UPLC-QTOF (ESI +) MS Analysis Applied for Targeted Profiling of Human Urine Amino Acids

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Abstract

Amino acids, the building blocks of proteins are providing fundamental information about metabolic status and early disease diagnosis. For an accurate interpretation of metabolic status, amino acid reference values for healthy humans are required. Most studies have pointed out that, the most accurate, reliable and sensitive tool to evaluate amino acids in biofluids (blood or urine) is liquid chromatography coupled with mass spectrometry (LC/MS). The aim of this study was to determine and optimize the urine amino acids profile by LC-QTOF (ESI+) MS, comparatively, using two generic samples from a healthy male and female. Urine aminoacids were purified using solid phase extraction, a pre-column derivatization and liquid/liquid extraction according to Ez-Faast kit (Phenomenex). The derivatized samples were analyzed by LC-QTOF-(ESI+)-MS and data were processed by Data Analysis-Extracted Ion Chromatogram software. A number of 16 amino acids were found in both samples, the male urine showed higher values for 10 amino acids (hydroxyproline, alanine, sarcosine, lysine, histidine, tryptophan, leucine, phenylalanine, cysteine and tyrosine), while glutamine and valine were higher in female urine. Most of the amino acids identified were essential amino acids found in both cases like: glutamine, proline, lysine, histidine, valine, tryptophan, leucine and phenylalanine. The main differences between the male vs female aminoacid fingerprints of urine were mainly quantitative and not qualitative. The amino acid concentrations ranged from 1.48 nmol/mg creatinine (proline) to 203.13 nmol/mg (histidine). The concentration of amino acids in man were 2 times higher than for female, while the percentages of essential amino acids were similar in both gender.

Keywords: *amino acids, urine LC-QTOF/MS, pre-column derivatization, targeted metabolomics*

INTRODUCTION

Amino acids, the building units of proteins, play an essential physiological role as metabolic precursors, regulators and intermediate metabolites, therefore they can potentially serve as indicators of the overall physiological status (Miyagi *et al.*, 2011) or specific biomarkers of different pathological. Of those 20 amino acids found in proteins, eleven can be synthesized in mammalian cells called non-essential and the other nine are obtained from extracellular sources named essential amino acids (Bhutia *et al.*, 2014). Amino acid concentrations in plasma and urine, especially the essential amino acids may vary

considerably depending on diet, geographic area, age and sex so they have a personalized profile (Pitkanen *et al.*, 2003).

Since the metabolic amino-acid balance changes in patients with various diseases, metabolomic fingerprint and quantitative profile may act as an indicator of the altered physiological status (Hassan, 2012; Shen, 2013). Several investigations have pointed out that changes in the level of some amino acids may be considered as risk factor for developing certain diseases such as cardiovascular diseases (Tang *et al.*, 2009), diabetes (Wang *et al.*, 2013), liver diseases (Baniasadi *et al.*, 2013).

Moreover, there are several research studies focused on the amino acids profile changes in relation to different types of cancer diagnosis. Consequently, in patients with lung and breast cancer, tryptophan concentration has been correlated with tumor presence (Lai *et al.*, 2005) while low levels of plasma arginine were correlated with breast and colorectal cancer (Okamoto, 2009; Visser, 2005). Also, it was reported that modified levels of amino acids in urine may be useful in ovarian and breast cancer detection at early stages (Slupsky *et al.*, 2010). Kami *et al.* (2013) showed that the correlation between different metabolites and amino acid profile can distinguish between normal and tumor tissue in patients with prostate and lung cancer. They found that leucine, isoleucine and valine were significantly increased in either cases (Kami *et al.*, 2013).

Generally, two types of amino acid profile alterations were observed in cancer, those reflecting metabolic changes common to many cancers, which are a limited to a set of amino acids, and those specific to each type of cancer which concerns a larger group of amino acids (Miyagi *et al.*, 2011). In these sense, metabolomics, both targeted and untargeted has a great potential to quantify biomarkers of interest from tissue or biofluides and to perform validation and implementation in clinical practice. There are two major techniques that are most commonly used in focused or open profiling methods for metabolites evaluation: nuclear magnetic resonance (NMR) and liquid chromatography coupled with mass spectrometry (LC/MS). The last one is the most accurate and reliable technique that can detect small metabolites that cannot be measured by other methods (Kasture *et al.*, 2012)

Many various techniques have been design for amino acids detection with a wide range of reagents that imply a very laborious sample preparation with protein precipitation, extraction and derivatization that are specially meant for LC/MS application (Meesters *et al.*, 2009). Liquid chromatography coupled with mass spectrometry (LC/MS) is one of the most widely used method for detection and quantification for amino acids (Grebe and Singh, 2011).

Therefore this study aims to apply targeted metabolomics on an healthy male and female urine for determination of amino acids profile by LC-QTOF (ESI+) MS using a pre-column derivatization

protocol. To reduce sample preparation and analysis time the amino acid profile of aliphatic and aromatic amino acids were determined using an commercially available kit from Phenomenex (EZ:faast™ amino acids analysis kit). The short time and relatively mild derivatization protocol, along with the large number of amino acids and dipeptides with detection limits about 1µM for most of the amino acids, makes the Phenomenex EZ:faast™ amino acids LC/MS free (physiological) amino acid analysis kit suitable for stable isotope trace studies (Wilson *et al.*, 2011).

