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A multiplexed LC-MS/MS assay for comprehensive screening of amino acid metabolism disorders

Jisha Chandran [a,b], Madan Gopal Ramarajan [a,b], Vykuntaraju Kammasandra Nanjundagowda [c], Aruna Gowdra [c], Jayesh Warade [d], Anikha Bellad [a,b], Kannan Rangiah [a,b,1,*]

[a] Manipal Academy of Higher Education, Manipal, Karnataka, India
[b] Institute of Bioinformatics, Bangalore, Karnataka, India
[c] Indira Gandhi Institute of Child Health, Bangalore, Karnataka, India
[d] Meenakshi Mission Hospital and Research Centre, Madurai, Tamil Nadu, India

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ABSTRACT

Advancements in liquid chromatography-tandem mass spectrometry (LC-MS/MS) are redefining the landscape of clinical diagnostics, particularly in the context of newborn screening for inborn errors of metabolism. Conventional analytical platforms are often limited by a small number of analytes and require multiple platforms for subsequent analyses, thereby impacting timely diagnosis and management. This study describes the development of a multiplexed targeted mass spectrometry-based assay for simultaneous detection and quantitation of amines, including amino acids and their derivatives, from plasma, urine and dried blood spots. The method utilizes a single step derivatization strategy based on one step click chemistry that simplifies sample preparation while improving analytical sensitivity allowing for detection of analytes at sub-picomolar concentrations. Furthermore, we adopted a strategy of generating heavy isotopically labeled standards by chemically modifying the corresponding light standards using a stable isotope labeled derivatizing agent, enabling their use as internal standards for quantification. This approach can offer a cost-effective and scalable solution for the early detection and management of inherited metabolic disorders, particularly in cases where accurate detection of amines is critical.

1. Introduction

Amino acids and amines are the building blocks of proteins, including enzymes and dipeptides, and are essential organic compounds needed as a source of energy for life activities [1]. Amino acids are necessary for several important functions as they serve as basic structural protein units and precursors of neurotransmitters, porphyrins and nitric oxide, which are neuroactive substances intricately related to a variety of physiological processes and diseases [1–3]. Thus, knowledge about changes in their levels in the body has diagnostic value in the context of diseases affecting related pathways. One of the key clinical applications of detection of amino acids and their derivatives is in newborn screening programs aimed at identifying amino acid disorders [4–7]. Amino acids and amines comprise a large category of metabolic disorders identified by standard investigations [8]. These disorders lead to accumulation of toxic metabolites along with deficiencies in essential metabolic products, resulting in clinical manifestations. Inborn errors of metabolism (IEMs) are a group genetic disorders with a global prevalence of about 1 in 800 globally which highlights the serious impact of these on pediatric health [9]. Exact prevalence data from India is limited; however, for certain well-studied disorders such as congenital hypothyroidism and glucose-6-phosphate dehydrogenase (G6PD) deficiency, available evidence suggests that the prevalence in some regions may be significantly higher than global averages [10]. Early diagnosis has significantly improved pediatric health outcomes globally, even though most affected newborns appear healthy at birth and only develop signs and symptoms as they grow older [11].

Liquid chromatography-mass spectrometry (LC-MS)-based assays have transformed newborn screening. They have shifted the paradigm from one test for one disease to multi-analyte panels that detect numerous conditions with high sensitivity and specificity, while also improving quality control and data standardization. In the United States,

* Corresponding author.
E-mail address: kannan.goref@gmail.com (K. Rangiah).
[1] **Current address:** Ganga Research Centre (GRC), Ganga Orthopaedic Research and Education Foundation (GOREF), 442 post, NGGO Colony, Vattamalaipalayam, Coimbatore- 641,022, Tamil Nadu, India

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4 million newborns undergo such screening annually. In contrast, in resource-limited countries like India, <3 % of the 23 million annual births are screened for a limited number of disorders due to lack of infrastructure and low public awareness [11,12]. Several studies pertaining to the measurement of amino acids and their derivatives using qualitative and quantitative approaches have been published [13–21]. However, a multiplexed assay targeting the entire amine metabolism pathway, suitable for newborn screening, is still lacking.

In this study, we describe the development of a multiplexed assay designed to measure amino acids, their derivatives and selected biogenic amines. Traditionally, for bioanalytical method development, the use of stable isotopically labelled internal standards (SIL-IS) is preferred, which share identical chemical properties with the target analytes and help mitigate the problems related to recovery, stability, and ion suppression. However, it can be challenging to include isotopically labelled analogs for every analyte in the assay due to limited availability, especially when the number of analytes in the panel is large. To overcome this limitation, we employed a strategy that uses a labelled derivatizing agent to generate internal standards for all analytes. Using a previously established protocol [22], we generated a derivatizing agent, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) and its labelled version (AQC-D6) from aminoquinolines/D6-aminoquinoline and N, N'-disuccinimidyl carbamate in-house. Additionally, a small set of ^{13}C-labelled amino acids was used to monitor and optimize the extraction efficiency and loss of analytes throughout the sample preparation process, and amines labelled with heavy isotope-labelled derivatizing agents were used as internal standards for quantification. AQC was used to label the samples while AQC-D6 labelled standards were used as the internal standards. This method has been standardized and validated by using 33 amino acid mix obtained from Sigma and subsequently used for the quantification of 62 amines in plasma, urine, and dried blood spots (DBS) by spiking amine specific labelled internal standards prepared by using labelled AQC.

2. Materials and methods

Control DBS samples (n = 18) and clinically suspected aminoacidemia samples (Table S1) were collected from the Meenakshi Mission Hospital and Research Centre, Madurai and Indira Gandhi Institute of Child Health (IGICH), Bengaluru, after obtaining informed consent from the parents/guardian. Ethical approval was obtained from the institutional ethics committees of the MMHRC (MMHRC/IEC/22Nov2022) and IGICH (IGCH/ACA/EC/03/2022–23). Pooled DBS, plasma, and urine samples were used as controls in each experiment.

All standards including 6-aminoquinoline (AMQ), N,N′-disuccinimidyl carbonate, histidine, hydroxyproline, histamine, asparagine, 3-methylhistidine, arginine, cysteic acid, serine, sarcosine, β-alanine, alanine, hypotaurine, taurine, glutamine, argininosuccinic acid, homoserine, glycine, agmatine, methionine sulfoxide, aspartic acid, citrulline, glutamic acid, threonine, hydroxylysine, γ-aminobutyric acid, n-acetyl cysteine, norepinephrine, cystathionine, proline, s-adenosylhomocysteine, amino adipic acid, N-acetyl lysine, cysteine, aminovaleric acid, epinephrine, ornithine, octopamine, phosphoserine, lysine, putrescine, cystine, dopa, 6-aminocaproic acid, cadaverine, dopamine, tyrosine, valine, methionine, serotonin, tyramine, 3-methoxytyramine, homocysteine, isoleucine, leucine, kynurenine, phenyl alanine, 6-methoxytryptamine, tryptophan, tryptamine, iodoacetic acid, ascorbic acid, ammonium bicarbonate, and sodium borate salt were obtained from Sigma-Aldrich.

