Phenylketonuria: Impact and Implications

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CHAPTER

Monitoring phenylalanine levels in phenylketonuria: differences between capillary blood spot and plasma analysis

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Submitted
ABSTRACT

Introduction
Phenylketonuria (PKU) is an autosomal recessive inborn error of phenylalanine (Phe) metabolism. Adherence to the protein restricted diet, aiming to maintain blood Phe levels within the advised range, is the strongest determinant for cognitive outcome. Frequent blood Phe monitoring, usually by analysis of capillary blood spots, is necessary to ensure that patients remain under good metabolic control. The aim of this study was to investigate the difference in Phe levels between plasma, analyzed by amino acid analyzer (AAA), and blood spots analyzed by tandem mass spectrometry (MS).

Methods
Fifteen adult patients participated, of whom five were studied twice. Three different methods were compared: 1) A heparinized whole blood sample was collected via venipuncture for determination of Phe in plasma; 2) before processing of this sample, a blood spot was prepared from the venous blood sample by pipetting on a filter card; 3) immediately after the venipuncture, a finger prick was done and a capillary blood spot was prepared. Plasma samples were analyzed by AAA, blood spots by tandem MS. Outcome measures were: difference in mean Phe concentration measured in plasma and blood spots from either venous blood or capillary blood.

Results
The mean Phe concentration in blood spots obtained from capillary blood and from venous blood were 40 μmol/L ($p = 0.032$) and 97 μmol/L ($p = 0.001$) lower respectively than the mean Phe concentration measured in plasma.

Conclusion
The observed significant differences in Phe levels due to different sampling techniques, warrant caution and the use of one and the same method during follow-up of individual PKU patients is advised.
INTRODUCTION

Phenylketonuria (PKU; MIM 261600) is an inborn error of phenylalanine metabolism in which, as a result of a deficiency of the enzyme phenylalanine hydroxylase (PAH; EC 1.14.16.1), phenylalanine (Phe) cannot be hydroxylated to tyrosine. If untreated, PKU leads to profound intellectual disability and neurological abnormalities. These sequelae can largely be prevented by early initiation of a protein restricted diet with supplementation of all amino acids except Phe. Management of Phe levels, aiming to maintain levels within the advised range by strict adherence to the diet, is the strongest determinant for the cognitive outcome of the patients (1-3). Therefore, regular monitoring of blood Phe levels is necessary to ensure that patients remain under good metabolic control. Several methods are available for the measurement of Phe levels. In many metabolic centers, including ours, patients collect capillary blood samples by finger prick at home at regular intervals, and send the dried blood spots by mail to the laboratory for determination of Phe concentration by tandem mass spectrometry (tandem MS). However, when patients visit their metabolic physician in the outpatient clinic, Phe levels are often determined in blood obtained by venipuncture. Plasma from the heparinized blood sample is analyzed either by the amino acid analyzer (AAA), when a whole amino acid profile is required, or by tandem MS, when only a Phe level is required. Few studies have examined the variability between these different methods for the quantitative analysis of blood Phe concentrations. Previously it was shown that Phe levels in venous blood spotted on filter paper and measured by tandem MS, were consistently almost 20% lower than Phe levels in plasma measured by the AAA (4). Differences between the various methods of Phe determination can be of clinical importance, especially in young children and pregnant women, in whom Phe levels should be maintained within a tight range. Differences in Phe levels due to different sampling and assay techniques may complicate the strict control of PKU patients and the assessment of effects of dietary adjustments. The aim of the present study was to evaluate the difference in Phe levels between plasma, analyzed by AAA, and blood spots obtained from a finger prick (capillary blood) and from venous blood, analyzed by tandem MS.
Chapter 7

PATIENTS AND METHODS

Patients
All patients aged 18 years and older, who are treated for PKU in the Academic Medical Center of Amsterdam (AMC), were informed of the study by mail and during the routine outpatient clinic visits. Patients signed an institutionally approved informed consent prior to enrolment. Approval for this study was granted by the Ethical Committee of the AMC, Amsterdam.

Sample preparation and analysis
At the time of an outpatient visit, a whole blood sample was collected via a venipuncture in a heparinized tube and within 5 minutes after collection, a blood spot was made via a finger prick on a filter paper. Subsequently, a blood spot was prepared from the venous blood sample by pipetting 40 µl of whole blood on a filter card prior to centrifugation for separation of the plasma.

Analysis of plasma amino acids
Plasma was prepared from heparinized blood by centrifugation at 1000 x g and stored at -20°C until analysis. Plasma amino acids were analyzed using the JEOL AminoTac amino acid analyzer JLC-500/V according to the manufacturer’s instructions.

