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Quantitative urine amino acid analysis using liquid chromatography tandem mass spectrometry and aTRAQ® reagents

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ABSTRACT

Ion-exchange chromatography (IEC) is the most widely used method for amino acid analysis in physiological fluids because it provides excellent separation and reproducibility, with minimal sample preparation. The disadvantage, however, is the long analysis time needed to chromatographically resolve all the amino acids. To overcome this limitation, we evaluated a novel liquid chromatography tandem mass spectrometry (LC–MS/MS) method, which utilizes aTRAQ® reagents, for amino acid analysis in urine. aTRAQ® reagents tag the primary and secondary amino groups of amino acids. Internal standards for each amino acid are also labeled with a modified aTRAQ® tag and are used for quantification. Separation and identification of the amino acids is achieved by liquid chromatography tandem mass spectrometry using retention times and mass transitions, unique to each amino acid, as identifiers. The run time, injection-to-injection, is 25 min, with all amino acids eluting within the first 12 min. This method has a limit of quantification (LOQ) of 1 μmol/L, and is linear up to 1000 μmol/L for most amino acids. The Coefficient of Variation (CV) was less than 20% for all amino acids throughout the linear range. Method comparison demonstrated concordance between IEC and LC–MS/MS and clinical performance was assessed by analysis of samples from patients with known conditions affecting urinary amino acid excretion. Reference intervals established for this method were also concordant with reference intervals obtained with IEC. Overall, aTRAQ® reagents used in conjunction with LC–MS/MS should be considered a comparable alternative to IEC. The most attractive features of this methodology are the decreased run time and increased specificity.

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1. Introduction

Quantitative analysis of amino acids in biological fluids is essential to the diagnosis and monitoring of inborn errors of metabolism (IEMs). Currently, the most widely used method for amino acid analysis is ion-exchange chromatography (IEC) with post-column ninhydrin derivatization. This method has been in use for over 40 years and it provides excellent separation and reproducibility, with minimal sample preparation [1,2]. The main disadvantage of IEC is the long run time (2.5 h per sample) resulting in low-throughput. In addition, particularly in urine samples, the specificity of this assay is affected by metabolites derived from medications or dietary supplements which interfere with the quantification of amino acids.

In recent years the use of tandem mass spectrometry for the identification of inborn error of metabolism has increased. One of the most successful applications is neonatal screening, which utilizes direct infusion tandem mass spectrometry for high throughput testing. Although this method is excellent for screening purposes, it does not allow the separation of isomeric and isobaric species and is therefore not amenable for diagnostic use [3]. Recently, the use of liquid chromatography prior to tandem mass spectrometry (LC–MS/MS) has been developed for amino acid analysis to overcome this limitation [4,5]. Accurate quantification of amino acids by tandem mass spectrometry also requires the use of internal standards, amino acids labeled with one or more stable isotopes. In the absence of specific internal standards, another labeled amino acid can be used. In this case, the measurement does not represent an absolute value, but rather a ratio between the responses of two compounds, resulting in less accuracy. At present, only selected labeled amino acids are commercially available to use as internal standards.

Isobaric tagging reagents, known as iTRAQ®, have been widely utilized with LC–MS/MS to label and quantify proteins in complex mixtures [6]. In a recent paper, iTRAQ® reagents were used for amino acid analysis [7]. Amino acids were labeled with an iTRAQ® tag which specifically associates with primary and

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Table 1
Gradient used for the separation of amino acids tagged with aTRAQ® reagents.

Time	Mobile phase A	Mobile phase B
00:00	98%	2%
00:30	98%	2%
06:00	75%	25%
12:00	50%	50%
13:00	20%	80%
16:00	20%	80%
17:00	98%	2%
25:00	98%	2%

secondary amino groups. The iTRAQ® tag contains a reporter ion with a mass to charge ratio (m/z) of 115. Internal standards for each amino acid were prepared by labeling amino acids with a modified iTRAQ® tag, containing a reporter ion that is different by one mass unit (m/z 114). The 115 tagged amino acids in physiological samples and 114 tagged internal standards have the same retention times, but can be distinguished by the unique mass transitions. The main advantage of this method is the availability of an internal standard for each amino acid, enabling an accurate quantification. However, the large number of mass transitions acquired within each window of time resulted in poor reproducibility [7]. Additionally, the isotopic overlap between the internal standard's and the sample's reporter ions can affect the results.

The iTRAQ® reagents have been modified to address these issues, resulting in a new generation of reagents: aTRAQ®. The principle is the same as with iTRAQ®, but the reporter ions for the tagged amino acids present in physiological samples and standards are now 8 mass units apart, with m/z = 121 for tagged amino acids in physiological samples and m/z = 113 for the tagged internal standards. The data processing software, Analyst 1.5.1 (AB Sciex), has also been modified to introduce a scheduled selective reaction monitoring (SRM) function, decreasing the number of transitions to be monitored within a given window of time and thereby improving the reproducibility. The objective of this study was to evaluate the improved aTRAQ® method for urine amino acid analysis in comparison to traditional ion-exchange chromatography. We have also evaluated the clinical performance of this assay and established method specific reference values for each amino acid in urine.

2. Materials and methods

2.1. Urine specimens

The protocols used for sample collection in this study were approved by the Institutional Review Board (IRB) of the University of Utah. Urine samples were collected from normal controls ranging in age from 1 week to 90 years. The samples were frozen within 24 h from collection and kept at −80 °C until analysis. In addition, urine samples from patients with impaired renal function or known metabolic disorders submitted to our laboratory were de-identified, according to a protocol approved by the IRB of the University of Utah, and used to evaluate the clinical performance of the assay. The samples were stored at −80 °C until analysis.