MATERIALS AND METHODS

Urine sample collection. Urine samples were obtained from two healthy people, one male of 51 and one female of 44 years of age, in order to determine the similarities and differences on amino acid profile between the genders. From each volunteer 10 ml of normal spot urine were collected in sterile urine containers. Homogenized samples were purified by 5 minutes centrifugation at 2000 revolutions per minute (RPM). After centrifugation the supernatant was aliquoted and stored at -20°C until analysis.

Extraction and derivatization of free amino acids. Urine sample preparation for UPLC-QTOF-(ESI+)-MS analysis was carried out by using the EZ:faast amino acid free (physiological) analysis kit. The EZ:faast™ amino acids analysis kit from Phenomenex procedure consist of a solid phase extraction (SPE) step followed by a derivatization and a liquid/liquid extraction step. 100 µl of thawed urine was pipetted into a glass sample preparation vial and then 100 µl of internal standard was added. The diluted sample was pulled slowly through a solid phase extraction tip by moving the piston of a 1.5 ml syringe attached to the sorbent tip. The internal standard solution included homoarginine (HARG), methionine-D₃ (MET-D₃) and homophenylalanine (HPHE) that are not naturally found in the samples. Then, 200 µl of washing solution (N-propanol) was added into the glass vial and slowly pulled through the SPE tip to remove urea and other components found in urine. The sorbent tip was detached into the vial and the liquid accumulated in the syringe was discarded. The next step was to discard the sample retained on the SPE tip by adding in the sample preparation vial 200 µl eluting/extraction solution consisting on a mix of reagent 1 (sodium

hydroxide) and reagent 2 (N-propanol, 3-picoline) (solution A/B: 3/2% (v/v)), next the SPE tip was attach to a 0.6 ml syringe and wet with the elution medium. The solid phase sorbent particles were then expelled into the sample preparation vial by pushing the piston down. Derivatization agent was added (50 μ l propyl chloroformate), was vortexed 10 s and let for 1 min. to react. Above 100 μ l of organic extraction solvent was added and the reaction procedure was repeated. The part of the upper layer was transferred into an autosampler vial for LC injection.

UPLC-QTOF (ESI+) MS analysis. UPLC-QTOF-(ESI+)-MS analysis was performed using a Thermo Scientific HPLC UltiMate 3000 system equipped with a quaternary pump delivery system Dionex and MS detection by a Bruker Daltonics MaXis Impact device. The column used for separation in LC/MS analysis was an EZ:faast AAA-MS 250x3.0 mm i.d. column from Phenomenex. The mobile phase consisted of 10 mM ammonium formate in water (A) and 10 mM ammonium formate in methanol. The flow rate was set at 0.250 ml/min, and the column temperature was 35°C. The gradient conditions used for the separation of amino acids derivatives were 68% B from 0 to 19 min followed by linear gradient to 93% B. The column was held at 93% B from 19.5 to 19.6 min. Then returned to initial conditions 68% B from 19.6 min and allowed to equilibrate for 4.4 min. Run-to-run time was 24 min. The injected volume was 5 μ L.

Mass spectrometric conditions as follows: the electrospray ion source (ESI) were operated under positive ion mode; using a nebulizing gas pressure set at 2.8 bar, the drying gas flow at 12 L/min, the drying gas temp at 300°C. Before each separation run, a calibrant solution of sodium formate was injected. The control of the instrument and the data processing were done using ToFControl 3.2 and Data Analysis 4.1 (Bruker Daltonics), respectively.

Quantitative evaluation of amino acids and ratios to creatinine. Quantification of free amino acids was made by reporting to internal standard HARG. Amino acids concentrations were calculated as formula: $C = (A_{AA} * C_{HARG}) / A_{HARG}$ where C represents the concentration of amino acids, A_{AA} represents the area of amino acids, C_{HARG} represents the concentration of internal standard and A_{HARG} represents the area of internal standard. The amino acids concentrations were reported

to creatinine. The standard curves for creatinine were validated over the concentration range of 0.1-0.0062 mg/ml from five dilutions. Average correlation coefficient (r^2) for the curve was 0.95.

Biostatistic interpretation of data. Chromatograms were processed using the Extracted Ion Chromatogram method from Data Analysis 4.1 (Bruker Daltonics) software.

RESULTS AND DISCUSSION

Chromatographic separation and MS identification of aminoacids using Extracted Ion chromatogram (EIC). Using UPLC-QTOF-ESI(+) MS analysis and a specific Data Analysis software (Extracted Ion chromatogram), 16 derivatized amino acids were separated and identified as adducts of amino acids with propyl chloroformate. *Figures 1A and 1B* shows the profiles obtained for male and female urine, respectively.

The general fingerprint showed 16 molecules identified (<http://www.phenomenex.com/Products/AminoAcidDetail/EZfaast>).

