Clinical Biochemistry 49 (2016) 1047–1050

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Short Communication

Validation of amino-acids measurement in dried blood spot by FIA-MS/MS for PKU management

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ARTICLE INFO

Article history:
Received 4 April 2016
Received in revised form 15 July 2016
Accepted 18 July 2016
Available online 21 July 2016

Keywords:
Mass spectrometry
Method validation
Phenylketonuria
Phenylalanine

ABSTRACT

Objectives: Phenylketonuria (PKU) is a metabolic disorder leading to high concentrations of phenylalanine (Phe) and low concentrations of tyrosine (Tyr) in blood and brain that may be neurotoxic. This disease requires a regular monitoring of plasma Phe and Tyr as well as branched-chain amino-acids concentrations to adapt the Phe-restricted diet and other therapy that may be prescribed in PKU. We validated a Flow Injection Analysis tandem Mass Spectrometry (FIA-MS/MS) to replace the enzymatic method routinely used for neonatal screening in order to monitor in parallel to Phe, Tyr and branched-chain amino-acids not detected by the enzymatic method.

Design and methods: We ascertained the performances of the method: linearity, detection and quantification limits, contamination index, accuracy. We cross validated the FIA-MS/MS and enzymatic methods and we evaluated our own reference ranges to monitor Phe, Tyr, Leu, Val on 59 dried blood spots of normal controls. We also evaluated Tyr, Leu and Val concentrations in PKU patients to detect some potential abnormalities, not evaluated by the enzymatic method.

Results: We developed a rapid method with excellent performances including precision and accuracy $<15\%$. We noted an excellent correlation of Phe concentrations between FIA-MS/MS and enzymatic methods ($p < 0.0001$) based on our database which are similar to references ranges published. We observed that 50% of PKU patients had lower concentrations of Tyr, Leu and/or Val that could not be detected by the enzymatic method.

Conclusion: Based on laboratory accreditation recommendations, we validated a robust, rapid and reliable FIA-MS/MS method to monitor plasma Phe concentrations but also Tyr, Leu and Val concentrations, suitable for PKU management. We evaluated our own reference ranges of concentration for a routine application of this method.

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1. Introduction

Phenylketonuria (PKU) is a metabolic disorder due to a deficiency in phenylalanine hydroxylase (PAH, RC 1.14.16.1), leading to high concentrations of phenylalanine (Phe) and low concentrations of tyrosine (Tyr) in blood and brain. Some countries recommend a lifelong specific phenylalanine-restricted diet; however the diet is frequently discontinued by adult patients, or teens [1,2]. Phe has been reported to cause neurological and neuropsychiatric perturbations, through pathophysiological mechanisms such as amino-acid (AA) imbalance, bioenergetics deficit or oxidative stress [3,4]. The low concentration of tyrosine may also be responsible of brain dysfunction inducing a depletion neurotransmitters. Phenylalanine-restricted diet may also contribute to other AA deficiencies that should be detected to be supplemented in treated patients. The management of PKU patients, including adequate diet and BH4 (tetrahydrobiopterin) administration is thus complex and marked by constraints [5]. Blood Phe concentrations are regularly measured in PKU patients [6,7], using dried spots to facilitate monitoring [8]. Moreover, a full AA profile is usually established once or twice a year for all PKU patients and every trimester during pregnancy, to monitor Tyr and branched-chain amino-acids [8]. The main analytical method available to measure Phe concentrations in dried blood

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http://dx.doi.org/10.1016/j.clinbiochem.2016.07.008
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spot is derived from the neonatal screening method based on an enzymatic technique. The main limitation is that only Phe concentrations are provided. Flow Injection Analysis tandem Mass Spectrometry (FIA-MS/MS) has become a robust technique to screen the inborn errors of metabolism on Guthrie filter cards. However, this technique has not been specifically adapted for PKU management. The heterogeneity of analytical methods to measure Phe and Tyr concentrations, the recommendations for laboratory accreditation as well as the physiological variability of amino acids concentrations led us to perform this present study. According to the French recommendations (COFRAC) of methods validation [9,10], we promoted a standard strategy for analytical validation by FIA-MS/MS to monitor concentrations of Phe, Tyr, Leucine (Leu) and Valine (Val). We compared this method with the enzymatic method and evaluated our own normal concentrations ranges, reflecting our methods of samples collection, pre-treatment and mass spectrometry. Moreover, we evaluated the interest to monitor Leu and Val in routine practice for the management of PKU patients.

