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Determination of underivatized amino acids in human plasma using ion pair liquid chromatography/tandem mass spectrometry

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ABSTRACT

Accurate quantification of amino acids (AA) is essential for several applications, including clinical research, food analysis, and pharmaceutical studies. In this study, we developed an analytical method based on liquid chromatography with electrospray ionization coupled to tandem mass spectrometry detection (LC-ESI-MS/MS). This method was devised to accurately quantify a spectrum of amino acids, notably taurine, creatinine, glutathione (GSH), and sulfur-containing amino acids (SAAs) such as methionine, cysteine, and homocysteine, using only 10 μL of human plasma. A stable isotope derivative of each AA is used as an internal standard (IS) for accurate quantification. For retention and separation on a C18 column, heptafluorobutyric acid (HFBA) was employed as an ion pair agent. Multiple reaction monitoring (MRM) in positive mode with the precursor-to-product ion transitions at m/z is used for quantification. The method showed excellent linearity for all AA with a high correlation coefficient ($r > 0.9927$). The linear fit indicates that the detector response is linear over the tested range of standard concentrations. The accuracy and precision of the method were within the acceptable range of 92–110% and < 15%, respectively. The limit of detection (LOD) and limit of quantification (LOQ) were in the range of 0.001–1.80 μM and 0.004–6.0 μM, respectively. No significant ion suppression or carry over was observed. In conclusion, the assay was validated and found to have adequate accuracy, precision, linearity, sensitivity and selectivity. The assay has been successfully applied to the analysis of human plasma.

1. Introduction

Amino acids play a fundamental role in supporting various physiological processes within the body [1]. They function as the building blocks for proteins, affect protein turnover and cellular biology, and are involved in regulation and transport mechanisms [2]. The catabolism of amino acids contributes significantly to the body's energy source through glycolytic and citric acid cycle pathways when carbohydrates and fats are limited [3,4]. Additionally, they contribute to cellular structure and function, act as neurotransmitter precursors, influence immune responses, and participate in cell signalling [1,5–7].

The concentration of plasma amino acids can be influenced by several factors such as diet, protein synthesis, and degradation. Essential amino acids obtained from dietary sources are vital for maintaining overall health [8]. Notably, certain dietary supplements, including amino acids, have demonstrated positive effects on diseases and have been emphasized in the medical field [9]. Moreover, genetic disorders caused by mutations in genes that affect the production, metabolism, or transport of amino acids, such as Phenylketonuria (PKU), Maple syrup urine disease (MSUD), Homocystinuria, Cystinuria, Tyrosinemia, and Argininosuccinic aciduria, are common [10–15].

Sulfur-containing amino acids (SAAs), including cysteine and methionine, play integral roles in various physiological processes and biochemical pathways within the body [16]. Notably, cysteine serves as a precursor for glutathione, an endogenous antioxidant that plays a crucial role in defending cells against oxidative stress and damage caused by reactive oxygen species [17]. Furthermore, SAAs are crucial in various cellular signalling pathways, influencing processes such as cell growth, differentiation, and gene expression [18]. Methionine contributes to methylation reactions, which are essential for processes like DNA synthesis, repair, and epigenetic regulation [19].

Accurate quantification of AA is crucial for understanding the role of AA and SAAs in clinical interventions, nutritional studies, and diseases [20–22]. However, quantifying amino acids from complex biological matrices in a time-efficient and comprehensive manner remains a challenge. The choice of analytical method is also important. To be

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useful in larger clinical research trials as part of a targeted metabolomics platform, the desired method needs to be cost-effective, robust, sensitive, and selective to enable validation of analyte signals in every sample. Historically, various chromatographic techniques have been used to separate and measure amino acids. Traditional techniques, such as ion-chromatography using ninhydrin post-column and derivatization, have been employed to increase sensitivity, selectivity, separation, and measurement of amino acids [23–25].

In this study, we utilized the 5500 Q TRAP linear MS/MS spectrometer tandem mass spectrometer (from AB Sciex) for separation and determination of plasma AA and SAAs. In summary, we developed an LC-MS/MS method for the simultaneous determination of amino acids, incorporating Heptafluorobutyric acid (HFBA) as an ion pair agent to enable retention and separation on a C18 column. We use homologous stable labeled isotopes for accurate quantification of the majority of analytes to correct for ion suppression from interfering compounds, mainly from the matrix. Sample consumption is kept to a minimum (10 µL) to avoid excessive usage. Sample pretreatment involves a simple protein precipitation step with 5-sulfosalicylic acid (SSA) and without the requirement for derivatization, which is a time-efficient and comprehensive way to quantify amino acids from complex biological matrices. Additionally, sample processing performed in a 96-well plate to reduce sample preparation time. Our method is rapid, sensitive, and specific, enabling us to include a large series of samples to increase the power of amino acid studies. Fig. 1 illustrates a representative mass chromatogram of plasma amino acids, showcasing their retention times.

