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Clinical relevance of the discrepancy in phenylalanine concentrations analyzed using tandem mass spectrometry compared with ion-exchange chromatography in phenylketonuria

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ABSTRACT

Introduction: Metabolic control of phenylketonuria (PKU) and compliance with the low-phenylalanine (phe) diet are frequently assessed by measuring blood phe concentrations in dried blood spots (DBS) collected by patients instead of plasma phe concentrations.
Objective: Our objective was to investigate the difference in blood phe concentrations in DBS collected by subjects and analyzed using either a validated newborn screening tandem mass spectrometry (MS/MS) protocol or ion-exchange chromatography (IEC) compared to plasma phe concentrations obtained simultaneously and analyzed using IEC.
Design: Three to four fasting blood samples were obtained from 29 subjects with PKU, ages 15–49 years. Capillary blood was spotted on filter paper by each subject and the DBS analyzed using both MS/MS and IEC. Plasma was isolated from venous blood and analyzed using IEC.
Results: Blood phe concentrations in DBS analyzed using MS/MS are 28% ± 1% ($n = 110$, $p < 0.0001$) lower than plasma phe concentrations analyzed using IEC resulting in a blood phe concentration of 514 ± 23 μmol/L and a plasma phe concentration of 731 ± 32 μmol/L (mean ± SEM). This discrepancy is larger when plasma phe is >600 μmol/L. Due to the large variability across subjects of 13.2%, a calibration factor to adjust blood phe concentrations is not recommended. Analysis of DBS using IEC reduced the discrepancy to 15 ± 2% lower phe concentrations compared to plasma analyzed using IEC ($n = 38$, $p = 0.0001$). This suggests that a major contributor to the discrepancy in phe concentrations is the analytical method.
Conclusion: Use of DBS analyzed using MS/MS to monitor blood phe concentrations in individuals with PKU yields significantly lower phe levels compared to plasma phe levels analyzed using IEC. Optimization of current testing methodologies for measuring phe in DBS, along with patient education regarding the appropriate technique for spotting blood on filter paper is needed to improve the accuracy of using DBS to measure phe concentrations in PKU management.

1. Introduction

Phenylketonuria (PKU, OMIM 261600), an autosomal recessive metabolic disorder caused by loss of function mutations of the gene encoding phenylalanine hydroxylase (EC 1.14.16.1, PAH), is characterized by hyperphenylalaninemia due to an inability to convert phenylalanine (phe) to tyrosine (tyr). Untreated PKU is typically characterized by elevated blood phe concentrations and severe cognitive impairment. Introduction of a low-phe diet shortly after birth and maintained lifelong is necessary to prevent cognitive impairment, seizures, eczema, behavior abnormalities, maternal PKU syndrome and other symptoms associated with untreated PKU [1]. The low-phe diet provides the cornerstone of PKU management by reducing phe levels and its metabolites in body fluids and protecting the brain [2,3]. Current recommended treatment for individuals with PKU of all ages in the United States [4] includes a low-phe diet with a goal of maintaining "generic" blood phe concentrations in the range of 120–360 μmol/L "specifically referencing the values

Abbreviations: PKU, phenylketonuria; pah, phenylalanine hydroxylase; phe, phenylalanine; AAA, amino acid analyzer; MS/MS, tandem mass spectrometry; IEC, ion-exchange chromatography; tyr, tyrosine; DBS, dried blood spot.

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obtained by classic venipuncture and amino acid analyzer (AAA) or HPLC analysis" (personal communication, Jerry Vockley). Thus, regular monitoring of blood phe levels to monitor dietary compliance and help assure good metabolic control is an essential aspect of the clinical care for PKU.

Several testing methodologies are available to measure concentrations of phe and tyr for identification, diagnosis and management of PKU. Determination of the free amino acid profile in deproteinized plasma samples using ion-exchange chromatography (IEC) with an AAA is considered the gold standard for diagnosis and management of PKU, as well as other disorders of amino acid metabolism [5]. Newborn screening, implemented in the United States in the 1960s, currently uses tandem mass spectrometry (MS/MS) to measure phe in dried blood spots (DBS) as a means of identifying patients at risk for PKU [6]. Measurement of phe concentrations in DBS with MS/MS has several advantages over plasma analysis because it is easier to obtain and transport than liquid specimens, the DBS sample preparation is minimal, phe and tyr are stable in DBS [7], and MS/MS offers a short analysis time and lower costs. Because of these advantages, many clinics are measuring phe concentrations in DBS specimens using newborn screening MS/MS protocols as a means for monitoring metabolic control in PKU patients.

A limited number of studies suggest lower accuracy of phe concentrations analyzed in DBS by MS/MS compared with analysis of phe in plasma samples [8–10]. The two studies most relevant to our approach conducted analyses of venous plasma and DBS sample pairs obtained at the same time from PKU subjects where trained staff spot the filter paper cards [8,9]. Results indicate that, when trained staff spot the filter paper cards, phe concentrations are consistently lower by 19–26% when measured in DBS and analyzed using MS/MS compared with phe concentrations in venous plasma analyzed using IEC [8,9]. There are no reports in the literature that reflect the clinical environment where patients prick their own finger and spot the capillary blood on the filter paper. The reported discrepancies of lower blood phe levels using MS/MS compared with plasma analyzed using IEC are likely to be higher when patients, instead of trained technicians, spot the filter paper with capillary blood, consistent with evidence of home blood glucose monitoring in diabetes mellitus [11].

