A Novel LC-MS/MS Method for the Direct Analysis of Underivatized Amino Acids In Human Plasma

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Abstract & Introduction

Amino acids are critical molecules for biological function as they serve as building blocks of peptides/proteins and intermediates of various metabolic pathways (e.g., urea cycle and citric acid cycle). Amino acids profiling in plasma is an important tool for the diagnosis of metabolic disorders, especially for inborn errors of metabolism. A fast and accurate large panel amino acids analysis is critical not only for disorder identification but also for continued monitoring and assessment of nutritional status of patients and treatment plans. Traditionally, amino acids were analyzed by either post-column or pre-column derivatization methodologies. The major shortcomings of these approaches are long chromatographic cycling time (post-column derivatization) and poor chromatographic separation (pre-column derivatization) for comprehensive amino acid quantification in plasma. The relative lack of applications for direct analysis of underivatized amino acids by LC-MS/MS is due to limited chromatographic retention and insufficient detection sensitivity. In this study, a simple workflow was developed and implemented for accurate quantification of a large panel of 45 amino acids in human plasma. A fast chromatographic analysis (13 minutes) was achieved using a unique hybrid HILIC/ion exchange column for high-throughput amino acids profiling in plasma.

Methods

Table 1: Analytical Conditions for Waters Xevo TQ-S with Acquity UPLC

Analytical Column	Raptor Polar X 2.7 µm 100 mm x 2.1 mm (Restek Cat.# 9311A12)	
Guard Column	Raptor Polar X EXP Guard Column Cartridge 2.7um, 5 mm x 2.1 mm (Cat.# 9311A0252)	
Mobile Phase A	0.5% formic acid, 1mM ammonium formate in water	
Mobile Phase B	0.5% formic acid, 1mM ammonium formate in 90:10 acetonitrile:water	
Gradient	**Time (min)**	**%B**
	0.00	96
	2.00	96
	10.00	30
	10.01	5
	11.00	5
	11.01	96
	13.00	96
Flow Rate	0.3 mL/min	
Injection Volume	5 µL	
Column Temp.	35°C	
Ion Mode	Scheduled MRM in positive ESI	

Standard and Control Plasma Materials

Two standard mixture solutions (A6282 & A6407) containing a total of 28 amino acids and 17 solid standards were all obtained from *Sigma-Aldrich*. A stock standard solution containing 45 amino acids was prepared in phosphate buffered saline (1x PBS) solution at 250-500 µmol/L. The internal standard working solution was prepared by mixing a stock solution of 17 isotopically labeled amino acids (MSK-A2-S, *Cambridge Isotope Laboratories*) with 12 individual deuterium isotopes of α-aminobutyric acid, β-aminoisobutyric acid, γ-aminobutyric acid, β-alanine, N-acetyltyrosine, asparagine, ethanolamine, glutamine, homocystine, 1-methylhistidine, pipecolic acid, and taurine (*CDN Isotopes*) in water at 125-250 µmol/L. MassChrom® Amino Acid Analysis Plasma Control Level I (0471), II (0472), and III (0473) were obtained from *Chromsystems*.

Calibration Standard Preparation

Calibration standards were prepared at the range of 1-500 µmol/L in 1x PBS solution. An aliquot of 25 µL of calibration standard was mixed with 2.5 µL of 30% sulfosalicylic acid solution, 2 µL of IS working solution, and 225 µL of mobile phase B for injection analysis.

Plasma Sample Preparation

An aliquot of 50 µL of control plasma was mixed with 5 µL of 30% sulfosalicylic acid solution for protein precipitation. Following centrifugation, took out 27.5 µL of clear supernatant and mixed with 2 µL of IS working solution and 225 µL of mobile phase B for injection analysis.