2.2. Reagents and aTRAQ® kit

All amino acid standards used for the preparation of calibrators and controls were purchased from either Beckman Coulter (STD® amino acid standard for hydrolysate analysis, AN+® amino acid supplement for physiological standards acidic and neutral, and B+® amino acid supplement for physiological standards basics) or from Sigma–Aldrich.

The aTRAQ® kit for analysis of amino acids in physiological fluids was provided by AB Sciex. The kit contains all reagents necessary for the labeling of amino acids with the aTRAQ® tag: aTRAQ® Reagent 121, 10% sulfosalicyclic acid (containing 400 pmol/μl of norleucine), borate labeling buffer (containing 20 pmol/μl of norvaline), 1.2% hydroxylamine solution, formic acid, heptafluorobutyric acid, and isopropanol. The aTRAQ® kit also contains a solution of forty four amino acids labeled with the 113 aTRAQ® tag (concentration of 5 pmol/μl for each amino acid, except for L-cystine present at 2.5 pmol/μl), to be used as internal standards. As a quality control parameter, non-physiological amino acids, norleucine and norvaline, added to sulfosalicyclic acid and borate labeling buffer, respectively, were used to assess the extraction and labeling efficiencies of the assay.

2.3. Separation and detection

Amino acids are separated by liquid chromatography using a Shimadzu Prominence HPLC system with an AB Sciex C18 (5 μm, 4.6 mm × 150 mm) column. Detection and identification of amino acids is achieved using an AB Sciex API 4000 tandem mass spectrometer, operated in selective reaction monitoring mode (SRM). All data acquisition and processing was performed using Analyst 1.5.1 software (AB Sciex).

Specifically, chromatographic separation of the amino acids was performed using the AB Sciex C18 column at a temperature of 50 °C. A binary gradient of water (mobile phase A) and methanol (mobile phase B), both containing 0.1% formic and 0.01% heptafluorobutyric acids, was delivered at a rate of 0.8 ml/min according to the program shown in Table 1. The run time, injection-to-injection, is 25 min and complete separation of all aTRAQ® tagged amino acids, with the exception of the isomers isoleucine and allo-isoleucine, is achieved within the first 12 min. aTRAQ® tagged isoleucine and allo-isoleucine cannot be separated due to structural constraints imposed by the tag, however the two amino acids can easily be separated in the untagged form (*mass transition 132.1 → 86.1, retention time of 6.9 min for allo-isoleucine and 7.2 min for isoleucine*) (Fig. 1B and C). Therefore, to identify allo-isoleucine, the diagnostic marker for the metabolic disorder Maple Syrup Urine Disease (MSUD), untagged amino acids are also monitored within the same run as the aTRAQ® tagged amino acids (see sample preparation below).

The API 4000 (AB Sciex) tandem mass spectrometer was operated in positive-ionization mode with the following parameters: ion spray voltage 1800 V, entrance potential 10 V, declustering potential 20 V, collision energy 30 V, collision cell exit potential 15 V. A 1-min window surrounding the expected retention time was applied for each analyte, allowing a minimum of 10 scans per peak, with a dwell time of 25 ms. Operating in selective reaction monitoring (SRM) mode, amino acids and internal standards are identified by a single mass transition, as well as the retention time (Table 2).

2.4. Controls and calibrators

Two urine control samples (QC low and QC high) were prepared and analyzed with each batch of samples. The QC low control was urine from a normal adult control with the addition of the amino acids argininosuccinic acid and homocitrulline. The QC high control was prepared by spiking urine from a normal adult control with amino acids at concentrations near the upper limit of linearity or encountered in pathological samples. Table 3 lists the amino acid concentrations in the controls used in this study.

An external four point calibration curve was generated for 42 amino acids to improve the accuracy of the assay. The calibration samples were prepared in a lithium citrate loading buffer (pH 2.2) (Biochrom) and the amino acid concentrations in the calibrators