2. Experimental

2.1. Chemical and reagents

Methanol, formic acid, acetonitrile and hydrochloric acid (HCl), all of pro analysis quality (LC-MS grade) were from E. Merck (Darmstadt, Germany) or Rathburn (Walkerburn, Scotland, UK). Potassium tetraborate were also from E. Merck. All aqueous solutions were prepared with Milli-Q ultrapure water. Dithioerythritol (DTE), 5-sulfosalicyclic acid (SSA), Heptafluorobutyric acid (HFBA) from Sigma-Aldrich (Darmstadt, Germany). Arginine, Valine, Proline, Isoleucine, Leucine, Phenylalanine, Tyrosine, Ornithine, Tryptophan, Homocysteine, Cystathionine, Glutathione, Cysteine, Methionine, Serine, Glutamine, Taurine, Creatinine Glutamic Acid, Asparagine, Aspartic Acid, Glycine, Alanine, Threonine, Histidine, Lysine, 2-Aminobutyric Acid (AMBA), 1-Methylhistidine, 3-methylhistidine and Citrulline were from Sigma-Aldrich. Labeled-AA were from Sigma-Aldrich, Cambridge Isotope Laboratories and CDN Isotopes. Quality controls (QC) were from Erndim (https://erndim.org/qa).

Polyether ether ketone (PEEK) tubing was from Upchurch Scientific (Oak Harbor, USA). 96-well microtiter plates wells and clear strong heat seals (# AB-0685) were both from AB-gene (Epsom, UK), (Argonaut Technologies Inc., Redwood City, CA).

2.2. Instrumentation

A Sciex5500 Q TRAP linear MS/MS spectrometer (Foster City, CA, USA) was used in combination with a Shimadzu LC20ADXR system consisting of a temperature regulated 96 well plate auto sampler, quaternary pump, vacuum degasser and column oven. An integrated compressor and gas generator (model NM20ZA, Peak Scientific Instruments, Renfrew, Scotland, and UK) supplied nitrogen, zero grade air and air needed for the mass spectrometer. Data acquisition and processing was controlled with Analyst version 1.6.1 software (Applied Biosystems). Infusion experiments such as tuning, optimization and post-column infusion were performed with an integrated syringe infusion pump.

2.3. Chromatography

Chromatographic separation was performed on Phenomenex Kinetex Core Shell C18 (100 × 4.6 mm, 2.6 µm) and temperature regulated at 30 °C. The mobile phases were (A) Water with 0.5% formic acid, 0.3 %

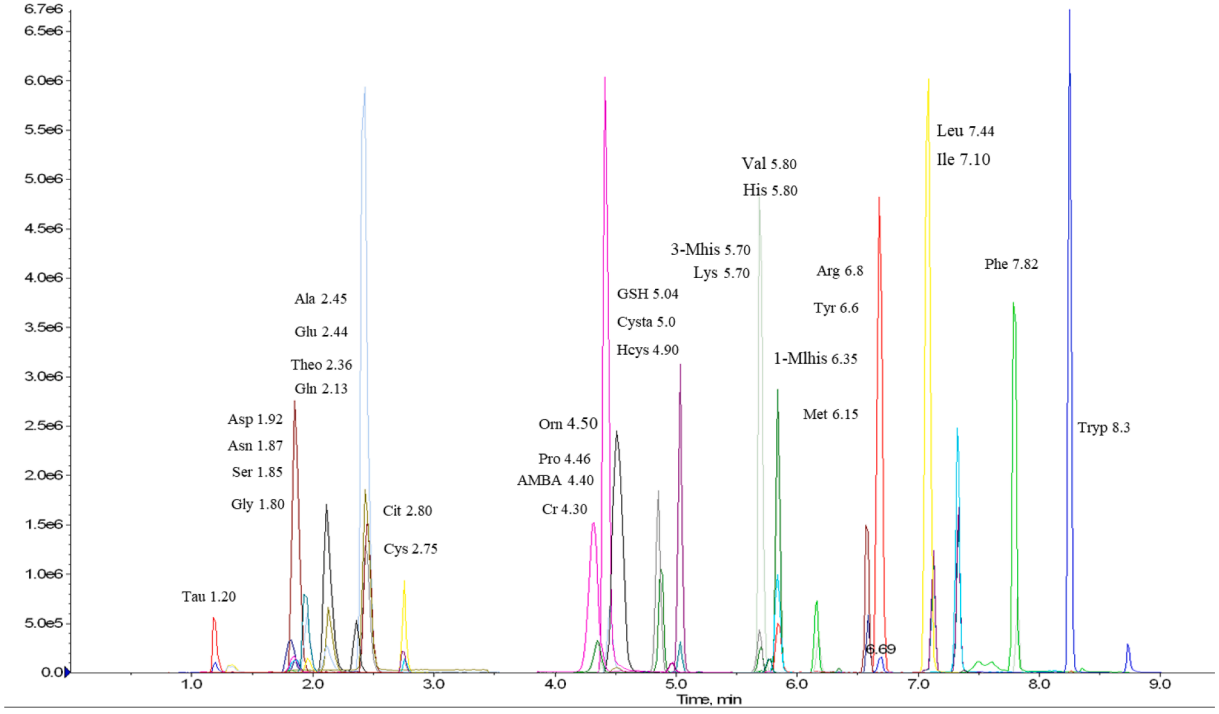

Fig. 1. Representative mass chromatogram of plasma of each AA and retention times. Taurine (Tau); Glycine (Gly); Serine (Ser); Asparagine (Asn); Aspartic acid (Asp); Glutamine (Gln); Glutamic acid (Glu); Threonine (Thr); Alanine (Ala); histidine (His); Cysteine (Cys); Citrulline (Cit); Creatinine (Cr); α-amino butyric acid (AMBA); Proline (Pro); Ornithine (Orn); Homocysteine (Hcys); Lysine (Lys); Cystathionine (Cysta); Glutathione (GSH); 3-metyl-histidine (1-MHis); Valine (Val); Methionine (Met); 1-metyl-histidine (3-MHis); Tyrosine (Tyr); Arginine (Arg); Isoleucine (Ile); Leucine (Leu); phenylalanine (Phe); and tryptophan (Tryp).