^{13}C-ISTD 17 amino acid mix (Product No 96,378) and 36 amine mix (amino acid standard, physiological, Product No A9906) were purchased from Sigma-Aldrich. The D6-labelled aminoquinoline was obtained from Toronto Research Chemicals (TRC, Canada). Hydrophobic lipophilic balance (HLB) solid-phase extraction (SPE) cartridges were obtained from Waters (SKU: 186,006,339). Strata RP SPE cartridges were obtained from Phenomenex (8B-S043-TAK).

AQC and AQC-D6 were generated from 6-aminoquinoline (AMQ) and labelled AMQ (AMQ-D6) by reaction with N, N'-disuccinimidyl carbonate (DSC) using a previously published protocol with some modifications (Fig. 1A and 1B). All amine stock solutions (5 mg/mL) were prepared in 0.1 N HCl and further working stock solutions (1 mg/mL and 10 μg/mL) were prepared by diluting the main stock using 0.1 % formic acid (v/v) in water. The 36-amine mix (0.5 mM) and a mixture of 17 ^{13}C-labelled amino acids (0.25 mM) were further diluted with 0.1 % formic acid.

Acetone with 1 % formic acid (v/v) was used for protein precipitation to extract amines from plasma and urine (10 μL). For DBS, a 4.5 mm punch was transferred to a 1.5 mL microcentrifuge tube and 200 μL of 80 % acetone with 1 % formic acid was used as the extracting solvent. The extracted amines were vacuum dried, derivatized and cleaned up using SPE (the detailed protocol is included in the Supplementary Information). Each amine (10 μg) was individually derivatized with AQC and the eluate from SPE was directly infused onto the mass spectrometer to standardize the compound specific parameters. The collision energy was optimized for the most prominent product ion of the precursor ion using an automated optimization function available on TSQ Altis. An unscheduled SRM method was created to determine the retention time of individual amines, and subsequently, a scheduled SRM was created for all amines by incorporating the retention time information into the method. UHPLC-MS/SRM analyses were performed using a Dionex 3000 UHPLC system interfaced with a TSQ Altis triple quadrupole mass spectrometer. The source conditions used were as follows: spray voltage, 3000 V; sheath gas, 50 (arb. units); auxiliary gas, 10 (arb. units); and sweep gas, 1 (arb. units); ion transfer tube temperature, 300 °C; and vaporizer temperature, 350 °C. Nitrogen was used as sheath, auxiliary and sweep gases and argon was used as collision gas. The compound specific parameters such as collision energy are provided in Table S2. The chromatographic separation was carried out under reversed-phase conditions using SB-C_{18} RRHD column (2.1 × 100 mm, 1.8 μm) from Agilent. A binary solvent system was used: (A) 0.1 % formic acid in water (v/v) and (B) 0.1 % formic acid in acetonitrile (v/v). The column was maintained at an ambient temperature, and a flow rate of 200 μL/min was used throughout the run. The gradient used is given as follows: 0–3 min, 2 % B; 20 min, 20 % B; 25 min, 35 % B; 25.1–27 min, 80 % B; 27.1–35 min (re-equilibration), 2 % B. All amine-AQC derivatives were analyzed in positive mode via electrospray ionization using SRM mode (Table S2).

Two sets of internal standards were employed: one was spiked at the beginning of sample preparation (^{13}C-AA ISTD), and the second one (Amine-AQC-D6 ISTD) towards the end. ^{13}C-AA ISTD mix was added to (50 pmol) the samples after extraction of metabolites to account for the loss, if any, in the sample preparation. Amine-AQC-D6 STD (5 pmol for Sigma AA mix) was spiked into the samples just before the final drying step after SPE for amine quantitation (Fig. 2). AQC-D6 labelled AA standards were produced by derivatizing the Sigma amine mix. The ratio between the peak areas of the AA-AQC and AA-AQC-D6 derivatives of the amines was plotted against the amine concentration to construct standard curves for quantitation. Method validation was performed following the CLSI (Clinical Laboratory Standards Institute) guidelines [23] assessing key parameters using the amine mix. Linearity, precision, accuracy and matrix effects were evaluated. The calibration curves ranged between 500 pmol/mL and 8 pmol/mL for the amine mix for validation. Accuracy and precision were assessed using four concentrations points across the standard curve, namely high (HQC), medium (MQC), low (LQC), and limit of quantification (LOQ). Limit of quantification (LOQ) was defined as the lowest concentration at which the coefficient of variation of three consecutive measurements was <20 %. and with an acceptable precision limit of <10 % for HQC, MQC, and LQC and <20 % for LOQ. Matrix effects and recovery were assessed using labelled internal standards, a ^{13}C-AA mixture (^{13}C-AA ISTD), with water as the blank matrix. The final amine quantification from samples was done by preparing in-house 62 amino acid mix in the same way as

