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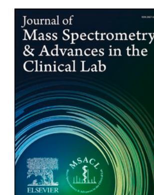

Quantitation of non-derivatized free amino acids for detecting inborn errors of metabolism by incorporating mixed-mode chromatography with tandem mass spectrometry

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ABSTRACT

Introduction: Amino acids are critical biomarkers for many inborn errors of metabolism, but amino acid analysis is challenging due to the range of chemical properties inherent in these small molecules. Techniques are available for amino acid analysis, but they can suffer from long run times, laborious derivatization, and/or poor resolution of isobaric compounds.
Objective: To develop and validate a method for the quantitation of a non-derivatized free amino acid profile in both plasma and urine samples using mixed-mode chromatography and tandem mass spectrometry.
Methods: Chromatographic conditions were optimized to separate leucine, isoleucine, and allo-isoleucine and maintain analytical runtime at less than 15 min. Sample preparation included a quick protein precipitation followed by LC-MS/MS analysis. Matrix effects, interferences, linearity, carryover, acceptable dilution limits, precision, accuracy, and stability were evaluated in both plasma and urine specimen types.
Results: A total of 38 amino acids and related compounds were successfully quantitated with this method. In addition, argininosuccinic acid was qualitatively analyzed. A full clinical validation was performed that included method comparison to a reference laboratory for plasma and urine with Deming regression slopes ranging from 0.38 to 1.26.
Conclusion: This method represents an alternative to derivatization-based methods, especially in urine samples where interference from metabolites and medications is prevalent.

Introduction

Amino acid analysis is critical for diagnosing and monitoring a broad range of inborn errors of metabolism [1]. Plasma amino acid analysis is typically performed for routine screening and evaluation of primary amino acid metabolic disorders, such as phenylketonuria, maple syrup urine disease (MSUD), and various urea cycle defects. Urine amino acid analysis, while not routinely performed for primary disorders, is also utilized for specific renal aminoacidurias, such as cystinuria and Hartnup disease [2]. However, the analysis of free amino acids is challenging because they are small (i.e., <300 Da) with a wide range of isoelectric points (2.77–10.76) and hydrophobicity [3]. Traditional methodologies separate amino acids using ion exchange chromatography combined with post-column derivatization, which typically have long run times (1–2 h); therefore, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is an attractive alternative. A significant LC-MS/MS advantage for amino acids and related compounds is the selectivity and sensitivity of mass spectrometry, which allows faster run times. Hence, chromatographic separation can focus on analytes that require extra resolution due to stereo- and structural isomers, such as leucine, isoleucine, and allo-isoleucine, three amino acids critical for the diagnosis of maple syrup urine disease (MSUD).

Abbreviations: AMR, analytical measurement range; AQC, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate; ASA, argininosuccinic acid; CEX, cation exchange; CS, Fcerebrospinal fluid; CV, coefficient of variation; GABA, gamma-aminobutyric acid; GC/MS, gas chromatography-mass spectrometry; HILIC, hydrophilic interaction liquid chromatography; HCl, hydrochloric acid; IS, internal standard; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLOQ, lower limit of quantitation; MM, mixed-mode; MS/MS, tandem mass spectrometry; MSU, Dmaple syrup urine disease; QC, quality control; RPL, Creversed phase liquid chromatography; ULO, Qupper limit of quantitation.

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Reversed-phase LC (RPLC), a commonly used mode of separation in LC-MS applications, is problematic for amino acid panels because of low retention on these columns due to the hydrophilicity and polarities of the analytes. Therefore, many labs utilize derivatization (for example, aTRAQ, AQC, bromobutane, etc.) to aid in retention and separation. While ready-to-use kits are commercially available for amino acid analysis [4–6], they are typically expensive and, similar to other derivation methods, increase possible errors, imprecision, sample complexity, and sample preparation time (10–60 min). Therefore, a non-derivatized amino acid analysis approach would be preferable for many labs looking for simplicity and cost savings.

Another possible solution to improve retention on reversed-phase columns is ion-pairing reagents, such as perfluorinated carboxylic acids [7]. However, ion-pairing reagents cause ionization suppression and retention time shifts, require dedication of the instrument to a single ESI polarity, and are known to require long LC equilibration times [7–9]. Theoretically, the hydrophilic nature of most amino acids would allow for a separation mode known as hydrophilic interaction liquid chromatography (HILIC), but, in practice, the chromatographic resolution of HILIC for leucine isomers is reportedly poor compared to what is typically achieved using RPLC and ion-pairing [10,11]. Additionally, HILIC methods often suffer from peak broadening [12], which can decrease accuracy due to poorly resolved isobaric compounds or cause inconsistent peak integration. An additional approach utilizes two-dimensional separation for amino acids. Two-dimensional separation provides good separation and sensitivity, but is challenging due to the need for multiple columns, switching valves, and the use of ion-pairing reagents [13]. An extensive review of various amino acid separation strategies is provided by Ferré et al. [14].

Recently, mixed-mode (MM) chromatography has been reported as an alternative strategy for amino acid analysis [15–20]. However, most MM amino acid methods published to date either do not effectively separate the leucine isomers or have smaller panel sizes. Within the biochemical genetics field, a comprehensive amino acid profile is necessary for a complete clinical picture. This can be achieved through a full amino acid assay or multiple targeted amino acid assays. Here, we report on the development and validation of an LC-MS/MS method for the analysis of 39 amino acids and related compounds in human plasma and urine using a MM column with cation exchange (CEX) and HILIC properties [15]. This new method offers a comprehensive amino acid panel within a short analysis time, while overcoming many of the limitations previously described, such as analyte retention and chromatographic resolution of critical isobaric components, namely allo-isoleucine, leucine, and isoleucine.

Materials and methods

Reagents

Acetonitrile was from Fisher Scientific (Waltham, MA, USA). Type 1 (18.2 MΩ-cm) water was produced from an Aqua Solutions system. The 10% sulfosalicylic acid solution and 0.1 M HCl were purchased from Waters (Milford, MA, USA). L-Citrulline-$^{13}C_5$, L-ornithine-$^{2}H_7$, β-alanine-$^{2}H_4$, and DL-homocystine-$^{2}H_8$ were purchased from CDN Isotopes (Pointe-Claire, QC, CA). Ammonium formate, allo-isoleucine, formic acid, and Amino acid standard solutions (A6282 and A6407) were from Millipore Sigma (St. Louis, MO, USA). Lyophilized amino acid calibrators, QC, and internal standard (IS) mixtures were purchased from Waters (Kairos™). The calibrators were lyophilized mixtures (7 levels) of amino acids and were reconstituted with 0.1 M HCl prior to use. Similarly, the QC samples (2 levels) were reconstituted with 0.1 M HCl prior to use. The components of the mixtures are listed in Tables S1 and S2 in Supplementary Data.

