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Simultaneous quantification of 48 plasma amino acids by liquid chromatography-tandem mass spectrometry to investigate urea cycle disorders

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ABSTRACT

Urea cycle disorders (UCD) are inborn errors of ammonia detoxification in which early diagnosis and treatment are critical to prevent metabolic emergencies. Unfortunately, the diagnosis was often and pronounced delayed. To improve diagnosis, we developed herein a liquid chromatography-tandem mass spectrometry method to investigate the disturbance of amino acid profile caused by UCD. The method enabled absolute quantification of 48 amino acids (AAs) within 20 min. Only 2.5 μL plasma was required for the analysis. The lower limits of quantification for most AAs were 0.01 μmol/L. Method accuracies ranged from 89.9% to 113.4%. The within- and between-run coefficients of variation were 0.8–7.7% and 2.6–14.5%, respectively. With this method, age-specific reference values were established for 42 AAs by analyzing 150 samples from normal controls, and patients with different subtypes of UCD were successfully distinguished. The data of patients revealed that UCD not only disturbed the metabolism of urea cycle AAs and induced accumulation of ammonia detoxification AAs, but also interfered the metabolism of some nervous system related AAs, such as pipecolic acid and *N*-acetylaspartic acid. This data may provide new insight into pathogenesis for UCD.

1. Introduction

Urea cycle disorders (UCD) represent one of the most common groups of inborn errors of metabolism, which are caused by deficiencies in enzymes or transporters involved in the conversion of ammonia to urea (Fig. 1) [1]. The overall prevalence of UCD in North America and Europe was roughly estimated to be 1/35,000 with 1/3rds presenting initial symptoms during the newborn period [2]. The incidence of UCD in Chinese populations is unclear, but recent publications demonstrated that these diseases were not “rare” in Chinese populations [3–5].

Patients with UCD have highly variable and unspecific symptoms, some common stimulus, such as infection, high protein intake, starvation or excessive exercises, could trigger metabolic crisis, patients would rapidly develop to coma, respiratory failure, hepatic failure and irreversible brain damage, even death [2]. Early diagnosis and treatment are critical to prevent metabolic emergencies and reduce mortality and neurological morbidity [6]. Unfortunately, the diagnosis is often and pronounced delayed according to the experience of our center and data from *E*-IMD (European Registry and Network for Intoxication Type Metabolic Diseases) consortium. A report from E-IMD consortium revealed that the median age of UCD patients at diagnosis was unexpectedly high (362 days) [7]. The delayed diagnosis could be attributed to two reasons: insufficient awareness of UCD by physicians, and lack of rapid and sensitive method for screening and diagnosis.

As shown in Fig. 1, six amino acids (AAs) directly involved in the urea cycle, different subtypes of UCD could lead to disease-specific

Abbreviations: UCD, urea cycle disorders; AAs, amino acids; *E*-IMD, European Registry and Network for Intoxication Type Metabolic Diseases; IEX, ion-exchange chromatography; CPSID, carbamoyl phosphate synthetase I deficiency; OTCD, ornithine transcarbamylase; ASSD, argininosuccinate synthetase deficiency; ARGID, arginase I deficiency; HHH, hyperornithinemia-hyperammonemia-homocitrullinuria syndrome; LPI, lysinuric protein intolerance; THP, tri(hydroxypropyl)phosphine; IS, internal standard; QCs, quality control samples; PCF, propyl chloroformate; ERNDIM, European Research Network for Evaluation and Improvement of Screening, Diagnosis, and Treatment of Inherited Disorders of Metabolism; MF, matrix factor

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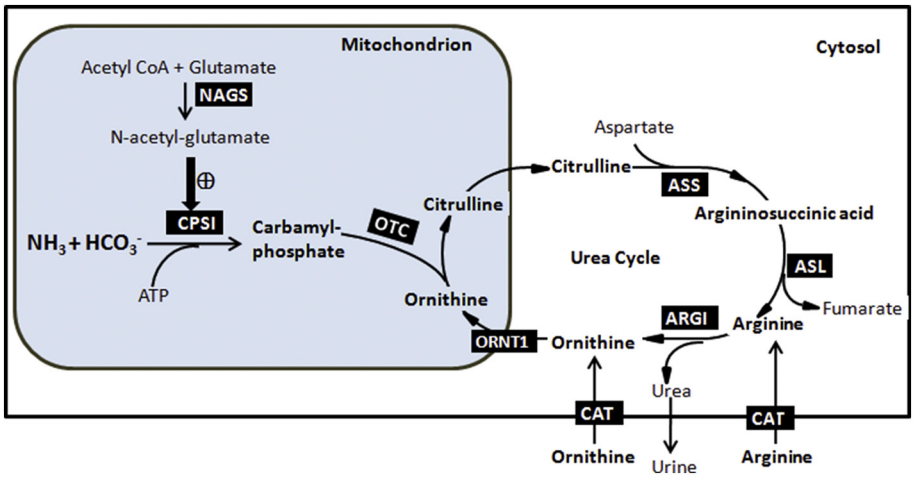

Fig. 1. The urea cycle. NAGS, *N*-acetylglutamate synthase; CPSI, carbamoyl phosphate synthase I; OTC, ornithine transcarbamylase; ASS, argininosuccinate synthase; ASL, argininosuccinate lyase; ARGI, aginase I; ORNT1, mitochondrial ornithine transporter 1; CAT, cationic amino acids transporter. ⊕ depicts the stimulatory effect of *N*-acetyl-glutamate on CPSI. This figure was adapted from reference [1].

amino acid profile [1]. So, quantification of AAs in plasma is the fastest way to confirm or exclude a UCD.

Today, the most widespread method for AAs quantification used in clinic is ion-exchange chromatography (IEX) followed by post-column derivatization with ninhydrin [8]. Despite its simplicity and successful use in UCD screening [9–11], it suffers from long data acquisition time (> 120 min) and unable to separate important biomarker homocitrulline from methionine [12]. In order to improve detection throughput, the butyl esterification-isotope dilution tandem mass spectrometry (MS/MS) has been used in clinic for AAs analysis since 1990s [13]. However, this method is powerless to distinguish proximal UCD (defects in *N*-acetylglutamate synthase, carbamoylphosphate synthetase I and ornithine transcarbamylase) due to its poor detection limits in amide- and imine groups containing AAs such as citrulline, arginine and asparagine, whereas, these AAs are biomarkers in diagnosis of UCD.

In addition to the derivatization methods, AAs could also be quantified without derivatization by using ion-pairing chromatography-MS/MS or hydrophilic interaction liquid chromatography (HILIC)-MS/MS [14,15]. These methods require least sample pretreatment, however, they also have the disadvantages of long equilibration time between runs and frequent column regeneration after a few injections [16], or have limited ability to resolve isomers [17]. These shortages limited their application in clinic.

Recently, a number of derivatization reagents had been developed, which coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) or gas chromatography mass spectrometry (GC–MS) expanded the analyte coverage, increased sample throughput and enhanced method sensitivity [18–22]. In summary, all these reagents target the amino group of AA except alkyl chloroformate and bromobutane, which react with amino-, carboxyl-, and phenolic hydroxyl-groups of AAs. Alkyl chloroformate derivatization coupled with GC–MS offered higher reproducibility and sensitivity compared with iTRAQ®-LC-MS/MS and the classic IEX method in the analysis of urinary AAs [23], but this method had no ability to detect imido-group containing AAs, such as biomarkers of UCD: arginine and homoarginine [17].

