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Direct Quantification of Amino Acids in Human Plasma by LC-MS/MS

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ABSTRACT

Purpose: Development and implementation of an analytical method for the quantification of 52 amino acids in plasma with a simple chromatographic approach without derivatization or ion pairing reagent addition

Methods: Plasma samples were protein precipitated and diluted with an internal standard solution prior to injection onto an LC-MS/MS triple quadrupole mass spectrometer. Separation of compounds was performed with an 18-minute gradient on a mixed mode chromatographic column.

Results: Sensitivity of the method was determined for 45 compounds in pure media. The method was also tested with external plasma quality controls for 27 compounds leading to accuracy and precision within the limit of 20% for the two quality controls in plasma for the 27 studied compounds.

INTRODUCTION

Amino acids are the building blocks of proteins and intermediaries to many biochemical pathways. They play an important role in biochemical regulation, and any abnormalities in abundance may be indicative of greater metabolic issues or inherited disorders. There is, therefore, an increasing interest in the rapid analysis of amino acids and other amino compounds in plasma samples and other biological fluids for clinical research. Typically, the expectation is to analyze a large panel of compounds in a single chromatographic run without adding ion pairing reagents and/or tedious sample preparation steps. Until now, this type of analysis has been performed with liquid chromatography (LC) methods based on post-column derivatization with ninhydrin (for UV-visible detection) or with pre-column derivatization with *o*-phthalaldehyde (for fluorescent detection). While these derivatization workflows are quite popular, they are time-consuming and require controlled conditions to achieve desired robustness, reliability, and reproducibility.

For the analysis by LC coupled to mass spectrometry (MS), chromatographic retention and the separation of certain isomers may be a challenge. The most popular techniques are based on either the use of ion pairing agents in the mobile phase or the use of derivatization prior to a reverse phase separation. The same issues with derivatization exist as compared to LC-UV techniques, as for the use of ion pairing agents, they alter the robustness of the method at the level of the separation, and their continuous injection to the MS irremediably leads to the contamination of the ion source.

Here we present a novel workflow that simplifies sample preparation by eliminating the use of a derivatization agent and is ion-pairing reagent-free for the analysis of a panel of 52 amino acids and related compounds by LC-MS/MS.

MATERIALS AND METHODS

Sample Preparation

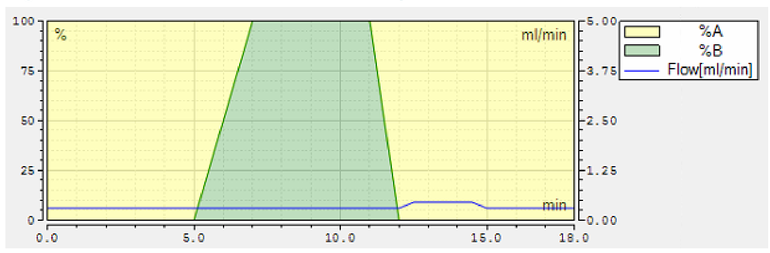
Calibrators were prepared by diluting in water some standard solutions containing all the amino acids. Two external quality control were used, and they consisted of Level 1 (L1) and Level 2 (L2) plasma controls from ERNDIM (http://www.erndim.org) with lot numbers Lot 2017.0061 and Lot 2017.0062, respectively.

One hundred microliters of each calibrator, quality control, and plasma donor sample were mixed with 10 µL of 30% sulfosalicylic acid in a 1.5 mL Eppendorf tube. After vortexing for 30 seconds, the mixture is refrigerated at 4 ° C for 30 minutes and centrifuged at 12,000 rpm for 5 minutes. Fifty microliters of supernatant were vortex mixed for 30 seconds with 450 µL of internal standard solution in 100% mobile phase A, and 4 µL of this final solution were injected into the LC-MS/MS system.

Chromatographic Conditions

Separation was performed with a mixed mode Thermo Scientific™ Acclaim™ Trinity column by gradient elution. Mobile phases consisted on ammonium formate in water at pH=2.8 for phase A, and a mixture of ammonium formate in water and acetonitrile (80/20 v/v) for phase B. The corresponding gradient appears in Figure 1.