IS working solution was prepared by preparing an aqueous solution comprising citrulline-$^{13}C_5$ (250 μmol/L), ornithine-$^{2}H_7$ (500 μmol/L), homocystine-$^{2}H_8$ (250 μmol/L), and β-alanine-$^{2}H_4$ (500 μmol/L). This solution (2 mL) was used to dissolve the contents of one vial of the Kairos IS lyophilized mixture prior to diluting to 10 mL with 10% sulfosalicylic acid. Working IS solution was aliquoted and stored at −70 °C prior to use.

Lc-MS/MS

Liquid chromatography was performed using a Waters Acquity UPLC system (binary solvent manager, sample manager with fixed loop injections, column manager) equipped with an Imtakt Intrada Amino Acid column (100 mm × 3 mm, 3 μmol/L, part no. WAA34). Two different column lots were used during validation to demonstrate robustness. Minimal retention time shifts with no disruption to the method were observed over the course of > 1000 injections and between lot changes. Mobile phase A was 40/60 (v/v) water/acetonitrile containing 0.5% formic acid, and mobile phase B was 80 mM ammonium formate in 80/20 (v/v) water/acetonitrile. The column temperature was 45 °C, and the initial flow rate was 600 μL/min. The injection volume was 20 μL. The mobile phase gradient is shown in Table 1.

Mass spectrometric analysis was performed on a Quattro Premier XE in ESI positive mode. The capillary voltage was 2.5 kV, desolvation temperature was 500 °C, source temperature was 110 °C, cone gas flow was 150 L/h, and desolvation gas flow was 1000 L/h. Analyte-specific tune parameters (Tables S3 and S4) were determined by infusing solutions of pure compounds into the instrument and optimizing MS response. This method was also cross-validated on an Acquity UPLC I-class system coupled to a Xevo TQ-XS using similar instrument parameters. The most significant difference in analysis on this instrument was the extracted samples had to be diluted 10-fold with acetonitrile containing 0.5% formic acid due to higher sensitivity in the Xevo model. All validation data reported here were collected on the Quattro Premier XE, but sensitivity, linearity, precision, and method comparison data were also acquired on the Xevo TQ-XS to demonstrate method transferability between similar instruments.

TargetLynx V4.1 SCN 805 was used for integration and quantitation. Although all analytes could be acquired in one 15-min run, two separate quantitation methods were required to process data from all 39 analytes due to the number of analytes in this panel (TargetLynx methods can only accommodate calibrator concentrations for 20 different compounds). Therefore, each batch of samples had to be processed twice to quantitate all amino acid compounds.

Extraction

A total of 50 μL of each sample (plasma or urine) and working IS solution was pipetted into microcentrifuge tubes. The samples were vortex mixed for 5 s, then 50 μL water was added to each tube before vortex mixing again for 5 s. The tubes were centrifuged for 15 min at 9000 × g. A total of 100 μL supernatant was combined with 300 μL acetonitrile containing 0.5% formic acid in an autosampler vial, and vials were vortex mixed prior to LC-MS/MS analysis. Extracted samples were stable at 2–8 °C for 3 days.

Table 1
LC-MS/MS HPLC Gradient.

Time (min)	Flow Rate (mL/min)	%B	Curve
Initial	0.600	15.0	Initial
4.00	0.600	15.0	6
4.75	0.600	20.0	6
4.85	0.700	40.0	6
7.00	0.700	100	6
12.00	0.700	100	6
12.10	0.700	15.0	6
14.50	0.600	15.0	11
15.00	0.600	15.0	6

Validation

Assay validation consisted of the following experiments: matrix effects, interferences, linearity, carryover, acceptable dilution, precision, accuracy, stability, and reference interval transfer. IRB approval was not necessary for this study because it was not deemed to be human subject research, since no record of patient information was kept, all plasma and urine specimens were pooled, and no interaction occurred between the individual and the researchers.

Matrix effects

Matrix effects were evaluated qualitatively through post-column infusion of stable isotope-labeled amino acids. Briefly, plasma and urine samples were extracted as described above, except no IS was added during extraction. Then, neat mixtures of stable isotope-labeled amino acids were infused into the flow path using a post-column tee connector during injections of the patient samples. The MRM transitions of the isotope-labeled amino acids were monitored during the entire 15-min run, and the traces of the patient sample injections were compared to that from a blank injection (free of matrix effects). Matrix effects were observed qualitatively through differences between the blank and patient sample injections.

Mixing study

A mixing study was performed to evaluate whether the developed method accurately accounts for any matrix effects between the matrix-free calibrators and patient samples. Admixtures of 0.1 M HCl with plasma and urine (ratios of 0, 0.33, 0.50, 0.67, 1) were prepared for 6 different lots of each matrix, and measured concentrations in the mixtures were compared to the expected concentrations. Similarly, admixtures of cerebrospinal fluid (CSF) with plasma (ratios of 0, 0.33, 0.50, 0.67, 1) were also prepared for 6 different lots of CSF to evaluate the congruence between the 2 matrices. Mixtures of endogenous interferences, such as icterus, lipemia, and hemolysis were also performed for plasma samples. Selectivity was evaluated by spiking in commercial drug mixtures (Cerilliant I-030), organic acid mixtures (Cambridge Isotope Laboratories MSK-OA-US-1), and small isobaric compounds (pyroglutamate, 5-aminolevulinic acid, 6-aminocaproic acid, estradiol, and dihydrotestosterone) into both plasma and urine and extracting to confirm that these endogenous and exogenous compounds did not interfere with analytes in the assay panel. The compounds in those commercial mixtures are listed in Table S5.

Linearity and AMR

Linearity samples for the plasma and urine validation were prepared by reconstituting the Kairos calibrator level 6 and/or 7 with pooled patient plasma and urine, respectively, and then serially diluted with 0.1 M HCl (concentrations of spiked amino acids ranged from ~0.5 to 1000 μmol/L when using Level 6 and from ~2 to 4000 μmol/L when using Level 7). These linearity samples were analyzed in triplicate, and accuracy and precision were evaluated at each level. Acceptable accuracy and precision was 85–115% of expected concentration and 15% CV, respectively, except at the limit of quantitation where those values were allowed 80–120% and 20% CV, respectively. Linearity was also evaluated with EP Evaluator 10 (EP6 Linearity module).

Dilutions

Acceptable dilution was determined by spiking in concentrations of amino acids beyond the upper limit of quantitation (ULOQ) and diluting back to within the AMR with 0.1 M HCl and ensuring accuracy was within 85–115% of the expected concentration. Carryover was assessed by injecting low-concentration samples before and after samples containing high concentrations of amino acids in triplicate. The second injections were considered acceptable if the standard deviation was within ±2SD, the CV < 15%, and difference <15%.

