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Short Communication

Optimization of an HPLC method for phenylalanine and tyrosine quantization in dried blood spot

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ABSTRACT

Objectives: Patients affected by Phenylketonuria (PKU) require lifelong management based on phenylalanine (Phe) and tyrosine (Tyr) restricted intake or tetrahydrobiopterin (BH_4) administration. Frequent monitoring of blood concentration of both amino acids during treatment is the key point for clinicians to achieve the best long-term neuropsychological outcome.

Results: The present study develops and validates a rapid and simple method for Phe and Tyr quantization in dried blood spot (DBS) since this specimen has the advantage of being low invasive, easily withdrawn even at home and stable if mail-delivered. The validation studies showed the robustness of the method.

Conclusions: Serum and DBS samples from PKU patients were analyzed and compared, finding a good correlation of Phe and Tyr concentrations between the two different matrixes.

Introduction

Hyperphenylalaninemia (HPA) is one of the most common inborn errors of metabolism caused by a primary defect either in the phenylalanine hydroxylase (PAH) gene or in its cofactor biosynthesis and regeneration (BH_4) with consequent elevated concentration of Phe in plasma and tissue. Besides, increased plasma levels of this amino acid can be secondary to maternal hyperphenylalaninemia [1].

A recent valuation of the PAH-dependent HPA reports an incidence of 1:10,000 and 1:15,000 for the European and the United States population, respectively [2,3].

The spread of newborn screening for Phenylketonuria (PKU) allows one nowadays to start early treatment preventing the severe mental retardation and behavioral disturbance due to the toxic effect for brain of Phe accumulation [4].

The diet therapy, based on a phenylalanine-restricted intake, though must be maintained for life, is, at the moment, the "gold standard" for the PKU management though BH_4-responsive patients have been identified [5]. The mainstay of the treatments is to maintain blood phenylalanine levels within the limits considered safe for the patient's age [6]. So frequent follow-up of patients, above all pregnant women with PKU, is necessary to monitor hyperphenylalaninemia and consequently adequate diet or BH_4 administration.

Many methods have been described for the quantitation of Phe and Tyr levels on serum or plasma for the follow-up of the patients [7–9]. However in recent years the advantage of using dried blood spot (DBS) as specimen for the development of a large number of bioanalytical methods has been highlighted [10] relying on being an only minimal-invasive technique which requires a very small amount of sample (few microliters). Stability of several compounds, amino acids as well, in DBS has been already investigated and ascertained even over a 15 year period [11] allowing storage and shipment at room temperature. As a consequence, methods for the determination of Phe and Tyr in DBS samples by HPLC have been described [12,13] and some studies have examined the validity of different methods (amino acid analyzer, HPLC with fluorescence detection and tandem mass spectrometry) for quantitative analysis of the absolute blood Phe concentration showing a correlation for Phe [14,15]. The purpose of the present paper is to develop a simple and rapid method for the extraction of Phe and Tyr from DBS and to show the correlation between the concentrations observed in the two different specimens in order to switch from one another at the occurrence and to be able to give the exact concentration of these amino acids to the clinicians for the therapy independently from the kind of sample.

Abbreviations: DBS, dried blood spot; HCl, hydrochloric acid; HPA, hyperphenylalaninemia; HPLC, high-performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantitation; OPA-3-MPA, *o*-phthaldehyde-3-mercaptopropionic acid; PAH, phenylalanine hydroxylase; Phe, phenylalanine; PKU, phenylketonuria; S/N, signal-to-noise ratio; Tyr, tyrosine.

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Material and methods

Reagents and chemicals

Amino acid calibration standards in 0.1 M HCl, borate buffer 0.4 M in water, pH 10.2, and *o*-phthaldehyde-3-mercaptopropionic acid (OPA-3-MPA) reagent in borate buffer 10 mg/mL in 0.4 M were obtained from Agilent Technologies (Waldbronn, Germany); sodium dihydrogen phosphate monohydrate RPE and HCl 37% were from Carlo Erba (Milano, Italy), high-performance liquid chromatography (HPLC)-grade methanol and acetonitrile from J.T. Baker (Deventer, Netherlands), L-Phe ≥98% and L-Tyr ≥98%, sodium azide, and sodium hydroxide from Sigma-Aldrich (Steinheim, Germany). Amicon Ultra-4 filters with low-protein-binding regenerated cellulose ultrafiltration membranes (10,000 molecular mass cut-off filters) were purchased from Merck Millipore (Darmstadt, Germany) and the filter paper used for sample collection was 903® Whatman (Dassel, Germany). Water used for HPLC buffers was produced with a Millipore Milli-Q system.