In this study, we sought to use alkyl chloroformate derivatization-HPLC-MS/MS to develop a rapid and sensitive method that could simultaneously quantify 48 disease-related plasma AAs, to determine age-based reference intervals for children, and to perform a limited clinical evaluation of the assay in patients with different subtypes of UCD.

2. Materials and methods

2.1. Subjects

This study was approved by the ethics committee of Guangzhou Women and Children's Medical Center.

Plasma samples from 150 children (94 boys and 54 girls, aged between 4 months to 15 years) were used to establish reference intervals. These children were hospitalized for health examination, and their plasma was mostly used for other tests, only 0.1 mL of spare blood was used for this study. No additional blood was collected for the purpose of this study. Informed consent was obtained from children and/or their parents. Children with signs of metabolic diseases, such as vomiting, seizure, development delay, liver damage, gastrointestinal symptoms and neurologic symptoms, were excluded from the control group.

The disease group included six patients with carbamoyl phosphate synthetase I deficiency (CPSID, OMIM 237300), ornithine transcarbamylase deficiency (OTCD, OMIM 311250), argininosuccinate synthetase deficiency (ASSD, OMIM 215700), arginase I deficiency (ARGID, OMIM 207800), hyperornithinemia-hyperammonemia-homocitrullinuria syndrome (HHH, OMIM 238970) and lysinuric protein intolerance (LPI, OMIM 222700) for each. Plasma samples from these patients were previously submitted to our lab for amino acid analysis by the traditional HPLC method [24]. The final diagnosis in all cases was based on gene mutation analysis results.

2.2. Chemicals

Standards of pipecolic acid (purity ≥97.0%) and α-aminobutyrate (purity ≥99.0%) were supplied by ANPEL Laboratory Technologies Inc. (Shanghai, China). Other standards of amino acids, and reagents tri (hydroxypropyl)-phosphine (THP) and ammonium formate were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Propyl chloroformate (PCF), 3-picoline, isooctane and n-propanol were obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). Stable isotope labeled D_3-methionine, D_4-cystathionine and $^{13}C_3$-propanol were from Cambridge Isotope Laboratories (Andover, MA, USA).

2.3. Calibrators, internal standards and quality control samples (QCs)

Standard stock solutions of AAs were gravimetrically prepared by dissolving the individual amino acid in deionized water or 0.5 mol/L HCl before mixing. Calibrators were prepared by mixing standard stock solutions and serially diluted with deionized water.

Two sets of internal standards were used: internal standard A (IS A) was an aqueous solution containing 5.0 μmol/L of D_3-methionine and 1.0 μmol/L of D_4-cystathionine, and internal standard B (IS B) was synthesized by derivatizing concentrated AA standard mixture according to the protocol described below except propanol was substituted by $^{13}C_3$-propanol, then derivatives were extracted and diluted with ethylacetate for use.

Three lyophilized plasma samples (no. 201701, 201,702, 201,703)

Table 1
MRM transitions for the 48 amino acids analyzed in this study. Analytes listed in order of retention time (R.T.) with peak number corresponding to Fig. 2.

Peak no.	R.T, min	Analyte	Full name	MRM transition Q1/Q3		DP*	EP*	CE*	CXP*
1	2.2	EA	Ethanolamine	MRM_1	148.1/62.1	36	6	16	6
				MRM_2	148.1/88.1	36	6	15	6
				IS	150.2/64.1	36	6	16	6
2	2.6	p-Glu	Pyroglutamic acid	MRM_1	172.2/84.1	35	7	24	2
				MRM_2	172.2/130.1	40	6	17	1
				IS	175.2/84.1	35	7	24	2
3	3.2	Arg	Arginine	MRM_1	303.2/70.1	54	7	57	8
				MRM_2	303.2/156.2	50	5	30	3
				IS	306.3/70.1	54	7	57	8
4	3.8	Harg	Homoarginine	MRM_1	317.2/84.1	56	4	50	2
				MRM_2	317.2/126.1	56	4	35	2
				IS	320.3/84.1	56	4	50	2
5	4.2	Gln	Glutamine	MRM_1	275.2/172.2	38	4	20	3
				MRM_2	275.2/84.1	38	5	30	2
				IS	278.3/175.2	38	4	20	3
6	4.3	Gly-Gly	N-glycyl-glycine	MRM_1	261.2/102.0	40	5	21	2
				MRM_2	261.2/144.1	40	5	21	2
				IS	264.3/102.0	40	6	16	2
7	4.3	Cit	Citrulline	MRM_1	304.2/156.1	33	10	24	2
				MRM_2	304.2/287.2	32	11	14	5.6
				IS	307.3/156.1	33	10	24	2
8	5	3Me-His	3-Methyl-Histine	MRM_1	298.2/96.1	50	4	50	2.5
				MRM_2	298.2/256.2	50	5	27	3.5
				IS	301.3/96.1	50	4	50	2.5
9	5	Ser	Serine	MRM_1	234.2/146.1	37	6	17	2.5
				MRM_2	234.2/174.1	35	5	14	2
				IS	237.2/146.1	37	6	17	2.5
10	5.1	Hcit	Homocitrulline	MRM_1	318.2/170.2	41	4	25	3
				MRM_2	318.2/127.1	41	3	30	1.5
				IS	321.3/170.2	41	4	25	3
11	5.6	Hyp	Hydroxyproline	MRM_1	260.2/172.2	40	5	19	3.5
				MRM_2	260.2/86.1	38	6	32	2.5
				IS	263.3/172.1	40	5	19	3.5
12	5.7	1Me-His	1-Methyl-Histine	MRM_1	298.2/124.1	50	4	38	2
				MRM_2	298.2/210.1	50	4	25	2
				IS	301.2/124.1	50	4	38	2
13	5.5, 5.9	Hlys	5-Hydroxylysine	MRM_1	334.2/125.1	25	3	36	2
				MRM_2	334.2/257.1	25	3	18	2
				IS	301.2/124.1	50	4	38	2
14	6.1	N-Asp	*N*-acetyl-aspartic acid	MRM_1	260.2/130.1	40	4	25	1.5
				MRM_2	260.2/172.2	40	4	25	1.5
				IS	266.3/133.2	37	5	18	1.5
15	6.2	Thr	Threonine	MRM_1	248.2/74.1	39	6	31	7
				MRM_2	248.2/160.2	39	5	16	2
				IS	251.3/74.1	39	6	31	7
16	6.6	Gly-Pro	N-glycyl-proline	MRM_1	301.2/158.2	42	3.5	21	2
				MRM_2	301.2/70.0	45	4.5	45	8
				IS	304.3/161.2	42	3.5	21	2
17	6.7	Gly	Glycine	MRM_1	204.2/102.0	45	6	16	2
				MRM_2	204.2/118.1	45	6	16	2.5
				IS	207.2/102.0	45	6	16	2
18	7.2	Asn	Asparagine	MRM_1	243.2/157.1	45	3.5	14	2
				MRM_2	243.2/115.0	44	3.5	19	1.5
				IS	246.2/160.2	45	3.5	14	2
19	7.4	Alev	δ-Aminolevulinic acid	MRM_1	260.2/158.1	41	5	18	2
				MRM_2	260.2/200.1	41	6	12	2
				IS	263.3/158.1	41	5	18	2
20	7.8	β-Ala	β-Alanine	MRM_1	218.2/116.1	39	5	19	1
				MRM_2	218.2/158.1	39	6	13	1.5
				IS	221.3/116.1	39	5	19	1
21	8.3	Ala	Alanine	MRM_1	218.2/130.1	40	5	17	1
				MRM_2	218.2/158.1	39	6	13	1.5
				IS	221.2/130.1	40	5	17	1
22	8.6	GABA	γ-Amino-butyric acid	MRM_1	232.2/172.2	36	4	13	3.5
				MRM_2	232.2/86.1	32	5	24	2
				IS	235.3/172.1	36	4	13	3.5
23	9.2	Sar	Sarcosine	MRM_1	218.2/116.0	37	5	16	2
				MRM_2	218.2/88.1	37	7	24	2
				IS	221.3/116.0	37	5	16	2
24	9.3	AIB	β-Aminoisobutyric acid	MRM_1	232.2/130.1	32	5	19	1
				MRM_2	232.2/172.2	36	5	14	2
				IS	235.3/130.1	32	5	19	1