HFBA and (B) Acetonitrile at a flow rate of 0.8 mL/min. The separation was achieved with a linear gradient from 100% (A) to 80% over 6 min, from 6 min to 9 min 40% and back to 100% (A) over 10.5 min, followed by a linear gradient back to 100% (A) over 12 min.

2.4. Mass spectrometry

The mass spectrometer operated in ESI positive mode. Q1 and Q3 operated in unit resolution. The precursor-to-product ion transitions used for quantitation in multiple reaction monitoring (MRM) mode. Mass spectrometer parameters were optimized; (curtain gas: 45 psi; Nebulizer current: 2 μA, Source temperature: 650 °C, Nebulizer gas: 60 psi; interface heater: on). Optimization of compound and MS dependent parameters was performed manually, and the best parameters were selected. The Scheduled MRM transitions, retention times, and compound IDs was used for acquisition method. Optimum MRM transitions and source parameters for of each AA and IS are shown in Table 1.

2.5. Preparation of standards and internal standards

Stock solutions (10 or 100 mM) of the individual amino acid standards and IS were prepared in HCl [0.1 M]. Sonic bath was used to aid solubilisation. Aliquot into 2 mL tubes (0.5–1 mL aliquots) and stored at −20 °C. An internal standard working solution of the combined analytes was prepared by diluting the stock solutions 1:1 (v/v) with aqueous potassium tetraborate, (17 mM) to achieve neutral pH and correct calibration standard concentration and stored at −20 °C. Further dilutions of working solutions were made fresh and stored at −80 °C. These solutions were made different concentrations for each AA and IS. These standard solutions were used for calibration.

2.6. Sample preparation procedure

Plasma samples were stored at −80 °C. After thawing and vortexing, 10 μL of plasma, calibration standards, QC samples, and blank matrix (water) were added to 96 well plates. Internal standard working solution (10 μL) added to all wells and then added 10 μL DTE [100 mM] in all wells. DTE serves as a reducing reagent for preventing and reversing cysteine disulfide bond formation and works at neutral PH or slightly alkaline. Well plates mixed on a plate shaker at room temperature for 15 min. 10 μL cold (4 °C) SSA (10%) was added to all wells to precipitate the plasma proteins. The solution in the wells mixed on a plate shaker and centrifuged at 4000 rpm for 15 min. Supernatant (15 μL) from the extraction plate diluted in 135 μL mobile phase A in a shallow 96 well analysis plate. Aliquots (15 μL) injected into the LC/MS/MS system for analysis. The injector was temperature regulated at 6 °C.

3. Method validation and results

Present method was validated for quantitative measurement methods, which are based on the linearity, accuracy, precision, limits of detection, lower limits of quantification, carry over and stability. The validation process adhered to the guidelines set forth by the US Food and Drug Administration (FDA) for bioanalytical method validation (https://www.fda.gov).

3.1. Linearity

The linearity of the detector response was measured with nine different concentrations from lower limit of quantification (LLOQ) to upper limit of quantification (ULOQ) for each of AA standards. The concentrations of analytes were determined from the ratio of analyte peak area / internal standard peak area against a linear calibration curve with a quadratic regression 1/x weighting used for Arginine, Glycine, Cysteine, Citrulline and 1/x weighting for all other AAs. Calibration curve ranges were made for each AA within the expected physiological concentrations levels of the AA in plasma. The correlation coefficient ($r > 0.9927$) for the linear fit indicates that the detector response is linear over the tested range of standard concentrations.

Table 1
Optimum MRM transitions and source parameters for of each AA and IS.