2. Material and methods

2.1. Analytical methods

2.1.1. Mass spectrometry method

A targeted metabolic fingerprint strategy by FIA-MS/MS in multiple reaction monitoring (MRM) mode was performed for the determination of amino acids based on Perkin Elmer Neobase Kit. Briefly, dried blood spot (3.2 mm diameter) were extracted with 100 μL of working solution containing the stable isotope labelled mix of internal standards for each analyte of interest (Phe, Tyr Leu, Val) and the extraction solution. The following internal standards were used: $^{13}C_6$-Phe, $^{13}C_6$-Tyr, $^{2}H_3$-Leu, $^{2}H_8$-Val. The plate was then covered and incubated (45 °C) during 45 min, with mixing. The supernatant was analyzed by flow injection analysis mass spectrometry (FIAMS). We used a tandem mass spectrometry detector, a Xevo TQ-D® autosampler (Waters Corporation, Milford, MA 01757 USA). The instrument operates in positive electrospray ionization mode using MassLynx V4.0 Software (Waters) with auto data processing by NeoLynx (Waters Corporation, Milford, MA, USA). The quality controls (QC) were provided by Perkin Elmer with the following concentrations for the low levels (LQC): 199 μM, 337 μM, 390 μM, 428 μM, and for the high levels (HQC): 587 μM, 1077 μM for, 816 μM, 981 μM for Phe, Tyr, Leu, Val, respectively.

2.1.2. Enzymatic method

The Quantase® Neonatal Phenylalanine Screening from Biorad is based on the phenylalanine dehydrogenase enzyme which catalyses the NAD^+ dependent oxidative deamination of L-phenylalanine. The NADH produced is measured indirectly by a colourimetric. Briefly, we extracted the dried blood spot on filter paper (3.2 mm diameter) with 50 μL of Elution Buffer. We added 100 μL of working enzyme reagent in each well and incubated at room temperature during 30 min. Then we added the colour reagent and incubated at room temperature with moderate shaking during 2 min. Absorption was red at 570 nm and 690 nm. For the quantification, the batch contained 1 blank, 4 standards (120; 300; 600; 1200 μM) and 2 quality controls (240 and 840 μM).

2.1.3. Assay validation

Validation was conducted according to the ICH guidelines [11], and French recommendation for laboratory accreditations (COFRAC) [9, 10]. Within-day precision and accuracy of amino-acids concentrations measurements were determined by analysis of 30 replicates of each quality control (HQC and LQC). The inter-assay reproducibility was assessed by repeated analysis of the two QC levels on 30 days, not necessarily consecutive. Precision was reported as CV (%) and accuracy as percent of the nominal value (% deviation). The lower limit of detection (LLOD) and quantification (LLOQ) were calculated by analyzing 30 blank samples (French Norm ISO 15189, Ambruster et al. [12]). The limit of linearity was evaluated by measuring the concentrations of high quality controls after several dilutions. The inter-samples contamination was evaluated by analyzing a high-concentrated sample (840 μM) 3 times consecutively followed by a low-concentrated sample (240 μM). The sequence was repeated 5 times and we calculated the index of contamination as analyzed by a paired Wilcoxon test.

2.2. Application to clinical samples

Before starting this study, we obtained a verbal permission from Persons Protection Committee's president of Tours to use blood spots obtained for routine check up without written consents of patients. No supplementary blood specimen collection was performed for this study. We collected 33 dried blood spots of PKU patients (routinely managed in Tours Hospital) that were analyzed by enzymatic method, and we measured the concentrations of Phe, Tyr, Leu, and Val by mass spectrometry method. We performed a cross validation of Phe concentration measurement.

We also analyzed values of these AA concentrations in 59 control patients, *i.e.* patients explored for acylcarnitines and amino acids profile because of suspected (but not confirmed) metabolic disorders. Based on COFRAC recommendations, we used these data to establish our own reference ranges for the 4 amino-acids (95% confidence interval (CI) of mean concentrations).

2.3. Statistical analysis

We compared the Phe concentrations of 33 dried blood spots from PKU patients between FIA-MS/MS and enzymatic methods. We analyzed the correlation of both methods using Spearman correlation and Bland and Altman plot with intraclass correlation coefficients (ICC). These analyses were performed by R version 2.8.0, an open source program developed by the R Foundation for Statistical Computing (Vienna, Austria). We evaluated the distribution of Phe, Tyr, Leu and Val concentrations in control patients to determine the normal values as mean ± standard deviation.

3. Results

3.1. Linearity and lower limit of quantification

According to the experiments performed with FIA-MS/MS, we obtained an excellent linearity from up to 650 μM, 1180 μM, 780 μM, and 990 μM ($R^2 > 0.98$, $p < 0.001$) for Phe, Tyr, Leu and Val, respectively. We noted a LLOQ at 1.25, 2.73, 4.61, 4.44 μM and LLOD at 0.43, 0.95, 1.47, 1.52 μM for Phe, Tyr, Leu, Val, respectively.

3.2. Precision, accuracy

Performances of accuracy and precision were acceptable according to the objectives of these explorations with all CV and biases <15% (Table 1).

3.3. Contamination

We observed no significant contamination, with an index of contamination of 1.23 for Phe, 0.54 for Tyr, 0.08 for Leu and 0.72 for Val ($p > 0.3$).

3.4. Method comparison

We obtained an excellent correlation between the FIA-MS/MS and the enzymatic methods (R2: 0.95, and $p < 0.0001$). The ICC was 0.95 [0.85–0.99]) and the Bland and Altman analysis confirmed the good correlation between both methods (data not shown).

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