(*continued on next page*)

Table 1 (*continued*)

Peak no.	R.T, min	Analyte	Full name	MRM transition Q1/Q3		DP*	EP*	CE*	CXP*
25	10.3	ABU	α-Amino-butyric acid	MRM_1	232.2/144.1	30	8	16	1.5
				MRM_2	232.2/172.1	30	6	11	1.5
				IS	235.3/144.1	30	8	16	1.5
26	10.5	Pro	Proline	MRM_1	244.2/156.1	42	4	18	1
				MRM_2	244.2/70.1	38	6	40	6
				IS	247.3/156.1	42	4	18	1
27	10.7	Orn	Ornithine	MRM_1	347.2/287.2	47	3.5	14	1
				MRM_2	347.2/156.1	47	3.5	26	2
				IS	350.3/290.2	47	3.5	14	1
28	11.4	Met	Methionine	MRM_1	278.1/190.2	41	4.5	17	3.5
				MRM_2	278.1/142.1	41	4.5	23	1.5
				IS	281.2/193.2	41	4.5	17	3.5
29	12.2	His	Histidine	MRM_1	370.2/196.2	40	6	27	3.5
				MRM_2	370.2/284.2	48	6	23	3
				IS	373.2/196.2	40	6	27	3.5
30	12.3	Sac	Saccharopine	MRM_1	429.3/369.2	36	8	19	1.5
				MRM_2	429.3/170.2	45	6	29	2.5
				IS	435.4/375.4	36	8	19	1.5
31	12.3	Lys	Lysine	MRM_1	361.3/170.2	46	6	26	2
				MRM_2	361.3/301.2	48	6	15	3.5
				IS	364.4/170.1	46	6	26	2
32	12.7	Val	Valine	MRM_1	246.3/158.2	35	6	15	3
				MRM_2	246.3/116.1	35	9	25	1.5
				IS	249.3/158.2	35	6	15	3
33	12.8	Asp	Aspartic acid	MRM_1	304.3/216.2	40	5	17	1.5
				MRM_2	304.3/130.1	40	5	27	1.5
				IS	310.4/219.2	40	5	17	1.5
34	13.5	γ-CG	γ-Carboxy-glutamic acid	MRM_1	344.2/258.2	42	4.5	15	1
				MRM_2	344.2/170.1	42	4.5	26	2.5
				IS	350.3/264.3	42	4.5	15	1
35	13.5	Glu	Glutamic acid	MRM_1	318.2/172.2	44	5.5	21	2.5
				MRM_2	318.2/84.1	44	5	38	8
				IS	324.3/175.2	44	5.5	21	2.5
36	13.5	Trp	Tryptophan	MRM_1	333.2/245.2	45	5.5	22	3
				MRM_2	333.2/273.1	45	6	16	1
				IS	336.3/245.1	45	5.5	22	3
37	14.8	GSH	Glutathione	MRM_1	564.3/162.1	55	6	30	2.5
				MRM_2	564.3/447.2	55	7	19	2
				IS	570.4/162.1	55	6	30	2.5
38	15	AAA	α-Aminoadipic acid	MRM_1	332.2/98.1	35	5.5	37	2
				MRM_2	332.2/244.2	35	4	18	1
				IS	338.3/98.1	35	5.5	37	2
39	15.1	Phe	Phenylalanine	MRM_1	294.2/206.1	50	5	17	2
				MRM_2	294.2/120.1	50	4	35	2
				IS	297.3/206.1	50	5	17	2
40	15.3	Leu	Leucine	MRM_1	260.2/172.2	45	6.5	18	2.5
				MRM_2	260.2/86.1	45	7	28	8
				IS	263.3/172.2	45	6.5	18	2.5
41	15.7	Ileu	Isoleucine	MRM_1	260.2/130.1	40	6	24	3.5
				MRM_2	260.2/172.2	43	5	16	1.5
				IS	263.3/130.1	40	6	24	3.5
42	15.8	Pip	Pipecolic acid	MRM_1	258.3/128.1	38	4	29	1.5
				MRM_2	258.3/170.2	38	5	19	2
				IS	261.3/128.1	38	4	29	1.5
43	16.6, 17.5	ASA	Argininosuccinic acid	MRM_1	529.3/469.3	47	6	23	2
				MRM_2	529.3/156.1	50	6	43	2.5
				IS	535.5/475.4	47	6	23	2
44	16.9	Cys	Cysteine	MRM_1	336.2/190.1	42	5.5	18	2
				MRM_2	336.2/248.2	48	6	17	4
				IS	339.3/193.2	42	5.5	18	2
45	18.1	Hcy	Homocysteine	MRM_1	350.2/142.1	46	5.5	24	1.5
				MRM_2	350.2/204.1	46	5.5	24	1.5
				IS	353.3/142.1	48	5.5	18	3
46	18.1	Cyt	Cystathionine	MRM_1	479.3/230.2	45	7	23	4
				MRM_2	479.3/142.1	45	7	28	4
				IS	483.2/234.2	45	7	23	4
47	18.6	Tyr	Tyrosine	MRM_1	413.3/136.1	31	3.5	45	2.5
				MRM_2	413.3/222.1	31	3.5	33	1
				IS	416.3/136.1	31	3.5	45	2.5
48	19.3	Kyn	Kynurenine	MRM_1	423.2/146.1	50	4.5	42	2
				MRM_2	423.2/260.1	50	4.5	21	1.5
				IS	426.3/146.1	50	4.5	42	2

* MRM_1, quantification transitions (m/z); MRM_2, confirmation transition (m/z)); IS, internal standard transition; DP, declustering potential (in V); EP, entrance potential (in V); CE, collision energy (in eV); CXP, collision cell exit potential (in V).

Table 2
Lower limit of quantification (LLOQ) and concentration ranges of calibrators in this study.

