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Comparison of tandem mass spectrometry and amino acid analyzer for phenylalanine and tyrosine monitoring—Implications for clinical management of patients with hyperphenylalaninemia

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ABSTRACT

Objectives: Regular and accurate monitoring of blood phenylalanine (Phe) and tyrosine (Tyr) levels is prerequisite for a successful management of patients with hyperphenylalaninemia (HPA). We aimed to compare the tandem mass spectrometry (MS/MS) and the amino acid analyzer (AAA) as methods to measure blood Phe and Tyr levels and Phe/Tyr ratio.

Methods: Venous blood samples were collected for the AAA analysis, using Pinnacle PCX (Pickering Laboratories), with HPLC Series 1200 (Agilent). Capillary blood was spotted directly on filter paper (Whatman 903) for the MS/MS analysis, using 3200 QTrap AB SCIEX and Perkin Elmer Series 200 HPLC system. The Bland–Altman test was used to compare agreement between the methods and Pearson correlation coefficient to assess the association between the methods.

Results: 207 pairs of measurements were performed. The Phe levels (range 0–2500 μM) obtained by the MS/MS were on average 26.1% (SD 13.9%) lower compared to those obtained by the AAA. The Tyr levels by the MS/MS were on average 15.5% (SD 20.6%) lower. The Phe/Tyr ratio by the MS/MS was on average 10.6% (SD 15.9%) lower. The Pearson correlation coefficients for Phe (range 0–2500 μM), Tyr and the Phe/Tyr ratio were 0.984 ($p < 0.001$), 0.841 ($p < 0.001$) and 0.987 ($p < 0.001$) respectively.

Conclusions: When monitoring blood Phe and Tyr levels in patients with HPA, clinicians need to be informed about the method used. Due to the considerable inter-assay variability, a single method is preferable for long-term follow-up of patients. When using MS/MS, on average 26% lower blood Phe levels were obtained as compared to the AAA. The guidelines and recommendations on HPA management should take into consideration the differences in laboratory methods.

Introduction

Hyperphenylalaninemia (HPA) is clinically most important in the context of phenylketonuria (PKU; MIM 261600), which is the most common inborn error of amino acid metabolism. Most frequently, the PKU is caused by the deficiency of hepatic phenylalanine hydroxylase (PAH; EC 1.14.16.1), which catalyzes the hydroxylation of phenylalanine (Phe) to tyrosine (Tyr) [1,2]. Consequently, various degrees of HPA are present, ranging from mild HPA (MHP), where treatment is frequently not required, to classic PKU, which causes severe mental retardation in untreated patients [3,4]. The Phe-restricted diet is well proven to prevent the neurotoxic effects of HPA if started soon enough after birth [1,4,5].

The dietary treatment of patients with HPA needs to be long-term, imposing a substantial burden on patients and their families [6]. According to most recommendations the blood Phe levels in children should not exceed 360 μM, and the Phe levels should be below 600–700 μM in adolescents and adults (the reference range: 50–120 μM) [6–8]. Moreover, regular monitoring of blood Phe levels and its maintenance within very strict limits (below 260–360 μM) is crucial during pregnancy of patients with HPA to prevent the maternal PKU syndrome [9]. Besides the Phe levels, it is also important to regularly monitor blood Tyr levels in patients with HPA. The Tyr levels indicate the adequacy of the dietary supplementation of Tyr as an essential amino acid [10]. In

Abbreviations: AAA, amino acid analyzer; DBS, dried blood spot; HPA, hyperphenylalaninemia; HPLC, high-performance liquid chromatography; MS/MS, tandem mass spectrometry; PAH, phenylalanine hydroxylase; Phe, phenylalanine; PKU, phenylketonuria; SD, standard deviation; Tyr, tyrosine.

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addition, the blood Phe/Tyr level ratio is lately recognized as a sensitive indicator of metabolic control in patients with HPA where the increased ratios are shown to be associated with cognitive deficits, perhaps even more strongly than increased Phe levels alone [10–12]. Thus, the dietary treatment of patients with HPA goes along with regular monitoring of the Phe and the Tyr levels, where the method of choice needs to be as accurate, simple, rapid and cheap as possible [13]. Guidelines and recommendations on management patients with HPA propose serial monitoring of blood Phe levels weekly in infants and toddlers and later on monthly at least in pre-pubertal children [7].

The most frequently applied method for the quantitative analysis of physiological amino acids in body fluids is ion-exchange chromatography with post-column derivatisation [14]. The ion-exchange chromatography amino acid analyzer (AAA) provides excellent separation and reproducibility with minimal sample preparation. The main disadvantage of the AAA is long run time resulting in low-throughput [15]. Alternative method to the AAA is the tandem mass spectrometry (MS/MS), a powerful technique providing simplicity and speed in the analysis of amino acids and also other small molecules important for diagnostic purposes [16]. The advantage of the MS/MS is simultaneous detection and quantification of multiple analytes from dried blood spot (DBS), which utilizes direct infusion MS/MS. The application does not require an HPLC column, enabling very short run times (approx. 2 min) [15].