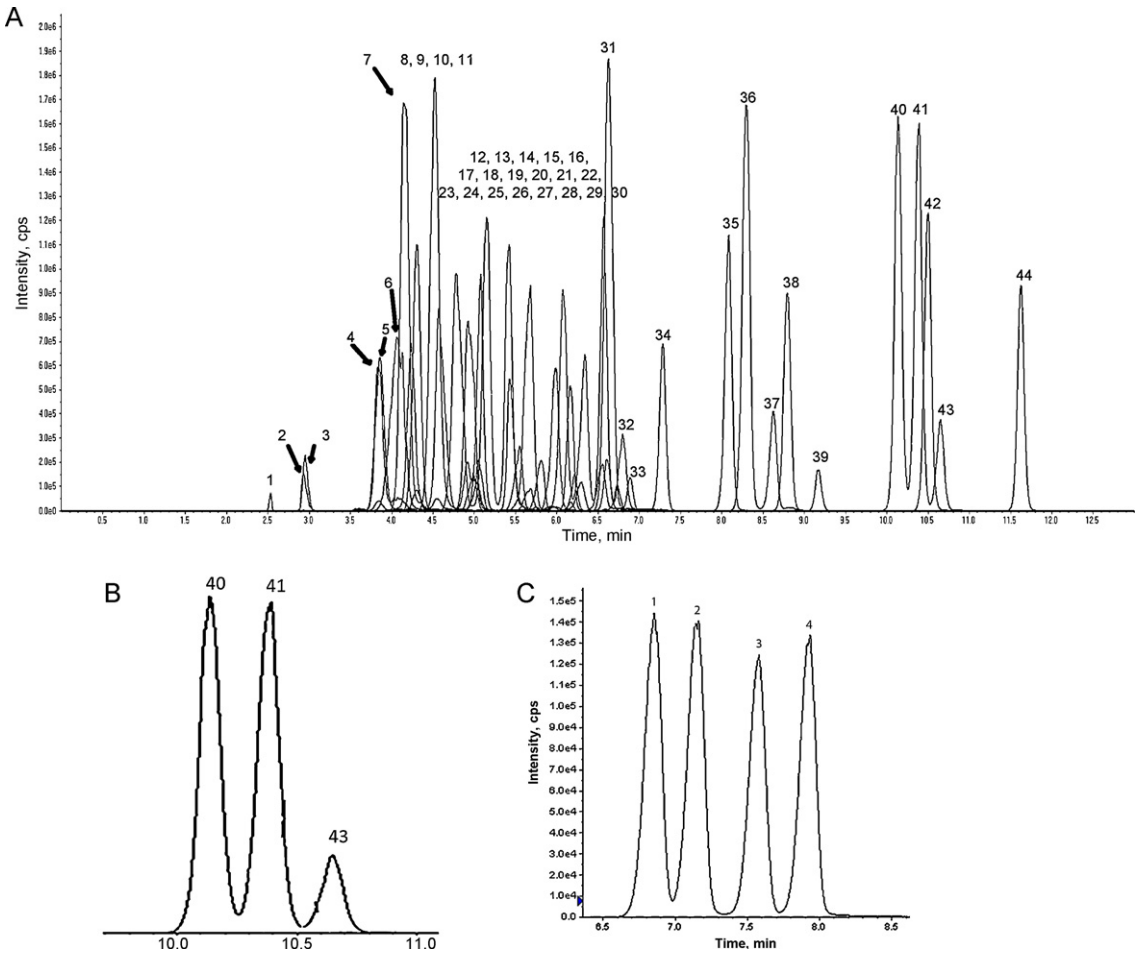

Fig. 1. (A) Profile of a mixture of amino acid standards labeled with aTRAQ® obtained by LC–MS/MS. 1, Phosphoserine; 2, phosphoethanolamine; 3, taurine; 4, asparagine; 5, serine; 6, hydroxyproline; 7, aspartate; 8, glycine; 9, glutamine; 10, ethanolamine; 11, histidine; 12, threonine; 13, citrulline; 14, 1-methyl-histidine; 15, 3-methyl-histidine; 16, argininosuccinic acid; 17, glutamate; 18, sarcosine; 19, β-alanine; 20, alanine; 21, carnosine; 22, arginine; 23, anserine; 24, homocitrulline; 25, hydroxylysine; 26, α-aminoadipic acid; 27, γ-aminobutyric acid; 28, β-aminoisobutyric acid; 29, α-aminobutyric acid; 30, ornithine; 31, proline; 32, cysthathionine; 33, cystine; 34, lysine; 35 methionine; 36, valine; 37, norvaline; 38, tyrosine; 39, homocystine; 40, isoleucine; 41, leucine; 42, phenylalanine; 43, norleucine; 44, tryptophan. (B) Enlarged region from (A) showing separation of the isobaric amino acids isoleucine and leucine. 40, Isoleucine; 41, leucine; 43, norleucine. (C) Profile of unlabeled amino acids showing separation of isomers allo-isoleucine and isoleucine. 1, Allo-isoleucine; 2, isoleucine; 3, leucine; 4, norleucine.

spanned the linear range (Table 4). The external calibration curves, in addition to the internal standards, were used to calculate the amino acid concentrations.

2.5. Sample preparation and labeling with aTRAQ® reagents

The creatinine concentration for each urine sample was measured by the kinetic Jaffe reaction on a Hitachi 911 analyzer. Aliquots of urine samples and QC high and low controls containing 0.1 mg of creatinine were lyophilized and then reconstituted in either 1 ml or 2.5 ml of lithium citrate loading buffer (pH 2.2) (Biochrom), depending upon the age of the patient. Throughout the remaining steps of the assay, the reconstituted urine samples and QC high and low controls, were treated identically to the calibrators. 40 μl aliquots of reconstituted urine samples, QC low and QC high controls, or calibrators were pipetted into a 1.5 ml eppendorf tube; 10 μl of 10% sulfosalicylic acid (containing 400 pmol/μl of norleucine) were added to precipitate any protein present. The samples were vortexed and then centrifuged for 3 min at 14,000 × *g*. 10 μl of the supernatant were then mixed with 40 μl of borate labeling buffer (containing 20 pmol/μl of norvaline) in a microfuge tube. The samples were vortexed and centrifuged for one minute at 14,000 × *g*. A 5 μl aliquot of the supernatant was set aside for the analysis of untagged amino acids allo-isoleucine and isoleucine, while 10 μl of the supernatant were pipetted into a 96 well plate and mixed with 5 μl of 121 aTRAQ® tag for labeling. The plate was incubated at room temperature for 30 min. To stop the labeling reaction, 5 μl of hydroxylamine were added to each well of the plate. The 5 μl aliquot of untagged amino acids was then added to each sample well. The samples were dried under nitrogen for 10 min and the residues were reconstituted with 32 μl of 113 aTRAQ® tagged internal standards.

2.6. Quantification of amino acids

Quantification of amino acids was performed using the Analyst 1.5.1 software (AB Sciex). Amino acid concentrations were determined by dividing the analyte peak area by the peak area of its corresponding internal standard and then multiplying by the slope of the external calibration curve. Appropriate dilutions were applied for samples that exceeded the linear range.

2.7. Statistical analysis of reference range data

EP® evaluator software [Release 8 (2007), David G. Rhoads Associates, Inc.] was utilized for analysis of reference range data.

Table 2
Mass transitions and retention times for amino acids and their corresponding internal standard.