Precision and stability

Precision samples were prepared by reconstituting various levels of the lyophilized Kairos kit with either plasma or urine to target a low (<10 μmol/L), medium (100–150 μmol/L), and high (200–300 μmol/L) spike concentration in addition to the endogenous amino acid levels. The precision samples were extracted (n = 5) over the course of 5 days, and within-run, between-run, and total precision were calculated. Total precision was considered acceptable if %CV was < 15% for compounds that had a matched isotope-labeled analogue IS and < 20% for the remaining analytes. The compounds with matched IS compounds were considered “quantitative” and the remaining compounds were considered “semi-quantitative”.

The low, mid, and high-level samples prepared for precision were also used for the stability experiment. The stability starting point zero was considered the time the samples were prepared, and one set of these samples was immediately extracted and analyzed. A separate set of time zero samples was placed in the freezer (-70 °C). The remaining sets of samples were kept at room temperature for up to 24 h or refrigerated (2–8 °C) for up to 7 days, and samples were frozen (-70 °C) at various time points until they were analyzed together as a batch. Samples were considered stable at each condition if they were within 85–115% of the initial concentrations.

Method comparisons

A total of 30 pooled plasma samples (spiked and unspiked) and 20 spiked, pooled urine samples were sent to a reference laboratory for a method comparison study to evaluate accuracy. The methodology listed by the reference laboratory for amino acid analysis was “Quantitative Liquid Chromatography/Tandem Mass Spectrometry”. Commercially available amino acid standard mixtures from Sigma (A6407 and A6282) were used to spike the urine samples, while five of the plasma samples were spiked with Sigma product A6407 (acidics and neutrals mixture) and three were fortified by dissolving lyophilized amino acid standards from Waters with pooled plasma. Additionally, most urine and plasma samples were spiked with a separately prepared 1 mg/mL allo-isoleucine solution. Results from our lab were plotted against those from the reference laboratory and Deming regressions were performed for analytes that had ≥10 data points between the two labs. Compounds were considered accurate if the average percent difference between the two labs was ≤20% and the slope was between 0.8 and 1.2 with a correlation coefficient of $R > 0.95$.

Additional samples were compared with Hitachi L-8800 Amino Acid Analyzers. The same 20 urine samples that were sent to the reference laboratory were compared with the L-8800 analyzers, while a different set of 20 plasma samples were used for comparison with the L-8800 analyzers.

Statistics

All statistics were performed using EP Evaluator 10 or Microsoft Excel.

Results and discussion

Development

The important parameters for peak shape, retention time, and separation using HILIC/CEX MM are defined by the salt concentration, acid modifier, and choice and composition of organic solvent(s) used in the mobile phases [16]. The priority during development was the separation of all 3 leucine isomers and separation of remaining amino acids for accurate quantitation (i.e., minimum of 12 data points across each chromatographic peak). Initial experiments showed that mixtures of acetonitrile and water were required to separate the leucine isomers from one another, and that substituting methanol for acetonitrile resulted in poor peak resolution. Acetonitrile also beneficially produced shorter retention times and increased MS response compared to

methanol. The ammonium formate in mobile phase B worked well as an MS-compatible salt for CEX, whereas formic acid in mobile phase A (0.5% v/v, or approximately 125 mM) was sufficient to keep the amino acids fully protonated at the start of the LC gradient.

Under the developed LC conditions, the selectivity factors for allo-isoleucine/leucine, allo-isoleucine/isoleucine, and leucine/isoleucine were 1.07, 1.15, and 1.08, which was considered acceptable for a large panel with many other analytes. Resolution between leucine and isoleucine was calculated to be approximately 1.15 based on peak widths of ~ 0.22 min, which would result in an approximately 1% peak overlap at similar peak heights and Gaussian peak shapes [21]. Additional selectivity was obtained by using different quantitative MRM transitions (132 > 69 for isoleucine and allo-isoleucine and 132 > 43 for leucine, see Fig. 1). All three isomers used 132 > 86 as a qualitative ion, which produced the best response out of all transitions. Under these chromatographic conditions, sulfocysteine was not retained and taurine had a low retention factor (~0.92), but all other amino acids had retention factors greater than 1 (Fig. 2). Sulfocysteine was not included in this panel, but it was evaluated during initial development, and it is present in all calibrators and QC samples.

The Kairos kit comes with 20 labeled amino acids (Table S2), which were supplemented with 4 additional isotope-labeled amino acids to generate the working IS solution. Ideally, every amino acid in the panel would be quantified using its own isotope-labeled analogue for accurate quantitation, but it became impractical and cost-prohibitive to include isotope-labeled analogues for all amino acids. Initially, $^{2}H_{7}$-citrulline was selected as an IS for citrulline, but was changed to $^{13}C_{5}$-citrulline during validation due to matrix effects. Deuterated citrulline and citrulline did not have identical retention times, and the IS experienced significant ionization suppression from homocitrulline, which impeded accuracy. The retention time of ^{13}C-labeled citrulline was nearly identical to citrulline, and, hence, matrix effects for both analyte and IS were better matched.

The amino acid compounds without matched isotope-labeled analogues were considered “semi-quantitative”. While accuracy, precision, linearity, sensitivity, and other validation parameters were evaluated for all compounds, those analytes without isotope-replaced analogues cannot guarantee that matrix effects would be corrected to the same degree as those analytes for which an isotope replaced internal standard was available. It is not uncommon for assays with large numbers of analytes to substitute an alternative IS in lieu of a homologous isotope-labeled IS [8,11,13,15]. Whenever possible, the IS chosen for the semi-