The comprehensive list of derivatized amino acids identified by their molecular mass and weight and retention time measured on under the conditions specified above are presented in *Table 1*. The identification of the amino acids in the chromatograms are given by peak number assigned.

Quantitative analysis. From a total of 27 amino acids, we quantified a number of 16, out of which 13 were found in both cases (*Fig. 2*). Amino acids concentrations were expressed in nmol per g of urinary creatinine.

Male showed higher levels in 10 of the 16 investigated amino acids while only 2 amino acids were higher on female urine profile. Compared with women, men had higher concentration of essential amino acids including histidine (203.13 nmol/mg creatinine), lysine (38.53 nmol/mg creatinine), phenylalanine (23.67 nmol/mg creatinine) and tryptophan (60.18 nmol/mg creatinine). Alanine (18.31 nmol/mg creatinine), cysteine (27.75 nmol/mg creatinine) and tyrosine (118.07 nmol/mg creatinine) were the non-essential amino acids that were higher among the male in this study. Also, a non-proteinogenic amino acid, hydroxyproline (HYP) was significantly higher in man. Glutamine (24.72 nmol/mg creatinine) and valine (19.42 nmol/mg creatinine) were the essential amino acids which showed higher levels in

Fig. 1. Extracted ion chromatograms (EIC) for amino acids on male (A) and female (B) urine

Tab. 1. The peak numbers, m/z values assigned for derivatized amino acids and molecular weight (MW) amino acids identified by LC/MS and EIC.

Peak number		m/z [M+H ⁺]	Amino acids MW	Amino acid identified
Fig. 1A	Fig. 1B			
21	9	218	89.1	Sarcosine
40	25	317	188.2	Homoarginine-IS
53	34	275	146.2	Glutamine
NI	44	234	105.1	Serine
90	56	260	131.1	4-Hydroxyproline
NI	65	248	119.1	Threonine
110	74	218	89.1	Alanine
136	104	281	152.2	Methionine-d3 -IS
142	102	244	115.1	Proline
154	112	361	146.1	Lysine
157	114	370	155.1	Histidine
158	115	246	117.1	Valine
161	NI	318	147.1	Glutamic acid
170	122	333	204.2	Tryptophan
188	133	260	131.2	Leucine
191	135	294	165.2	Phenylalanine
204	144	336	121.2	Cysteine
219	151	308	179.2	Homophenylalanine-IS
232	160	396	181.2	Tyrosine

NI- non-identified; IS-internal standard

Fig. 2. Comparative concentrations of the 16 identified amino acids, expressed in nmol/g creatinine by LC-QTOF-(ESI+)-MS in urine of an healthy male and female.

Tab. 2. Quantitative ratios of essential, non-essential and non-proteinogenic amino acids

	Male	Female
TFAA (nmol/mg creatinine)	679.2265	313.0746
EAA (%)	50.24081	50.3984
NEAA (%)	27.31762	28.80036
NP (%)	22.44157	20.80124

TFAA-total free amino acid, EAA-essential amino acid, NEAA-non-essential amino acid, NP-non-proteinogenic amino acids.

female. No serine and threonine were detected in male and no glutamic acid in female. Our findings were correlated with other studies (Hassan, 2011, Pitkanen, 2003). Significantly higher levels of histidine and tryptophan were reported in males vs females while the female serum had higher concentration of valine (Caballero *et al.*, 1991). No significant differences in amino acid profile between gender were found in case of proline.

Quantitative ratios of essential, non-essential and non-proteinogenic amino acids from total free amino acids were quantified, as presented in Table 2.

From the total free amino acids around 50% were essential amino acids, this ratio being similar in male and female. The non-essential amino acids represented 27-28%, while the non-proteinogenic ones represented around 20-22%. No significant differences were noticed between genders.

Such evaluation provides information about a good nutrition and health, but lower levels can reflect digestion deficiencies (ex. hystidine decrea-

se), inadequate stomach acid secretion (lower concentration of tryptophan, phenylalanine and tyrosine) as reported (Bongiovanni *et al.*, 2003).

Disturbances in aminoacid profiles can indicate also pathologic processes like cancer, by modification of cell metabolic pathways and synthesis (Vissers *et al.*, 2005; Slupsky *et al.*, 2010) or cardiovascular diseases (Tang *et al.*, 2010).

CONCLUSION

Using a rapid UPLC-QTOF-ESI(+)MS method, including apre-column derivatization for amino acids, the comparative profile of male amnd female urine was obtained. By extract ion chromatogram, the quantitative composition of 16 amino acids was evaluated.

The general fingerprint of the two samples was similar, while the concentrations of male amino acids were generally higher (aprox. 2 times) as compared with women, mainly for essential amino acids. The most abundant amino acid was hystidine, two times higher in male than female.

Also, tyrosine and tryptophan were much higher in man.

Considering the percentages of each subclass of amino acids, the values were not significantly different among gender, the essential amino acids representing around 50% from the total free amino acids.

The method applied is very useful to identify modifications in the amino acid profile in different pathologies and metabolic diseases.

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