Reliable Quantitation on 52 L-Amino Acids in Human Plasma for Clinical Research by LC-MS/MS

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ABSTRACT

Purpose: An analytical method was developed that quantitatively detects 52 L-amino acids in human plasma for clinical research. An offline protein precipitation of the plasma sample without derivatization was performed prior to dilution with internal standard and followed by LC-MS/MS analysis with a triple quadrupole mass spectrometer. Separation and quantitation of compounds were performed with an 18-minute reverse phase gradient and internal or external calibration of eight standards ranging from 0.1 to 500 µM. Problematic issues such as observed carryover of the basic amino acids as well as retention time shifts were addressed by optimizing the LC method.

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 aqueous/organic reverse phase gradient. Issues with carryover that originated from the mobile phases were observed in blank samples and were addressed with the use of a cation exchange trap column for their removal. Retention time shifts were kept to a minimum for excellent reproducibility by limiting pH differences to < 0.1 batch to batch.

Results: The lower limit of quantification (LLOQ) for each amino acid was set to the lowest calibration standard analyzed that yielded < 20 % inaccuracy and < 20% CV for 3 replicate injections of the same standard.

Figure 1. Thermo Scientific™ Vanquish™ Flex and TSQ Quantis™ Triple Quadrupole Mass Spectrometer.

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. Here we present a novel workflow that simplifies sample preparation by eliminating the use of a derivatization reagent to detect 52 L-amino acids for clinical research in human plasma with excellent reproducibility in 18 minutes.
- Current methods most widely accepted in the routine clinical research laboratory require several steps which are highly cumbersome and time consuming. The method presented is simple, straightforward, and reproducible.

MATERIALS AND METHODS

Sample Preparation

- All labeled and unlabeled standards were purchased from Cambridge Isotope Laboratories Inc., Sigma Aldrich, or Wako Pure Chemical Industries. Standard solutions were prepared at the appropriate concentrations and used for dilution of the sample prior to injection onto the LC-MS/MS system.
- 100 µL of calibrator, control, or plasma were mixed with 100 µL of precipitation reagent and then vortexed for 60 seconds, refrigerated for 30 minutes, and centrifuged at 12000 rpm for 5 minutes. The supernatant (50 µL) was added to 200 µL of the dilution solution containing both labeled and unlabeled standards. Samples were vortexed and then injected onto the LC-MS/MS system.

Table 1. List of 52 quantifiable L-Amino Acids. The listed amino acids are detectable in human plasma using this method.

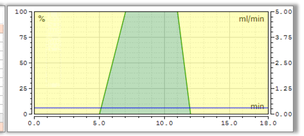
1	Alanine	27	Homocystine	Internal Standards	
2	β-Alanine	28	Hydroxylysine	1	Alanine 13C3 15N
3	α-Aminoadipic Acid	29	Hydroxyproline	2	Arginine 13C6 15N4
4	α-Aminobutyric Acid	30	Isoleucine	3	Asparagine 13C4 D3 15N2
5	β-Aminoisobutyric Acid	31	Allo-Isoleucine	4	Aspartic Acid 13C4 15N1
6	γ-Aminobutyric Acid	32	Kynurenine	5	Citrulline 13C D4
7	5-Aminolevulinic Acid	33	Leucine	6	Cystine 13C6 15N2
8	Anserine	34	Lysine	7	Glutamic Acid 13C5 15N1
9	Arginine	35	Methionine	8	Glutamine 13C5 D5 15N2
10	Argininosuccinic Acid	36	1-Methylhistidine	9	Glycine 13C2 15N1
11	Asparagine	37	3-Methylhistidine	10	Histidine 13C6 15N3
12	Aspartic Acid	38	Ornithine	11	Isoleucine 13C6 15N1
13	Carnosine	39	Phenylalanine	12	Leucine 13C6 15N1
14	Citrulline	40	Phosphoethanolamine	13	Lysine 13C6 15N2
15	Cystathionine	41	Phosphoserine	14	Methionine 13C5 15N1
16	Cysteine	42	Pipecolic Acid	15	Ornithine 13C5 15N2
17	Cysteine-Homocysteine Disulfide	43	Proline	16	Phenylalanine 13C9 15N1
18	Cystine	44	Sarcosine	17	Pipecolic Acid 13C6 15N
19	Ethanolamine	45	Serine	18	Proline 13C5 15N1
20	Glutamic Acid	46	Serine	19	Serine 13C3 15N1
21	Glutamine	47	Sulfocysteine	20	Taurine 13C2 15N
22	Glutathione Reduced	48	Taurine	21	Threonine 13C4 15N1
23	Glycine	49	Threonine	22	Tryptophan 13C11 15N2
24	Histidine	50	Tryptophan	23	Tyrosine 13C9 15N1
25	Homocitrulline	51	Tyrosine	24	Valine 13C5 15N1
26	Homocysteine	52	Valine	25	γ-Aminobutyric Acid 13C4

Liquid Chromatography Conditions

- Mobile phases consisted of an organic/aqueous composition.
- Columns used for this method consisted of a trap column to remove basic amino acids seen consistently in the background, a guard column to increase column lifetime, and an analytical column for amino acid separation.
- Column Temperature: 30 ° C
- Injection volume: 4 µL

Figure 2. Liquid chromatography gradient used for analysis.