Results & Discussion

(1) Chromatographic Performance: A fast chromatographic method was established for simultaneous analysis of 45 amino acids with a 13-minute total cycling time (Figure 1a, control plasma L2). The isobaric compounds leucine/isoleucine/allo-isoleucine (Figure 1b) and alanine/sarcosine (Figure 1c) are chromatographically separated for definitive and accurate quantification in plasma. The chromatographic retention of 3 analytes, β-aminoisobutyric acid, γ-aminobutyric acid, and β-alanine, is sensitive to the salting condition in final injection solution. Therefore, PBS was added to the standard solution to match the salting condition in plasma sample solution and resulted in consistent retention time of these 3 compounds between standard and sample solutions.

(2) Linearity: Various calibration ranges were determined considering different MS detection sensitivity and the suitability of diagnostic measurement of all 45 analytes (see Table 2). All compounds showed acceptable linearity with r^2 value > 0.990 and deviations <20% with quadratic regression (1/x weighted). While the highest quantifiable concentration of 500 µmol/L is suitable for most of the analytes, it is necessary to dilute the plasma sample for the analysis of glutamine and alanine due to their relatively higher physiological concentration of > 500 µmol/L.

(3) Accuracy & Precision: Three control plasma samples containing low to high concentration of all 45 analytes were used to evaluate the method accuracy and precision. The acceptable criteria for method accuracy is to have measured concentration within the specified concentration range. A total of 3 batches of analyses were performed on different days. To compensate for the plasma matrix effect, 28 isotopes were carefully examined to act as the best fitted internal standards for the quantification of various analytes. Table 2 shows the accuracy and precision results averaged from the collection of all 3 batches of data (n=9) and internal standards used for the quantification of each analyte. The method accuracy was demonstrated with concentration values of within the nominal range for all 45 analytes. The %RSD was <20% indicating acceptable method precision.