Amino acid	Analytes				Internal standards			
	Abbreviation	Q1 mass	Q3 mass	Retention time	Abbreviation	Q1 mass	Q3 mass	Retention time
1-Methyl-histidine	1MHis	318.2	121.1	5.1	IMHisIS	310.2	113.1	5.1
3-Methyl-histidine	3MHis	318.2	121.1	5.4	3MHis IS	310.2	113.1	5.4
α-Aminoadipic acid	Aad	310.2	121.1	6.5	Aad IS	302.2	113.1	6.5
α-Aminobutyric acid	Abu	252.2	121.1	7.1	Abu IS	244.2	113.1	7.1
Alanine	Ala	238.2	121.1	5.6	Ala IS	230.2	113.1	5.6
Anserine	Ans	389.2	121.1	6.0	Ans IS	381.2	113.1	6.0
Arginine	Arg	323.2	121.1	6.1	Arg IS	315.2	113.1	6.1
Argininosuccinic acid	Asa	439.2	121.1	5.3	Asa IS	431.2	113.1	5.3
Asparagine	Asn	281.2	121.1	3.8	Asn IS	273.2	113.1	3.8
Aspartate	Asp	282.1	121.1	4.4	Ans IS	274.1	113.1	4.4
β-alanine	bAla	238.2	121.1	5.3	bAla IS	230.2	113.1	5.3
β-aminoisobutyric acid	bAib	252.2	121.1	6.7	bAib IS	244.2	113.1	6.7
Carnosine	Car	375.2	121.1	5.8	Car IS	367.2	113.1	5.8
Citrulline	Cit	324.2	121.1	5.1	Cit IS	316.2	113.1	5.1
Cystathionine	Cth	519.3	121.1	7.0	Cth IS	503.3	113.1	7.0
Cystine	Cys	537.2	121.1	7.1	Cys IS	521.2	113.1	7.1
Ethanolamine	EtN	210.2	121.1	4.5	EtN IS	202.2	113.1	4.5
γ-Aminobutyric acid	GABA	252.2	121.1	6.1	GABA IS	244.2	113.1	6.1
Glutamate	Glu	296.2	121.1	5.3	Glu IS	288.2	113.1	5.3
Glutamine	Gln	295.2	121.1	4.4	Gln IS	287.2	113.1	4.4
Glycine	Gly	224.1	121.1	4.2	Gly IS	216.1	113.1	4.2
Histidine	His	304.2	121.1	4.7	His IS	296.2	113.1	4.7
Homocitrulline	Hcit	338.2	121.1	6.4	Hcit IS	330.2	113.1	6.4
Homocystine	Hcy	565.3	121.1	9.4	Hcy IS	549.3	113.1	9.4
Hydroxylysine	Hyl	459.3	121.1	6.5	Hyl IS	443.3	113.1	6.5
Hydroxyproline	Hyp	280.1	121.1	4.1	Hyp IS	272.1	113.1	4.1
Isoleucine	Ile	280.2	121.1	10.6	Ile IS	272.2	113.1	10.6
Leucine	Leu	280.2	121.1	10.8	Leu IS	272.2	113.1	10.8
Lysine	Lys	443.3	121.1	7.5	Lys IS	427.3	113.1	7.5
Methionine	Met	298.2	121.1	8.4	Met IS	290.2	113.1	8.4
Norleucine	Nle	280.2	121.1	11.0	Nle IS	272.2	113.1	11.0
Norvaline	Nva	266.2	121.1	8.6	Nva IS	258.2	113.1	8.6
Ornithine	Orn	429.3	121.1	6.8	Orn IS	413.3	113.1	6.8
Phenylalanine	Phe	314.2	121.1	10.8	Phe IS	306.2	113.1	10.8
Phosphoethanolamine	PEtN	290.1	121.1	2.8	PEtN IS	282.1	113.1	2.8
Phosphoserine	PSer	334.1	121.1	2.5	PSer IS	326.1	113.1	2.5
Proline	Pro	264.2	121.1	6.9	Pro IS	256.2	113.1	6.9
Sarcosine	Sar	238.2	121.1	5.0	Sar IS	230.2	113.1	5.0
Serine	Ser	254.2	121.1	3.9	Ser IS	246.2	113.1	3.9
Taurine	Tau	274.1	121.1	2.8	Tau IS	266.1	113.1	2.8
Threonine	Thr	268.2	121.1	5.2	Thr IS	260.2	113.1	5.2
Tryptophan	Trp	353.2	121.1	11.9	Trp IS	345.2	113.1	11.9
Tyrosine	Tyr	330.2	121.1	9.1	Tyr IS	322.2	113.1	9.1
Valine	Val	266.2	121.1	8.8	Val IS	258.2	113.1	8.8

Reference intervals were generated by non-parametric analysis and represent the central 95% (2.5–97.5%) of the populations evaluated.

3. Results

3.1. Imprecision

The intra-assay and inter-assay variation was determined by 5 consecutive analyses of the two control samples (QC low and QC high) repeated on 5 different days (5 × 5). The intra-assay coefficient of variation (CV) for the QC low control was less than 10% and the inter-assay CV was less than 20% for most amino acids. The intra-assay CV for the QC high control was less than 5% for all amino acids, with an inter-assay CV of less than 10% (Table 3).

3.2. Limit of quantification and upper limit of linearity

The limit of detection (LOD) was determined by analyzing samples containing amino acids spiked in lithium citrate loading buffer at progressively lower concentrations until a minimum signal-to-noise ratio of 3 was achieved, while the limit of quantification was determined at a signal-to-noise ratio of 10. The limit of quantification ranged from 0.5 to 5 μmol/L depending on the amino acid. The upper limit of linearity ranged from 375 to 2500 μmol/L (Table 4).