No	Time	Flow [ml/min]	%B	Curve
1	0.000		Run	
2	0.000	0.300	0.0	5
3	5.000	0.300	0.0	5
4	7.000	0.300	100.0	5
5	11.000	0.300	100.0	5
6	12.000	0.300	0.0	5
7	12.500	0.300	0.0	5
8	14.500	0.300	0.0	5
9	15.000	0.300	0.0	5
10	18.000	0.300	0.0	5
11	New Row			
12	18.000		Stop Run	



Conditions for TSQ Quantis Mass Spectrometer

Table 2. HESI Source Parameters.

HESI Source Parameters			
Polarity	Positive	**Sheath Gas**	45
Spray Voltage	3.5 kV	**Auxiliary Gas**	15
Capillary Temp	270 ºC	**Sweep Gas**	0
Vaporizer Temp	370 ºC	**Source Fragmentation**	15.0 V

Table 3. Scan Properties used for SRM experiment type.

SRM Scan Properties			
Use Cycle Time	True	**Collision Gas Pressure**	1.5 mTorr
Cycle Time	0.4 sec	**Use Calibrated RF**	False
Chrom Filter	3.0 sec	**Q1 Resolution (FWHM)**	0.7
Data Mode	centroid	**Q3 Resolution (FWHM)**	0.7

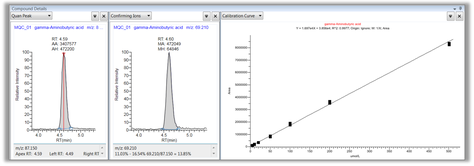
RESULTS

Table 4. SRM Table and Calibration Curves.

Amino Acid	Precursor (m/z)	Product (m/z)	RT	R^2	Range µmol/L	Type	Weighting
1-methylhistidine	170.12	124.11	10.41	0.9915	2-200	Linear	1/X
3-methylhistidine	170.12	126.1	10.27	0.9945	2-100	Linear	1/X
alanine	90.274	44.44	2.03	0.9969	20-500	Linear	1/X
α-aminioadipic	162.152	98.22	2.35	0.9949	5-250	Linear	1/X
α-aminobutyric	104.213	58.33	2.43	0.9819	10-250	Linear	1/X
anserine	241	109	10.93	0.9966	5-200	Linear	1/X
arginine	175.12	70.26	12.12	0.9912	5-200	Linear	1/X
asparagine	133.2	87.15	1.9	0.9977	20-500	Linear	1/X
aspartic acid	134.11	74.22	2.03	0.9949	10-500	Linear	1/X
β-aminoisobutyric acid	104.252	86.15	4.22	0.9942	5-500	Linear	1/X
carnosine	227.183	110.17	11.21	0.9953	5-200	Linear	1/X
citrulline	176.213	159.04	2.87	0.9962	5-200	Linear	1/X
cystathionine	223.152	134.11	8.94	0.9985	2.5-250	Linear	1/X
cystine	241.1	152	8.71	0.9973	5-500	Linear	1/X
ethanolamine	62.365	62.37	3.75	0.9938	10-200	Linear	1/X
γ-aminobutyric acid	104.252	87.15	4.69	0.9968	5-200	Linear	1/X
glutamic acid	148.1	84.15	2.11	0.9968	5-500	Linear	1/X
glutamine	147.12	130.04	1.98	0.9926	5-500	Linear	1/X
glycine	76.3	76.3	2.16	0.9989	50-500	Linear	1/X
histidine	156.13	110.1	10.46	0.9594	5-100	Linear	1/X^2
hydroxylysine	163	128	10.15	0.9909	2-100	Linear	1/X^2
isoleucine	132.18	69.26	3.52	0.9974	5-500	Linear	1/X
leucine	132.18	86.22	3.74	0.9917	20-200	Linear	1/X
lysine	147.17	84.22	10.49	0.9906	5-500	Linear	1/X
methionine	150.098	133.07	3.08	0.9981	5-500	Linear	1/X
ornithine	133.243	70.26	10.22	0.9933	20-500	Quadratic	1/X
phenylalanine	166.12	103.17	6.85	0.9885	2-500	Linear	1/X^2
phosphoethanolamine	142.07	44.44	1.56	0.9946	2.5-100	Linear	1/X
phosphoserine	186	88	3.72	0.9957	2.5-250	Linear	1/X
pipecolic acid	130.16	84.22	2.98	0.9987	10-500	Quadratic	1/X
proline	116.243	70.26	2.13	0.9942	5-500	Quadratic	1/X
serine	106.191	60.33	1.88	0.9982	20-500	Linear	1/X
sulfocysteine	202.091	120.06	6.44	0.9989	2-500	Linear	1/X
taurine	126.152	126.15	1.54	0.9917	10-100	Linear	1/X
threonine	120.16	74.28	1.89	0.9965	10-500	Linear	1/X
tryptophan	205.078	187.99	9.64	0.998	5-500	Linear	1/X
tyrosine	182.078	136.04	5.95	0.9995	5-500	Linear	1/X
valine	118.17	72.26	2.62	0.9942	20-500	Linear	1/X

