A Proposed Strategy for the Detection of Metabolic Disorders: High Resolution Screening with Reflex to Targeted Quantitation by Tandem Mass Spectrometry

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Introduction

Amino acids are essential for every metabolic process. Amino acid analysis is required for the diagnoses and screening of primary and secondary aminoacidopathies as well as for the monitoring of therapeutic responses and assessment of nutritional status. Phenylketonuria (PKU) is characterized by accumulation of Phenylalanine (Phe) in the blood due to mutations in the Phe hydroxylase gene, resulting in low levels of the enzyme Phe hydroxylase which prevents the conversion of excess Phe to tyrosine. Untreated PKU can lead to seizures and intellectual disabilities. Considering the complexity required for sample preparation, separation of analytes of interest from interferences and required detection levels with existing LC-MS/MS methods in amino acid analysis, we explored the possibility of using a LC-QTOF-MS system run in TOF mode as a higher throughput, simplified, qualitative screen for the detection of metabolic disorders such as PKU in human plasma. The results were analyzed using the Agilent Mass Hunter Qualitative Analysis and compared with an existing quantitative LC-MS/MS method for amino acid measurement.

Methods

- **Sample selection:** 50 human plasma samples with abnormally high levels of Phe (196-1263 µmol/L; age range 0-64 years) and 10 samples with normal concentrations of Phe (45-80 µmol/L; age range 0-43 years).
- **Sample preparation:** 100 µL of the samples were subjected to protein precipitation using equal volume of 0.4N $HClO_4$, shaken and allowed to sit for 15 min at room temperature and filtered using AcroPrep Advance 0.2 µm 94 well filter plates (PALL Life Sciences USA).
- Abnormal samples were diluted 10, 20, 30, 40 and 50 times; normal samples were diluted 30 times using 0.1N HCl before being subjected to LC-QTOF-MS analysis. To account for technical variation each normal sample was injected twice. Area counts of serially diluted abnormally high Phe samples were compared with concentrations of samples measured by a quantitative LC-MS/MS assay ((API 4000 mass spectrometer; using an 18 min HPLC-MS/MS aTRaq™ reagent method).
- **Data analysis:** Data were analyzed with Agilent Mass Hunter Qualitative Analysis (Qual) B.07.00 software using the find by formula search algorithm, comparing results to a customized database containing the analyte of interest. Exact mass match, isotope pattern and spacing, and retention time match were used for identification.

MS Parameters (Agilent 6550 TOF System)

Ion Polarity	Positive
Gas temperature	250ºC
Drying gas	15 L/min
Sheath gas	12 L/min
Nebulizer	45 psi
Sheath gas temperature	400ºC
Capillary voltage	3500V
Nozzle voltage	0V
MS range	50-1000 m/z
Acquisition rate	1 spectra/sec
MS/MS range	NA
Collision energy	NA
Reference mass	121.0509, 149.0233, 322.048, 922.0098
Reference pump flow rate	0.500 mL/min

LC Parameters (Agilent 1290 Infinity LC system)

Analytical column	Intrada Amino Acid 50 mm x 3 mm x 3 µm (Imtakt USA)
Guard column	Pre column 2 µm filter
Column temp	350ºC
Autosampler temp	4ºC
Mobile phase	A: 100mM ammonium formate; B: 0.1% formic acid in acetonitrile
LC conditions (10 min)	0-3 min 14% A, 3.1-10 min 100% A
LC conditions (1 min)	0-1min 14% A, 1.1-1.5 min 100% A
Flow rate	0.6mL/min
Stop time	10 min
Post time	0.5min

Results

- Phe ($C_9H_{11}NO_2$) eluted at 2.56 and 0.92 min using the 10 and 1 min gradient methods respectively. It was detected within a mass and retention time tolerance of ±15 ppm and ±0.05 min in Mass Hunter Qualitative Analysis using Find by Formula search in the specified database (Fig 1A, B, C).
- Linearity of response of serially diluted abnormal Phe samples was found between 20 and 40 factors of dilution with correlation coefficients being 0.77 and 0.89 respectively (Fig 2).
- Comparison of corresponding area counts for Phe for 2 and 40 fold dilution on the LC-QTOF-MS using the 1 and 10 min gradient methods vs concentrations measured by the quantitative LC-MS/MS method showed acceptable correlation (Fig 3A,B).
- The LC-TOF-MS method was able to detect DL-β-aminoisobutyric acid ($C_4H_9NO_2$, retention time 4.66 min, mass tolerance ±17 ppm and retention time tolerance ± 0.05 min) in all samples while the quantitative LC-MS/MS method failed to detect the analyte in 30% of the normal samples (Fig 4).