Amino acid	LLOQ, μmol/L	Range, μmol/L
EA	0.01	0.01–10.00
p-Glu	0.01	0.10–100.04
Arg	0.01	0.10–100.00
Harg	0.01	0.02–25.02
Gln	0.01	0.20–200.00
Gly-Gly	0.01	0.02–20.00
Cit	0.01	0.10–100.06
3Me-His	0.05	0.02–20.00
Ser	0.05	0.10–100.00
Hcit	0.01	0.02–24.96
Hyp	0.01	0.04–40.08
1Me-His	0.02	0.02–20.00
Hlys	0.01	0.02–25.03
N-Asp	0.01	0.03–25.00
Thr	0.01	0.10–100.00
Gly-Pro	0.01	0.02–19.57
Gly	0.05	0.20–200.00
Asn	0.05	0.05–50.03
Alev	0.02	0.02–20.00
β-Ala	0.01	0.02–20.00
Ala	0.05	0.20–200.00
GABA	0.01	0.01–10.00
Sar	0.01	0.05–50.08
AIB	0.01	0.02–20.00
ABU	0.01	0.02–20.00
Pro	0.01	0.10–100.03
Orn	0.01	0.05–50.04
Met	0.01	0.05–50.00
His	0.01	0.05–50.00
Sac	0.01	0.01–10.00
Lys	0.01	0.10–100.00
Val	0.01	0.10–100.07
Asp	0.01	0.02–20.00
γ-CG	0.10	0.20–10.01
Glu	0.01	0.05–50.03
Trp	0.01	0.05–49.53
GSH	0.01	0.07–70.00
AAA	0.01	0.02–20.00
Phe	0.01	0.10–100.00
Leu	0.01	0.10–100.00
Ileu	0.01	0.10–100.00
Pip	0.01	0.02–20.00
ASA	0.01	0.01–10.00
Cys	0.01	0.15–75.00
Hcy	0.01	0.06–60.00
Cyt	0.01	0.01–10.02
Tyr	0.01	0.05–50.03
Kyn	0.02	0.02–20.04

from ERNDIM (www.erndim.org) were used as QCs to assess method precision and accuracy at analyzing low, median and high levels of AAs.

2.4. Sample pretreatment

Plasma samples and QCs were diluted 20 fold with water at first. 50 μL each of blank (water)/calibrator/diluted QC/diluted plasma sample was added into a 1 mL glass vial to mix with 10 μL of IS A and 10 μL of THP aqueous solution (0.5 mg/mL). A derivatization reaction was initiated by the addition of 40 μL of 3-picoline/propanol solution (23:77, *v*/v) followed by the addition of 25 μL of PCF/isooctane/dichloromethane mixture (17:11:72, v/*v*/v). The composition of derivatization reagent was based in part on the EZ: faast kit (Phenomenex, Torrance, CA) [21]. Samples were vortex mixed for 3 s, and allowed to stand for about 1 min, then mixed again after the addition of 50 μL saturated sodium chloride solution and 200 μL IS B. The mixture was centrifuged (2000 rpm, 1 min), and the organic phase was then transferred to a 96-vial plate and dried under a gentle nitrogen stream. Residues were reconstituted with 100 μL of methanol/water (4:6, v/v) solution and filtered through 0.2 μm GHP membrane (AcroPrep™ 96-well filter plate, Pall, USA) before LC-MS/MS analysis.

2.5. LC-MS/MS method

A Shimadzu Ultrahigh Pressure Nexera chromatograph system (Kyoto, Japan) interfaced to an AB Sciex 3200 Q TRAP mass spectrometer (Foster City, CA, USA) was used for this study.

The chromatographic separation was achieved on an Agilent Zorbax Elipse AAA column (150 × 3.0 mm i.d., 3.0 μm) with column temperature set at 35 °C. Mobile phase consisted of solvent A (5 mmol/L ammonium formate aqueous solution) and solvent B (acetonitrile/methanol/water, 70:25:5, *v*/v/v), and was eluted at 0.4 mL/min following the gradient: 40% B maintained for 1.0 min, increased to 60% at 7.0 min, increased to 70% at 16.0 min, and increased to 100% at 18.0 min and held for 2.0 min, then decreased to 40% at 20.1 min followed by 2.0 min for equilibration. The inject volume was 5 μL.

The mass spectrometer was operated under positive electrospray ionization (ESI^+) mode. The ionization source parameters were optimized as following: ionspray voltage, 5500 V; desolvation temperature, 500 °C; curtain gas, 30 psi; CAD gas, medium; nebulizer gas (GS1), 50 psi; auxiliary gas (GS2), 40 psi. Data was collected in time scheduled multiple reaction monitoring (MRM) mode. Two MRM transitions for each AA were monitored for quantitative and qualitative purpose respectively, and one transition was monitored for each internal standard. Optimized parameters for each MRM transition were listed in Table 1.

2.6. Method validation

Method validation was executed according to the US Food and Administration (FDA) guidelines on bioanalytical method validation [25], method sensitivity, selectivity, calibration curves, accuracy, precision, matrix effect and stability were evaluated.

Lower limit of quantification (LLOQ), defined as the concentration level with detection imprecision and inaccuracy < 20%, was determined by analyzing low concentrations of standard mixture as there was no blank matrix available.

Two MRM transitions (MRM_1 and MRM_2) for each amino acid were employed in this study. Method selectivity was assessed by calculating variation coefficients of peak area ratios of MRM_1/MRM_2 in all plasma samples and comparing the ratios in samples with their ratios in standards.

Calibration curves were obtained by analyzing a blank sample (water) and eight levels of calibrators. The calibration range for each AA was prepared based on physiological and pathological concentrations of the AA in plasma (Table 2). A standard addition experiment was performed as described by Minkler [26] to justify whether water could be used as proxy for plasma in this method: increasing amounts of standard mixture were added to aliquots of plasma before they were processed. Concentrations of AAs in spiked plasma samples were calculated using calibrators prepared in water. The relationship between added concentration and detected concentration was studied for each AA.

Accuracy and precision of the method were evaluated by analyzing QCs in 12 analytical runs ($n = 6$ for the first run, and $n = 2$ for other runs at each concentration level). Accuracy was calculated by comparing values obtained by the present method with values submitted by > 250 ERNDIM participants worldwide who used mostly IEX and HPLC methods, and the results were expressed as recoveries ((our value/median of participants) * 100%). The precisions were expressed as the coefficients of variance (CVs) of within- and between-run analytical results.

The matrix effect was assessed by postextraction spike method: the internal standard mixture solution (consisted of derivatized IS A and IS B) was spiked to ethylacetate extract of 6 plasma samples from 6 people. Matrix factor (MF) defined as the ratio of internal standard peak