Figure 1. Gradient used for amino acids separation



Mass Spectrometry Conditions

Compounds were detected by electrospray ionization on a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer in Selected Reaction Monitoring (SRM) acquisition mode. Sheath gas was set at 45 arbitrary units, auxiliary gas at 15 arbitrary units, and spray voltage at 3500 V for positive ionization and at 2700 V for negative ionization. Vaporizer temperature was set to 370 ° C and transfer tube temperature to 270 ° C, while source fragmentation was applied at 15 V. SRM transitions for target compounds appear in Table 1.

Table 1. SRM transitions and retention times for target compounds

Compound	Retention Time (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
1-Methylhistidine	10.80	Positive	170.12	124.11	14	75
3-Methylhistidine	10.60	Positive	170.12	126.1	13	75
5-Aminolevunilic Acid	4.5	Positive	132.183	114.18	10	67
Alanine	2.07	Positive	90.274	90.274	5	56
alpha-Aminoadipic Acid	2.16	Positive	162.152	98.22	16	72
alpha-Aminobutyric Acid	2.20	Positive	104.213	58.33	10	47
Anserine	11.70	Positive	241	109	20	90
Arginine	13.36	Positive	175.12	70.26	22	83
Argininosuccinic Acid	9.5	Positive	291.183	70.29	31	151
Asparagine	1.82	Positive	133.2	87.15	10	60
Aspartic Acid	2.02	Positive	134.11	74.22	15	65
Beta-Alanine	3.52	Positive	90.252	72.21	10	46
beta-Aminoisobutyric Acid	3.78	Positive	104.252	86.15	10	51
Carnosine	12.13	Positive	227.183	110.17	22	95
Citrulline	2.37	Positive	176.213	159.04	10	71
Cystathionine	8.68	Positive	223.152	134.11	14	91
Cysteine	4.93	Positive	122.131	59.28	21	48
Cysteine-Homocysteine Disulfide	7.00	Positive	255	134	20	100
Cystine	5.78	Positive	241.1	152	13	91
Ethanolamine	3.54	Positive	62.365	62.37	5	51
gamma-Aminobutyric Acid	4.29	Positive	104.252	87.15	10	51
Glutamic Acid	2.02	Positive	148.1	84.15	15	62
Glutamine	1.88	Positive	147.12	130.04	10	64
Glutathione Reduced	7.00	Positive	308.335	179	12	110
Glycine	2.01	Positive	76.3	76.3	5	46
Histidine	10.73	Positive	156.13	110.1	13	73
Homocitruline	3.20	Positive	190.098	173.06	10	75
Homocysteine	3.50	Positive	136.13	90.17	10	61
Homocystine	9.57	Positive	269.152	136.04	10	83
Hydroxylysine	10.21	Positive	163	128	13	65
Hydroxyproline	1.73	Positive	132.04	86.22	11	65
Isoleucine	3.06	Positive	132.18	69.26	16	55
Kynurenine	9.40	Positive	209.183	192.06	10	82
Leucine and allo-isoleucine	3.21 / 2.91	Positive	132.18	86.22	10	55
Lysine	10.73	Positive	147.17	84.22	16	64
Methionine	2.68	Positive	150.098	133.07	10	61
Ornithine	10.35	Positive	133.243	70.26	17	62
Phenylalanine	5.61	Positive	166.12	120.17	11	62
Phosphoethanolamine	1.71	Positive	142.07	44.44	10	53
Phosphoserine	5.04	Positive	186	88	15	60
Pipecolic Acid	2.59	Positive	130.16	84.22	15	67
Proline	2.00	Positive	116.243	70.26	15	67
Saccharopine	7.50	Positive	277.077	84.21	24	105
Sarcosine	1.91	Positive	90.274	44.44	10	56
Serine	1.82	Positive	106.191	60.33	10	47
Sulfocysteine	6.37	Positive	202.091	120.06	11	79
Taurine	1.69	Positive	126.152	126.15	5	82
Threonine	1.83	Positive	120.16	74.28	10	58
Tryptophan	9.65	Positive	205.078	187.99	10	71
Tyrosine	4.72	Positive	182.078	136.04	12	73
Valine	2.35	Positive	118.17	72.26	10	45

Data Analysis

The limit of quantification (LOQ) for each analyte was determined as the lowest value in the calibration curve giving an average % bias between nominal and back calculated concentration within ±20% and a %CV below 20% on 10 replicate injections of calibrators. Intra- and inter-assay precision was performed for the 27 compounds that are present in the AMI-02.1 and AMI-02.2 plasma control material from ERNDIM (Lot 2017.0061 and Lot 2017.0062). For intra-assay precision, the controls were prepared and analyzed 30 times in the same day. For inter-assay precision, they were prepared 30 times in different days.