3.3. Analytical validation

Amino acids concentrations in urine samples from both normal controls and patients with known metabolic disorders or impaired renal function were measured by LC–MS/MS and compared to values obtained by ion-exchange chromatography (IEC). Fig. 2 shows the Deming regression plots for two amino acids, glycine and leucine. Glycine was selected to represent amino acids whose normal excretion is well above the limit of detection, while leucine was selected to represent amino acids which have a very minimal excretion, typically near the limit of detection, in normal controls. Fig. 2A demonstrates good concordance between glycine concentrations obtained by both methods (slope 0.995, *R* value 0.9904). Fig. 2B shows the Deming regression plot for leucine where the data is significantly more scattered and has a positive bias for IEC (slope 0.864, *R* value 0.9151). Urine samples contain, in addition to free amino acids, other metabolites that are derived from diet and/or medications. These metabolites, if they contain an amino group, can react with ninhydrin and co-elute with amino acids when IEC is used for analysis. In patients with normal renal function, where

Table 3
Intra and inter assay variation for OC low and QC high controls.

Amino acid	QC Low control			QC high control		
	Concentration	Intra-assay	Inter-assay	Concentration	Intra-assay	Inter-assay
1-Methyl-histidine	3500	2.9	3.5	904	3.4	4.0
3-Methyl-histidine	298	2.7	4.6	574	2.2	2.1
α-Aminoadipic acid	45	5.3	4.5	126	2.9	2.3
α-Aminobutyric acid	15	12.0	14.3	131	2.9	7.8
Alanine	141	3.5	5.8	2181	2.9	5.0
Anserine	305	2.2	4.6	480	3.7	3.9
Arginine	16	7.7	10.1	1403	2.0	3.6
Argininosuccinic acid	2254	3.1	9.2	0	0.0	0.0
Asparagine	62	4.7	5.3	242	3.0	6.1
Aspartate	0	0.0	0.0	526	3.1	2.8
β-Alanine	0	1.8	0.0	547	3.4	5.3
β-Aminoisobutyric acid	139	3.1	13.3	573	2.3	4.2
Carnosine	113	3.5	3.3	474	2.8	3.4
Citrulline	0	0.0	0.0	122	3.2	4.3
Cystathionine	45	7.9	5.6	522	5.1	3.0
Cystine	52	5.5	4.9	1875	2.5	3.0
Ethanolamine	236	8.3	23.9	583	4.0	3.6
γ-Aminobutyric acid	0	0.0	0.0	512	2.7	3.7
Glutamate	0	0.0	0.0	695	3.1	6.0
Glutamine	271	3.6	4.8	463	2.5	2.8
Glycine	719	3.5	2.9	4393	2.2	1.4
Histidine	920	2.4	6.1	676	2.4	6.7
Homocitrulline	928	2.7	5.5	0	0.0	0.0
Homocystine	0	0.0	0.0	493	1.6	4.6
Hydroxylysine	0	0.0	0.0	491	3.8	3.9
Hydroxyproline	0	0.0	0.0	520	2.1	4.8
Isoleucine	16	8.8	6.0	554	0.7	2.6
Leucine	44	2.2	3.6	552	1.1	2.5
Lysine	358	2.3	2.6	1364	1.8	2.5
Methionine	9	2.9	8.0	520	1.8	6.1
Ornithine	16	8.1	14.5	1232	3.5	6.5
Phenylalanine	43	1.3	3.0	551	1.0	2.1
Phosphoethanolamine	46	10.2	10.1	250	4.9	7.7
Phosphoserine	0	0.0	0.0	253	3.1	6.4
Proline	7	6.1	19.0	561	1.9	3.8
Sarcosine	3	4.3	10.1	544	3.0	5.1
Serine	231	3.0	5.9	600	2.2	4.9
Taurine	556	2.7	6.5	365	3.3	5.7
Threonine	86	4.7	4.6	565	2.3	2.5
Tryptophan	58	1.0	7.3	12	2.6	4.2
Tyrosine	74	1.7	7.7	548	1.7	3.3
Valine	37	3.8	5.9	571	1.6	2.0

Concentrations are expressed in μmol/g creatinine and the intra and inter assay variation is expressed as %CV.

the excretion of most amino acids is minimal, these interferences can lead to a significant overestimation in the concentration, as seen with leucine. The LC–MS/MS method monitors only the transitions associated with the specific amino acids, leading to a more specific and accurate quantitative result.

3.4. Clinical performance

Quantitative analysis of urine amino acids is typically used to identify patients with disorders of amino acid transport or to assess renal tubular function. Therefore, to evaluate the clinical performance of the aTRAQ LC–MS/MS method, urine from twenty-seven patients with known conditions affecting urinary amino acid excretion were analyzed. The amino acid profiles from three samples were compared to age matched controls in Fig. 3. Fig. 3A shows the amino acid profile of a patient with cystinuria with the characteristic increased excretion of cystine, lysine, ornithine, and arginine, and Fig. 3B shows markedly increased excretion of lysine in a patient with lysinuric protein intolerance, as compared to the normal controls. The elevated excretion of multiple amino acids in a patient with impaired renal tubular function is shown in Fig. 3C. For all patients' samples analyzed by LC–MS/MS, there was a 100% concordance with the IEC analysis.

3.5. Reference intervals

Reference intervals were established for 42 amino acids using urine samples from 249 normal controls ranging in age from 1 week to 90 years (Table 5). The reference intervals were determined as the central 95% of the values observed within each age group population and are expressed in μmol/gram creatinine. The highest excretion and greatest variation in amino acid concentrations were observed in infants, reflecting the variability of renal tubular function in the first few months of life [8]. As the renal function improves with age, the concentration as well as the variability for most amino acids decreased. For some amino acids, such as proline and hydroxyproline, there was a sharp drop in excretion after the first two months of life, while for other amino acids (glycine and alanine) the change in concentration was more gradual. Several amino acids such as isoleucine, leucine, and methionine showed minimal changes in concentrations with age. Interestingly, a few amino acids, such as 1-methyl-histidine, increased in concentration during the first few years of life. These amino acids are abundant in poultry; therefore the increased excretion with age

Table 4
Calibration curve parameters.