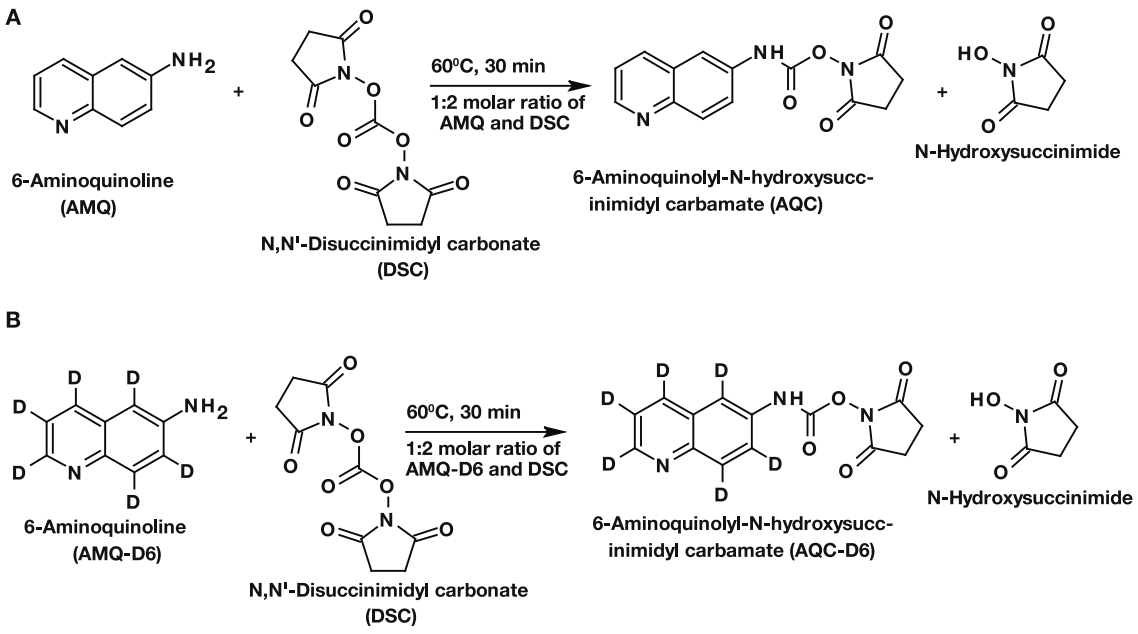

Fig. 1. Generation of 6-Aminoquinolyl-N-hydroxysuccinimidyl Carbamate (AQC) and deuterated AQC. (A) Generation of AQC. AQC was generated by reacting 6-aminoquinoline (AMQ) with N,N′-disuccinimidyl carbonate (DSC) in a 1:2 molar ratio at 60 °C for 30 min. The reaction involves the nucleophilic attack of the primary amine of AMQ on the central carbonyl group of DSC, resulting in the formation of the carbamate linkage and the release of N-hydroxysuccinimide as a byproduct. (B) Generation of deuterated AQC (AQC-D6). The deuterated analog, d_6-AQC was synthesized by reacting 6-aminoquinoline-D6 (AMQ-D6) with DSC in a 1:2 molar ratio at 60 °C for 30 min. As in the unlabelled synthesis, the amine group of AMQ-D6 forms a carbamate linkage with DSC, releasing N-hydroxysuccinimide.

mentioned for Sigma amine mix.

3. Results

3.1. Advantages of AQC derivatization

Quantification of amines directly without any derivatization has many difficulties such as low binding to reversed-phase columns, ionization effect in the mass spectrometry analysis and thiol oxidation during sample processing. To address these issues, a range of pre-column derivatization methods using different reagents have been developed. Some pre-column derivatization agents include 5-(dimethylamino) naphthalene-1-sulfonyl chloride (dansyl-Cl) [24,25], O-phthalaldehyde (OPA) [26], 9-fluorenylmethylchloroformate (FMOC—Cl) [27,28], 6-amino-quinolyl-N-hydroxysuccinimidyl carbamate (AQC) [29], p-N, N, N-trimethylammonioanilyl N′-hydroxysuccinimidyl carbamate iodide (TAHS) [30], and phenylisothiocyanate (PITC) [31]. The major benefits of these derivatization agents are (i) increasing hydrophobicity, (ii) improving the retention of analytes on reversed-phase chromatographic columns, (iii) enhancing ionization that leads to improve the sensitivity in MS. Additionally, derivatization facilitates multiplexing of amino acids with other hydrophobic amines such as tryptamine, histamine, and dopamine. In the present study AQC and AQC-D6 were prepared in-house (. 1A and 1B) and checked in MS (Fig. S1) prior to use in the method.

Derivatization with AQC has been found to be superior to other derivatization strategies for primary and secondary amines due of its high reactivity under ambient conditions, stability of the amine-AQC derivative, and a 10–1000-fold increase in the sensitivity of detection by LC-MS/MS [29,32]. All primary and secondary amines reacted with AQC under alkaline conditions (pH ~9) at room temperature. Amine-AQC derivatives resemble asymmetric urea in structure, which is highly ionizable in positive-ion mode. The amines in the present study were selected based on clinical relevance to cover the primary and secondary markers of amino acid metabolism disorders along with biogenic amines. Each amine was individually tested for derivatization. All amines with one primary/secondary amine group generated a singly charged ion, whereas those with two amine groups produced doubly charged ions. All amine-AQC derivatives exhibited a characteristic fragment ion at *m/z* 171 and 177 (Fig. S1) for AA-AQC and AA-AQC-D6, respectively, in the positive mode. These ions were the most intense fragment ions for all amines except histidine, its methyl derivatives, and S-adenosylhomocysteine (SAH). Thus, *m/z* 171 and 177 were targeted for all AA-AQC and AA-AQC-D6 amine derivatives for SRM-based quantification (Table S2).

3.2. Application of AQC-D6-labelled standards as internal standards

In the present study, we prepared the isotope labelled version of the amine-AQC derivative to be used as internal standard. The amine standard mixture was derivatized using AQC-D6 which was used as internal standards. The ratios of amine-AQC (amines in the sample) to corresponding amine-AQC-D6 (amine standards) were used for the quantification of amines (Fig. 2). For the accurate quantitation, these ratios (amine-AQC to amine-AQC-D6) should be consistent and linear over the analytical measurement range. We used the Sigma amine mix to verify this. A list of the individual amines in the mixture is provided in Supplementary Table S3. Sigma amine mix was derivatized with AQC and AQC-D6 separately and mixed and analyzed by LC-MS/MS. We spiked 5 pmol of the amine-AQC-D6 mixture into 50 pmol of the amine-AQC mixture. The expected ratio was ~12.5, as the isotopic purity of the D6-AMQ was 80 %. We considered a reference ratio range of 10 to 15 (±20 %), within which most of the amines were found to fall. However, five amines such as histidine, hydroxyproline, anserine, carnosine, and ethanolamine exhibited ratios exceeding this range. (Fig. S2).

Because the amine-AQC-D6 internal standards were spiked at the final stages of sample preparation, they cannot account for any sample loss throughout the process. To overcome this, a mixture of stable isotope-labelled amino acids (^{13}C-AA mix, 50 pmol) was spiked into the sample after protein precipitation (Fig. 2). The ratio, ^{13}C-AA-AQC/^{13}C-AA-AQC-D6 was monitored for all the ^{13}C-amino acids in the mixture. All ^{13}C-amino acids were found to have a ratio of ~10 and we considered the ratio from 8 to 12 (± 20 %), except for lysine and aspartic acid, which showed a slightly lower value of 7 (Fig. S3). The ratio ^{13}C-AA-