Figure 1A: Phe in normal patient sample (10 min method)

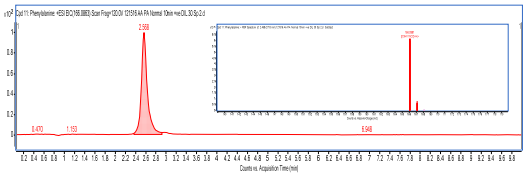


Figure 1B: Phe in abnormal patient sample (10 min method)

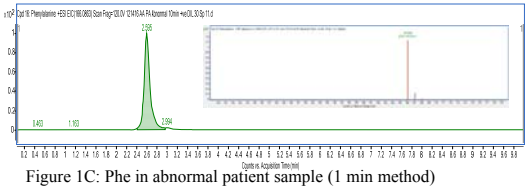


Figure 1C: Phe in abnormal patient sample (1 min method)

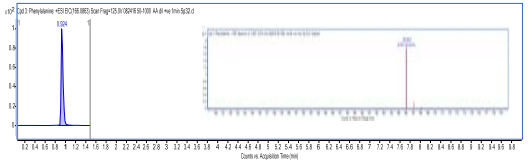


Figure 2: Area count (LC-TOF-MS) in patient samples diluted by 10-50 factors containing abnormally high concentrations of Phe vs concentration of Phe using LC-MS/MS

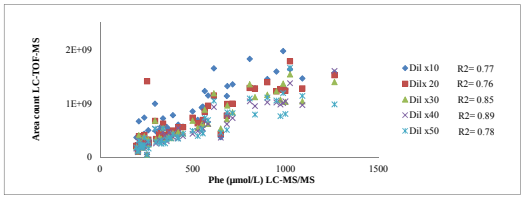


Figure 3A: Correlation between area counts of LC-TOF-MS (1 min method) and concentration of Phe using LC- MS/MS; LC-QTOF-MS = $1.30e^5$ LC-MS/MS + $1.26e^7$, n= 50, r = 0.88, Sy/x = 98.52

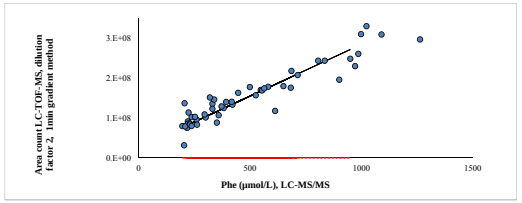


Figure 3B: Correlation between area counts of LC-TOF-MS (10 min method) and concentration of Phe using LC-MS/MS; LC-QTOF-MS = $1.0e^6$ LC-MS/MS -$1.30e^8$, n= 50, r = 0.89, Sy/x = 92.43

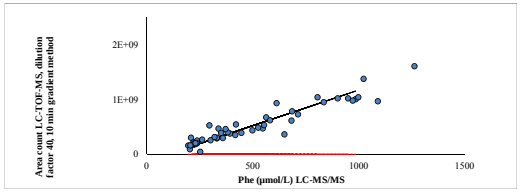
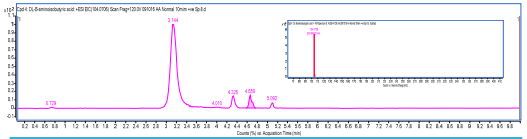


Figure 4: DL-β-aminoisobutyric acid detected at 4.66 min by LC-TOF-MS in normal patient sample



Conclusion

- The described LC-TOF-MS method is characterized by ease of work flow when compared with the LC-MS/MS method.
- There is acceptable agreement between the two methods. Due to the high mass accuracy of the QTOF, the screening technique enables easy method set up without precursor ion selection and fragmentation conditions.
- Use of Q-TOF platform as the screen allows the retrospective interrogation of collected MS without re-injection of sample.

Acknowledgements

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