Amino acid	Slope + intercept	*R* squared	LLOD	LLOQ (%CV)	Linear range
1-Methyl-Histidine	0.972 (0.089)[a]	1.0000	1.00	2.50 (8.3)[b]	500
3-Methyl-Histidine	0.866 (0.051)	0.9993	1.00	2.50(13.9)	500
α-Aminoadipic acid	1.046 (0.056)	0.9999	1.00	2.50(13.5)	188
α-Aminobutyric acid	0.968 (0.058)	0.9998	1.00	2.50 (5.6)	188
Alanine	0.939 (0.081)	1.0000	1.00	2.50(12.9)	1000
Anserine	0.856 (0.072)	0.9997	0.50	1.00 (5.0)	500
Arginine	0.948 (0.068)	0.9998	0.50	1.00 (4.7)	1000
Argininosuccinic acid	0.835 (0.047)	0.9998	2.50	5.00(17.8)	500
Asparagine	1.258(0.131)	0.9999	2.00	5.00 (11.4)	1000
Aspartate	0.877 (0.072)	0.9999	0.25	1.00(16.2)	1000
β-Alanine	0.743 (0.043) + 0.232 (0.060)	0.9994	5.00	10.00(15.6)	750
β-Aminoisobutyric acid	0.801 (0.048)	0.9998	0.50	1.00(2.3)	750
Carnosine	0.998 (0.073)	0.9999	0.50	1.00 (4.7)	500
Citrulline	0.998 (0.063)	0.9997	0.50	1.00(13.2)	188
Cystathionine	0.796 (0.051)	0.9998	0.50	1.00 (3.3)	750
Cystine	1.067(0.081)	0.9999	0.50	1.00 (11.8)	500
Ethanolamine	0.993 (0.052)	0.9997	2.50	5.00(16.9)	500
γ-Aminobutyric acid	0.852 (0.058)	0.9997	0.25	1.00 (7.0)	500
Glutamate	0.927 (0.054)	0.9997	0.50	1.00(13.3)	1000
Glutamine	1.152(0.175)	0.9999	2.00	5.00(11.7)	1000
Glycine	0.917(0.050)	0.9999	1.00	5.00 (7.3)	2500
Histidine	0.970 (0.069)	0.9996	0.50	1.00 (11.7)	1000
Homocitrulline	0.871 (0.052)	1.0000	2.00	5.00(10.3)	500
Homocystine	0.852 (0.058)	0.9999	1.00	2.50 (7.0)	750
Hydroxylysine	1.006 (0.048)	0.9999	0.50	1.00 (4.6)	500
Hydroxyproline	1.210(0.056)	0.9999	0.25	1.00 (4.5)	750
Isoleucine	0.908 (0.053)	0.9999	1.00	2.50(11.6)	1000
Leucine	0.900 (0.049)	0.9999	0.50	2.50 (7.3)	1000
Lysine	1.067(0.071)	1.0000	0.50	1.00 (1.8)	1000
Methionine	0.880 (0.038)	0.9999	1.00	2.50(12.5)	1000
Ornithine	1.027 (0.069)	0.9998	0.50	1.00(10.4)	500
Phenylalanine	0.911 (0.051)	0.9998	0.50	1.00 (8.6)	1000
Phosphoethanolamine	0.994 (0.054)	0.9999	1.25	2.50(12.2)	375
Phosphoserine	0.900 (0.047)	0.9998	1.25	2.50(12.4)	375
Proline	1.047 (0.060)	0.9999	0.25	1.00 (6.6)	1000
Sarcosine	1.256(0.118)	0.9998	1.00	2.50(13.0)	750
Serine	0.910(0.062)	0.9999	1.00	2.50(10.9)	1000
Taurine	0.811 (0.057)	0.9998	1.00	2.50(19.3)	1000
Threonine	0.908 (0.053)	1.0000	1.00	1.00 (9.6)	1000
Tryptophan	0.854 (0.042)	1.0000	0.50	1.00 (5.7)	500
Tyrosine	0.894 (0.061)	0.9999	0.50	1.00 (5.0)	1000
Valine	0.912 (0.060)	0.9999	1.00	2.50 (6.5)	1000

LLOQ, LLOD, and linear range expressed in μmol/L.
[a] Standard deviation for the calculated slope and intercept.
[b] %CV at the lowest limit of quantification.

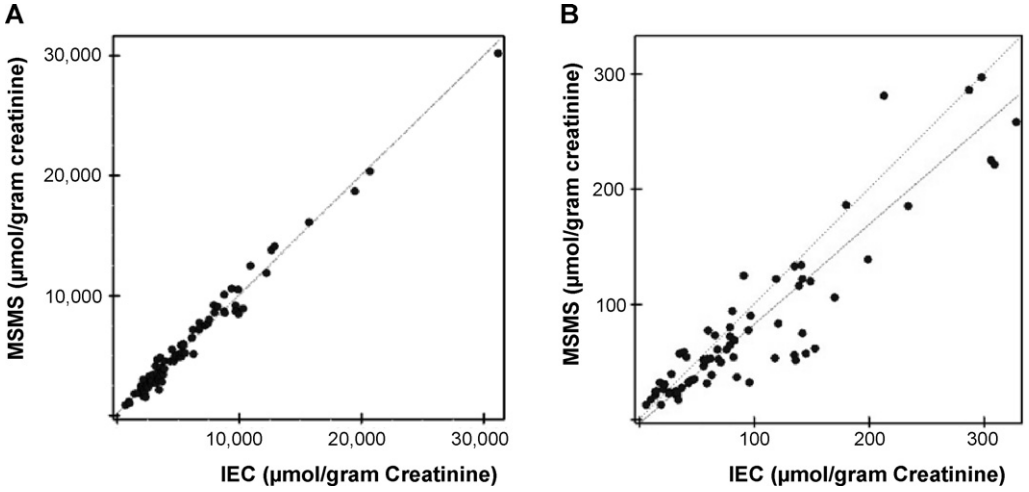

Fig. 2. (A) Deming regression model comparing glycine concentrations obtained by IEC to tandem mass spectrometry (MS/MS). (B) Deming regression model comparing leucine concentrations obtained by IEC to tandem mass spectrometry (MS/MS).