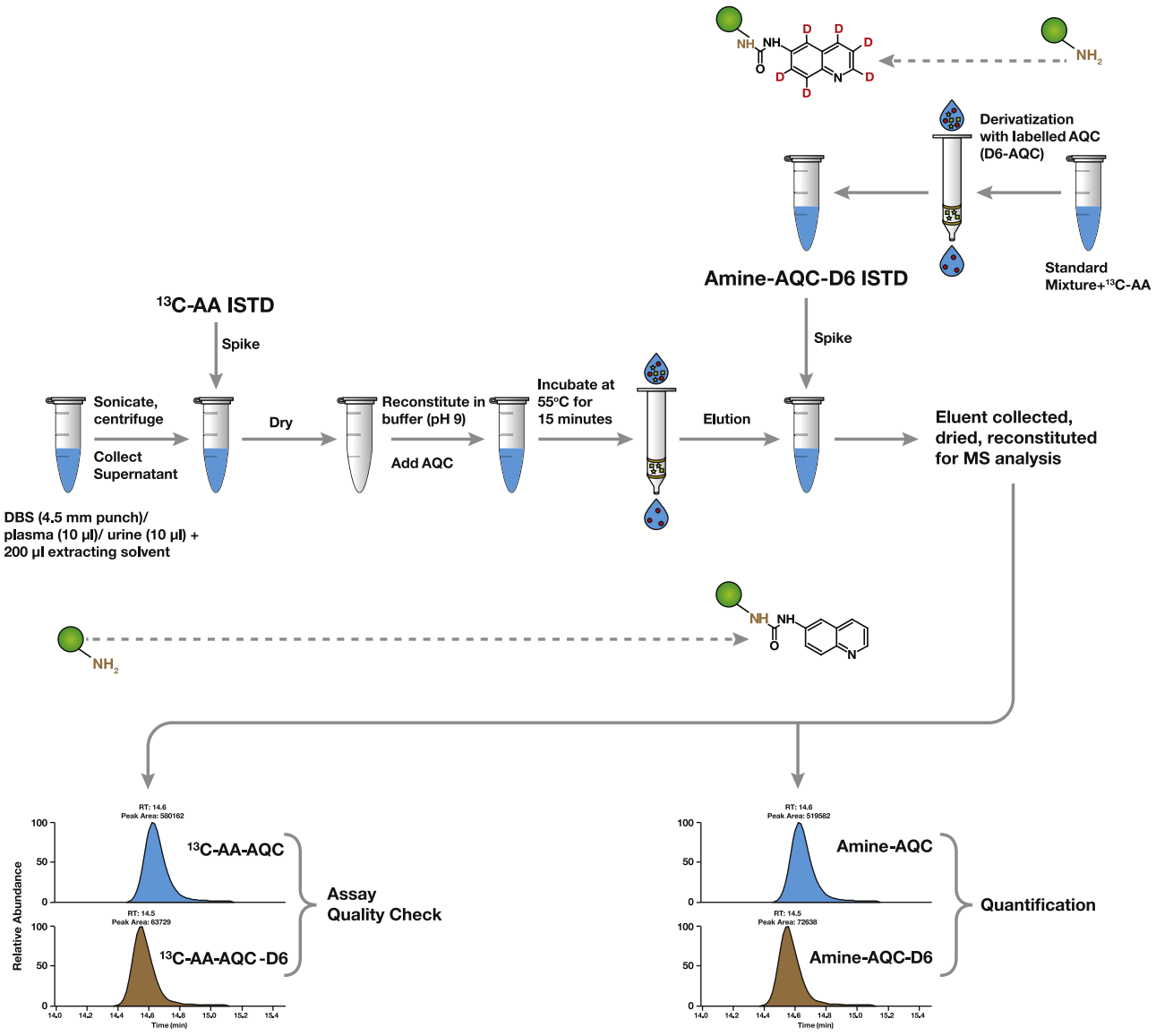

Fig. 2. Schematic overview of the sample preparation and quantification for amine analysis. The diagram illustrates the overall workflow for extraction, derivatization, and quantification of amines from dried blood spots (DBS), plasma, and urine samples. Metabolites were first extracted from the biological matrices, followed by spiking with a mixture of uniformly ^{13}C-labelled amino acid internal standard (^{13}C-AA ISTD) for quality check. Samples were dried and reconstituted prior to derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), followed by incubation at 55 °C for 15 min. After derivatization, the amine mixture labelled with deuterated AQC (AQC-D6) internal standards were added. The combined eluate was vacuum-dried and stored at −80 °C until mass spectrometry (MS) analysis. Quantitation was performed by comparing the area under the curve (AUC) of each AQC-derivatized amine to its corresponding AQC-D6-labelled internal standard, enabling quantitative measurement across samples.

AQC/^{13}C-AA-AQC-D6 of ~10 indicates >90 % recovery of amino acids in the assay and we monitored this ratio in the sample as a quality control.

3.3. Analytical method validation

To validate the analytical method, 36 amine standard mixture was used. Out of these 36 amines, 33 showed good linearity under AQC derivatization conditions which were validated using the CLSI guidelines. Since we used a small volume of serum and urine (10 µL) and one punch of DBS spot (4.5 mm in diameter), we spiked 10 µL of water into the reaction mixture as a control matrix for method development. The matrix effect was assessed by comparing the ^{13}C-AA-AQC-D6 peak areas in water and the different matrices. The matrix effect was found to be <20 % for all matrices (Fig. S4 a-c). For DBS, it was found to be <10 %. Ideally, blank matrices matching biological matrices should be used to nullify the effects of the matrix in an LC-MS/MS method. Because the matrix effect was minimal, we used water spiked with extraction solvents as a matrix for method development. Additionally, there are no suitable matrices for DBS. Standard curves were constructed ranging from 500 pmol/mL to 7.8 pmol/mL. The four quality control sample concentrations were: HQC-400 pmol/mL, MQC-250 pmol/mL, LQC-50 pmol/mL, and LOQ-25 pmol/mL. A standard curve was constructed by plotting the ratio of AA-AQC to AA-AQC-D6 against amine concentrations. All 33 amines showed linear response over the concentration range of the calibration curve with a regression coefficient >0.997 when the ratio of AA-AQC to AA-AQC-D6 was plotted against amine concentration. Both LOQ and quality control samples (LQC, MQC, and HQC) were used to determine the method validation parameters. The LOQ (defined as the lowest standard that could be analyzed with an accuracy of 85–120 % and precision of ±15 % for three replicates on three different days) was 25 pmol/mL for each amine. The whole assay was

repeated for three different days and overall accuracy and precision were within the permissible range for all amines (Table 1). Inter-day accuracy ($n = 3$) for the LQC (50 pmol/mL) for all 33 amines ranged from 85–102 %, for MQC (250 pmol/mL) from 91–115 % and for HQC (400 pmol/mL) from 94 to 112 %. The inter-day precision ($n = 3$) ranged from 0.2 to 14.70 % (for LQC), 0.8–11.8 % (for MQC) to 0.4–14.5 % (for HQC).

3.4. Quantification of amines in plasma, DBS and urine

An optimized workflow has been devised for the quantitation of amines from biological samples as depicted in Fig. 2. After extraction of amines, ^{13}C-AA ISTD mixture was spiked followed by drying and derivatization. Amine-AQC-D6 ISTD was added after SPE of the amine derivatives from samples. The ratio, Amine-AQC/Amine-AQC-D6 was used for quantification and ^{13}C-AA-AQC/^{13}C-AA-AQC-D6 was used for assay quality check. We assessed the applicability of the workflow by quantifying 33 amines from control plasma, urine and DBS prepared by pooling samples from five healthy individuals using Sigma amine mix. The amine concentrations were calculated using standard curves. Except for glycine, all 33 amines in the mixture were quantified in DBS, plasma, and urine. The levels of amines were found to be consistent across replicate experiments in plasma, DBS and urine (Fig. S5). Alanine showed the highest concentration:145.4 ± 11.8 μM in plasma, 221.1 ± 25.4 μM in DBS. In the case of urine, alanine concentration was 77.3 ± 5.5 μM, which is ~3-fold lower than both DBS and plasma.