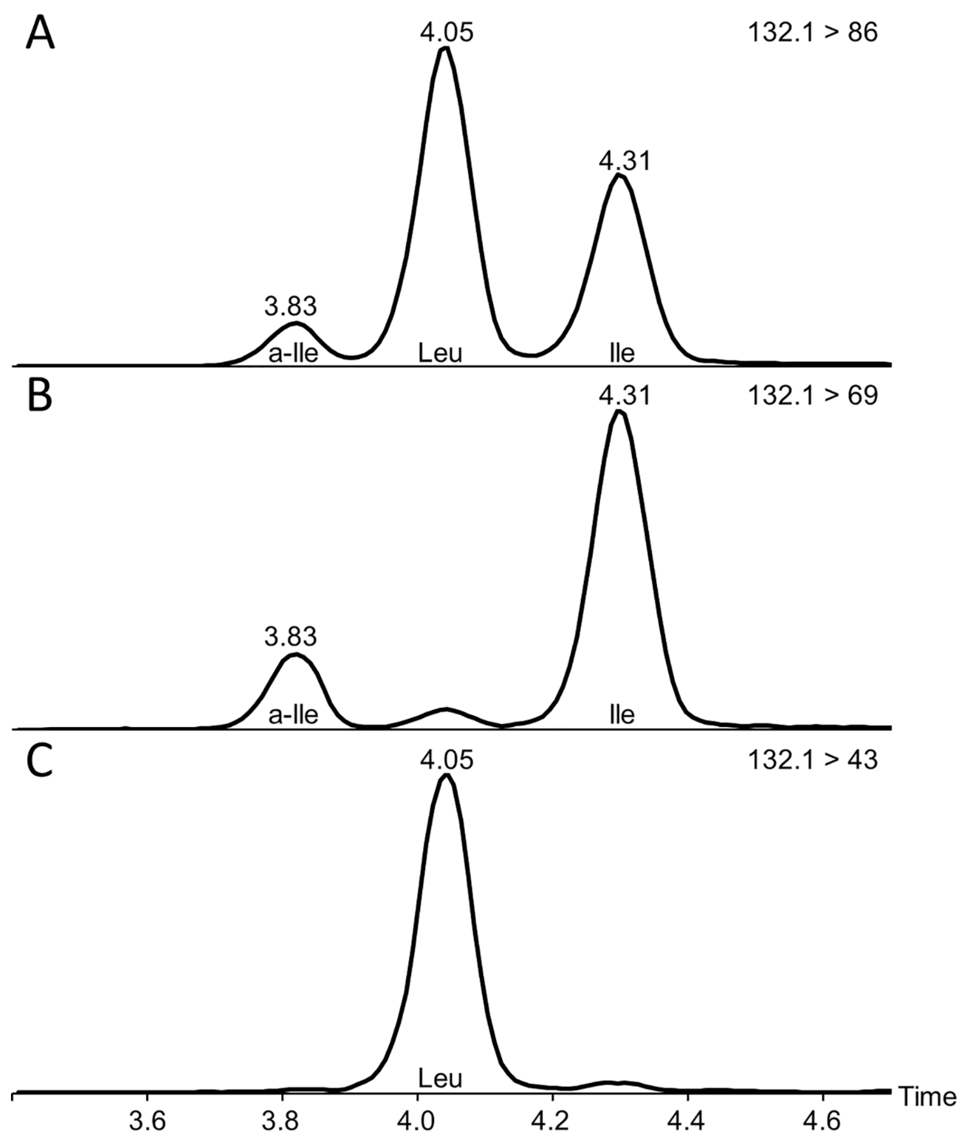

Fig. 1. Leucine isomers in plasma (leucine 96 μmol/L, isoleucine 39 μmol/L, and alloisoleucine 7 μmol/L). A) 132 > 86 transition, used as qualitative ion for all three isomers, showing degree of separation obtained by this method. B) 132 > 69 transition, which is selective for allo-isoleucine and isoleucine. C) 132 > 43 transition, which is selective for leucine.

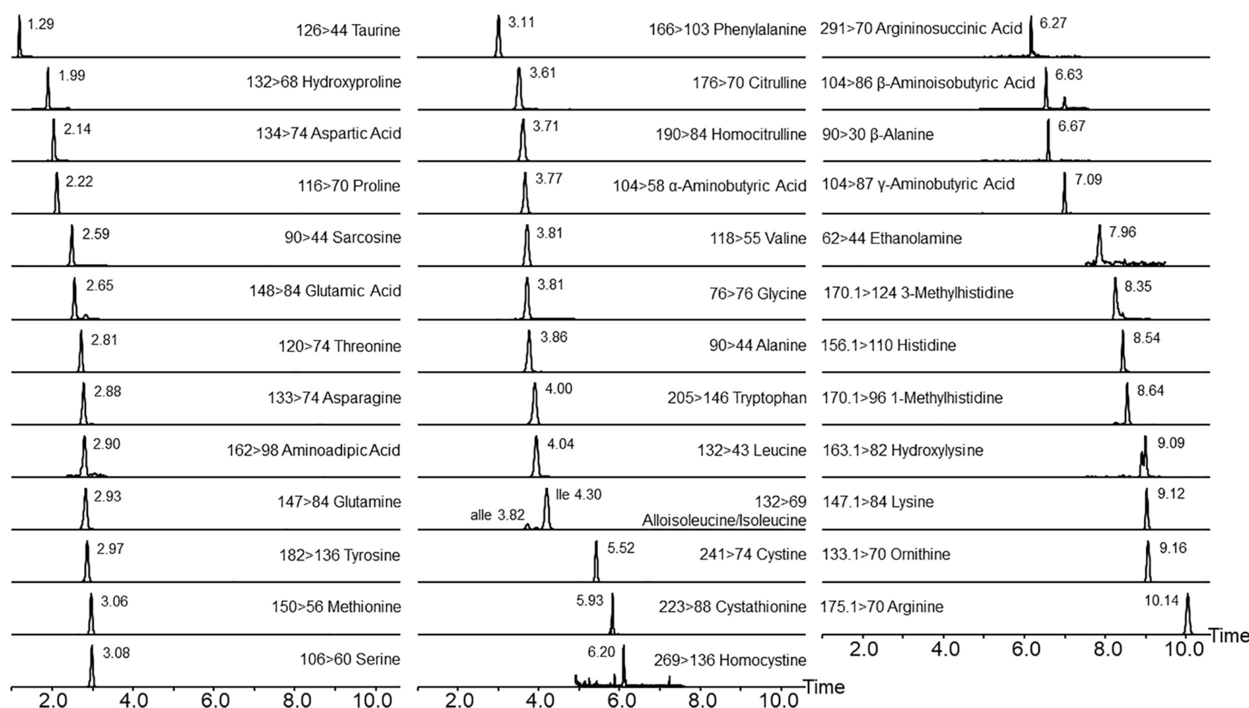

Fig. 2. Chromatogram of all components of LC-MS/MS amino acid method. Plasma sample spiked with ~5 μmol/L of each amino acid in addition to the exogenous level.

quantitative compounds had a similar or identical retention time. With the exception of allo-isoleucine, the semi-quantitative compounds in our assay served more of a supporting role in the diagnosis and monitoring of inborn errors of metabolism. Allo-isoleucine utilized leucine-$^{13}C_6$, ^{15}N as the IS, which was both chemically similar and had a similar retention time.

It became apparent early in development that the analysis of argininosuccinic acid (ASA) would need to be qualitative due to the absence of a stable isotope-labeled ASA in this method. There was likely a combination of matrix effects and loss of ASA during deproteinization [17], and results were inaccurate without an appropriate IS to correct. ASA is also known to be labile through conversion to ASA anhydrides at low pH [22]. The protein precipitation method and LC-MS mobile phases were acidic, so it was not surprising to observe poor calibration curves and unreproducible QC measurements for ASA. Both plasma and urine specimens from patients with argininosuccinate lyase deficiency were evaluated to verify the clinical utility of qualitative assessments of ASA. Elevated ASA (plasma: >100 μmol/L; urine >1000 μmol/L) was clearly distinguishable from normal levels (plasma: <5 μmol/L; urine typically <50 μmol/L), and ASA anhydrides were also monitored and detected (MRM: 273.1 > 70.1) as confirmation of elevated ASA.