Figure 1: Analysis of Control Plasma

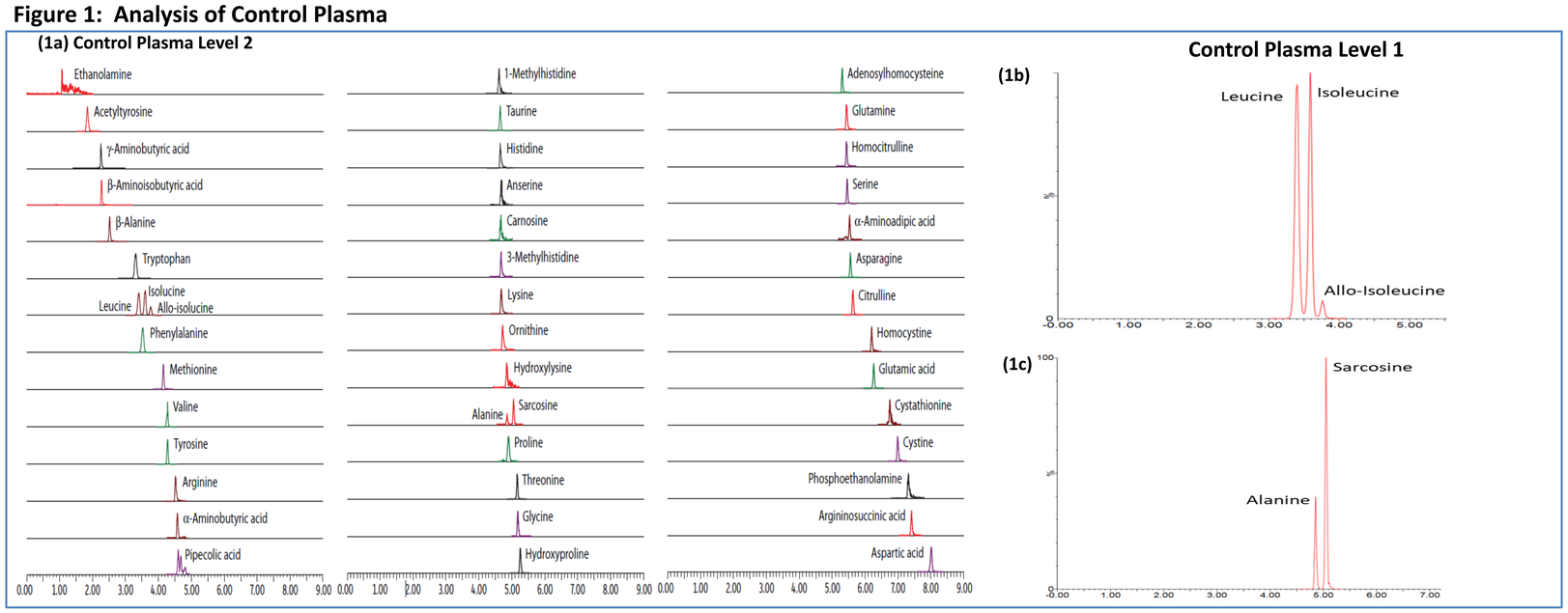

Table 2: Accuracy & Precision Analysis

Analytes	Retention Time	IS	Linearity Range (µmol/L)	Control Level 1 Nominal Conc. Range (µmol/L)	Control Level 1 Average Conc. (µmol/L) (%RSD)	Control Level 2 Nominal Conc. Range (µmol/L)	Control Level 2 Average Conc. (µmol/L) (%RSD)	Control Level 3 Nominal Conc. Range (µmol/L)	Control Level 3 Average Conc. (µmol/L) (%RSD)
Acetyltyrosine	1.83	Acetyltyrosine-d3	0.5 - 250	4.10 - 6.15	4.84 (9.76)	32.5 - 48.8	40.5 (9.76)	58.5 - 87.7	73.9 (15.1)
Adenosylhomocysteine	5.30	Threonine $^{13}C_4$ ^{15}N	2.5 - 250	2.77 - 4.16	3.16 (15.0)	17.8 - 26.7	20.4 (15.4)	32.5 - 48.8	42.9 (9.98)
Alanine	4.86	Alanine $^{13}C_3$ ^{15}N	5 - 500	159 - 239	191 (15.0)	524 - 786	655* (20.0)	890 - 1334	979* (19.4)
β-Alanine	2.52	ß-Alanine-d4	1 - 500	10.4 - 15.5	10.8 (3.13)	42.7 - 64.0	47.9 (3.12)	74.0 - 111	81.1 (3.01)
α-Aminoadipic acid	5.53	Asparagine-d3	1 - 500	4.98 - 7.46	5.74 (7.53)	9.92 - 14.9	11.2 (6.75)	14.7 - 22.1	17.9 (11.7)
α-Aminobutyric acid	4.57	α-Aminobutyric acid-d2	1 - 500	3.69 - 5.54	4.47 (11.4)	39.5 - 59.2	52.1 (10.9)	73.6 - 110	97.7 (7.43)
β-Aminoisobutyric acid	2.27	β-Aminoisobutyric acid-d3	1 - 500	3.82 - 5.73	4.40 (9.85)	17.0 - 25.5	19.2 (6.10)	29.7 - 44.5	35.3 (7.75)
γ-Aminobutyric acid	2.25	γ-Aminobutyric acid-d4	1 - 500	3.79 - 5.69	4.75 (5.35)	7.98 - 12.0	9.60 (3.45)	12.0 - 17.9	14.1 (5.60)
Anserine	4.67	Histidine $^{13}C_6$ $^{15}N_3$	1 - 500	3.85 - 5.77	4.71 (12.5)	7.65 - 11.5	10.8 (5.82)	11.5 - 17.3	15.9 (7.71)