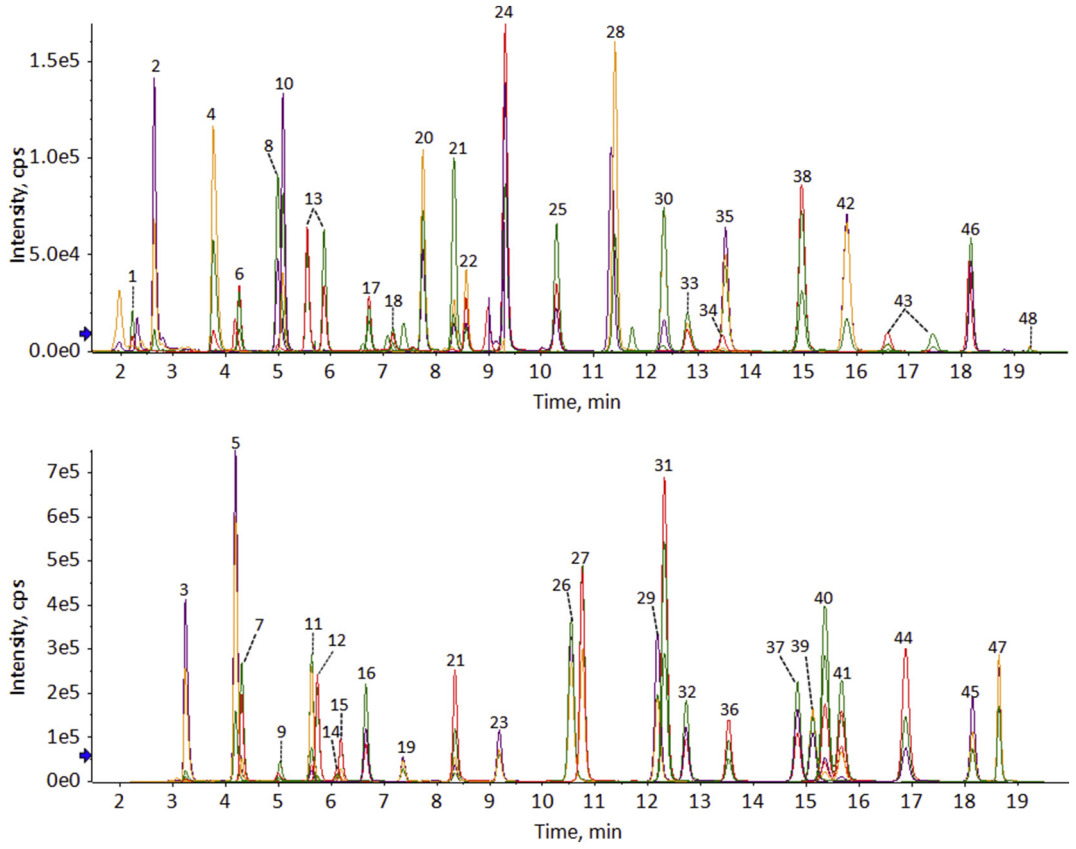

Fig. 2. Extracted ion chromatograms of 48 amino acids.
Information related to retention time, peak shape and color-coded *m/z* used for analysis was indicated. The 48 amino acids were demonstrated in two chromatograms separately to demonstrate details of their chromatographic behavior. Peaks were numbered as listed in Table 1.

area in presence of matrix to the internal standard peak area in absence of matrix was calculated.

The stability of AAs during sample collection and handling, after short- term and long-term storage, and after freeze and thaw cycles et al., had been studied by many researchers [27,28]. This study hasn't repeated these researches. Only the stabilities of IS B and post-preparative samples were evaluated by monitoring peak intensities of IS B during a period of 52 days, and re-analyzing three samples on autosampler in each day during a period of 3 days, respectively.

2.7. Data process and statistical analysis

Peak integration and calibration curve fitting were processed with MultiQuant 2.1.1 software (AB Sciex). Quantification was performed against a calibration curve using analyte-to-internal standard peak area ratios. A dilution factor of 20 was multiplied to obtain the final concentrations of AAs in QCs and plasma samples.

Statistical analysis was performed by using SPSS 16.0 software (SPSS Inc.). Normal controls were divided into four groups according to the age of children (< 1 year, 1 year to 3 years, 3 years to 6 years and > 6 years). Descriptive statistics were used to determine mean, SD, median and 2.5th, 97.5th quantile values of AAs for each group, and intergroup differences were analyzed by Mann-Whitney *U* non-parameter tests. *P* values < .05 were considered significant.

Amino acid results of patients were transformed into standard scores (Z scores) to investigate the changes caused by UCD. Z score was defined as: $Z = {}^{(x-\mu)}/_{\sigma}$, where x was the amino acid value of patient, and μ and σ were the mean value and SD, respectively, in controls of the same age group.

3. Results and discussion

3.1. Method development and optimization

A set of strategies were applied to address challenges in simultaneous quantifying 48 analytes with different chemical properties in a complex biological matrix. PCF/propanol was chosen as the derivatization reagent because it could react with amino-, carboxyl-, and phenolic hydroxyl-groups of AAs under aqueous solution within several seconds. The derivatization process eliminated most active hydrogen of AAs, leading to strong retention of derivatives on a reverse phase column. Besides it also provided a chance to introduce an isotope-coded group to analyte by using isotope-labeled derivatization reagent, enabling synthesis of internal standard for each analyte in the lab. Due to commercially isotope-labeled PCF was unavailable, ^{13}C-labeled propanol was considered. $^{13}C_3$-propanol labeled all AAs except ethanolamine and 5-hydroxylysine. Ethanolamine has no carboxylic-group to label, and 5-hydroxylysine lost its carboxylic-group in a dehydration reaction during derivatization. So, the nearest eluted internal standards were used as their's (Table 1).

In this study, five groups of isomers were included (sarcosine, alanine and β-alanine; α-aminobutyrate, β-amino isobutyrate and γ-aminobutyrate; hydroproline and *N*-acetyl aspartate; 1-methyl-histidine and 3-methyl-histidine; leucine and isoleucine), they are all disease-related biomarkers. Efforts were made to separate these isomers. By the use of ammonium formate aqueous solution as solvent A and the mixture of acetonitrile/methanol/water as solvent B, satisfactory performance was obtained in terms of peak shape and retention time for analytes. Results showed that 48 AAs, including the five groups of isomers, could be well-separated within 20 min (Fig. 2).

Table 3
Data for accuracy and precision of the method.