After establishing an optimized workflow using Sigma amine mix, we tested it on 62 amines panel, consisting of biogenic amines and neurotransmitters in addition to amino acids and derivatives. The total ion chromatogram of AQC-derivatives of 62 amines in the panel has been given in Fig. 3A. All the amines were optimized for collision energy giving maximum intensity for the characteristic ions m/z 171 and 177 to increase the sensitivity of detection. The chromatography was optimized in such a way that isomers, such as leucine and isoleucine, α, β and γ-aminobutyric acids, 1 and 3-methylhistidines, sarcosine, alanine, and beta-alanine showed baseline separation (Fig. 3B). In addition, there were distinctive changes in the profiles of α- and γ-aminobutyric acids and β-aminoisobutyric acid in urine compared to plasma and DBS (Fig. 4A-C). Their levels were similar in plasma and DBS, with α-aminobutyric acid being the most abundant (~4 μM). However, the concentration of β-aminoisobutyric acid was found to be ten times greater in urine than in plasma or DBS.

As the method of quantitation using the two sets of internal standards was found to be highly reproducible, we tested calculating the concentration values from the peak area ratios, without standard curve. Concentrations of the 33 amines were calculated using both the calibration curve and the amine-AQC/amine-AQC-D6 peak area ratios from the standards (spiked in the ratio of 10:1) (Table 2). The values were found to be comparable, with an error of <15 %, except for hydroxyproline. As explained earlier, ^{13}C-AA-AQC/^{13}C-AA-AQC-D6 was used as quality control. If the ^{13}C-AA-AQC/^{13}C-AA-AQC-D6 ratio was close to 10, the quantification using the amine-AQC/amine-AQC-D6 ratio was found to be accurate and reliable. In addition, the amine levels determined using the present assay were comparable to those of previously published data [33–35]. Hence, for routine analysis in a clinical setting, we propose the use of amine-AQC from sample to spiked amine-AQC-D6 standard ratio for quantitation. To assess, we made a mix of 62 amines with labelled AQC and used for analyzing the DBS samples from patients with IEM.

Table 1
Interday precision and accuracy for the 33-amine mix.

		HQC (400 pmol/mL)			MQC (250 pmol/mL)			LQC (50 pmol/mL)			LOQ (25 pmol/mL)		
		Mean	Precision	% Accuracy	Mean	Precision	% Accuracy	Mean	Precision	% Accuracy	Mean	Precision	% Accuracy
1	Histidine	395.6	9.7	98.9	267.0	2.2	107.1	43.4	7.0	86.9	30.0	13.7	120.1
2	Hydroxyproline	383.6	14.5	95.8	234.8	11.2	93.9	47.9	4.6	95.8	25.6	11.4	102.7
3	3-Methylhistidine	383.0	6.1	95.7	242.3	5.9	96.9	45.8	1.6	91.6	25.1	6.9	100.4
4	1-Methylhistidine	405.2	7.0	101.3	250.0	5.5	100.0	42.4	13.6	84.8	21.1	18.5	84.4
5	Carnosine	425.3	13.2	106.3	227.3	0.7	90.9	46.4	5.2	92.9	23.3	13.5	93.4
6	Arginine	375.1	8.6	93.7	235.4	3.1	94.1	44.5	6.8	89.1	21.8	11.3	87.3
7	Ethanolamine	394.0	2.4	98.6	247.4	4.2	98.9	50.7	5.5	101.4	25.5	1.2	102.3
8	Serine	403.4	3.6	100.8	247.7	3.9	99.1	47.4	5.1	94.8	27.6	1.8	110.5
9	Taurine	413.3	3.2	103.3	256.1	1.3	102.4	49.5	3.1	99.0	23.5	5.9	94.2
10	Sarcosine	410.9	4.3	102.7	256.0	5.4	102.4	50.9	2.1	101.9	26.1	13.2	104.4
11	Aspartic acid	413.9	6.3	103.4	254.8	5.1	101.9	49.6	9.6	99.2	24.5	9.3	98.1
12	Citrulline	414.4	11.7	103.6	254.2	6.1	101.6	51.1	2.3	102.2	24.5	6.3	98.3
13	Beta alanine	402.9	4.7	100.7	255.7	4.3	102.2	50.1	3.2	100.2	24.9	8.2	99.6
14	Gluatamic acid	391.3	8.3	97.8	246.7	5.8	98.6	48.5	7.0	97.1	24.2	2.2	96.8
15	Threonine	384.8	3.2	96.2	229.8	4.8	91.9	47.1	4.8	94.2	24.5	6.8	97.9
16	Alanine	390.2	8.2	97.5	231.3	11.8	92.5	45.2	14.7	90.3	21.1	25.6	84.4
17	γ- aminobutyric acid	397.1	10.3	99.2	247.2	6.0	98.9	47.2	2.1	94.4	29.0	20.8	116.1
18	Hydroxylysine	450.0	5.4	112.5	286.7	5.9	114.7	48.5	7.0	97.6	29.4	7.6	117.7
19	Proline	379.9	2.7	94.9	247.2	3.1	98.8	46.6	2.9	93.2	24.6	19.0	98.4
20	β- aminoisobutyric acid	394.2	2.7	98.5	251.6	6.5	100.6	49.8	6.4	99.7	26.7	1.2	107.1
21	Ornithine	399.5	0.4	99.8	252.2	4.0	100.9	48.6	7.4	97.3	35.1	17.7	140.7
22	Cystathionine	389.4	1.9	97.3	241.5	2.5	96.6	48.9	6.6	97.9	27.1	19.9	108.5
23	α-aminobutyric acid	389.5	1.1	97.3	246.4	2.7	98.5	48.7	3.7	97.4	27.0	9.1	108.2
24	Lysine	377.1	3.1	94.2	239.7	7.5	95.8	50.5	7.1	101.1	24.8	4.0	99.1
25	Cystine	379.3	7.5	94.8	241.9	3.3	96.7	46.1	3.8	92.2	23.3	5.3	93.3
26	Tyrosine	398.8	2.8	99.7	243.0	1.2	97.2	46.7	1.5	93.5	24.2	2.7	96.8
27	Methionine	405.0	0.8	101.4	247.8	3.9	99.1	49.1	6.4	98.2	28.2	4.4	113.1
28	Valine	389.7	4.1	97.4	243.1	1.9	97.2	47.2	4.1	94.4	26.5	5.0	106.6
29	Homocystine	386.8	1.3	96.70	248.5	7.3	99.4	50.1	8.3	100.3	22.6	18.3	90.4
30	Isoleucine	393.3	4.4	98.3	246.7	4..5	98.7	48.1	1.8	96.2	24.5	4.9	98.0
31	Leucine	393.9	4.3	98.5	246.0	4.0	98.4	49.2	0.2	98.5	24.9	3.2	99.9
32	Phenylalanine	401.2	4.6	100.3	248.3	1.5	99.3	47.8	2.6	95.6	24.1	12.1	96.7
33	Tryptophan	383.8	6.0	95.9	245.8	1.3	98.3	47.0	3.6	94.1	25.3	7.9	101.4

