass
C2	NA (NA - NA)**	ND	NA	Pass	2.4 (0.9 - 3.8)	1.3	2.6	Pass
C3	0.7 (0.4 - 1.0)	0.6	5.9	Pass	9.1 (5.9 - 12.2)	8.6	3.6	Pass
C4	0.2 (0.1 - 0.3)	0.2	7.7	Pass	0.7 (0.5 - 0.9)	0.5	3.6	Pass
C5	0.5 (0.3 - 0.7)	0.4	7.5	Pass	2.1 (1.3 - 2.9)	2.0	2.1	Pass
C5DC	1.3 (0.5 - 2.2)	1.4	6.0	Pass	4.0 (1.6 - 6.3)	4.6	2.3	Pass
C6	0.1 (0.1 - 0.2)	0.1	7.6	Pass	0.8 (0.5 - 1.1)	0.7	4.2	Pass
C8	0.5 (0.2 - 0.7)	0.4	6.9	Pass	2.5 (1.4 - 3.6)	2.3	3.6	Pass
C10	0.2 (0.1 - 0.4)	0.2	7.8	Pass	1.0 (0.6 - 1.5)	0.9	3.6	Pass
C12	0.4 (0.3 - 0.6)	0.4	7.5	Pass	5.5 (3.9 - 7.2)	5.4	3.9	Pass
C14	0.5 (0.3 - 0.7)	0.4	8.5	Pass	3.1 (1.9 - 4.3)	2.8	3.5	Pass
C16	1.5 (0.7 - 2.2)	1.2	8.8	Pass	11.2 (6.2 - 16.2)	9.9	3.4	Pass
C8	0.7 (0.4 - 1.1)	0.6	8.0	Pass	4.7 (2.6 - 6.8)	4.2	3.9	Pass

* The measured Orn level was slightly lower than the target range, but this was determined to be acceptable (see the text for explanations).

** C0 in Control Level I was not detected. NA, not available

Table 5 (part 1). Inter-day and intra-day precision for Control Level I (acceptance criteria: %CV < 25)

Analytes	Control Level I							
	Day-1		Day-2		Day-3		Inter-day	
	Conc. (µM)	%CV	Conc. (µM)	%CV	Conc. (µM)	%CV	Conc. (µM)	%CV
Ala	425.1	7.4	417.2	2.5	419.8	4.7	420.7	1.0
Arg	3.7	3.9	3.5	4.8	3.6	4.9	3.6	2.5
Cit	18.5	4.5	18.5	10.5	18.8	2.6	18.6	1.1
Glu	477.1	3.8	469.3	2.0	486.1	2.0	477.5	1.8
Gly	361.0	5.9	359.3	4.4	356.5	2.4	359.0	0.6
Leu	201.8	6.1	205.6	1.9	199.7	3.9	202.4	1.5
Met	15.6	7.1	16.4	5.6	16.3	3.0	16.1	2.6
Orn	65.7	4.2	64.3	3.4	68.6	3.7	66.2	3.4
Phe	76.2	6.0	75.9	5.3	75.1	4.5	75.7	0.7
Pro	196.1	6.2	200.2	2.1	197.9	2.2	198.1	1.0
Tyr	65.8	4.7	65.9	3.9	65.2	7.2	65.6	0.6
Val	117.7	6.2	120.4	2.8	117.2	5.1	118.4	1.4
C0	29.6	5.1	30.5	2.4	29.4	4.8	29.9	2.0
C2	ND	NA	ND	NA	ND	NA	ND	NA
C3	0.6	5.9	0.6	4.1	0.6	5.0	0.6	0.7
C4	0.2	7.7	0.2	4.6	0.2	5.6	0.2	1.4
C5	0.4	7.5	0.4	4.9	0.4	6.3	0.4	0.7
C5DC	1.4	6.0	1.4	7.2	1.4	8.6	1.4	1.4
C6	0.1	7.6	0.1	6.2	0.1	5.5	0.1	1.7
C8	0.4	6.9	0.4	3.8	0.4	4.5	0.4	1.9
C10	0.2	7.8	0.2	9.0	0.2	5.4	0.2	2.7
C12	0.4	7.5	0.4	4.4	0.4	5.6	0.4	0.4
C14	0.4	8.5	0.4	4.2	0.4	10.6	0.4	0.7
C16	1.2	8.8	1.3	2.4	1.2	2.3	1.2	1.9
C8	0.6	8.0	0.6	3.0	0.6	4.1	0.6	2.2

Table 5 (part 2). Inter-day and intra-day precision for Control Level II (acceptance criteria: %CV < 25)

	Control Level II							
	Day-1		Day-2		Day-3		Inter-day	
Analytes	Conc. (µM)	%CV	Conc. (µM)	%CV	Conc. (µM)	%CV	Conc. (µM)	%CV
Ala	965.9	4.2	983.0	3.4	877.5	4.8	942.1	6.0
Arg	9.6	2.7	9.1	2.8	9.2	5.5	9.3	3.1
Cit	175.7	2.6	191.7	3.7	184.9	4.4	184.1	4.4
Glu	615.8	2.4	625.7	5.3	595.7	3.0	612.4	2.5
Gly	937.8	4.1	954.9	5.3	875.4	4.0	922.7	4.5
Leu	580.6	6.4	587.5	7.0	547.1	1.9	571.7	3.8
Met	305.7	2.9	310.3	7.7	283.5	4.6	299.8	4.8
Orn	269.2	5.5	262.1	4.4	274.6	10.1	268.6	2.3
Phe	637.4	2.6	650.6	5.5	599.4	5.1	629.1	4.2
Pro	505.5	2.7	528.4	7.1	481.8	2.7	505.3	4.6
Tyr	515.3	3.0	530.3	8.3	479.8	3.5	508.5	5.1
Val	340.4	6.1	350.8	6.8	312.1	5.7	334.4	6.0
C0	176.8	3.9	184.5	6.4	168.9	2.7	176.7	4.4
C2	1.3	2.6	1.3	10.9	1.3	2.9	1.3	2.6
C3	8.6	3.6	8.7	9.3	8.9	5.2	8.4	4.7
C4	0.5	3.6	0.5	8.6	0.5	4.3	0.5	1.7
C5	2.0	2.1	2.1	8.9	1.9	4.0	2.0	3.8
C5DC	4.6	2.3	4.8	7.6	4.7	5.5	4.7	1.5
C6	0.7	4.2	0.7	7.3	0.7	3.8	0.7	2.8
C8	2.3	3.6	2.4	9.5	2.2	8.9	2.3	3.6
C10	0.9	3.6	0.9	11.6	0.9	2.4	0.9	4.7
C12	5.4	3.9	5.6	9.7	5.0	2.4	5.4	5.8
C14	2.8	3.5	2.9	9.3	2.5	3.9	2.7	7.8
C16	9.9	3.4	10.2	8.3	9.1	2.8	9.7	5.8
C8	4.2	3.9	4.3	7.5	3.8	2.5	4.1	7.0

Conclusions

We developed a Vanquish MD HPLC and TSQ Quantis MD MS-based FIA-MS/MS method to verify the quantitation of 12 amino acids and 13 acylcarnitine from the ClinSpot LC-MS/MS Complete Kits, Amino Acids and Acylcarnitines in Dried Blood Spots (DBS) (non-derivatized). The method showed good accuracy and precision measurements that meet the needs of clinical laboratories performing primary screening for many treatable metabolic disorders in newborns.

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*Not for Clinical Diagnosis.