Analyte	Q1 Mass	Q3 Mass	Internal Standard	Q1 Mass	Q3 Mass	DP (volt)	EP (volt)	CE (volt)	CXP (volt)	RT/min
1-Methylhistidine	170.001	126.001	D3-His	159.062	113.1	51	10	10	8	6.35
3-Methylhistidine	170.000	126.000	D3-His	159.062	113.1	51	10	10	8	5.70
Alanine	90.002	44.000	D3-Asp	137.048	76.9	41	10	5	10	2.45
AMBA	103.980	57.900	D3-Asp	137.048	76.9	31	10	5	6	4.40
Arginine	175.000	70.000	D7-Arg	182.000	77.0	36	8	11	12	6.80
Aspargine	133.100	74.100	D3-Asp	137.048	76.9	36	8	24	8	1.87
Aspartic acid	134.100	74.100	D3-Asp	137.048	76.9	36	8	10	8	1.92
Citrulline	176.069	158.900	D2-Cys	124.000	107.0	41	10	5	10	2.80
Creatinine	113.986	86.000	D3-Creatinine	116.986	89.0	56	15	10	10	4.30
Cystathionine	223.000	134.000	D4-Cysta	227.000	138.0	56	8	18	8	5.00
Cysteine	122.000	105.000	D2-Cys	124.000	107.0	91	8	5	4	2.75
Glutamic acid	148.101	102.000	D3-GA	151.064	105.0	36	10	7	6	2.44
Glutamine	146.900	130.000	D5-Gln	152.000	135.0	36	8	27	8	2.13
Glutathione	308.000	179.000	13C215N-GSH	311.000	182.0	56	8	15	10	5.04
Glycine	76.038	30.100	D3-Asp	137.048	76.9	26	10	23	4	1.80
Histidine	156.062	110.100	D3-His	159.062	113.1	51	10	5	8	5.80
Homocysteine	136.000	90.000	D4-Hcy	140.000	94.0	36	6	17	6	4.90
Isoleucine	132.001	86.001	D10-Ile	142.000	96.0	36	8	5	6	7.10
Leucine	132.000	86.000	D3-Leu	135.000	89.0	41	8	10	14	7.44
Lysine	147.103	84.000	D8-Lys	155.103	92.0	41	10	5	10	5.70
Methionine	150.063	133.000	D4-Met2	154.063	137.0	96	10	8	8	6.15
Ornithine	133.000	70.000	D6-Orn	139.000	76.0	41	8	7	12	4.50
Phenylalanine	166.000	120.000	D5-Phe	171.000	125.0	36	8	5	12	7.82
Proline	116.000	70.000	D7-Pro	123.000	77.0	41	8	5	10	4.46
Serine	106.000	60.000	D3-Ser	109.000	63.0	50	10	5	10	1.85
Taurine	125.880	108.000	D4-Taurine	129.880	112.0	36	10	17	10	1.20
Threonine	120.064	74.000	D2-Thr	122.064	76.0	31	10	5	14	2.36
Tryptophan	205.000	146.000	D5-TRP2	210.000	151.0	46	10	27	12	8.30
Tyrosine	182.000	136.000	D2-Tyr	184.000	138.0	51	8	7	8	6.60
Valine	118.000	72.000	D8-Val	126.000	80.0	36	8	5	10	5.80

3.2. Sensitivity and carryover

The LOD defined as the lowest amount of substance that generates a signal with a signal-to-noise (S/N). Measurement of all LOQ were 10 times above S/N. The LOD and LOQ were in the range of 0.001–1.80 μM and 0.004–6.0 μM for all AA, respectively. LOD, LOQ, calibration range of each AA are shown in Table 2.

The carryover assessed by injecting blank sample after ULOQ sample of the calibration standard. The experiment were repeated six times. Carry over from top calibration standard was < 0.4%.

3.3. Accuracy, precision and recovery

The accuracy of an analytical method defines the confidence of mean test results obtained by the method to the actual value (concentration) of the analyte. The intra-day accuracy and precision of the assay were determined by analyzing replicates (n = 6) at nine different calibration range for each AA in four concentration levels and calculating the mean value and coefficient of variation (CV). Table 2 includes four concentration levels; LLOQ, Low (LQC), Mid (MQC), and High (HQC). Accuracy is expressed as [(found concentration)/ (added concentration)] × 100%. To assess the inter-day precision, two different quality controls (QC) were analyzed every day together with standards and clinical samples (n = 6). The intra-day accuracy were within the acceptable range of 90.90–112.60%. The intra-day and inter-day precision were < 10%. The results are presented in Table 3 demonstrating accuracy and precision of amino acids in human plasma.

The recovery of AAs obtained spiked and unspiked plasma or blank (n = 6), performed by comparing extracted samples to standards. The extraction recoveries for all amino acids (AAs) were found to exceed 89%. Notably, a pivotal factor in enhancing these recovery values was the utilization of cold (4 °C) SSA solution.

3.4. Selectivity and assessment of matrix effect

Selectivity is the ability of an analytical method to differentiate the analyte in the presence of other components in the sample.

Analyses of blank samples tested for interference, and ensured at the LLOQ and there were no detectable interferences. In addition, several other MRM transitions were selected to test selectivity of AA and IS in samples and standards. The MRM transitions selected for quantification showed no interferences at the retention times in question. Fig. 2 illustrates chromatograms obtained from the selectivity study, incorporating blank samples, standards, and sample analyses. These chromatograms serve to visually validate the method's selectivity by illustrating the lack of interference from endogenous components. LC–MS/MS is often the most sensitive and selective analytical technique for biological samples. This technique, however, suffers from matrix effects that suppress ionization of analytical compounds. This is often a significant problem with ESI and results in decreased precision and accuracy. It would also improve the method to use homologous IS to avoid potential ion suppression issues. Where a homologous IS was not used due to lack of availability or expense then a close structural analyte or analytes with a close retention time was used. By comparing the peak areas of the analyte standards, standards spiked before and after extraction into different lots of plasma, and the peak area ratios of analytes to an IS, ion suppression or enhancement associated with a given lot of plasma were assessed. No significant ion suppression or enhancement was observed. Furthermore, a comparison was undertaken between the linearity of slopes obtained from the standard calibration and standard addition calibration, aiming to quantify the matrix effect. Notable disparities in slope values between these two calibration curves are indicative of the existence of matrix effects. A greater discrepancy in slope values signifies a more pronounced matrix effect.

In the context of our investigation, the slopes of calibration curves derived from both standards and standard additions exhibited close alignment. This convergence implies a negligible imposition of matrix effects. This observation underscores that the composition of the sample

Table 2
LOD, LLOQ, low QC (LQC), mid QC (MQC), high QC (HQC) and calibration range of each AA.