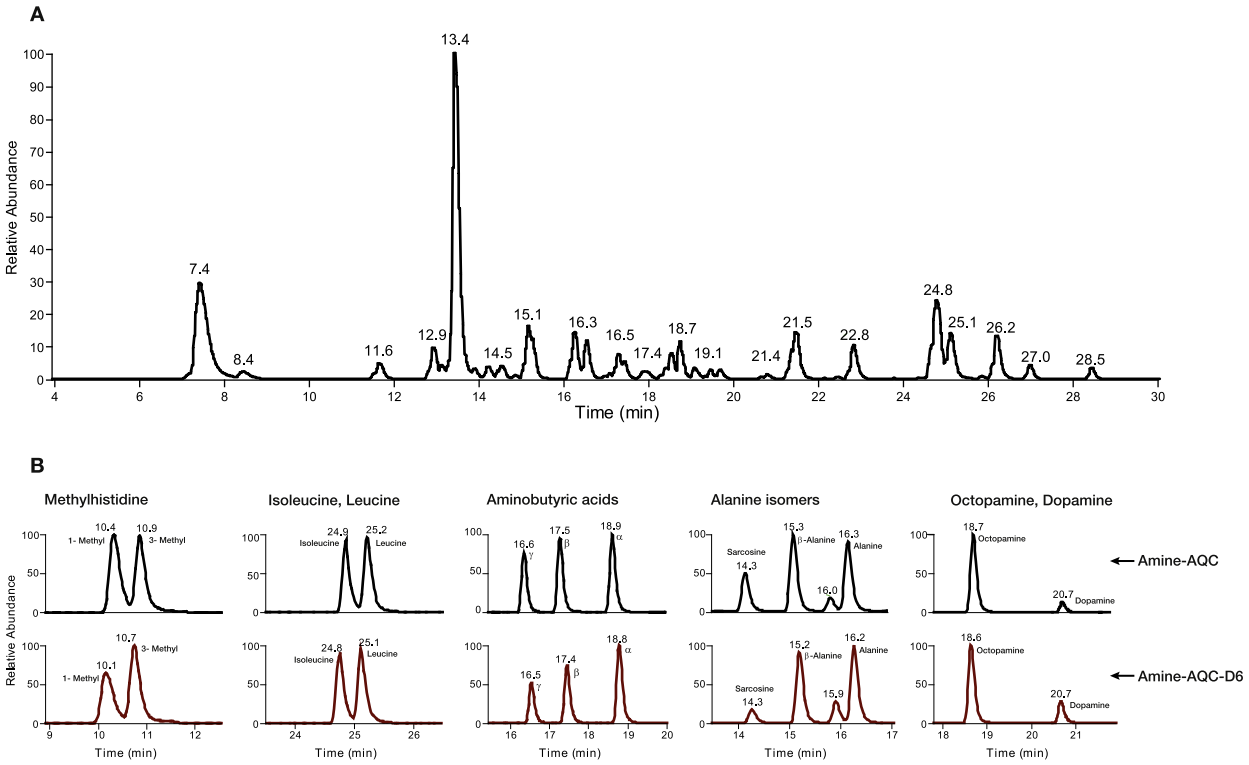

Fig. 3. Chromatographic profile of a standard mixture of 62 amines using LC-MS/MS. (A) Total ion chromatogram (TIC) of a standard mixture of 62-amines by LC-MS/MS analysis. The TIC represents the total ion current detected over time during the chromatographic separation of 62 amine standards. Each peak corresponds to the compounds eluting from the column. The x-axis denotes the retention time (minutes), while the y-axis shows the relative signal intensity. This chromatogram provides an overview of the separation of the amine mixture. (B) Extracted ion chromatograms (EICs) of selected isomeric amines highlighting chromatographic resolution for isomers. The EICs of the isomers included show 1-methylhistidine and 3-methylhistidine; isoleucine and leucine; α-, β-, and γ-aminobutyric acid (GABA); alanine, β-alanine and sarcosine; as well as octopamine and dopamine. The upper panel displays EICs for AQC-derivatized isomers, while the lower panel presents those derivatized with AQC-D6. Clear baseline separation was achieved for most isomeric pairs, with relative abundances depicted along y-axis and retention times (RT in minutes) indicated along x-axis for all isomeric pairs. This demonstrates the method's ability to resolve structurally similar amines.

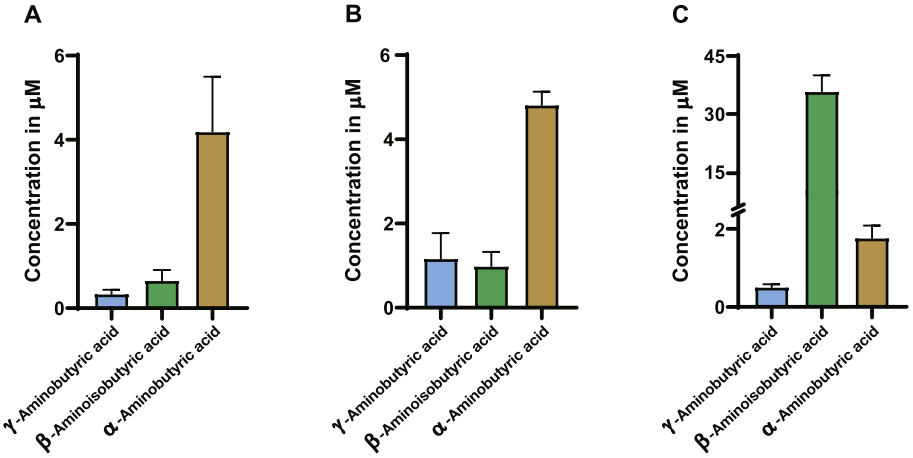

Fig. 4. Levels of γ-aminobutyric acid, β-aminoisobutyric acid, and α-aminobutyric acid in different biological matrices. Bar plots depicting the measured levels of the α-, β- and **γ-** aminobutyric acid isomers in (A) plasma, (B) dried blood spots (DBS) and (C) urine samples. The y-axis represents concentration in micromoles (µM).

3.5. Application of the assay for screening inborn errors of metabolism

To evaluate the clinical utility of the developed LC-MS/MS assay as a potential screening tool for inborn errors of metabolism, DBS from two confirmed cases of urea cycle disorder (UCD) and 18 blinded control subjects were analyzed. Out of the 62 amines analyzed, 42 could be reliably quantified from a 4.5 mm diameter punch of the DBS card. Urea cycle is a crucial metabolic pathway in the liver converting ammonia, the toxic byproduct of protein metabolism, into urea which is excreted in urine. Fig. 5A depicts the steps in the urea cycle with the corresponding

Table 2
Comparison of the concentration of amines in pooled DBS samples.