Plasma amino acids

Matrix effects

A total of six plasma samples were evaluated for matrix effects, and ionization suppression was present in varying degrees compared to the blank injection for all studied amino acids (only 21 isotope-labeled amino acids were studied due to the difficulty in obtaining amino acid-free matrix). Signal suppression appeared to be more significant during the latter half of the run (i.e., >6 min). Most of the compounds showed a prominent dip in signal at 8 min, around the time that ethanolamine elutes off column (see Fig. S1 for representative data).

Mixing studies between CSF and plasma showed that there were no significant differences between results from the two matrices. Mixing studies showed that isotope-replaced IS corrected for matrix effects for their respective analytes. However, certain amino acid compounds that did not have isotope-replaced IS, such as cystathionine, hydroxylysine, and ethanolamine, showed bias in the mixing study, which indicated that matrix effects were present and were not accounted for due to lack of acceptable IS. Likewise, additional experiments evaluating endogenous interferences from visibly icteric, hemolytic, and lipemic samples showed that taurine, sarcosine, ethanolamine, hydroxylysine, gamma-aminobutyric acid (GABA), and 1-methylhistidine all experienced discrepant results due to lack of matched IS. No interferences were detected from commonly prescribed drugs, organic acids, or targeted isobaric compounds that were spiked into plasma samples.

Linearity

Generally, amino acids with isotope-replaced IS were both accurate and precise across the AMR (Table 2). The ULOQ and LLOQ of the AMR were truncated for a few amino acids due to nonlinear behavior and poor accuracy, respectively.

Dilution

Recoveries for amino acids ranged from 85.1 to 113.8% for 100-fold dilutions with 0.1 M HCl. In most cases, carryover limits were set near the ULOQ, which ranged from approximately 1000 to 4000 μmol/L.

Precision and stability

All quantitative compounds that had stable isotope-replaced IS had acceptable total precision except for homocystine (%CV = 14.0 – 34.5%) The biggest contribution to the high %CV in homocystine was the between-day precision due to its poor storage stability, whereas its within-run precision was acceptable (data not shown). Other compounds with matched IS typically had <10% CV, except for β-alanine with 12.3% at <10 μmol/L concentrations. Most semi-quantitative compounds that did not have a direct match for IS still had acceptable precision. Compounds with higher imprecision (ranging from 21.2 to 30.6 %CV) included 1-methylhistidine, 3-methylhistidine, cystathionine, homocitrulline, and hydroxylysine. Precision values in this assay were comparable to other MM studies that had performed precision studies in matrix-free samples [15,20] and were better than precision in plasma samples using ion-pairing reagents [8].

Table 2
Plasma amino acid validation results ("N/A" indicates Deming regression not performed due to <10 data points).

		Total Precision		Method Comparison				
Compound	AMR (μmol/L)	Mean (μmol/L)	%CV	Slope	Intercept	Corr. Coef. (R)	Meas.Range (μmol/L) (Reference Laboratory Range)	N
1-MeHis‡	22–1000	14.7	**30.6**	Compound not included in reference laboratory's panel.				
		214	11.8					
		393	10.4					
3-MeHis ‡	5–1000	7.1	17.7	Compound not included in reference laboratory's panel.				
		85.1	17.4					
		184	**21.2**					
α-aminobutyric acid ‡	1–985	26.6	6.6	**1.234**	−1.7	0.9993	9–653	29
		135	5.1				(8–443)	
		264	5.8					
α-aminoadipic acid ‡	2–1000	5.3	10.1		N/A		<2–511	9
		105	6.1				(<2–450)	
		233	6.4					
Alanine	5–4210	378	4.0	1.189	−12.6	0.9919	260–1440	30
		535	2.9				(225–1241)	
		713	4.5					
Allo-isoleucine ‡	2–985	6.8	19.8	1.079	0.1	0.9921	<2–552	19
		118	3.6				(<2–458)	
		241	3.8					
Arginine	1–1080	79.6	3.7	0.985	2.6	0.9984	51–516	30
		189	4.3				(50–518)	
		315	4.2					
Asparagine	1–1054	56.0	5.5	1.012	−1.2	0.9891	31–537	30
		161	5.4				(34–559)	
		287	6.5					
Aspartic Acid	1–981	9.8	5.7	1.159	−0.7	0.9953	2–659	10
		108	4.2				(<5–556)	
		226	4.9					
β-alanine	5–1038	8.3	12.3		N/A		<5–750	8
		137	6.4				(<25–872)	
		285	9.3					
β-aminoisobutyric acid ‡	3–1004	18.2	11.6		N/A		<3–587	8
		332	6.2				(<5–471)	
		744	6.7					
Citrulline	2–1049	169	9.4*	1.005	−3.7	0.9991	19–491	10
		272	6.1*				(18–482)	
		421	8.8*					
Cystathionine ‡	5–750	5.3	**25.9**		N/A		<5–593	8
		94.4	9.8				(<5–463)	
		216	7.7					
Cystine	1–525	30.8	5.6	**1.234**	3.0	0.9973	13–304	30
		79.4	6.1				(9–248)	
		127	7.0					
Ethanolamine ‡	5–100	9.5	19.4	**0.639**	−1.2	0.9970	<5–270	12
		80.3	17.0				(8–442)	
		148	16.0					
Gamma-Aminobutyric acid ‡	3–1003	7.1	13.1		N/A		<3–528	3
		141	6.3				(<5–434)	
		293	8.1					
Glutamic Acid	2–1114	75.4	6.0	**1.230**	−11.8	0.9967	33–635	30
		181	3.0				(24–509)	
		318	4.8					
Glutamine	2–4392	579	4.9	1.154	−4.6	0.9730	454–1470	30
		732	5.6				(395–1311)	
		919	6.6					
Glycine	20–4014	310	6.3	1.196	−24.4	0.9822	170–1470	30
		537	4.9				(163–1153)	
		772	7.6					
Histidine	5–1000	91.7	5.4	0.806	10.5	0.9974	43–698	30
		278	5.6				(54–882)	
		515	6.4					
Homocitrulline ‡	5–1000	18.2	**27.5***		N/A		<5–698	4
		221	8.3*				(<5–470)	
		408	9.6*					
Homocystine	2–967	1.0	**34.5**		N/A		<2–441	5
		36.5	**22.7**				(<2–233)	
		159	14.0					
Hydroxylysine ‡	2–1028	4.5	**22.4**		N/A		<2–551	3
		124	**26.1**				(<5–433)	
		311	14.8					
Hydroxyproline ‡	5–1000	24.6	12.2	0.890	−0.6	0.9964	<5–465	30
		118	9.2				(10–487)	
		238	8.0					
Isoleucine	5–777	71.9	4.7	1.041	−7.7	0.9948	39–613	29