Amino acids	LOD (μM)	LLOQ (μM)	LQC (μM)	MQC (μM)	HQC (μM)	Calibration range (μM)
1-Methylhistidine	0.50	1.7	5.1	250	500	1.7–625
3-Methylhistidine	0.08	0.3	0.8	250	500	0.3–625
Alanine	0.60	2.0	6.0	250	500	2–625
AMBA	0.30	1.1	3.0	25	50	1.1–63
Arginine	0.15	0.5	1.5	100	200	0.5–250
Asparagine	0.85	2.9	8.5	75	150	2.9–190
Aspartic Acid	0.67	2.4	6.7	25	50	2.4–63
Citrulline	1.30	4.4	13	50	100	4.4–125
Creatinine	0.75	2.5	7.5	100	200	2.5–250
Cystathionine	0.001	0.004	0.01	1	2	0.004–2.5
Cysteine	0.07	0.2	0.7	350	700	0.2–875
Glutamic Acid	0.45	1.5	4.5	75	150	1.5–195
Glutamine	0.41	1.4	4.1	600	1200	1.4–1500
Glutathione	0.01	0.02	0.1	7.5	15	0.02–19
Glycine	1.80	6.0	18	600	1200	6–1500
Histidine	0.54	1.8	5.4	125	250	1.8–315
Homocysteine	0.02	0.07	0.2	25	50	0.07–63
Isoleucine	0.05	0.2	0.5	100	200	0.2–250
Leucine	0.09	0.3	0.9	125	250	0.3–315
Lysine	0.45	1.5	4.5	250	500	1.5–625
Methionine	0.18	0.6	1.8	40	80	0.6–100
Ornithine	0.27	0.9	2.7	100	200	0.9–250
Phenylalanine	0.01	0.04	0.1	175	350	0.05–440
Proline	0.13	0.4	1.3	250	500	0.4–625
Serine	1.50	5.0	15.0	150	300	5–375
Taurine	0.90	3.0	9.0	150	300	3–375
Threonine	0.38	1.2	3.8	250	500	1.2–625
Tryptophan	0.10	0.3	1.0	150	300	0.3–375
Tyrosine	0.10	0.3	1.0	62	125	0.3–160
Valine	0.75	2.5	7.5	250	500	2.5–625

Table 3

	Concentration Nominal	Determined	Accuracy	Precision Intra-day		Inter-day	
Amino acids	(μM)	(μM)	% [a]	%CV (n=6)	%CV QC1 (n=6)	%CV QC2 (n=6)	
1-Methylhistidine	1.70	1.55	91.1	8.11	7.90		8.14
	5.10	5.00	98.0	5.34	4.08		3.89
	250	253	101	3.06	3.09		3.50
	500	495	99.0	1.51	1.85		2.11
3-Methylhistidine	0.29	0.30	103	8.32	8.86		9.27
	0.87	0.98	113	7.99	9.44		8.77
	250	249	99.6	2.33	3.13		3.50
	500	505	101	1.94	1.95		2.00
Alanine	2.00	1.90	95.0	3.45	5.38		4.99
	6.00	5.90	98.3	3.45	3.92		3.90
	250	258	103	1.28	2.09		1.97
	500	509	102	0.46	0.69		0.70
AMBA	1.10	1.00	90.9	8.82	9.25		8.88
	3.30	3.40	103	5.86	4.08		4.10
	25.0	26.0	104	3.37	5.70		5.10
	50.0	55.0	110	4.11	5.55		5.20
Arginine	0.50	0.51	102	6.96	7.61		7.10
	1.50	1.60	107	2.67	2.76		2.20
	100	100	100.	1.47	1.11		1.33
	200	201	101	1.13	1.54		1.23
Aspargine	2.90	3.00	103	3.48	4.56		4.33
	8.70	9.00	103	1.24	4.48		4.30
	75.0	77.0	103	1.86	1.66		1.90
	150	148	98.7	1.46	2.09		2.10
Aspartic Acid	2.50	2.40	96.0	3.11	4.11		3.95
	6.70	7.00	104	0.90	1.36		2.50
	25.0	26.0	104	2.25	2.83		2.44
	50.0	52.0	104	1.92	2.43		2.20
Citrulline	4.40	4.40	100	1.64	1.97		2.22
	13.2	13.5	102	2.41	2.37		2.54
	50.0	55.0	110	2.30	2.30		1.96
	100	102	102	1.92	2.16		2.00
Creatinine	2.50	2.40	96.0	4.74	4.32		4.00
	7.50	7.30	97.3	0.58	1.84		2.10
	100	99.0	99.0	1.49	1.49		1.40
	200	198	99.0	0.83	1.08		1.10
Cystathionine	0.004	0.0037	92.5	1.64	9.44		8.87
	0.012	0.011	91.6	1.41	1.37		3.44
	1.00	1.02	102	2.30	2.59		2.60
	2.00	1.95	97.5	1.92	4.55		3.67
Cysteine	0.23	0.22	95.7	4.87	4.17		3.88
	0.70	0.68	97.1	1.49	4.96		4.62
	350	344	98.2	2.02	1.73		2.00
	700	688	98.2	0.72	0.32		0.56
Glutamic Acid	1.50	1.45	96.6	4.96	3.70		3.43
	4.50	4.40	97.8	1.24	1.05		1.22
	75.0	77.0	103	3.36	2.35		1.98
	150	155	10	1.48	2.13		1.90
Glutamine	1.37	1.42	104	0.62	5.83		5.55
	4.10	4.10	100	4.48	2.88		2.70
	600	601	100	0.19	1.23		1.10
	1200	1198	99.8	0.37	0.18		0.23
Glutathione	0.02	0.022	110	6.23	9.85		9.50
	0.06	0.063	105	7.60	4.43		4.30
	7.50	7.60	101	2.27	1.19		1.30
	15.0	15.0	100	5.09	3.43		2.78
Glycine	6.00	6.05	101	3.87	2.51		3.55
	18.00	18.00	100	3.12	1.46		1.67
	600	604	101	1.40	1.24		2.00
	1200	1190	99.1	0.45	0.14		0.12
Histidine	1.80	1.78	98.9	3.00	4.46		5.66
	5.40	5.50	102	2.87	1.48		2.43
	125	127	102	1.48	2.06		2.44
	250	255	102	1.07	1.15		1.09
Homocysteine	0.07	0.07	97.1	4.40	3.16		5.33
	0.20	0.22	110	6.21	6.27		5.48
	25.0	26.0	104	3.32	1.04		2.69
	50.0	50.0	100	2.26	3.93		2.90
Isoleucine	0.17	0.18	106	5.42	8.22		7.74
	0.50	0.52	104	4.20	3.67		6.50