Calibrators and quality control

A 1200 μM calibrator was made by spiking a healthy donor whole blood sample with Phe and Tyr from a 30 mM stock solution prepared in 0.1 M HCl. Subsequently, serial dilutions in water were performed to obtain a calibration curve in the range of 12–1200 μM (the exact concentrations were 12 μM, 60 μM, 300 μM, 600 μM, 1200 μM). The enriched blood samples (20 μL) were then spotted onto a filter paper and let dry overnight at room temperature. A set of homemade DBS, prepared by adding each amino acid to obtain final concentrations of 100, 200, 300, 500 μM, was used as control. Our laboratory is involved in an external quality assurance (EAQ) program. A set of eight serum samples provided once a year by ENRDIM (European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism) [16] is performed for the improvement of accuracy, reliability and comparability of the results of amino acid quantification.

Sample collection and preparation

Peripheral venous blood without anticoagulant and DBS were obtained from a group of patients affected by PKU (n = 25).

Serum samples separated from formed elements by centrifugation at 3000 ×*g* for 10 min were then ultrafiltered at 4000 ×*g* for 20 min at room temperature using Amicon Ultra-4 filters. The ultrafiltrates underwent a precolumn derivatization procedure in the needle of the autosampler with OPA-3-MPA reagent [17] according to the sequence shown in Table 1 and finally injected in HPLC.

A 3.2 mm diameter disk (containing 3.3 μL of blood) [18] was punched from each DBS and extracted with 150 μL of a solution containing methanol/water 50:50 (v/v) for 20 min while shaking. Then, 50 μL of cold methanol (−20 °C) was added. The samples, after being vortexed, were centrifugated at 10,000 ×*g* for 10 min. The supernatant was transferred into conical vial inserts for precolumn derivatization and HPLC analysis as for previous described serum samples. Amino acid concentration was determined from the calibration curve. Phe and Tyr were identified and quantified by their retention time and their absorption ratio comparatively to the ratio of authentic compounds in the calibration solution [19].

Instrumentation

Experiments were performed by using an Agilent Technologies 1200 Series LC System equipped with a binary pump delivery system, an autosampler to automate the pre-column derivatization and injection procedure, a heated column compartment and a programmable fluorescence detector, all of them being controlled from Agilent ChemStation.

Table 1
Injection program for pre-column derivatization.

Function	Amount (μL)	Vial [a]	Reagent
Draw	2.5	From vial 1	Borate buffer
Draw	0.5 (serum)/25 (DBS)	Choose vial position for sample	From sample
Mix × 2 times	3 (serum)/27.5 (DBS)	In air	
Draw	0.0	From vial 2 (needle wash using water in uncapped vial)	
Draw	0.5	From vial 3	OPA
Mix × 6 times, max. speed	3.5 (serum)/28.0 (DBS)	In air	
Draw	0.0	From vial 2 (needle wash using water in uncapped vial)	
Draw	32.0	From vial 4	Water
Mix × 2 times, max. speed	20.0	In air	
INJECT			

[a] The positioning of reagent vials in the autosampler is designed for the listed injector program.

An Agilent Zorbax Eclipse XDB-C18 analytical column 5 μm, 4.6 × 150 mm, was used in conjunction with an Agilent Eclipse XDB-C18 analytical Guard column 5 μm, 4.6 × 12.5 mm for chromatographic separation.

HPLC injection program and gradient

Table 1 summarizes the steps of the set-up injection program. The chromatographic separation was carried out at a flow rate of 1.3 mL/min with a gradient starting from 75% of mobile phase A (40 mM sodium dihydrogen phosphate monohydrate, 0.005% sodium azide, adjusted to pH 7.8 by the addition of sodium hydroxide 10 M) and 25% of mobile phase B (acetonitrile/methanol/water 40:40:20, v/v/v). 54% of mobile phase A was reached within 10 min and 100% of mobile phase B in other 2 min for a total run of 20 min (including wash and re-equilibration). During run the column compartment temperature was maintained at 40 °C. Under these conditions, Tyr and Phe were monitored at excitation wavelength (λ_{ex}) of 340 nm and emission wavelength (λ_{em}) of 450 nm, and eluted at 3.8 min and at 8.5 min, respectively.

Results and discussion

Method validation

The pre-column derivatization and reversed-phase liquid chromatography method applied to serum and urine amino acid assay were validated previously as described by Turnell and Cooper [8]. Validation studies of our DBS-method were performed in order to determine its reliability and robustness. For this purpose linearity, intra- and interday variation and recovery were determined.

Linearity

Quantization of Phe and Tyr was performed in triplicate. The linear correlation between Phe and Tyr peak areas and nominal concentrations was assessed in the range of 12–1200 μM. The resulting r^2 shows an excellent fit of the obtained data to the regression line. Table 2 shows mean values ± standard deviations (SD) of slope and r^2.

Intra- and interday variation

To establish intra- and interday coefficients of variation five replicates of control DBS at four different concentrations of both Phe and Tyr were performed within the same day and in five consecutive days