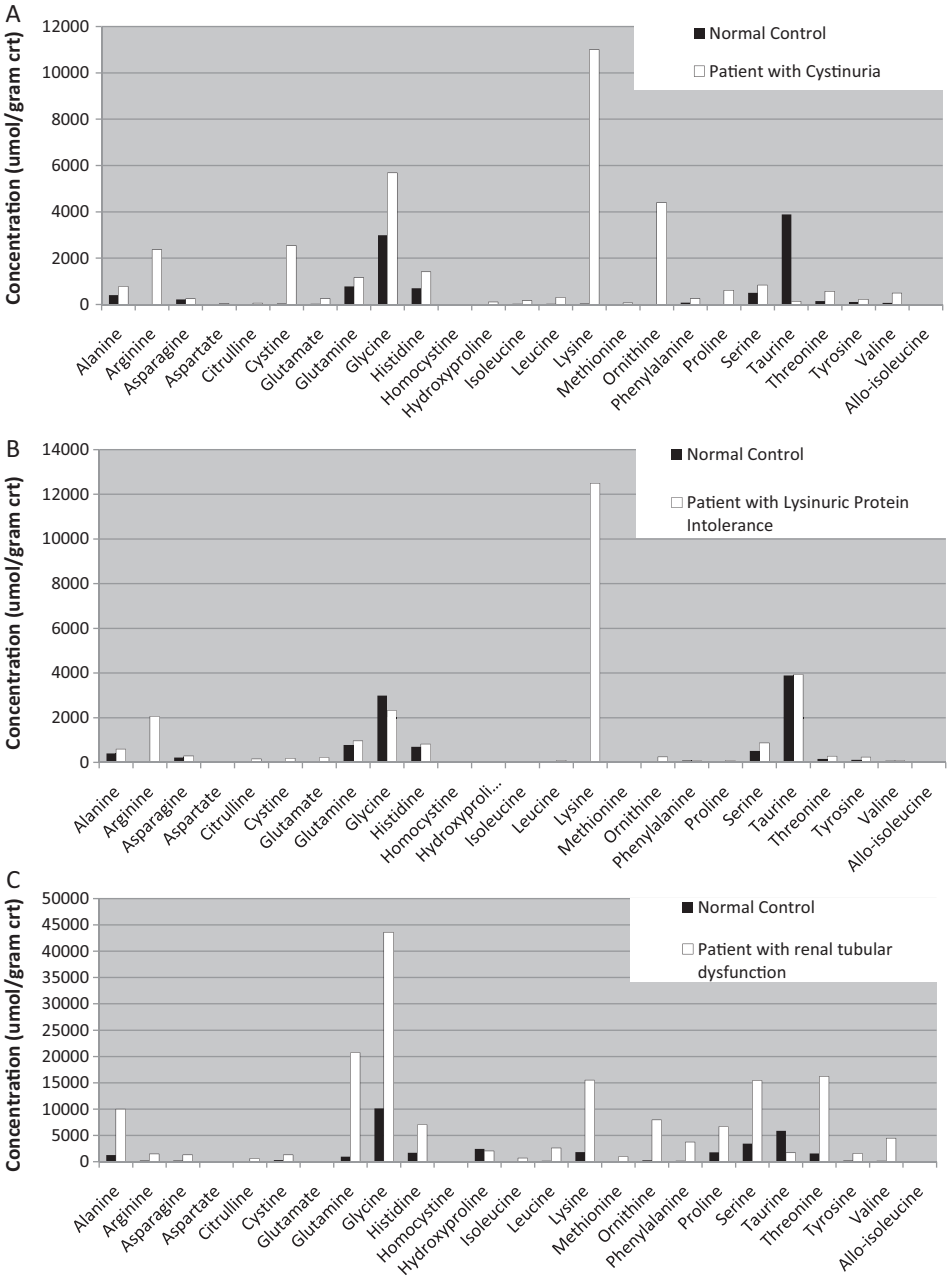

Fig. 3. Comparison of amino acid profiles in patients with metabolic disorders to age matched controls. (A) Elevated excretion of cystine, lysine, ornithine, and arginine consistent with cystinuria. (B) Markedly elevated excretion of lysine consistent with lysinuric protein intolerance. (C) Elevated excretion of most amino acids in patient with impaired kidney function.

in childhood likely reflects dietary changes. These overall trends in amino acid concentrations as well as the specific reference intervals are comparable with previously published reference data [9–13].

4. Discussion

Diagnosis and treatment of many inborn errors of metabolism requires quantitative amino acid analysis. For years laboratories have utilized ion-exchange chromatography (IEC) for amino acids analysis because of its reproducibility and high sensitivity. However, amino acid separation by IEC requires a long run time (2.5 h), which decreases the throughput. Additionally, interferences due to diet or medications, may affect the quantification of amino acids, especially in urine.

aTRAQ® reagent technology used in conjunction with liquid chromatography and tandem mass spectrometry (LC–MS/MS) is an attractive alternative to IEC. The two most significant advantages of this method are the reduction in chromatographic run time (25 min for LC–MS/MS) and the increased specificity. All amino acids elute within the first 12 min of the run and all physiological amino acids, including isomers, are clearly separated from each other. Mass transitions unique to each amino acid, along with retention times, are used to identify amino acids, eliminating the effect of interferences and increasing specificity. In urine samples from patients with normal renal tubular function, the excretion of certain amino acids is minimal and the presence of interferences resulted in overestimation of the concentration, when analyzed by IEC, as compared to LC–MS/MS. However, at high concentrations, where interferences, if present, contribute minimally to the overall concentration, there was excellent concordance between the two methods.