(*continued on next page*)

Table 3 (*continued*)

	Concentration		Accuracy	Precision			
	Nominal	Determined		Intra-day		Inter-day	
Amino acids	(μM)	(μM)	% [a]	%CV (n=6)	%CV QC1 (n=6)	%CV QC2 (n=6)	
	100	101	101	0.96	2.28		2.55
	200	202	101	0.62	1.16		1.00
Leucine	0.25	0.24	96.0	3.99	3.33		4.00
	0.75	0.79	105	5.51	2.77		3.04
	125	120	96.0	0.77	1.19		0.99
	250	245	98.0	0.77	0.22		0.38
Lysine	1.50	1.60	107	8.89	8.60		7.80
	4.50	4.70	104	5.34	4.55		5.00
	250	256	102	3.37	3.44		4.50
	500	501	100	1.66	1.89		2.28
Methionine	0.60	0.66	110	8.78	9.73		8.88
	1.80	1.88	104	5.33	1.30		2.62
	40.0	42.0	105	1.89	2.57		2.68
	80.0	79.0	99.8	1.88	1.65		2.06
Ornithine	0.90	0.98	109	9.20	3.80		4.36
	2.70	2.78	103	5.14	4.29		3.89
	100	101	101	1.58	3.47		3.90
	200	199	99.5	0.66	1.24		2.00
Phenylalanine	0.05	0.05	100	7.45	7.66		6.70
	0.13	0.13	100	5.55	3.86		4.22
	175	170	97.1	1.52	0.90		1.09
	350	341	97.4	0.74	1.03		1.09
Proline	0.42	0.40	95.2	7.52	9.15		9.23
	1.25	1.20	96.0	1.17	1.04		2.44
	250	246	98.4	3.30	3.74		2.56
	500	500	100	1.56	1.80		2.08
Serine	5.00	5.10	102.0	8.68	7.99		8.10
	15.00	15.20	101.0	3.30	2.13		3.12
	150	155	103.0	1.55	0.70		0.88
	300	298	99.3	0.67	0.90		0.97
Taurine	3.00	3.00	100	4.48	5.39		4.55
	9.00	8.90	98.9	2.45	2.35		3.95
	150	153	102	2.39	2.36		1.96
	300	300	100	2.36	0.66		0.80
Threonine	1.25	1.30	104	8.89	8.60		8.25
	3.75	3.85	107	5.34	4.55		4.56
	250	253	101	3.37	3.44		4.00
	500	503	101	1.66	1.89		2.02
Tryptophan	0.34	0.33	97.0	6.74	5.41		6.80
	1.00	1.02	102	6.54	4.76		6.26
	150	153	102	2.12	2.34		3.54
	300	298	99.3	0.51	0.85		2.23
Tyrosine	0.35	0.35	100	3.27	3.81		5.36
	1.05	1.04	99.0	6.13	5.12		4.00
	62.0	63.0	102	2.04	3.93		4.09
	125	124	99.2	1.87	2.28		2.25
Valine	2.50	2.50	100	5.48	2.79		4.66
	7.50	7.45	99.3	0.96	3.58		3.26
	250	247	98.8	3.29	3.13		2.84
	500	496	99.2	1.66	2.03		1.95

[a]Accuracy is expressed as [(found concentration)/ (added concentration)] × 100.

matrix exerts minimal influence on the signal generated by the analyte.

3.5. Processed sample stability

In the present method, we were able to analyze an entire microtiter plate consisting of 96 samples in a single run sequence. However, a prerequisite for this is the stability of analytes in the processed samples throughout the entire duration of the run. Furthermore, extra time is frequently necessary to account for pauses in the run sequence caused by issues like excessive pressure build-up and leaks. Stability of the AA are evaluated using samples of the low and high level QC samples. A set of samples consisting of a standard curve and QC samples (n = 4) were processed and analyzed. The variance between the 0-day and 3-day are reported regarding the highest diversity.

(a) Freeze and Thaw Stability

The samples were Freeze and thaw for 0, 1, 2 and 3 cycles. Difference between time 0 and 3 cycles was (94 % to 103 %) for all AAs.

(b) Bench-Top Stability

In addition, the samples were left at room temperature for 0, 1, 2 and 3 days. Difference between time 0 and 3 days was less than 6 % for all AAs except for glutamine (9%).