Amino acid	Targeted*, μmol/L	Detected, μmol/L	Accuracy,%	Precision,CV %	
				Within-run (n = 6)	Between-run (*n* = 12)
ABU	101.0	97.5	96.5	1.9	7.5
	23.0	22.3	97.1	2.7	7.5
	54.4	50.4	92.6	2.3	6.7
Ala	925.0	955.8	103.3	3.3	5.1
	181.0	187.3	103.5	2.8	4.9
	308.0	317.3	103.0	2.7	4.5
Arg	767.0	691.8	90.2	4.2	5.6
	99.8	98.7	98.9	2.6	6.3
	245.0	229.2	93.6	2.3	3.7
Asn	17.0	16.4	96.5	4.2	7.3
	93.0	84.6	91.0	6.6	8.8
	109.0	101.1	92.8	3.5	6.3
Asp	23.0	22.8	99.1	2.2	9.0
	85.0	89.8	105.6	3.8	9.1
	167.0	180.3	108.0	5.5	6.8
Cit	16.0	15.4	96.3	2.3	4.1
	722.0	711.1	98.5	2.5	4.0
	1896.0	1930.8	101.8	3.0	8.2
Cys*	/	293.6	/	1.6	3.7
	/	447.2	/	3.1	4.2
	/	535.1	/	1.6	2.3
Gln	406.0	402.7	99.2	1.9	3.8
	1214.0	1240.7	102.2	2.3	5.5
	94.0	90.9	96.7	2.8	4.8
Glu	106.0	113.0	106.6	3.0	9.9
	211.0	229.6	108.8	4.7	9.6
	62.0	67.3	108.5	3.6	7.4
Gly	142.0	137.9	97.1	4.0	6.4
	719.0	713.1	99.2	3.9	5.9
	86.9	84.1	96.8	4.6	5.5
His	81.0	83.6	103.2	0.8	3.9
	280.0	297.2	106.1	1.9	3.6
	49.2	51.7	105.1	0.9	3.2
Hcy*	17.5	24.3	138.9	2.5	4.9
	31.0	45.3	146.1	3.2	4.2
	45.1	52.6	116.6	2.6	4.2
Hyp	71.2	72.2	101.4	1.7	5.6
	24.4	25.4	104.1	2.3	5.8
	48.0	48.0	100.0	3.0	4.5
Ileu/Alloleucine*	1503.6	1531.3	101.8	1.8	6.0
	118.7	126.8	106.8	2.1	4.0
	522.0	550.8	105.5	2.8	4.5
Leu	460.0	468.3	101.8	2.0	4.3
	52.0	53.4	102.7	2.8	4.1
	102.0	101.8	99.8	2.2	3.8
Lys	542.0	548.2	101.1	1.5	5.7
	84.0	86.2	102.6	3.7	3.9
	279.0	280.7	100.6	2.2	3.9
Met	456.0	472.1	103.5	7.7	14.5
	45.0	45.7	101.6	4.6	8.9
	140.0	130.7	93.4	5.4	8.5
Orn	701.0	679.4	96.9	2.3	8.8
	79.0	80.8	102.3	2.2	5.3
	223.0	227.6	102.1	2.5	5.4
Phe	919.0	976.6	106.3	2.6	6.7
	102.0	105.7	103.6	2.7	3.4
	375.0	390.2	104.1	2.9	3.8
Pro	52.0	54.2	104.2	2.2	7.6
	332.0	345.2	104.0	2.8	6.2
	672.0	682.0	101.5	2.2	5.4
Ser	14.8	13.8	93.2	6.4	9.4
	195.0	189.4	97.1	3.6	6.6
	388.0	385.5	99.4	4.5	5.0
Thr	149.0	146.1	98.1	1.9	5.0
	488.0	487.5	99.9	2.5	5.3
	52.0	51.5	99.0	2.2	3.9
Tyr	112.0	114.1	101.9	4.2	3.9
	670.0	739.7	110.4	4.7	7.7
	32.0	32.2	100.6	2.8	2.6
Val	105.0	103.2	98.3	3.0	4.6
	775.0	776.6	100.2	4.0	6.8
	52.2	51.5	98.7	2.2	4.4

* targeted value was the median value of the data supplied by > 250 ERNDIM quality control scheme participants worldwide who use mostly ion-exchange chromatography and HPLC methods. Cys*, the determined cysteine was the sum of reduced cystine and albumin-bounded cysteine. Hcy*, the concentration of homocysteine detected here was total homocysteine instead of free homocysteine. Ileu/Alloleucine*, the detected value was the sum concentration of isoleucine and alloisoleucine.

3.2. Analytical validation

To make sure this method had good selectivity, two MRM transitions (MRM_1 and MRM_2) for each AA were employed. The variation coefficient of MRM_1/MRM_2 of AAs in 150 plasma samples were in the range of 0.96%–11.13%, and the deviations of ratios in samples to the ratios in standards were in the range of 0.05% to 9.92% (Supplementary material S1), which indicated that no significant interference was detected.

The lower limits of quantification of were 0.01 μmol/L for most AAs (Table 2), which were apparently lower than their physiological levels, therefore samples could be diluted 20 fold before pretreatment, and only 2.5 μL of plasma was required for the analysis. The amount of protein in 2.5 μL of plasma was low enough to be neglected instead of competing for derivatization reagent, so samples could be derivatized directly without deproteinization.

The calibration curves for most AAs had dynamic range of 10^3 (Table 2). Quadratic regression with a weighting factor of 1/x was used to describe the concentration-response relationship for AAs. All points on the calibration curves were back-calculated, and the residuals were less than ± 20% at LLOQs and less than ± 15% at other concentration levels. The standard addition experiment results showed that most AAs in plasma displayed linear relationships between added and detected concentrations, with slopes and linear regression coefficient values (r^2) at 0.913 ± 0.063 and 0.996 ± 0.003 (mean ± SD), respectively (Supplementary material S2). Only cysteine had slope of 0.751 with linear regression coefficient of 0.996, which indicated that the aqueous calibration curve underestimated the concentration of cysteine in plasma. Nevertheless, the data in method precision assessment indicated that the measurement of free cysteine was reproducible with between-run CVs < 5% (Table 3) and the reference range of cysteine in our study was in agreement with the result reported by the published literature [29] ((113.8–238.3) μmol/L vs (120–226 μmol/L), our data vs literature). The blank sample (no cysteine added plasma sample) used for standard addition experiment maybe the reason that leaded to the low slope between added and detected concentrations due to its high background level of cysteine (296.2 μmol/L).

The QCs from ERNDIM contain 26 AAs, and 23 of them were detected with accuracies ranged between 89.9% and 113.4%, and the CVs of within- and between-run were in the ranges of 0.8–7.7% and 2.6–14.5%, respectively (Table 3). Two AAs in QCs couldn't be detected, one was taurine due to it couldn't be derivatized by PCF/propanol, and the other was cystine, which had been converted to free cysteine by disulfide reducing agent THP. THP also converted homocystine and albumin-bound homocysteine to free homocysteine [30], leading to recovery of homocysteine > 115%. Whereas, the measurement of total homocysteine was more valuable and reproducible for disease diagnosis [31]. The present method had no ability to completely

Table 4
Age-related reference intervals for plasma amino acids (unit, μmol/L).