Few previous studies comparing the MS/MS to the AAA indicate that the Phe levels are consistently lower when measured by the MS/MS (10–19% difference). However, the precise cause is not elucidated [13,17,18]. Furthermore, scarce data exist on comparisons of the methods for measuring the Tyr levels [18]. Thus, the aims of this study were: to compare the MS/MS, using DBS samples, and the AAA, using plasma samples, as methods for measuring blood Phe and Tyr levels and determining Phe/Tyr ratio; to demonstrate suitability of the MS/MS as a method for diagnosing and monitoring patients with various degrees of the HPA.

Material and methods

Blood sample collection

Venous blood samples (2 mL) were collected into heparinized tubes and plasma was used for the AAA analysis. Capillary blood, obtained from the finger prick collection, was spotted directly on filter paper (Whatman 903) for the MS/MS analysis. Blood spots were allowed to dry at room temperature for at least 4 h before the analysis. Blood samples were obtained on the occasion of ongoing long-term follow-up study of patients with mild HPA and of another study assessing BH4-responsiveness—the later cohort, protocol and results were described previously [19,20]. The samples were derived from altogether 51 patients, followed-up at our center. The study was approved by the National Medical Ethics Committee. Written informed consent/assent was obtained from all the participants and/or their parents.

Phe and Tyr level determination by the MS/MS

The MS/MS measurements were performed using 3200 QTrap AB SCIEX (Framingham, USA) and Perkin Elmer Series 200 HPLC system (Waltham, USA). ESI-positive ionization and MRM mode were used for detection of the Phe and the Tyr using following parameters; ion spray voltage (IS) 5500 V; auxiliary gas temperature (TEM) 550 °C; curtain gas (CUR), nebulizer gas (GS1) and auxiliary gas (GS2) 20, 60 and 60 arbitrary units, respectively; collision gas (CAD) medium; entrance potential (EP) 3.5, 3 V, for the Phe and the Tyr, respectively; declustering potential (DP) 26, 31 V, for the Phe and Tyr, respectively; collision cell entrance potential (CEP) 26, 18 V, for the Phe and the Tyr, respectively; collision energy (CE) 23 V; collision cell exit potential (CXP) 4 V. LC isocratic flow was 100 μL min^{-1} and the run time is 2 min. The application does not require an HPLC column.

DBS samples were refrigerated (2–8 °C) prior the analysis. For the determination of the Phe and the Tyr levels in DBS, Chromsystems MassChrom® Aminoacid and Acylcarnitines from Dried Blood reagent kit was used. The sample preparation was based on extraction followed by derivatisation to butyric esters. The DBS controls Level I and Level II were included in every analytical batch to monitor accuracy and precision within the system. Inter-laboratory variation was assessed by participation in the two external quality control schemes, ERNDIM (www.erdnimqa.nl) and INSTAND (www.instandev.de) [21].

Phe and Tyr level determination by the AAA

The AAA with post-column ninhydrin-derivatization consisted of HPLC pump Series 1200 (Agilent, Tokyo, Japan) and Pinnacle PCX (Pickering Laboratories, Mountain View, USA) was used for the determination of the Phe and the Tyr levels in plasma samples. All buffers and ninhydrin reagents were obtained from Pickering Laboratories, USA. Amino acid standard solution used for calibration was purchased from Sigma (Deisenhofen, Germany) and prepared according to the manufacturer's protocol.

Samples were deproteinized by mixing equal portions of plasma and Seraprep (Pickering Laboratories, Mountain View, USA) and after 5 min incubation at room temperature centrifuged at 10,000 rpm for 5 min. Supernatant was filtered through a 0.2 μm syringe filter (Sartorious Minisart, Germany). Samples were stored at −20 °C prior to the analysis. The injection volume of the sample was 100 μL. The separation program was performed using manufacturer standard protocols. Run time was 120 min.

The accuracy and precision of the determination of the Phe and the Tyr levels in plasma were checked by the analysis of the intra quality control material (pool plasma sample) and amino acid control material (SKML, MCA Laboratories). Inter-laboratory variation was assessed by participation in the ERNDIM (www.erdnimqa.nl) external quality control scheme [21].

Statistical analysis

GraphPad Prism software v6.0 (GraphPad Software Inc., USA) was used for all statistical analyses. To compare both methods for results of the Phe and the Tyr levels and the Phe/Tyr ratio, we calculated the percent difference Bland–Altman test to determine the 95% limits of agreement between the methods. The Pearson correlation coefficient was determined to assess the association between two methods. Additionally, the unpaired *t*-test with Welch correction was used to determine the differences of measurement bias between 20 samples with lowest and highest Phe levels.

Results

Altogether, 207 pairs of measurements of the Phe and the Tyr levels in plasma and DBS were performed by the MS/MS and by the AAA, respectively.

The Phe levels, the Tyr levels and the Phe/Tyr ratio obtained by the MS/MS were each compared to those obtained by the AAA. Firstly, the Bland–Altman analyses were performed, where the percent differences in blood Phe levels, Tyr levels and Phe/Tyr ratio between the two methods, were each plotted against their average blood levels (Fig. 1). For the Phe level range of 0–2500 μM, the Phe levels obtained by the MS/MS were on average 26.1% (SD 13.9%) lower as compared to those obtained by the AAA. For the Phe level range of 40–240 μM, the Phe levels obtained by the MS/MS were on average 22.1% (SD 14.6%) lower as compared to those obtained by the AAA. For the Phe levels higher than 1000 μM, the Phe levels obtained by the MS/MS were on average 34.1% lower as compared to those obtained by the AAA. The