Our objective was to investigate the difference in blood phe concentrations in DBS collected by patients and analyzed using either a validated newborn screening MS/MS protocol or IEC compared to plasma phe concentrations obtained at the same time as DBS and analyzed using IEC. We observed that blood phe concentrations are 28% lower in DBS analyzed using MS/MS compared with plasma phe concentrations analyzed using IEC. This discrepancy was reduced to 15% lower phe concentrations compared to plasma when DBS were analyzed using IEC. Lastly, a reliable calibration factor for adjusting the blood phe levels to better reflect plasma phe concentrations cannot be determined due to the large variability across subjects of 13.2% associated with subjects spotting the filter paper cards.

2. Methods

2.1. Study participants and experimental design

The University of Wisconsin-Madison Health Sciences IRB approved the protocol as part of our clinical trial to assess the nutritional management of PKU. The trial was registered at www.clinicaltrials.gov as NCT01428258. All 29 subjects had a diagnosis of PKU that required management with low-phe medical food. The subjects included 12 males and 17 females, 27.2 ± 8.6 years of age (mean ± SD). Three to four fasting blood samples were obtained from each of the 29 subjects with PKU over a period of 10 weeks, n = 110 total sample size. Blood was collected (5 mL) into a tube with EDTA and plasma was isolated and then analyzed using IEC (plasma-IEC). Immediately after the venous puncture was performed, subjects were asked to prick their fingers and spot the capillary blood on a filter paper card for analysis of DBS using MS/MS (DBS-MS/MS). A sub-study including 16 of the 29 subjects was conducted, using the original samples, to compare phe measurement using three methods (n = 38 for each method); 1) Plasma-IEC, 2) DBS-MS/MS, and 3) blood phe extracted from DBS and analyzed using IEC (DBS-IEC). All analyses were conducted in the Wisconsin State Laboratory of Hygiene (Madison, WI) consistent with the standard of care for patients with PKU in Wisconsin. The Wisconsin State Laboratory of Hygiene is accredited by the College of American Pathologists (CAP) and participates in proficiency testing administered through CAP and the Centers for Disease Control for data quality and surveillance.

2.2. Plasma amino acid analysis using IEC

The Hitachi High-Technologies L-8900 Amino Acid Analyzer (Tokyo, Japan) and corresponding buffer components for the instrument were used for separation and quantitation of amino acids in plasma [5]. Frozen plasma samples were thawed and analyzed at the same time for each of the 3–4 samples obtained from the subjects. A 150 μL aliquot of plasma was mixed with 15 μL of a 35% sulfosalicyclic acid solution. After vortexing and centrifugation for 14,000 × *g* for 3 min, the supernatant was filtered using a 1 cm^3 syringe with a 0.2 μm syringe filter. The eluent was mixed 1:1 with 4 nmol aminoethylcysteine internal standard and then 20 μL was injected onto the ion-exchange column. Amino acids were selectively eluted from the column by buffers of increasing pH along with a programed method of varying flow rates and temperatures. After elution, ninhydrin was mixed with the buffer–amino acid solution, heated to develop the purple color and read at 570 nm for amino acids phe and tyr. The total run time was 2.5 h per sample. The concentration of each amino acid was calculated by comparing the peak areas of the amino acid to the peak area of the internal standard, aminoethylcysteine.

2.3. Dried blood spot amino acid analysis using IEC

The Hitachi L-8900 Amino Acid Analyzer and corresponding buffer components for the instrument were used for separation and quantitation of amino acids in DBS. Four 3 mm (1/8th inch) punches (equivalent to 12.4 μL of blood) were removed from the DBS and placed into a 0.2 μm filter tube. Twenty five microliters of a 10% sulfosalicylic acid solution was added to the filter tube along with 100 μL of 4 nmol aminoethylcysteine internal standard diluted in water. The tube was vortexed for 15–20 s and then centrifuged at 14,000 × *g* for 3 min. to allow the eluent to flow into the collection vial. The eluent was then removed from the collection vial and placed over the filter containing the DBS. Again the tube was vortexed for 15–20 s and then centrifuged at 14,000 × *g* for 3 min. This step was repeated for a total of 3 times to maximize extraction of amino acids from the filter paper. Afterwards, the 20 μL solution was injected onto the ion-exchange column and amino acids were selectively eluted from the column using the same method as plasma amino acid analysis. The total run time was 2.5 h per sample. Phe and tyr were quantified by comparison of the peak areas to the internal standard, aminoethylcysteine, with adjustments made for the amount of blood contained within the DBS.

2.4. Dried blood spot amino acid analysis using MS/MS

The routine, newborn screening, non-derivatized flow-injection analysis—tandem mass spectrometry (FIA-MS/MS) method was used for quantification of phe and tyr in DBS [12]. Amino acids were extracted from a 3 mm (1/8th inch) punch of the DBS (equivalent to 3.1 μL of blood) after the addition of 100 μL of methanol containing internal standards for phe ($^{13}C_6$-Phenylalanine) and tyr ($^{13}C_6$-Tyrosine) followed by shaking at room temperature for 10 min. The AB Sciex API 4000 tandem mass spectrometer with a TurboV electrospray ionization source was operated in positive-ionization mode (Framingham, MA). The mobile