	< 1 year, n = 44		1–3 years, n = 40		3–6 years, n = 39		6–15 years, n = 27	
Analyte	Median	2.5th–97.5th percentile	Median	2.5th–97.5th percentile	Median	2.5th–97.5th percentile	Median	2.5th–97.5th percentile
Glutamine[a]	475.2	287.7–588.5	420.2	331.3–565.5	435.2	360.2–566.3	438.5	294.3–625.6
Alanine	303.7	221.8–550.0	298.5	156.9–475.1	312.2	167.3–475.6	312.6	191.6–522.9
Asparagine	51.9	32.6–71.3	46.3	24.2–71.3	47.6	29.8–76.7	43.2	28.0–79.9
Ornithine	80.6	41.3–136.4	77.7	38.1–117.7	84.3	41.0–135.5	81.2	59.1–156.4
Citrulline	23.9	14.7–40.0	25.2	13.6–32.8	25.5	17.4–41.2	27.0	15.1–34.5
Arginine	61.1	30.6–115.7	44.9	14.7–119.0	57.6	29.0–113.7	53.7	23.5–120.1
Homoarginine	1.1	0.2–2.2	1.1	0.2–2.0	1.3	0.2–2.7	1.3	0.3–2. 8
Homocitrulline	0.3	< 0.9	0.4	0.1–1.1	0.4	< 0.8	0.2	< 0.7
Glutamic acid[a]	76.6	39.8–134.2	67.2	36.4–97.4	58.6	36.8–95.6	58.2	31.5–113.1
Pyroglutamic acid[a]	50.0	29.3–113.3	43.1	22.1–74.7	43.8	22.0–99.7	46.1	24.6–60.9
Aspartic acid[a]	5.8	3.5–11.0	5.2	2.8–12.9	4.7	2.2–12.7	4.5	3.0–7.4
N-acetyl-aspartic acid	0.5	< 0.8	0.4	< 0.8	0.3	< 0.6	0.3	< 0.4
γ-Amino-butyric acid[b]	0.4	0.2–0.5	0.3	0.2–0.5	0.3	0.1–0.5	0.3	0.1–0.3
Proline[a]	179.1	109.1–283.7	152.8	74.5–304.7	154.2	99.0–331.1	151.9	80.1–358.1
Hydroxyproline[a]	26.3	17.8–48.1	19.9	12.1–37.4	21.2	12.1–44.0	24.6	8.7–51.2
Leucine	121.0	65.7–250.7	111.4	75.9–196.9	114.5	70.7–248.9	122.6	89.0–257.5
Isoleucine	75.1	39.8–149.9	62.4	39.8–115.4	67.8	37.4–163.9	66.4	50.8–154.5
Valine	222.8	107.7–353.3	201.2	140.0–360.3	213.1	115.8–376.3	200.2	152.1–362.0
Phenylalanine	57.2	40.1–94.7	56.6	47.2–80.8	59.9	47.0–95.2	62.1	48.2–88.9
Threonine[a]	137.2	79.0–233.2	99.9	46.7–164.2	109.7	74.5–211.7	110.8	53.4–195.3
Tryptophan	62.9	40.9–87.2	58.6	36.9–84.1	60.3	36.1–86.3	55.9	34.7–94.8
Histidine	83.4	60.0–116.3	81.0	61.0–116.6	82.7	59.2–108.9	87.8	50.1–116.5
Lysine	159.9	105.2–271.5	147.8	71.8–242.1	159.6	117.6–272.2	157.4	111.5–213.6
Pipecolic acid	1.2	0.4–2.6	0.8	0.5–2.8	1.0	0.5–2.6	1.1	0.6–2.1
α-Aminoadipic acid[a]	1.4	0.5–2.8	1.0	0.5–2.4	0.9	0.6–2.9	0.8	0.5–1.6
Methionine[a]	25.93	13.4–59.6	21.2	9.5–50.9	23.0	11.1–51.8	19.4	10.7–36.1
Cystathionine[a]	0.1	< 0.29	0.1	< 0.2	0.1	< 0.3	0.1	< 0.3
Total homocysteine[c]	6.9	4.3–10.5	6.6	4.6–10.4	7.2	4.3–11.9	8.1	5.8–11.1
Total cysteinine[c]	170.3	116.5–211.4	172.8	113.8–237.4	171.6	138.5–230.9	186.4	145.2–238.3
Total glutathione[a]	7.4	4.3–12.6	9.6	3.8–14.4	8.2	4.9–13.5	9.2	5.4–13.0
Glycine[c]	164.3	110.5–271.7	172.4	92.7–307.4	184.4	136.9–252.4	191.4	90.1–286.4
Serine	143.7	101.3–206.4	131.4	77.6–172.5	135.3	94.7–217.7	127.6	93.3–212.6
Sarcosine	2.3	0.9–4.5	1.76	0.8–3.0	2.2	0.9–4.4	2.0	1.0–3.2
α-Amino-butyric acid	23.7	11.7–42.1	19.79	9.5–38.8	22.1	12.8–35.7	20.5	9.5–39.8
β-Aminoisobutyric acid[a]	2.4	0.6–5.9	2.66	0.5–5.9	2.0	0.2–5.5	1.7	0.3–4.2
β-Alanine[a]	3.6	2.5–12.6	2.47	1.7–7.6	2.7	1.8–9.9	2.6	1.8–9.9
Ethanolamine	12.8	< 23.4	11.33	< 21.1	9.0	< 22.0	4.9	< 17.3
N-glycyl-glycine	0.3	< 0.6	0.2	< 0.3	0.1	< 0.2	0.2	< 0.3
Tyrosine	73.8	45.4–136.9	63.78	37.9–104.9	73.0	45.2–113.5	64.2	52.3–125.9
1-Methyl-Histine[d]	2.1	1.3–5.1	2.3	1.3–5.6	3.1	1.8–6.0	3.3	1.8–6.6
3-Methyl-Histine[d]	0.5	0.2–5.3	0.6	0.2–6.8	1.1	0.3–13.7	1.3	0.4–15.4
Kynurenine[c]	2.8	1.1–4.5	2.2	0.9–4.4	2.1	1.3–4.6	1.7	1.0–2.4

a-d, Statistical significant differences between age groups were observed. a, (≤1 year) vs (> 1 year); b, (≤1 year) vs (1–6 years) vs > 6 years; c, (≤6 years) vs (> 6 years); d, (≤3 years) vs (> 3 years);

separate derivatives of chiral isomers isoleucine and alloisoleucine, so the determined isoleucine was in fact included the concentration of alloisoleucine.

Absolute matrix effect and relative matrix effect were both evaluated in this study. For absolute matrix effect, the data of MFs (Supplementary material S3) revealed that amino acids, including arginine, homoarginine, glutamine, aspartic acid, tryptophan, tyrosine and kynurenine had response suppressed by matrix with MFs in the range of 0.38–0.82, whereas, the responses of glycine, alanine, β-alanine, γ-amino-butyric acid, sarcosine, β-aminoisobutyric acid, proline, methionine, valine, glutamic acid, leucine, isoleucine and pipecolic acid, were enhanced by matrix with MFs ≥ 1.15. No significant absolute matrix effect was observed for the rest amino acids as indicated by MFs (0.85–1.14). The variability in MFs, as measured by the coefficient of variation (CV, %) was used to evaluate relative matrix effect. Our results showed that all amino acids had CVs of MFs ≤15%. In summary, 20 amino acids had their responses affected by matrix, and the low relative matrix effect indicated that matrix effect was consistent from sample to sample.

The post-preparative samples were stable for at least 3 days with CVs of peak areas for all AAs in the range of 2.0% to 13.3%. For IS B, all $^{13}C_3$-labeled AA derivatives were stable for at least 52 days in ethylacetate except $^{13}C_3$-methionine and $^{13}C_3$-cystathionine derivatives, which degraded along with time, so D_3-methionine and D_4-cystathionine were supplemented as internal standards for methionine and cystathionine quantification, respectively.

Carryover was evaluated by injecting a blank following the highest calibrator. No carryover was detected. The column performance did not deteriorate after 1000 chromatographic runs.

3.3. Clinical validation

In this study, 150 plasma samples from children aged between 4 months to 15 years were analyzed to establish the reference intervals. For the 48 AAs, there were 6 AAs (argininosuccinic acid, γ-carboxyglutamate, saccharopine, N-glycyl-proline, 5-hydroxylysine, and δ-aminolevulinic acid) were found had levels lower than LLOQs in normal controls, and the other 42 AAs all had levels above LLOQs. Table 4 listed age-related reference intervals for the 42 AAs. To the best of our knowledge, age-specific distributions of β-amino isobutyrate, γ-amino butyate, α-aminoadipic acid, kynurenine, and *N*-acetylaspartic acid, total reduced glutathione, 1-methyl-histidine and 3-methyl-histidine, were reported here for the first time. The reference ranges of other AAs were in agreement with published literatures [32,33].