Amine	Concentration in DBS in μM								%error in calculation using area ratio
	From calibration curve				From area ratio				
	DBS1	DBS2	DBS3	Mean	DBS1	DBS2	DBS3	Mean	
Histidine	7.4	6.4	7.5	7.1	7.5	6.5	7.7	7.2	1.5
Hydroxy proline	3.1	2.8	3.8	3.2	3.0	5.4	3.8	4.1	25.7
1-Methylhistidine	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	−14.0
3-Methylhistidine	1.3	1.3	1.2	1.3	1.1	1.2	1.1	1.1	−10.3
Arginine	12.6	12.7	13.7	13.0	12.7	12.8	13.8	13.1	0.7
Ethanolamine	2.4	2.1	2.7	2.4	2.5	2.2	2.7	2.5	2.7
Serine	48.7	49.7	52.2	50.2	43.1	44.0	46.2	44.4	−11.5
Taurine	59.7	56.8	67.5	61.3	60.1	57.1	67.9	61.7	0.6
Sarcosine	6.1	6.1	7.8	6.7	6.0	6.1	7.7	6.6	−1.1
Aspartic acid	34.1	33.5	31.9	33.2	32.7	32.2	30.6	31.8	−4.0
Citrulline	9.8	9.4	9.7	9.6	9.7	9.3	9.6	9.6	−0.3
Glutamic acid	62.2	59.4	59.4	60.4	60.6	57.8	57.8	58.7	−2.7
Beta alanine	3.1	2.9	2.9	3.0	3.2	2.9	3.0	3.0	1.4
Threonine	39.0	38.1	43.4	40.2	37.9	37.1	42.2	39.1	−2.8
Alanine	218.7	204.9	229.0	217.5	201.4	188.8	210.9	200.4	−7.9
Gamma amino butyric acid	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	1.4
Proline	85.9	82.3	93.7	87.3	84.1	80.5	91.7	85.5	−2.1
Beta amino butyric acid	0.4	0.3	0.4	0.4	0.3	0.3	0.3	0.3	−11.8
Ornithine	27.6	25.9	22.6	25.4	26.2	24.6	21.5	24.1	−5.1
Alpha amino butyric acid	3.8	3.5	4.1	3.8	3.7	3.5	4.0	3.7	−1.5
Lysine	41.6	37.1	33.9	37.5	40.6	36.2	33.0	36.6	−2.4
Tyrosine	36.9	34.7	37.6	36.4	36.8	34.6	37.5	36.3	−0.3
Methionine	19.3	17.6	15.3	17.4	19.9	18.0	15.5	17.8	2.2
Valine	84.5	82.5	94.4	87.1	82.3	80.4	92.0	84.9	−2.5
Isoleucine	28.9	27.2	33.7	29.9	28.4	26.8	33.1	29.4	−1.7
Leucine	55.1	53.2	62.0	56.8	53.8	51.9	60.6	55.4	−2.3
Phenylalanine	25.6	24.1	28.1	25.9	25.4	23.9	27.8	25.7	−0.8
Tryptophan	19.5	16.3	17.6	17.8	19.3	16.2	17.4	17.7	−1.0

enzymes involved. UCDs are a group of inherited metabolic disorders caused by deficiencies in these enzymes, leading to impaired conversion of ammonia to urea, and resulting in hyperammonemia [36]. The panel described here includes all metabolites in the urea cycle. Given the complexity of diagnosing UCDs, we applied the developed assay to two specific cases, to demonstrate its diagnostic utility. The assay effectively differentiated between the two UCD cases and controls by identifying significant elevations in specific metabolites associated with each disorder, according to established metabolic markers.

The first case recruited was a diagnosed case of citrullinemia type I, caused by a deficiency of the enzyme, argininosuccinate synthetase 1 (ASS1). ASS1 is a key enzyme in the urea cycle and catalyzes the conversion of citrulline and aspartate to argininosuccinate. A deficiency in this enzyme hence leads to decreased levels of argininosuccinate and increased levels of its substrate, citrulline. The newly developed LC-MS/MS-based assay showed a profound elevation in citrulline levels over 100-fold higher than those in the control group, reflecting the metabolic blockade at the ASS1 step (Fig. 5B). In addition, the argininosuccinic acid was below the detectable limits in the samples along with lower levels of downstream urea cycle intermediates, such as arginine. This highlights the assay's ability to identify the disease specific metabolic disruption.

The second case was a diagnosed case of arginase deficiency (ARG1). Arginase, the final enzyme in the urea cycle, catalyzes the conversion of arginine into urea and ornithine. In arginase deficiency, this step is impaired, leading to increased levels of arginine and decreased levels of its product, ornithine. Analysis using our newly developed LC-MS/MS-based assay exhibited a 35-fold increase in arginine levels relative to controls, along with a marked decrease in ornithine levels (Fig. 5C) demonstrating the ability of the assay to identify argininemia. Notably, this patient also showed an approximately 4-fold elevation in agmatine. Elevation of agmatine, a decarboxylated derivative of arginine [37,38], is again indicative of arginase deficiency.

Both patient samples showed clear biochemical abnormalities that were readily distinguished from the 18 control samples (Fig. 5D). The ability to resolve these clinically relevant patterns from a small volume of DBS samples highlights the assay's feasibility for integration into routine newborn screening program for amino acid-related metabolic disorders.

4. Discussion

The approach described in this paper is particularly relevant for aminoacidemia screening, especially in countries such as India, where the prevalence of inherited metabolic disorders is high [39,40]. The current method integrates multiple metabolic pathways involving amino acids and amines, providing crucial insights into secondary metabolite changes linked to these disorders (Table S4). This is beneficial as it provides insights into secondary metabolite changes associated with these disorders, which may be crucial for understanding their pathomechanisms. Using the present assay, we observed elevated agmatine levels in a sample, along with increased arginine and decreased ornithine levels. While agmatine, synthesized from arginine serves as a precursor to polyamine [41], has neuromodulatory functions [42,43], further research is required to elucidate its role in argininemia.

The use of a labelled derivatizing agent instead of labelled internal standards for individual analytes has the potential to reduce the overall cost of the assay. The assay demonstrated high sensitivity (LOQ of $\geq$25 pmol/mL) and reproducibility, and the method has been optimized for plasma, urine and DBS. Proof-of-concept data generated from confirmed cases of urea cycle disorders and unaffected controls demonstrate the robust applicability of this panel for screening amino acid disorders.