RESULTS

Calibration Results

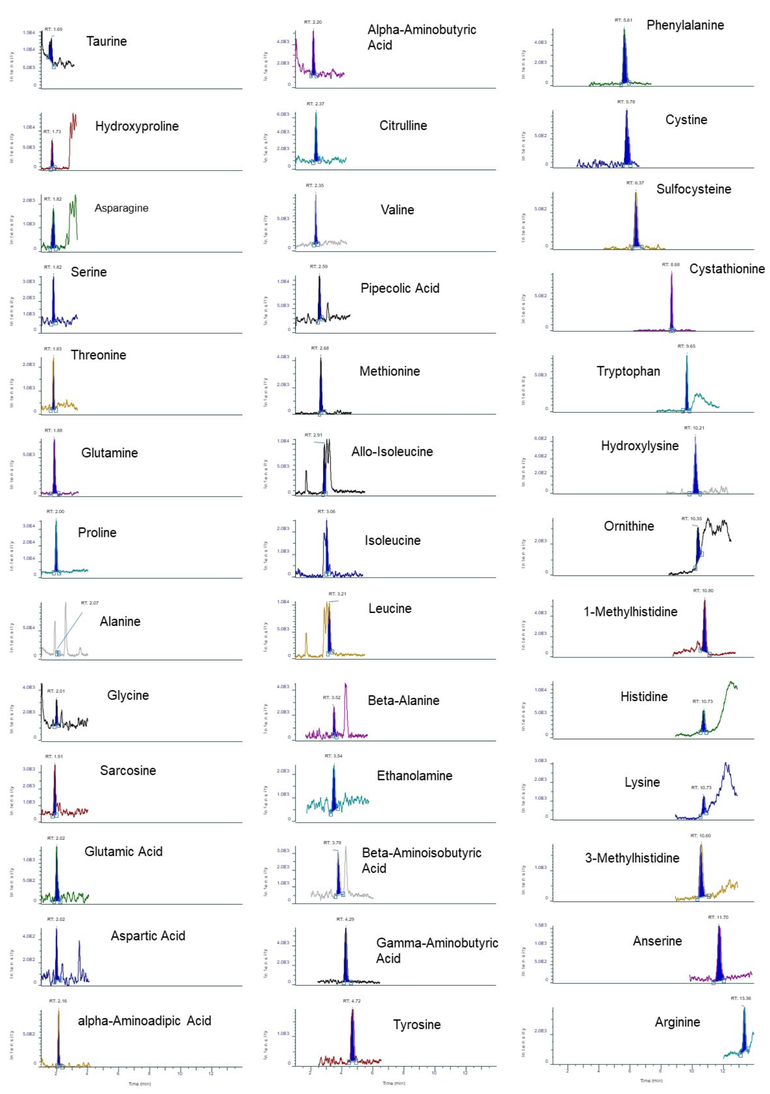
Internal calibration was used for 37 compounds, 25 using the corresponding isotopically labeled internal standards. External calibration (no internal standard) was used for five additional analytes. Qualitative detection was achieved for the remaining compounds. Details of calibration approach, linearity range, and LOQ for each analyte are reported in Table 2.