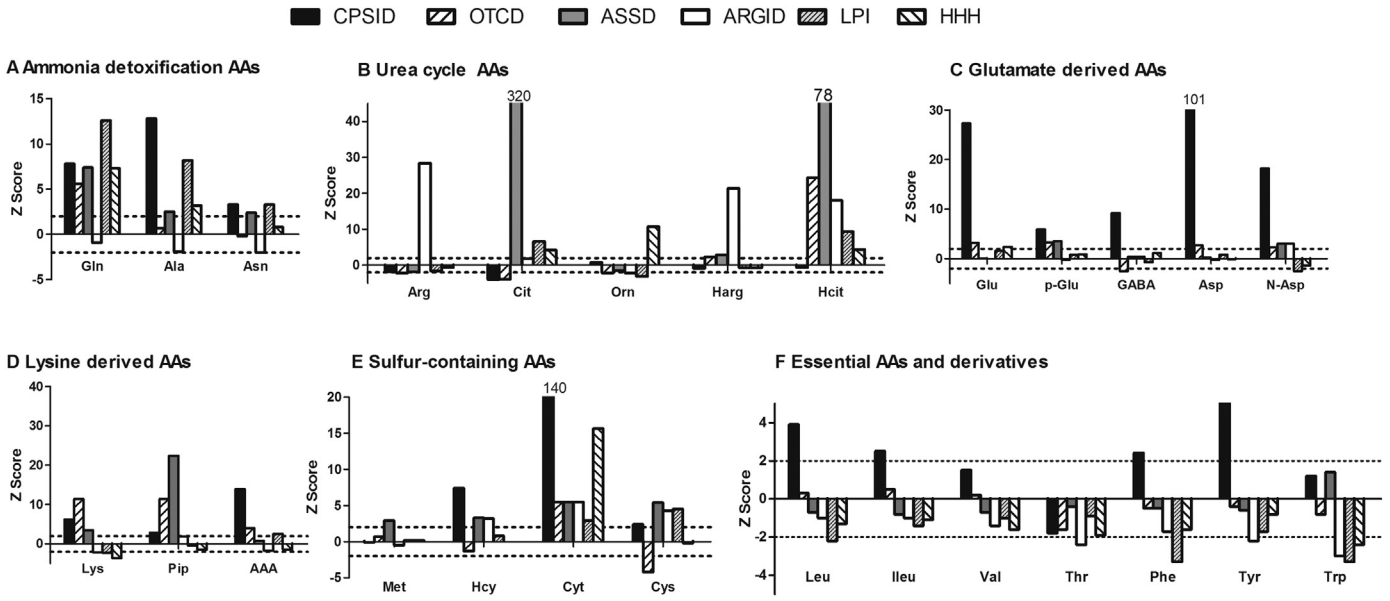

Fig. 3. Z scores of amino acids for patients with different subtypes of UCD. Z score indicated the deviation of amino acids concentrations in patients from that in normal controls. Amino acid with Z score ≤ −2 or ≥ 2 was considered as clinical significance, and the dotted lines labeled the boundaries of −2 and 2.

Samples from six patients with different subtypes of UCD were analyzed. Clinical presentations of these patients included recurrent vomiting, seizure, somnolence, irritability, dysphoria, jaundice, psychomotor retardation, abnormal gait and coma. The time to diagnosis after onset of first symptoms ranged from 2 days to 4.7 years for them. Patients with ASSD and HHH syndrome both had long been misdiagnosed as encephalopathy. Fig. 3 depicted the disturbances of plasma AAs of these patients before treatment.

The patient with CPSID fell into a coma at the second day of illness, his blood ammonia elevated to 4300 μmol/L (reference interval: < 50 μmol/L). With the exception of urea cycle AAs, all other AAs elevated with Z score > 2 (Fig. 3A, C and E), indicating acute metabolic decompensation of the patient. Low levels of urea cycle AAs (Fig. 3B), especially citrulline, suggested the patient had proximal UCD (CPSID or OTCD) instead of secondary amino acidemia induced by diseases like sepsis. However, it was a challenge to make differential diagnosis of CPSID from OTCD due to similar clinical presentation and amino acid profile of patients [1]. In this study, the patient with OTCD had mild symptom, his amino acid profile also showed increased ammonia detoxification AAs, glutamate- derived AAs and decreased citrulline (Fig. 3A, B and C). Traditionally, additional assays were required to differentiate CPSID and OTCD, such as urinary orotic acid and/or uracil test [1]. In this study, the strikingly elevated homocitrulline in plasma of patient with OTCD distinguished OTCD from CPSID (Fig. 3B). Homocitrulline was a bypath metabolite of carbamoylphosphate (Fig. 1), it was deficient in body fluids of patients with CPSID and accumulated in patients with OTCD [34].

ASSD patients were characterized by citrullinemia [35], and the patient in this study had Z score of citrulline elevated to 320. In addition, ASSD also leaded to remarkably high levels of all ammonia detoxification AAs and homocitrulline (Fig. 3A and B), indicating hyperammoniemia and interruption of carbamylphosphate metabolism.

Patients with ARGID were usually not characterized by hyperammoniemia, which was different from other UCD [36]. In this study, the patient with ARGID had normal to low levels of ammonia detoxification AAs (Fig. 3A), which was coincided with his normal blood ammonia level (20 μmol/L). The obviously elevated arginine, and arginine precursor argininosuccinic acid (0.30 μmol/L) as well as arginine bypath metabolite homoarginine, indicated ARGI catalyzed reaction was blocked (Fig. 3B).

Defects in cationic amino acids transporter y + LAT-1 reduced or blocked the absorption of lysine, arginine and ornithine from intestine, and leaded to LPI [37]. The patient with LPI in this study had long been misdiagnosed as malnutrition and nephritis according to his short stature, edema in the face and extremities and high levels of protein in urine. High levels of plasma ammonia detoxification AAs and homocitrulline as well as low levels of plasma arginine, ornithine and lysine revealed the etiology of the patient (Fig. 3A, B, and C).

HHH syndrome is a disorder of mitochondrial ornithine transporter 1, in which ornithine couldn't be transported into mitochondrial, but trapped in the cytosol [38]. The patient with HHH syndrome in this study had ornithine, homocitrulline and ammonia detoxification AAs all elevated (Fig. 3A and B), which was consistent with the specific profile of HHH syndrome.

The effects of UCD are systemic, current therapeutic strategies prolong survival, however, development outcomes remain suboptimal, most cases had neurological sequelae even undergo liver transportation [39,40]. The pathogenesis is complex and not completely understood. In addition to differential diagnosis, this study also had findings related to pathogenesis, which may provide hint for further investigation: Fig. 3C and D showed that several nervous system related AAs, including pipecolic acid [41,42] *N*-acetylaspartic acid [43], and homocitrulline [44,45] and their precursors, had Z scores > 2 in some patient samples. In addition, Fig. 3E demonstrated that the catabolism of methionine was disturbed by UCD with the concentrations of methionine metabolites homocysteine, cystathionine and/or cysteine increased. Due to their important role in transmethylation reactions [46], it deserves further research to investigate whether or not UCD interfered with transmethylation reactions.

Low levels of essential AAs were observed in plasma of most UCD patients (Fig. 3F), indicating protein deficiency of these patients. This was agreed with earlier publication [9], and supported the opinion that supplementation with essential AAs for these patients should be considered.

4. Conclusions

To improve diagnosis of UCD and better understanding of the pathogenesis, we developed herein a LC-MS/MS-based method that could simultaneously monitor the dynamic changes of 48 AAs in plasma. The method was fully validated in terms of the sensitivity, precision and accuracy and specificity. With the advantages of rapidity, sensitivity

and robust, this method is very suitable for screening, diagnosis and researches on UCD as well as other AAs related disorders.

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Appendix A. Supplementary data

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