(*continued on next page*)

Table 2 (*continued*)

Compound	AMR (μmol/L)	Total Precision		Method Comparison				
		Mean (μmol/L)	%CV	Slope	Intercept	Corr. Coef. (R)	Meas.Range (μmol/L) (Reference Laboratory Range)	N
		222	5.0				(38–614)	
		391	5.7					
Leucine	2–781	129	4.5	1.120	−9.7	0.9842	74–688	29
		280	4.1				(69–555)	
		450	5.2					
Lysine	1–1099	172	6.8	1.132	−8.5	0.9940	99–648	30
		266	6.7				(97–565)	
		392	6.5					
Methionine	1–1011	30.8	4.0	1.125	−5.2	0.9963	19–584	30
		130	3.5				(18–486)	
		255	5.2					
Ornithine	1–1057	68.5	4.6	1.062	−0.5	0.9972	29–534	30
		169	5.3				(27–514)	
		297	5.7					
Phenylalanine	5–776	83.5	5.5	1.086	−3.9	0.9954	46–601	29
		237	4.8				(39–573)	
		406	5.4					
Proline	1–1239	257	5.8	1.022	25.5	0.9885	153–804	30
		426	6.0				(129–791)	
		603	8.4					
Sarcosine ‡	5–700	7.9	11.8		N/A		<5 – 574	9
		120	7.0				(<5–461)	
		254	7.1					
Serine	2–3693	117	5.9	1.047	−6.2	0.9950	73–1040	30
		296	5.2				(69–962)	
		452	5.5					
Taurine ‡	5–1041	79.3	5.9	**1.258**	−2.3	0.9932	43–643	30
		191	6.8				(38–537)	
		327	8.1					
Threonine	5–783	125	4.5	1.058	5.8	0.9831	102–498	25
		283	5.0				(99–426)	
		458	5.6					
Tryptophan	5–1000	63.7	4.1	1.189	−7.0	0.9978	13–582	30
		166	4.5				(14–487)	
		291	6.3					
Tyrosine	2–792	77.2	3.2	1.165	−11.6	0.9946	46–606	29
		236	4.7				(40–500)	
		416	4.7					
Valine	5–817	231	4.2	1.123	−7.8	0.9847	136–785	29
		384	3.5				(120–701)	
		557	4.2					

‡Indicates compound is “semi-quantitative”.
*Used $^{2}H_7$-citrulline as IS here instead of $^{13}C_5$-citrulline.
Bolded values are outside the acceptance criteria.

A previous study indicated that plasma samples must be stored at −80 °C as soon as possible to prevent concentration changes in certain amino acids, most notably arginine, glutamic acid, and cystine [23]. Room temperature and refrigerated conditions were investigated as well. The most notable changes were with arginine and glutamic acid, which showed significant increases in concentration at room temperature at time points greater than 6 h and at 2–8 °C within 2 days, and homocystine, carnosine, and anserine, which degraded within those time points. Lysine, histidine, methylhistidines, and β-alanine also showed increases at 2–8 °C within 5 days. The increases in histidine, methylhistidines, and β-alanine were attributed to the degradation of carnosine and anserine by serum carnosinase [24]. All other plasma amino acid concentrations appeared to be stable for at least 24 h at room temperature and 7 days at 2–8 °C. Low cystine concentrations (~30 μmol/L) decreased by more than 20% within 18 h at room temperature, but higher concentrations (>80 μmol/L) only showed a slight decrease in concentration over a 24-h period. Once samples were extracted, concentrations were stable for at least 7 days at 2–8 °C.

Method comparison

Results from a total of 30 (20 initially plus 10 extra) plasma samples were compared with those from a reference laboratory. After the initial comparison, it was necessary to perform a method comparison for citrulline using a new IS, citrulline-$^{13}C_5$, as the first 20 samples highlighted a need for a more appropriate IS. Additionally, the majority of the first 20 samples were not spiked with anything other than allo-isoleucine. Therefore, while most analytes were present in measurable amounts in the first 20 samples sent to the reference laboratory, the measurements of certain compounds were below the lower limit of quantitation (LLOQ) for both laboratories. Therefore, an additional 10 samples (5 unspiked, 5 spiked with “neutrals and acidics” Sigma product A6407) were compared with a reference laboratory, which increased the number of method comparison data points for some amino acids due to the spiking solution. Certain compounds such as GABA, homocitrulline, homocystine, and hydroxylysine were not present in the spike solution, so some compounds still had fewer than 10 comparison data points with measurable values after 30 samples were analyzed. A Deming regression was performed on amino acids with ≥10 non-“less than” samples. The two methods compared favorably (Table 2), though cystine and glutamic acid both had slopes of 1.23. Additional amino acids with higher bias were taurine, alpha-aminobutyric acid, and ethanolamine (slopes of 1.26, 1.23, and 0.64, respectively). A total of 27 amino acids had ≥10 samples for comparison. Out of those 27 samples, the slopes, intercepts, and correlation coefficients for the 22 quantitative compounds ranged from 0.81 to 1.23, −24.4 to 25.5 μmol/L, and 0.9730 to 0.9991, respectively. The slopes, intercepts, and correlation coefficients for the remaining 5 semi-quantitative compounds ranged from 0.64 to 1.26, −2.3 to 0.1 μmol/L, and 0.9921 to 0.9993, respectively (Table 2).

To evaluate the newly developed LC-MS/MS assay against the lab's established amino acid methodology, a comparison was also performed between the new LC-MS/MS method and the Hitachi L-8800 Amino Acid Analyzers, which utilize ion exchange chromatography with post-column ninhydrin derivatization. The purpose of this comparison was to baseline the new assay from an analytical perspective to ensure a smooth method transfer from the L-8800 Analyzers. All compounds except for asparagine, citrulline, and tryptophan had slopes between 0.8 and 1.2 relative to the L-8800 Analyzers. A summary of the results are found in Table S6.

Because the above comparison was evaluated primarily from an analytical perspective using spiked, pooled specimens, the clinical utility of the assay was also confirmed by separately analyzing samples from patients with various inborn errors of metabolism to ensure that expected amino acid profiles were measured. Some examples of the disorders that were analyzed included phenylketonuria, maple syrup urine disease, isovaleric academia, citrullinemia, arginosuccinate lyase deficiency, and ornithine transcarbamylase deficiency. Additional proficiency testing samples from previous years were also analyzed and evaluated relative to peer group measurements and expected interpretations with good agreement (data not shown).