Table 2. Calibration approach, linearity range , and LOQ for each analyte

Compound	Calibration	ISTD	LOQ (µmol/L)	Linearity Range (µmol/L)	Type	Weighting	Origin
1-Methylhistidine	Internal	Histidine 13C6 15N3	2	2-500	Quadratic	1/X	Ignore
3-Methylhistidine	Internal	Histidine 13C6 15N3	5	5-500	Linear	1/X	Ignore
Alanine	Internal	Alanine 13C3 15N	20	20-500	Linear	1/X	Ignore
Allo-Isoleucine	Internal	Isoleucine 13C6 15N1	5	5-500	Quadratic	Equal	Ignore
Anserine	Internal	Lysine 13C6 15N2	5	5-500	Linear	1/X	Ignore
Arginine	Internal	Arginine 13C6 15N4	5	5-500	Linear	1/X	Ignore
Asparagine	Internal	Asparagine 13C4 D3 15N2	24	24-600	Linear	1/X	Ignore
Aspartic Acid	Internal	Aspartic Acid 13C4 15N1	10	10-200	Linear	1/X2	Ignore
Citrulline	Internal	Citrulline 13C D4	5	5-200	Linear	1/X	Ignore
Cystathionine	Internal	Phenylalanine 13C9 15N1	2.5	2.5-100	Linear	1/X	Ignore
Cystine	Internal	Cystine 13C6 15N2	10	10-500	Linear	1/X	Ignore
Ethanolamine	Internal	Tyrosine 13C9 15N1	2	2-500	Linear	1/X	Ignore
Glutamic Acid	Internal	Glutamic Acid 13C5 15N1	5	5-500	Linear	1/X	Ignore
Glutamine	Internal	Glutamine 13C5 D5 15N2	10	10-500	Linear	1/X	Ignore
Glycine	Internal	Glycine 13C2 15N1	50	50-500	Linear	1/X	Force
Histidine	Internal	Histidine 13C6 15N3	5	5-500	Linear	1/X2	Ignore
Hydroxylysine	Internal	Tyrosine 13C9 15N1	10	10-500	Linear	1/X	Ignore
Hydroxyproline	Internal	Proline 13C5 15N1	10	10-500	Linear	1/X	Ignore
Isoleucine	Internal	Isoleucine 13C6 15N1	10	10-500	Linear	1/X	Ignore
Leucine	Internal	Leucine 13C6 15N1	5	5-500	Linear	1/X	Ignore
Lysine	Internal	Lysine 13C6 15N2	5	5-500	Linear	1/X2	Ignore
Methionine	Internal	Methionine 13C5 15N1	5	5-500	Linear	1/X	Ignore
Ornithine	Internal	Ornithine D6	20	20-500	Quadratic	1/X	Ignore
Phenylalanine	Internal	Phenylalanine 13C9 15N1	2	2-500	Linear	1/X2	Ignore
Pipecolic Acid	Internal	Pipecolic Acid 13C6 15N	2	2-500	Linear	1/X2	Ignore
Proline	Internal	Proline 13C5 15N1	5	5-500	Linear	1/X	Ignore
Sarcosine	Internal	Phenylalanine 13C9 15N1	12.5	12.5-1250	Linear	1/X	Ignore
Serine	Internal	Serine 13C3 15N1	20	20-500	Linear	1/X2	Ignore
Taurine	Internal	Taurine 13C2 15N	25	25-500	Linear	1/X	Ignore
Threonine	Internal	Threonine 13C4 15N1	10	10-500	Linear	1/X	Ignore
Tryptophan	Internal	Tryptophan 13C11 15N2	5	5-500	Linear	1/X2	Ignore
Tyrosine	Internal	Tyrosine 13C9 15N1	5	5-500	Linear	1/X	Ignore
Valine	Internal	Valine 13C5 15N1	5	5-500	Linear	1/X	Ignore
α-Aminoadipic Acid	Internal	Alanine 13C3 15N	5	5-250	Linear	1/X	Ignore
α-Aminobutyric Acid	Internal	γ-Aminobutyric Acid 13C4	10	10-100	Linear	1/X	Ignore
β-Alanine	Internal	Tyrosine 13C9 15N1	10	10-500	Linear	1/X	Ignore
β-Aminoisobutyric Acid	Internal	GABA 13C4	5	5-500	Linear	1/X	Ignore
γ-Aminobutyric Acid	Internal	GABA 13C4	2	2-500	Linear	1/X	Ignore
Carnosine	External	N/A	2	2-500	Linear	1/X^2	Ignore
Phosphoethanolamine	External	N/A	10	10-250	Linear	Equal	Ignore
Phosphoserine	External	N/A	10	10-250	Linear	Equal	Ignore
Sulfocysteine	External	N/A	5	5-500	Quadratic	1/X	Ignore
5-Aminolevunilic Acid	No calibration performed for this compound						
Argininosuccinic Acid	No calibration performed for this compound						
Cysteine-Homocysteine Disulfide	No calibration performed for this compound						
Glutathione Reduced	No calibration performed for this compound						
Homocitrulline	No calibration performed for this compound						
Homocysteine	No calibration performed for this compound						
Homocystine	No calibration performed for this compound						
Kynurenine	No calibration performed for this compound						
Saccharopine	No calibration performed for this compound						
Cysteine	Non applicable, converted to cystine						