Arginine	4.52	Arginine $^{13}C_6$ $^{15}N_4$	1 - 500	8.63 - 12.9	9.55 (11.5)	124 - 186	133 (3.60)	237 - 356	257 (3.32)
Argininosuccinic acid	7.41	Cystine $^{13}C_6$ $^{15}N_2$	0.5 - 250	2.35 - 3.53	2.96 (13.3)	59.7 - 89.6	72.3 (11.1)	115 - 172	141 (7.99)
Asparagine	5.54	Asparagine-d3	1 - 500	21.4 - 32.1	31.3 (2.46)	97.0 - 145	143 (1.39)	173 - 259	250 (2.22)
Aspartic acid	8.00	Aspartic Acid $^{13}C_4$ ^{15}N	1 - 500	18.5 - 27.7	19.2 (1.83)	101 - 152	105 (3.54)	182 - 274	189 (2.89)
Carnosine	4.67	Histidine $^{13}C_6$ $^{15}N_3$	1 - 500	7.69 - 11.5	10.3 (9.66)	18.9 - 28.3	23.7 (10.1)	29.2 - 43.8	36.6 (10.2)
Citrulline	5.61	Asparagine-d3	5 - 500	12.7 - 19.1	15.6 (13.9)	83.8 - 126	88.8 (4.17)	153 - 230	161 (1.63)
Cystathionine	6.75	Glutamic Acid $^{13}C_5$ ^{15}N	1 - 500	4.86 - 7.29	5.76 (6.47)	20.1 - 30.1	23.1 (8.07)	35.3 - 52.9	40.7 (9.63)
Cystine	6.99	Cystine $^{13}C_6$ $^{15}N_2$	0.5 - 250	8.54 - 12.8	10.3 (9.50)	62.7 - 94.0	78.8 (6.33)	116 - 174	144 (9.05)
Ethanolamine	1.06	Ethanolamine-d4	5 - 250	12.3 - 18.4	15.2 (11.1)	98.5 - 148	125 (11.3)	182 - 273	206 (3.53)
Glutamic acid	6.26	Glutamic Acid $^{13}C_5$ ^{15}N	1 - 500	45.8 - 68.7	53.7 (6.16)	304 - 456	335 (8.12)	553 - 829	593* (8.49)
Glutamine	5.43	Glutamine-d5	1 - 500	286 - 429	371 (3.58)	748 - 1122	975* (5.46)	1209 - 1814	1587* (8.40)
Glycine	5.19	Threonine $^{13}C_4$ ^{15}N	25 - 500	154 - 231	177 (10.2)	517 - 775	613* (7.54)	864 - 1295	995* (10.9)
Histidine	4.65	Histidine $^{13}C_6$ $^{15}N_3$	1 - 500	35.9 - 53.9	47.6 (5.27)	121 - 182	157 (7.27)	210 - 316	266 (4.35)
Homocitrulline	5.43	Glutamine-d5	2.5 - 250	7.88 - 11.8	9.87 (2.05)	22.6 - 33.9	31.3 (3.83)	37.1 - 55.6	53.7 (2.69)