Urine amino acids

Matrix effects

A total of six urine samples were evaluated for matrix effects, and ionization suppression was present for all studied amino acids (see Fig. S2 for representative data). Similar to plasma samples, mixing studies showed that appropriate isotope-replaced IS corrected for matrix effects. However, 1-methylhistidine showed significant bias in the mixing study, indicating the matrix effects were not corrected for in this particular compound. No interferences were detected from commonly prescribed drugs, organic acids, and targeted isobaric compounds that were spiked into urine samples.

Linearity

Amino acids with their own isotope-labeled analogues were generally both accurate and precise across the AMR. The ULOQ and LLOQ of the AMR were truncated for a few amino acids due to nonlinear behavior and poor accuracy, respectively, and AMRs are shown in Table 3 for urine.

Dilution

The recoveries of amino acids diluted by up to 100-fold with 0.1 M HCl ranged from 92.7 to 113.7%. Carryover limits were typically set at the ULOQ for most amino acids. In specific cases, the carryover limit was greater than the ULOQ for compounds with truncated AMRs.

Precision and stability

All compounds that had stable isotope-labeled internal standard analogues had acceptable precision (within 15% CV). Most semi-quantitative compounds that did not have a direct match for IS still had acceptable precision. At low concentrations, hydroxylysine and 3-methylhistidine both had higher imprecision at 26.1% and 23.1%, respectively. The $^{13}C_5$-citrulline was not in use in the IS mixture at the time this experiment was performed, and the imprecision for the citrulline at low concentrations was 21.1%, but CV from QC data collected since implementation of $^{13}C_5$-citrulline has been <10%. Urine amino acids appeared to be stable over the course of the stability experiment (i.e., room temperature for 24 h, 2–8 °C for 7 days).

Method comparison

Results from a total of 20 urine samples were compared with those from reference laboratory. The results were normalized to the creatinine content in the sample, and creatinine measurements for the samples ranged from 8 to 106 mg/dL. While the two methods compared favorably, the reference laboratory had higher LLOQ values for some amino acids; as a result, many amino acids had fewer than 20 samples for comparison. Amino acids with higher relative bias included asparagine, citrulline, histidine, and ethanolamine (slopes 0.69, 0.79, 0.80, and 0.38, respectively), and correlation with ethanolamine was also poor (R = 0.8884), summarized in Table 3. A total of 30 amino acids had ≥10 samples for comparison. Out of those 30 samples, the slopes, intercepts, and correlation coefficients for the 22 quantitative compounds ranged from 0.69 to 1.12, −250 to 74.5 μmol/g creat, and 0.9815 to 0.9996, respectively. The slopes, intercepts, and correlation coefficients for the 8 semi-quantitative compounds, excluding ethanolamine above, ranged from 0.88 to 1.12, −44.4 to 162 μmol/g creat, and 0.8884 to 0.9981, respectively (Table 3). Increased intercepts were observed for the Deming regression in urine due to creatinine normalization and higher average concentrations. For example, glycine measurements ranged from 422 to 2630 μmol/L, which converted to 895–17,900 μmol/g creat. For all sample measurements the average difference ranged from −6 to 17%, yet the intercept was −250 μmol/g creat due to the lack of glycine measurements at lower normalized concentrations. This was also seen with glutamine, histidine, and lysine.

The new LC-MS/MS method was also compared to the L-8800 analyzers for urine samples. There were some notable differences in the results between the two methodologies for urine samples, which were attributed to well-known challenges of urine amino acid analysis on the Hitachi instrument. Many compounds in urine, including metabolites, medications, and supplements, react with ninhydrin yielding higher baselines and additional interfering peaks that make accurate quantitation challenging. Most amino acids that eluted before 30 min or after 90 min compared reasonably well, but amino acids within that 30–90 min elution window appeared to suffer from more interferences compared to the LC-MS/MS method. For example, branched chain amino acids, Phe, Tyr, Met, and Cit did not compare well with results from the L-8800, whereas amino acids such as Gln, Glu, Ser, Thr, Orn, and Arg showed good agreement. Results for Trp showed no correlation because Trp co-elutes with ammonia on the L-8800. Ammonia is typically present in large concentration in urine and, relative to Trp peak, is significant. This resulted in skewing of the results from the L-8800s. A summary of the L-8800 comparison results for urine samples is shown in Table S7.

Conclusions

Most reported LC-MS/MS methods for amino acids have not reported on the separation of Leu, Ile, and allo-Ile, which could be due to analytical limitations or differing clinical needs. While there are reported LC-MS/MS methods with the ability to chromatographically resolve these three isomers, they tend to be narrowly focused on a small numbers of amino acids [17,18,25,26]. However, given the need for a comprehensive amino acid panel, it was more appropriate to develop a complete assay that could provide an inclusive panel while simultaneously resolving critical structural and stereoisomers in a short period of time. This assay also exhibited advantages inherent to LC-MS/MS compared to more traditional assays. Notably, runtimes were decreased from >1 h to 15 min and common co-eluting peaks such as ASA/leucine and tryptophan/ammonia were resolved. Additionally, this method didn't suffer from derivatization-related interferences commonly observed with urine amino acid analysis.

We describe in this work a comprehensive amino acid panel that includes the separation of Leu, Ile, and allo-Ile in addition to 36 other amino acids that are commonly included in amino acid profiles used to diagnose a wide range of inborn errors of metabolism. While there are analytical challenges from the large number of amino acids, the use of stable isotope-replaced IS for the majority of panel members corrected for matrix effects, a primary cause of inaccuracy and imprecision. Primary amino acid biomarkers that are critical for diagnosing inborn errors of metabolism (e.g., branched chain amino acids, Phe, Tyr, Gly, Met,

Table 3
Urine amino acid validation results ("N/A" indicates Deming regression not performed due to < 10 data points).