The chromatograms at the LOQ are presented in Figure 2.

Figure 2. Chromatograms at the LOQ



Precision and Accuracy Results

The results obtained for intra-assay accuracy and precision are presented in Table 3, and the same results for inter-assay study are presented in Table 4.

Table 3. Intra-assay accuracy and precision

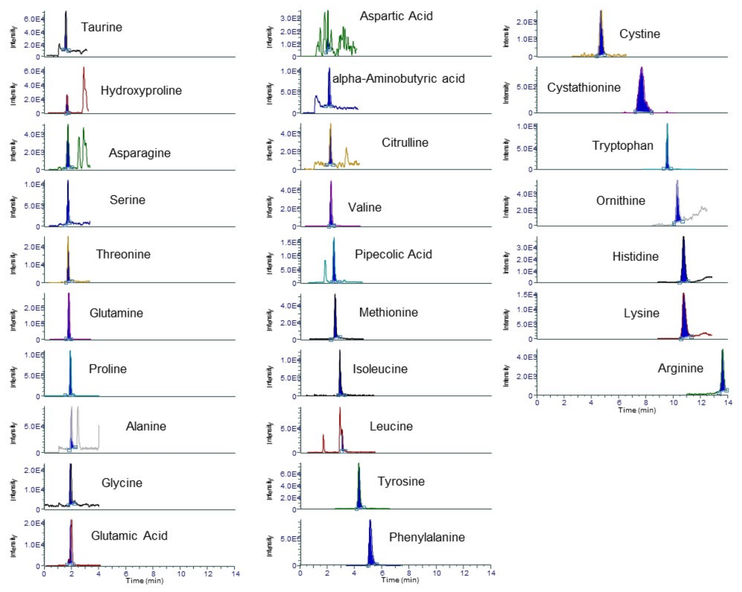
Compound	Control L1				Control L2			
	Theoretical Concentration (µmol/L)	Average Concentration (µmol/L)	Bias (%)	CV (%)	Theoretical Concentration (µmol/L)	Average Concentration (µmol/L)	Bias (%)	CV (%)
Alanine	318	297	-6.6	10.8	925	1078	16.5	8.0
α-Aminobutyric Acid	31.2	26	-17.6	7.5	94.7	82	-13.3	4.2
Arginine	16.59	16	-5.8	4.7	519	512	-1.4	1.3
Asparagine	107.7	87	-19.4	8.1	222	189	-15.1	5.7
Aspartic Acid	12	10	-13.8	43.1	99.6	104	4.0	8.9
Citrulline	4.63	5	4.9	15.0	415	422	1.8	1.8
Cystathionine	9.95	8	-21.8	4.9	29.6	25	-15.9	4.8
Cystine	32.5	27	-17.2	6.0	69.4	63	-9.7	16.7
Glutamic Acid	107	103	-3.8	5.4	223	202	-9.4	8.4
Glutamine	575	604	5.0	2.6	1165	1238	6.3	3.5
Glycine	516	479	-7.2	7.2	1021	935	-8.5	8.8
Histidine	203	187	-7.7	3.1	398	395	-0.7	1.5
Hydroxyproline	48	46	-4.3	4.3	98	106	7.9	5.1
Isoleucine	52.1	44	-14.8	6.6	398	386	-3.0	3.4
Leucine	26.8	25	-6.7	5.4	890	803	-9.8	2.6
Lysine	271	245	-9.8	5.6	534	522	-2.2	2.8
Methionine	79.9	75	-6.2	4.4	241	244	1.0	3.3
Ornithine	159	151	-5.1	7.6	639	610	-4.6	6.1
Phenylalanine	341	335	-1.8	1.7	681	693	1.8	0.8
Pipecolic Acid	44.6	47	6.4	1.8	92.6	99	7.1	1.3
Proline	301	292	-3.0	1.9	602	608	0.9	2.0
Serine	154	157	2.2	8.2	463	480	3.6	5.2
Taurine	213	231	8.6	5.9	417	475	14.0	7.6
Threonine	205	205	0.0	6.5	408	386	-5.3	3.1
Tryptophan	116	112	-3.4	6.0	292	289	-0.9	1.3
Tyrosine	234	222	-5.2	1.6	927	953	2.8	1.6
Valine	416	395	-5.1	2.2	823	815	-1.0	2.2