(c) Re-injection Stability

Furthermore, extracted samples were left in the autosampler at 6 °C for 0, 1, 2, and 3 days and re-injected. The variance between the 0-day and 3-day intervals showed a discrepancy of under 11% for low and less than 7% for high QC across all amino acids. No significant difference in accuracy or precision between the initial and re-injected samples over a

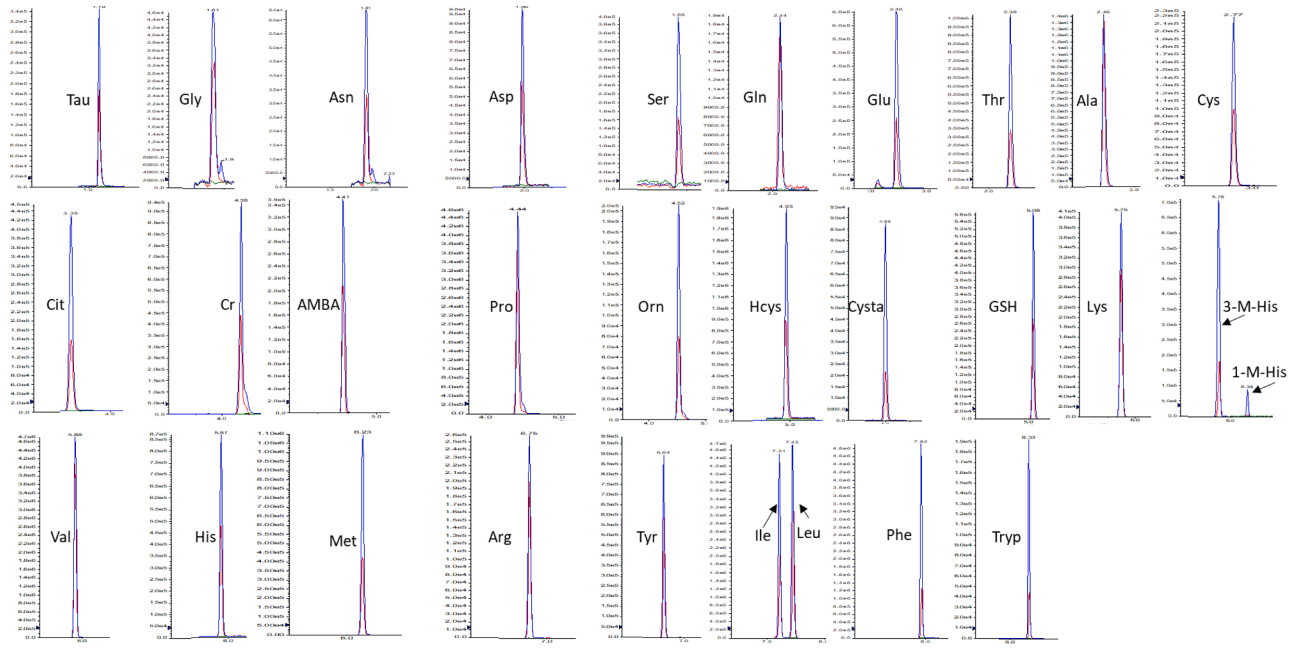

Fig. 2. A representative LC-MS/MS chromatograms of amino acids in the selectivity study. Green: blanks, Blue: standards, and Red: endogenous plasma samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

48 h period observed and extracted samples are stable for at least 48 h at 6 °C in the autosampler.

(d) Long-term freezer stability

Long-term freezer stability studied at storage temperature − 80 °C (6 months). The mean concentration at each level was within 14.6 % of the nominal concentration for low and 5.9 % for high QC.

These results support and confirm that the stability of AA is sufficient for analyzing.

3.6. Application of the assay

The LC/MS/MS assay developed has been utilized successfully to investigate the profiles of AA in thousands of plasma samples over several year in different clinical studies, the results of which will be published elsewhere [16,26–30]. The method was found to be robust throughout the duration of the study.

4. Discussion

Previously we successfully developed method for analyzing sulfur-containing amino acids (SAAs), including methionine, cysteine, homocysteine, taurine, creatinine, glutathione (GSH) [31,32]. These methods have found application in various contexts [32,33].

In this study, our aim was to expand and explored feasibility of analyzing multiple amino acids, including SAAs using LC/MS/MS. Remarkably, we accomplished this using just a minute 10 μL plasma sample without the need for derivatization. To facilitate this analysis, we employed the ion pair agent HFBA for chromatography.

Traditionally, HPLC with UV or fluorescence detection stood as the primary method for amino acid analysis. It relied on the absorption of the carboxyl group (–COOH) and the presence of benzene rings in certain amino acids. However, direct analysis of amino acids faces challenges due to their specific chemical properties, necessitating adequate sensitivity and selectivity. GC detection can also separate volatile amino acid derivatives, yet all these methods require a pre-column or post-column derivatization step. While derivatization enhances accuracy and sensitivity, it introduces drawbacks like increased analysis time, higher costs, and the potential for sample loss due to chemical reactions or precipitation. Notably, not all compounds or sample matrices are amenable to derivatization, complicating analyses. The process can lead to instability, loss of analyte specificity, and reduced analytical reliability.

Efficient compound separation is pivotal in LC/MS-based assays. The diverse concentration levels of free amino acids, spanning a wide range, demand methods that detect trace amounts and quantify higher concentrations accurately. Isomeric amino acids like isoleucine and leucine require resolution for accurate quantification. Overcoming interference necessitates specific methods, especially in complex matrices. Ion pair agents like HFBA are suitable for mass spectrometry, enhancing positive ionization.