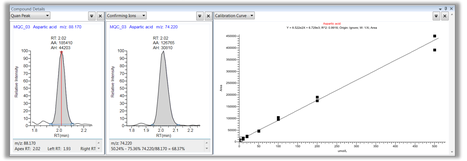
Amino acids quantitated with retention times, calibration R2 values, calibration ranges, calibration type, and calibration weighting. Limit of Quantification (LOQ) was determined as the lowest value in the calibration curve giving <20%Diff and <20% CV for 3 injections of calibrators in neat solution.

Figure 3. Aspartic Acid Mid Level QC with calibration curve.



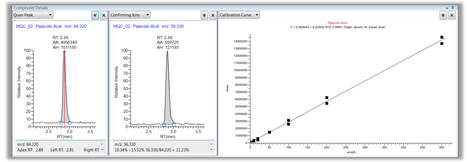
Example Chromatograms and Calibration Curves

Figure 3. Aspartic Acid Mid Level QC with calibration curve.



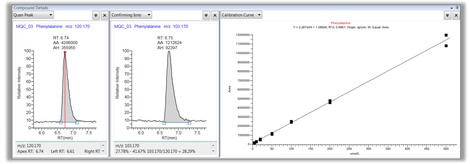
Chromatogram of quantitative and confirming ion peaks. Mid Level QC (200 µmol/L) was extracted using the protein precipitation method.

Figure 4. Pipecolic Acid Mid Level QC with calibration curve.



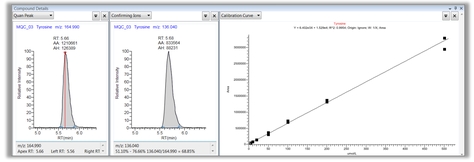
Chromatogram of quantitative and confirming ion peaks. Mid Level QC (200 µmol/L) was extracted using the protein precipitation method.

Figure 5. Phenylalanine Mid Level QC with calibration curve.



Chromatogram of quantitative and confirming ion peaks. Mid Level QC (200 µmol/L) was extracted using the protein precipitation method.

Figure 6. Tyrosine Mid Level QC with calibration curve.



Chromatogram of quantitative and confirming ion peaks. Mid Level QC (200 µmol/L) was extracted using the protein precipitation method.

Repeatability

Table 5. Repeatability of Mid Level QC (Control L1), High Level QC (Control L2), and Plasma. Controls, quality controls, and plasma samples were prepared using the protein precipitation method. Calibration curves were determined as described in Table 4. Controls and Plasma samples were analyzed using five replicate injections with % RSD and % Difference reported here for ten of the amino acids.

	Control L1				Control L2				Plasma	
Amino Acid	Theoretical µmol/L	AVG µmol/L	%RSD	%Diff	Theoretical µmol/L	AVG µmol/L	%RSD	%Diff	AVG µmol/L	% RSD
Aspartic Acid	200	194	4.23	-3.06	500	474	2.67	-5.17	44.4	17.7
Pipecolic Acid	200	178	3.98	-11.0	500	493	3.35	-1.46	N/F	-
Phenylalanine	200	180	3.63	-9.86	500	460	3.39	-8.10	37.1	5.78
Tyrosine	200	176	4.77	-12.0	500	443	2.37	-11.4	44.0	3.39
γ-aminobutyric acid	200	188	4.28	-6.08	500	474	2.67	-5.17	N/F	-
alanine	200	180	6.13	-10.2	500	467	2.71	-6.63	30.3	12.3
α-Aminoadipic acid	100	86.7	5.79	-13.3	250	223	8.37	-10.6	5.20	8.23
α-Aminobutyric acid	100	88.9	9.33	-11.1	250	236	4.55	-5.52	N/F	-
β-Aminoisobutyric acid	200	175	2.18	-12.3	500	478	3.91	-4.31	N/F	-
citrulline	200	186	4.53	-7.03	500	442	3.97	-11.5	21.2	9.12

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, representing a significant improvement over conventional methods.

The method has been evaluated for 37 amino acids in neat standard and in human plasma, and will be further assessed for more extensive reproducibility and repeatability for all 52 amino acids.

TRADEMARKS/LICENSING

PO65510-EN0519S

ThermoFisher SCIENTIFIC