Table 4. Inter-assay accuracy and precision

Compound	Control L1				Control L2			
	Theoretical Concentration (µmol/L)	Average Concentration (µmol/L)	Bias (%)	CV (%)	Theoretical Concentration (µmol/L)	Average Concentration (µmol/L)	Bias (%)	CV (%)
Alanine	318	330	3.8	14.8	925	1066	15.3	18.6
α-Aminobutyric Acid	31.2	28	-10.3	8.8	94.7	86	-9.2	8.3
Arginine	16.59	17	4.2	9.2	519	524	1.0	6.7
Asparagine	107.7	88	-18.0	9.4	222	194	-12.6	8.4
Aspartic Acid	12	12	-3.0	37.0	99.6	109	9.6	11.6
Citrulline	4.63	5	1.0	20.6	415	425	2.5	5.4
Cystathionine	9.95	8	-18.7	5.5	29.6	26	-13.4	6.5
Cystine	32.5	28	-14.9	6.6	69.4	61	-11.5	5.4
Glutamic Acid	107	101	-5.4	8.2	223	229	2.9	14.1
Glutamine	575	609	6.0	7.4	1165	1255	7.7	6.8
Glycine	516	497	-3.7	13.5	1021	946	-7.3	13.3
Histidine	203	196	-3.5	5.4	398	411	3.3	8.2
Hydroxyproline	48	50	3.5	5.4	98	111	13.3	5.6
Isoleucine	52.1	47	-8.9	7.8	398	387	-2.8	7.0
Leucine	26.8	25	-7.5	8.7	890	811	-8.9	4.8
Lysine	271	256	-5.4	6.4	534	537	0.5	9.1
Methionine	79.9	78	-2.9	4.7	241	248	3.1	9.5
Ornithine	159	152	-4.2	11.4	639	575	-10.1	7.8
Phenylalanine	341	340	-0.4	2.9	681	693	1.7	4.5
Pipecolic Acid	44.6	49	8.8	5.2	92.6	101	9.0	5.8
Proline	301	300	-0.2	4.5	602	619	2.9	5.7
Serine	154	141	-8.3	11.4	463	449	-3.0	7.9
Taurine	213	232	9.0	13.3	417	492	18.1	6.9
Threonine	205	197	-4.0	6.8	408	407	-0.3	5.7
Tryptophan	116	114	-1.9	6.2	292	303	3.8	7.8
Tyrosine	234	224	-4.1	3.7	927	956	3.1	5.3
Valine	416	409	-1.7	3.7	823	826	0.4	4.6

The extracted chromatograms of the 27 compounds for L1 are presented in Figure 3.

Figure 3. Chromatograms of L1 sample



CONCLUSIONS

- The elimination of derivatization simplifies the sample preparation and increases overall throughput with shorter preparation and analysis time, and also minimizes the need for costly reagents.
- The LC-MS/MS method was able to simultaneously quantify 52 amino acids within 18 minutes, providing favorable performance relative to existing, long duration HPLC methods.
- The method has been evaluated for 37 amino acids in neat standard and in human plasma but will need to be further assessed for more extensive reproducibility and repeatability for all 52 amino acids.
- Accuracy and precision studies confirm the possibility of using aqueous calibrators to quantify at least 27 compounds in plasma.

TRADEMARKS/LICENSING

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