Numerous methodologies for ion pair chromatography have been published. For instance, Koletzko et al. [34] and Liu et al. [35] involve derivatization agents, while Hiroshi et al. employed pre-column derivatization [36]. Other approaches include ion pair and without derivatization, but often they incorporate two columns or overlook cysteine, and report only cystine as sulfur amino acid [37,38]. While sulfur amino acids tend to be present in lower concentrations, derivatization effectively boosts sensitivity and selectivity. The presence of sulfur compounds like sulfides and thiols might introduce interference during analysis.

In recent years, Hydrophilic Interaction Liquid Chromatography (HILIC) has gained increasing interest due to its proficiency in separating highly polar compounds, such as amino acids. It offers benefits such as improved retention and simplified sample preparation by reducing derivatization [39–41]. However, HILIC presents challenges, including matrix effects, retention time shifts, short column lifetime, extended equilibration times, and the use of a high proportion of acetonitrile, as reported [42–44]. On the contrary, Reverse Phase Liquid Chromatography (RPLC) offers versatility for various analytes and maintains a stable baseline during analysis [45,46]. The choice between HILIC and RPLC depends on the sample nature and analytical objectives, as each technique possesses distinct strengths and limitations.

Traditionally, amino acids, being polar, exhibit weak retention in non-polar RPLC columns, impeding efficient separation and quantification. In our extensive application of the method, involving the analysis of several thousand samples, we consistently observed stable and

reliable performance using the Phenomenex Kinetex Core Shell C18 column with the ion-pairing reagent HFBA. The enhanced retention is primarily attributed to the incorporation of HFBA as an ion-pairing agent. HFBA facilitates ion pairing with amino acids, resulting in stronger interactions and improved retention on the C18 column.

To address concerns related to ion-pairing reagents and potential retention time shifts, we implemented a cautious approach by the first few injections. This strategy was guided by insights derived from previous research in the field [34]. Moreover, we carefully considered the potential impact of the presumed aqueous phase on the column packing and associated risks like phase collapse. These considerations led us to integrate specific factors into our method aimed at mitigating such risks and ensuring a consistent level of reproducibility and sustained performance. In summary, the integration of outlined factors within our method significantly contributes to the retention and stability of the column under the prescribed experimental conditions.

This study achieved exceptional amino acid retention in RPLC, effectively overcoming the challenge posed by their polar nature. Tailoring the stationary phase and optimizing the mobile phase significantly enhanced amino acid retention, resulting in sharper peaks, improved sensitivity, and precise quantification. Notably, our validated method sidesteps these limitations, presenting no issues with retention-time drift, low sensitivity, or ion suppression. Despite the very simple preparation of our method on 96- well plate, detection limits comparable to, or better than, those of the other methods have been achieved. The LOD and LOQ were in the range of 0.001–1.80 μM and 0.02–6.0 μM, respectively and even better for SAAs LOD and LOQ were in the range of 0.001–0.18 μM and 0.02–0.6 μM (Table 2).

In general, it is advantageous to use an internal standard for quantifying compounds in LC/MS-based assays. For the majority of amino acids, we used deuterated (or similar) equivalent isotopes as internal standards. However, for a few amino acids, we used other internal standards with almost the same retention time. For instance, we used D3-Aspartic acid as the internal standard for aspartic acid, asparagine, glycine, alanine, and AMBA. Additionally, we used D2-Cysteine as the internal standard for cysteine and citrulline. Using labeled standards for quantitation not only reduces ion suppression issues but also significantly improves the accuracy of quantification In addition, we used MRM scheduling timing to a specific moment in time which is another way to enhance specificity. This greatly reduces false positives and improves overall quantitative statistics. We observed that glutamine and glutamic acid are significantly unstable at room temperature and in the fridge, as well as due to repeated freeze–thaw cycles. Glutamine levels decrease due to conversion to glutamic acid.

Separating amino acid isomers in a short amount of time is challenging, but we were able to successfully separate isomers such as Isoleucine, Leucine, Histidine, 1-Methylhistidine, and 3-methylhistidine. We did not observe any significant carryover, but we always used a blank sample after the highest calibration standard to prevent any potential issues. Although we added several other transitions for the detection of related compounds, we did not validate these compounds due to the specific needs of our study, and the corresponding data are not shown.

We included creatinine in our method, as it is an important indicator of renal function and a byproduct of muscle metabolism that is excreted unchanged by the kidneys. This allowed us to establish correlations in some of our studies on sport science, although the data have not been published. The LC/MS/MS assay we developed has been successfully used to investigate the profiles of amino acids in over thousands of plasma samples in different clinical studies, as mentioned in the application of the assay. Throughout the duration of the study, the method was found to be robust without any changes to the analytical column. We also used internal quality control and compared it to external quality control (Erndim), which showed excellent correlation.

5. Conclusions

The HPLC/MS/MS assay developed and validated in this study is a robust and reliable method for quantifying amino acids in human plasma/serum. The accuracy and precision of the method make it well-suited for AA characterization and large-scale studies. The use of stable isotopically labeled AA as an internal standard compensates for potential issues such as ion suppression or injection volume changes. This method has already been successfully applied in the investigation of plasma AA profiles in clinical studies. Overall, this LC/MS/MS assay could be a valuable tool for researchers studying plasma/serum samples.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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