	AMR (μmol/L)	Total Precision		Method Comparison				
Compound		Mean (μmol/L)	%CV	Slope	Intercept	Corr. Coef. (R)	Meas. Range (μmol/g creat) (Reference Laboratory Range)	N
1-MeHis ‡	7–1150	21.2	15.2	Compound not included in reference laboratory's panel.				
		209	9.0					
		391	7.7					
3-MeHis ‡	4–1142	49.3	**23.1**	Compound not included in reference laboratory's panel.				
		106	**21.4**					
		159	18.4					
α-aminobutyric acid ‡	5–742	7.7	6.4	1.119	26.2	0.9915	17–2777	12
		130	5.1				(<20–2509)	
		257	6.4					
α-aminoadipic acid ‡	2–630	14.5	9.3	0.919	4.6	0.9980	27–2250	20
		121	6.9				(24–2486)	
		237	6.8					
Alanine	2–4198	171	5.0	0.912	11.1	0.9950	173–3069	20
		325	3.7				(189–3232)	
		501	5.6					
Allo-isoleucine ‡	1–984	5.9	14.7	Compound not included in reference laboratory's panel.				
		126	5.7					
		245	5.6					
Arginine	1–1031	22.7	2.6	0.946	7.1	0.9986	15–1893	13
		130	1.7				(<50–2014)	
		243	4.1					
Asparagine	5–1000	45.2	7.8	**0.685**	23.3	0.9959	46–2155	20
		149	5.0				(51–3166)	
		260	7.7					
Aspartic Acid	5–1000	9.4	4.5	1.064	7.3	0.9977	<5–2609	10
		113	3.8				(<50–2436)	
		229	4.3					
β-alanine	5–1000	11.2	19.7		N/A		<5–2518	7
		123	7.6				(<250–2603)	
		248	6.4					
β-aminoisobutyric acid ‡	2–1058	502	5.1	1.053	23.8	0.9834	24–3795	17
		594	5.4				(<50–3680)	
		716	4.4					
Citrulline	5–1000	8.80	**21.1***	**0.786**	20.7	0.9971	<5–2027	16
		123	4.8*				(<10–2606)	
		235	5.3*					
Cystathionine ‡	5–750	39.3	7.6	0.997	−14.8	0.9981	5–2259	10
		125	7.5				(<50–2275)	
		220	5.7					
Cystine	3–500	13.6	6.8	0.932	6.6	0.9943	25–1227	19
		66.9	6.7				(<50–1314)	
		124	6.9					
Ethanolamine ‡	3–1365	87.6	9.9	**0.382**	141.8	**0.8884**	205–1296	20
		171	11.0				(331–3126)	
		261	13.6					
Gamma-Aminobutyric acid ‡	4–1000	9.7	10.3		N/A		5–1992	9
		168	6.6				(<50–1967)	
		320	5.1					
Glutamic Acid	2–1101	15.9	7.4	0.962	8.3	0.9944	15–2523	14
		131	5.9				(<50–2627)	
		258	6.7					
Glutamine	3–4285	121	4.6	0.994	37.9	0.9944	446–3159	20
		276	6.0				(426–3039)	
		448	5.9					
Glycine	20–4518	2269	12.6	1.116	−250	0.9927	895–17,923	20
		2386	14.1				(798–15,176)	
		2465	13.4					
Histidine	1–879	88.9	4.2	**0.799**	74.5	0.9939	360–3964	20
		194	4.2				(397–5004)	
		301	6.0					
Homocitrulline ‡	5–1000	71.8	7.4*		N/A		17–631	8
		190	5.8*				(<50–505)	
		313	5.3*					
Homocystine	5–800	6.9	14.6	Compound not included in reference laboratory's panel.				
		129	14.2					
		263	12.5					
Hydroxylysine ‡	5–1000	6.4	**26.1**	1.016	9.0	0.9955	<5–2039	13
		83.7	12.2				(<50–1949)	
		171	10.4					
Hydroxyproline ‡	5–1000	37.8	8.6	1.034	−44.4	0.9973	<5–5185	18

(*continued on next page*)

Table 3 (*continued*)

Compound	AMR (µmol/L)	Total Precision		Method Comparison				
		Mean (µmol/L)	%CV	Slope	Intercept	Corr. Coef. (R)	Meas. Range (µmol/g creat) (Reference Laboratory Range)	N
		114	6.2				(<20–4816)	
		208	6.6					
Isoleucine	2–3283	9.8	4.5	0.980	21.0	0.9967	14–2395	11
		164	4.2				(<50–2477)	
		327	4.5					
Leucine	3–2853	20.9	10.2	0.914	−10.6	0.9991	30–2500	20
		186	5.1				(31–2690)	
		357	5.7					
Lysine	5–996	32.4	4.6	1.075	−90.2	0.9981	54–14,167	20
		132	4.2				(52–12,381)	
		245	5.7					
Methionine	1–996	8.5	5.4	0.881	20.2	0.9981	<5–2268	15
		112	3.7				(<20–2608)	
		227	4.5					
Ornithine	1–1020	15.1	9.5	1.091	13.5	0.9985	13–1968	12
		126	5.0				(<50–1840)	
		235	5.5					
Phenylalanine	2–3884	19.4	4.1	0.925	4.4	0.9995	41–2632	20
		174	4.6				(40–2868)	
		345	4.3					
Proline	1–1083	49.9	3.3	1.030	–22.7	0.9980	7–3215	18
		159	3.3				(<20–2990)	
		282	4.5					
Sarcosine ‡	5–1000	13.3	10.8		N/A		<5–2459	9
		143	7.3				(<50–2382)	
		272	7.2					
Serine	3–3998	130	4.0	0.905	43.2	0.9966	247–3686	20
		278	3.7				(221–4187)	
		446	5.4					
Taurine ‡	2–1154	162	8.3	0.882	161.6	0.9940	275–8273	20
		289	8.9				(219–9652)	
		426	10.4					
Threonine	5–3888	82.2	3.0	0.934	13.1	0.9996	98–2564	20
		236	3.6				(87–2736)	
		403	5.3					
Tryptophan	5–1000	17.7	5.6	1.028	−8.6	0.9994	46–2082	20
		130	4.1				(46–2020)	
		250	6.0					
Tyrosine	5–3000	26.1	5.3	0.964	12.8	0.9815	58–2659	20
		187	3.7				(59–2853)	
		360	4.6					
Valine	2–3885	17.3	4.1	0.979	4.4	0.9987	31–2532	20
		174	3.5				(29–2621)	
		352	4.1					

‡Indicates compound is "semi-quantitative".
*Used $^{2}H_7$-citrulline as IS here instead of $^{13}C_5$-citrulline.
Bolded values are outside the acceptance criteria.

cystine, homocystine) and urea cycle disorders (e.g., Arg, Orn, Citr, Ala, Lys, and Gln) were represented well by the IS mixture, and these compounds were more reliably accurate and precise. In contrast, other ancillary amino acid compounds, such as hydroxylysine, ethanolamine, and methylhistidines experienced more matrix effects and were considered semi-quantitative as a result of the lack of a matched IS. The semiquantitative amino acids with the most issues with precision and accuracy tended to be near the second half of the run, where signal suppression was observed to be more prevalent.

In conclusion, we developed a rapid, sensitive, non-derivatized LC-MS/MS method that resolved issues commonly encountered with ion exchange chromatography combined with post-column derivatization, as well as derivatized and ion-pairing LC-MS/MS methods.

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.

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Author contribution

PD and DRB contributed to the conception, writing, and manuscript approval. PD conducted the experiments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2022.05.002.

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