Table 5
Age-related reference intervals for urinary amino acid excretion in μmol/gram creatinine.

Amino acid	0–2 months (n = 47)	3–8 months (n = 45)	9 months–2 years (n = 43)	3–12 years (n = 60)	13+ years (n = 54)
1-Methyl-histidine	0–246	0–206	2–1452	0–1887	0–1926
3-Methyl-histidine	64–450	86–489	68–509	103–293	0–256
α-Aminoadipic acid	6–522	34–353	120–425	15–271	7–57
α-Aminobutyric acid	0–84	0–70	1–96	0–51	0–20
Alanine	658–3424	601–2918	224–2210	176–1255	78–587
Anserine	0–109	0–218	0–635	0–600	0–67
Arginine	6–313	29–307	20–424	11–54	5–34
Argininosuccinic acid	0–102	0–139	0–129	0–64	0–68
Asparagine	0–1260	0–854	0–758	49–466	22–170
Aspartate	0–866	0–299	0–209	0–35	0–26
β-alanine	28–705	0–731	0–218	0–195	0–130
β-aminoisobutyric acid	0–8918	91–7140	64–9100	18–4923	2–922
Carnosine	89–1544	132–1446	49–1216	12–927	0–149
Citrulline	0–165	0–82	0–76	0–22	0–12
Cystathionine	0–227	0–171	0–131	0–66	0–34
Cystine	14–573	28–461	34–186	26–98	12–81
Ethanolamine	0–2362	0–2251	156–1462	60–714	123–610
γ-Aminobutyric acid	0–31	0–16	0–16	0–6	0–4
Glutamate	35–1441	65–1155	19–321	8–100	4–38
Glutamine	117–4086	0–2916	480–3070	300–1896	110–609
Glycine	1970–16,940	1613–14,465	837–6625	595–5432	296–4419
Histidine	405–4186	421–3393	435–3986	259–2070	100–1004
Homocitrulline	0–308	0–111	12–141	0–140	3–52
Homocystine	0	0	0	0	0
Hydroxylysine	0–383	0–205	0–143	0–70	0–55
Hydroxyproline	83–5430	0–2561	0–1191	0–54	0–15
Isoleucine	1–186	22.3–159	14.3–107	9–59	10–32
Leucine	9–336	37–253	22–215	20–129	7–50
Lysine	58–2246	52–1716	49–953	37–292	16–271
Methionine	0–84	0–69	0–59	2–34	1–22
Ornithine	0–216	2–151	1–100	2–75	0–24
Phenylalanine	30–356	61–357	53–364	39–187	12–92
Phosphoethanolamine	0–377	0–510	32–595	8–239	0–71
Phosphoserine	0–8	0–5	0–4	0–4	0–15
Proline	50–2484	74–1200	10–662	6-117	3–23
Sarcosine	11–431	1–138	1–54	1–10	0–4
Serine	51–2910	173–3399	265–1827	211–947	64–501
Taurine	142–9594	100–7410	62–5572	60–2198	19–2859
Threonine	54–2334	67–1380	118–1318	74–585	33–209
Tryptophan	40–402	76–388	60–386	36–183	13-94
Tyrosine	70–857	126–775	76–659	59–410	18–170
Valine	41–428	60–280	30–338	26–191	10–73

n = total number of samples evaluated for each reference interval.

In a recent review article by Kaspar et al., several different methodologies for amino acid analysis including iTRAQ® chemistry were compared [14]. The advantage of iTRAQ® over other tandem mass spectrometry methods is the availability of internal standards. The disadvantages included poor reproducibility due to the large number of mass transitions and insufficient recovery of sulfur-containing amino acids. Significant improvements have been made to the data acquisition methods and to the reagents since the publication of this article. First, implementation of scheduled selective reaction monitoring (SRM) with narrow windows of acquisition considerably improved the reproducibility of the assay. The coefficient of variation (CV) for intra- and inter-assay comparison is now comparable to the CV obtained by IEC. Secondly, the use of aTRAQ® reagents, instead of the iTRAQ® reagents, improves the sensitivity of the method and the dynamic range. aTRAQ® reagents are designed with 8 mass units difference between the tagged labeled internal standards and the tagged natural amino acids, reducing considerably the background noise. Lastly, in our method, the use of an external calibration curve, in addition to the labeled internal standards, reduced the lot-to-lot variability and improved the overall accuracy of the assay.

A remaining challenge for the aTRAQ® LC–MS/MS method is data analysis and interpretation. Unlike IEC, each amino acid is analyzed separately and a full chromatographic representation of all the amino acids is not possible, making recognition of patterns of amino acids difficult. Utilization of graphing programs in conjunction with the data analysis software should be considered to facilitate the integration of this method in clinical laboratories. Also, rare amino acids or other ninhydrin-reacting compounds with transitions not monitored in this in the assay will be missed. Laboratories should evaluate the need for including additional compounds in their routine methods.

5. Conclusion

Overall, aTRAQ® chemistry used with LC-MS/MS represents a valid alternative to IEC. The increased preparation time is very well compensated by a significant reduction in run time, in addition to the high specificity. The recovery and quantification of 42 amino acids are comparable to the data obtained by other methods, including IEC, as demonstrated by the reference ranges values. This method can be easily integrated in clinical laboratories for amino acids analysis.

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