5. Conclusions

In summary, our newly established multiplexed screening assay based on labelled AQC derivatization shows promise as a method for the comprehensive detection of amino acid disorders, analyzing 62 amines, including both primary and secondary markers. The use of isotopically labelled internal standards prepared in-house with a labelled

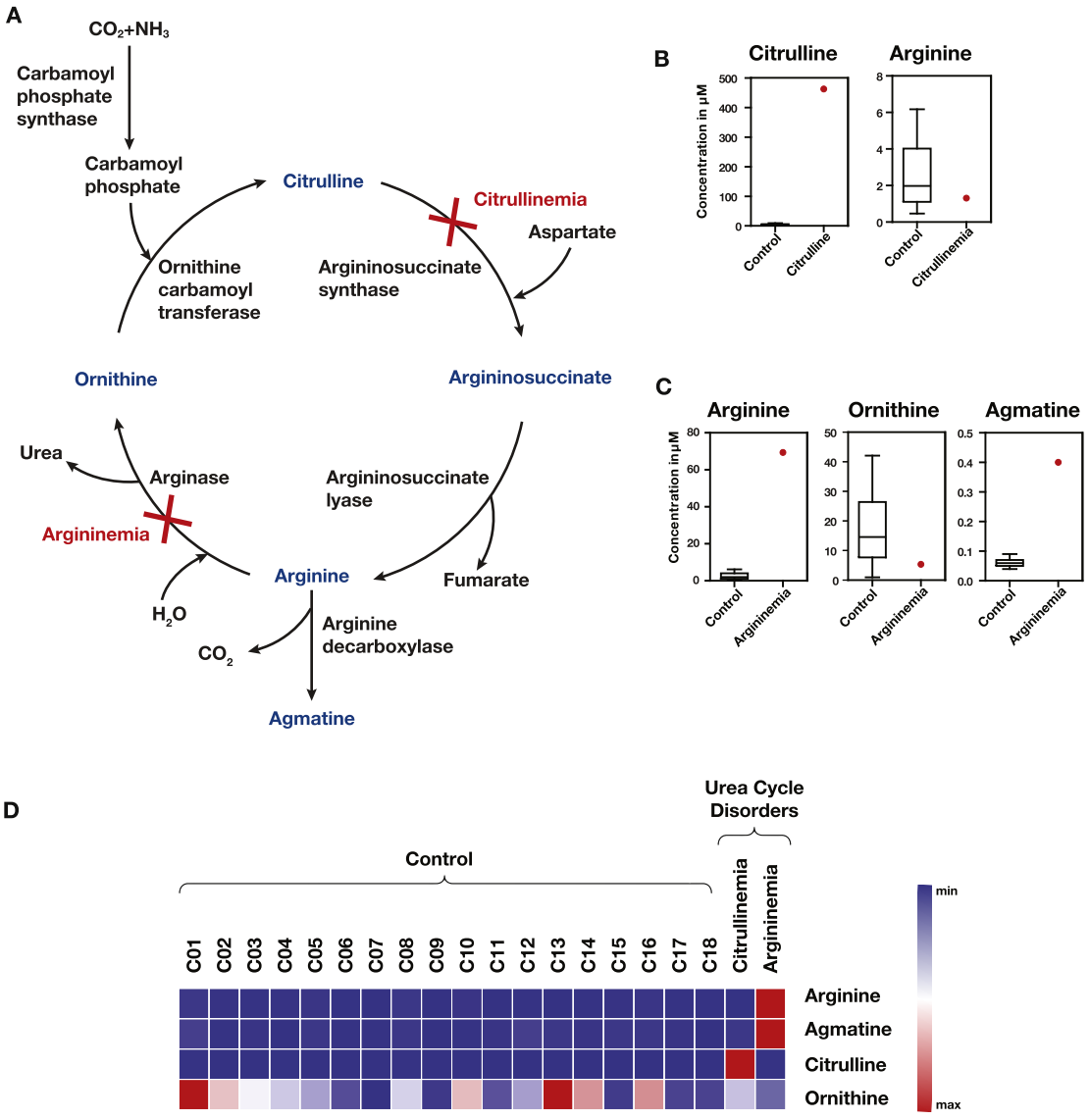

Fig. 5. Amine profile and pathway mapping in argininemia and citrullinemia cases. (A) Schematic representation of the urea cycle depicting key enzymatic steps and highlighting the enzyme defects in the urea cycle disorders. The figure illustrates the urea cycle, where ammonia and carbon dioxide are converted into carbamoyl phosphate by carbamoyl phosphate synthetase I (CPSI). CPS then combines with ornithine to form citrulline via ornithine transcarbamylase. Citrulline then combines with aspartate to form argininosuccinate, catalyzed by argininosuccinate synthase 1 (ASS1). A blockade at ASS1 results in citrullinemia type I. Argininosuccinate is then cleaved by argininosuccinate lyase (ASL) into arginine and fumarate. Arginine is finally converted into urea and ornithine by the enzyme arginase 1 (ARG1). A blockade at ARG1 leads to argininemia, marked by the accumulation of arginine. Ornithine is transported back into the mitochondria to continue the cycle. Red "X" marks indicate the enzymatic blockades at ASS1 and ARG1 responsible for the respective disorders. **(B)** Boxplots Detection of metabolite markers in a case of citrullinemia. The graphs depict an increase in citrulline levels but not arginine in a case of citrullinemia as compared to controls. Concentrations (µM) of individual metabolites are indicated on the y-axis. **(C)** Detection of metabolite markers in a case of argininemia. The graphs depict an increase in arginine and agmatine levels with a concomitant reduction in ornithine levels in a case of argininemia as compared to controls. Concentrations (µM) of individual metabolites are indicated on the y-axis. **(D)** A heatmap depicting the variation in levels of key metabolite markers associated with argininemia and citrullinemia across controls and the two patients. The heatmap shows elevated levels of arginine and agmatine in cases of argininemia, along with a corresponding decrease in ornithine compared to controls. In citrullinemia, citrulline levels were significantly increased relative to the control group.

derivatizing agent, along with addition of ^{13}C-AA to the sample preparation, significantly improved the reliability and accuracy of quantitation, while suppressing matrix effects. Although tested on a limited number of positive samples, the findings highlight the feasibility and robustness of this method for improving the diagnosis and monitoring of amino acid disorders in clinical settings. Further efforts will aim to increase the sample size to further evaluate and validate the assay's performance for broader clinical applications, with the potential to enhance patient care strategies.

CRediT authorship contribution statement

Jisha Chandran: Writing – review & editing, Validation, Methodology, Formal analysis. **Madan Gopal Ramarajan:** Writing – review & editing, Methodology, Formal analysis. **Vykuntaraju Kammasandra Nanjundagowda:** Writing – review & editing, Resources. **Aruna Gowdra:** Writing – review & editing, Resources. **Jayesh Warade:** Writing – review & editing, Resources. **Anikha Bellad:** Writing – review & editing, Methodology, Formal analysis. **Kannan Rangiah:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization.

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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.talo.2025.100523.

Data availability

Data will be made available on request.

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