Analytes	Retention Time	IS	Linearity Range (µmol/L)	Control Level 1 Nominal Conc. Range (µmol/L)	Control Level 1 Average Conc. (µmol/L) (%RSD)	Control Level 2 Nominal Conc. Range (µmol/L)	Control Level 2 Average Conc. (µmol/L) (%RSD)	Control Level 3 Nominal Conc. Range (µmol/L)	Control Level 3 Average Conc. (µmol/L) (%RSD)
Homocystine	6.20	Homocystine-d8	1 - 500	3.29 - 4.93	3.60 (9.17)	10.5 - 15.8	12.8 (5.69)	18.0 - 27.0	22.0 (4.71)
Hydroxylysine	4.84	Threonine $^{13}C_4$ ^{15}N	5 - 500	3.97 - 5.96	5.50 (7.60)	14.3 - 21.4	16.58 (11.2)	24.3 - 36.5	28.4 (14.2)
Hydroxyproline	5.26	Threonine $^{13}C_4$ ^{15}N	5 - 500	7.94 - 11.9	10.1 (14.2)	72.8 - 109	97.52 (8.40)	136 - 204	198 (5.02)
Allo-isoleucine	3.77	Methionine $^{13}C_5$ ^{15}N	0.5 - 250	2.86 - 4.29	3.55 (3.12)	65.5 - 98.2	79.15 (4.56)	128 - 192	150 (5.87)
Isoleucine	3.60	Isoleucine $^{13}C_6$ ^{15}N	5 - 500	18.4 - 27.6	20.1 (3.12)	143 - 215	164.41 (3.01)	260 - 389	282 (2.69)
Leucine	3.40	Leucine $^{13}C_6$ ^{15}N	5 - 500	40.2 - 60.3	43.3 (2.57)	239 - 358	288.87 (4.37)	444 - 666	512* (4.67)
Lysine	4.68	Lysine $^{13}C_6$ $^{15}N_2$	1 - 500	21.9 - 32.9	28.2 (5.79)	231 - 346	315.89 (4.13)	436 - 655	621* (4.61)
Methionine	4.15	Methionine $^{13}C_5$ ^{15}N	1 - 500	6.80 - 10.2	7.46 (5.29)	64.8 - 97.1	72.02 (3.83)	121 - 181	139 (2.32)
1-Methylhistidine	4.62	1-Methylhistidine-d3	1 - 500	2.06 - 3.08	2.52 (8.18)	6.17 - 9.25	7.12 (9.84)	10.3 - 15.4	12.4 (9.84)
3-Methylhistidine	4.68	Histidine $^{13}C_6$ $^{15}N_3$	5 - 500	5.82 - 8.73	8.03 (8.31)	36.2 - 54.3	43.81 (10.2)	66.3 - 99.5	85.1 (4.10)
Ornithine	4.72	Lysine $^{13}C_6$ $^{15}N_2$	5 - 500	13.6 - 20.4	15.2 (3.13)	161 - 242	168.76 (4.35)	301 - 452	322 (5.76)
Phenylalanine	3.53	Phenylalanine $^{13}C_9$ ^{15}N	1 - 500	32.2 - 48.3	36.5 (3.38)	248 - 373	297.96 (1.80)	461 - 692	547* (4.31)
Phosphoethanolamine	7.32	Cystine $^{13}C_6$ $^{15}N_2$	5 - 500	8.53 - 12.8	9.64 (12.2)	57.4 - 86.1	64.32 (9.09)	105 - 158	127 (9.93)
Pipecolic acid	4.60	Pipecolic acid-d9	0.5 - 250	1.62 - 2.43	1.85 (9.98)	13.4 - 20.0	17.25 (8.08)	24.6 - 36.9	31.0 (7.45)
Proline	4.89	Proline $^{13}C_5$ ^{15}N	1 - 500	50.7 - 76.1	58.6 (4.05)	268 - 403	322.45 (3.46)	482 - 722	578* (4.99)
Sarcosine	5.05	Threonine $^{13}C_4$ ^{15}N	1 - 500	3.29 - 4.94	4.00 (7.35)	13.5 - 20.3	19.59 (6.51)	23.7 - 35.5	34.8 (4.19)
Serine	5.46	Serine $^{13}C_3$ ^{15}N	5 - 500	102 - 153	126 (3.65)	295 - 443	383.86 (6.51)	484 - 726	627* (5.89)
Taurine	4.65	Taurine-d4	5 - 500	14.7 - 22.1	18.2 (6.28)	173 - 260	234.74 (3.51)	325 - 488	433 (6.26)
Threonine	5.15	Threonine $^{13}C_4$ ^{15}N	1 - 500	36.7 - 55.1	42.6 (2.76)	203 - 304	246.86 (7.01)	366 - 549	464 (4.16)
Tryptophan	3.31	ß-Alanine-d4	1 - 500	13.5 - 20.2	16.9 (11.5)	88.4 - 133	107.52 (7.72)	158 - 237	188 (10.3)
Tyrosine	4.27	Tyrosine $^{13}C_9$ ^{15}N	1 - 500	22.3 - 33.5	25.4 (5.35)	129 - 194	148.82 (2.58)	232 - 348	264 (3.40)
Valine	4.27	Valine $^{13}C_5$ ^{15}N	1 - 500	74.4 - 112	91.3 (6.73)	256 - 384	318.69 (7.67)	442 - 664	532* (9.56)

*Concentration is outside of the calibration range

Conclusions

It was demonstrated that simultaneous measurement of 45 amino acids in plasma can be achieved with a simple sample preparation procedure and a fast 13-minute LC-MS/MS analysis using the Raptor Polar X column. The established method provides high-throughput and accurate determination for amino acid profiling in human plasma.