Table 1 Mean, standard deviation, median, range of different methods of Phe determination

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe plasma (µmol/L)</td>
<td>654</td>
<td>417</td>
<td>659</td>
<td>27-1392</td>
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<tr>
<td>Phe BSP cap (µmol/L)</td>
<td>614</td>
<td>378</td>
<td>630</td>
<td>21-1223</td>
</tr>
<tr>
<td>Phe BSP ven (µmol/L)</td>
<td>557</td>
<td>330</td>
<td>561</td>
<td>19-1029</td>
</tr>
<tr>
<td>Tyr plasma (µmol/L)</td>
<td>74</td>
<td>45</td>
<td>70</td>
<td>16-183</td>
</tr>
<tr>
<td>Tyr BSP cap (µmol/L)</td>
<td>67</td>
<td>42</td>
<td>65</td>
<td>21-177</td>
</tr>
<tr>
<td>Tyr BSP ven (µmol/L)</td>
<td>64</td>
<td>40</td>
<td>56</td>
<td>17-160</td>
</tr>
</tbody>
</table>

*Tandem MS analysis of Phe in blood spots
Blood spots were stored at 4°C. The method used to analyze Phe was essentially as described previously (5). Briefly, a blood spot punch was extracted using a methanolic
solution containing known concentrations of stable isotope-labeled internal standards (d₅-Phe and d₄-Tyr). The extract was taken to dryness using N₂ and the residue was butylated for 15 min at 60°C. Subsequently, the butylation reagent was removed under N₂-flow and the residue resuspended in 100 µl of 7:3 acetonitril:water. An aliquot of 10 µl of this solution was used for loop injection tandem mass spectrometry on a Waters Quattro – Premier XE tandem mass spectrometer.

Table 2 Difference between plasma and blood spot analysis of Phe/Tyr levels

<table>
<thead>
<tr>
<th></th>
<th>Difference</th>
<th>95% CI</th>
</tr>
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<tbody>
<tr>
<td>Phe: plasma vs BSP cap*</td>
<td>39.7</td>
<td>5.4 – 74.0</td>
</tr>
<tr>
<td>Phe: plasma vs BSP venb</td>
<td>96.6</td>
<td>42.1 – 151.1</td>
</tr>
<tr>
<td>Phe: BSP cap vs BSP ven'</td>
<td>57</td>
<td>21.2 – 92.5</td>
</tr>
<tr>
<td>Tyr: plasma vs BSP cap*</td>
<td>6.9</td>
<td>2.0 – 11.9</td>
</tr>
<tr>
<td>Tyr: plasma vs BSP venb</td>
<td>9.5</td>
<td>1.4 – 17.6</td>
</tr>
<tr>
<td>Tyr: BSP cap vs BSP ven'</td>
<td>2.6</td>
<td>-4.0 – 9.2</td>
</tr>
</tbody>
</table>

* Phe/Tyr plasma versus Phe/Tyr measured from capillary dried blood spots  
* Phe/Tyr plasma versus Phe/Tyr measured from dried blood spots from venous blood  
* Phe/Tyr measured from capillary dried blood spots versus Phe/Tyr measured from dried blood spots from venous blood

Statistical analysis

The Statistical Package for Social Sciences (SPSS) Windows version 16.0 was used for the analyses. Since the data were not normally distributed the comparisons between the different parameters were tested non-parametrically by Wilcoxon signed-rank-tests.

Alpha was set at 0.05. For the creation of the Bland-Altman plots, to compare the different methods of Phe determination, the statistical software Analyse-it for Excel was used.

RESULTS

A total of 20 venous blood samples, 20 blood spots prepared from the venous blood sample and 20 capillary blood spots were collected. 15 patients consented to participate in the study, 6 males and 9 females with a mean age of 28 years (range 21-34 years). Five patients (1 male and 4 females) were studied twice, on two separate outpatient clinic visits. Results of the Phe analysis in these samples are summarized in Table 1. Table 2 shows the differences between the different methods of Phe
The comparison of the plasma Phe values with the Phe values from capillary blood spots, showed a mean difference (bias) of 39.7 µmol/L with limits of agreement (mean difference ± 2 SD) from 183.4 to -103.9 µmol/L. The Bland-Altman plot of the plasma Phe values with the Phe values from venous blood spots, showed a mean difference of 96.6 µmol/L with limits of agreement from 324.9 to -131.8 µmol/L.

The mean Phe level measured in the capillary blood spots was approximately 40 µmol/L (6%) lower than the mean Phe level measured in the plasma samples ($z = -2.15, p = 0.032, r = -.34$). In addition, Phe levels measured in blood spots spotted from the venous blood, were approximately 97 µmol/L (15%) lower than plasma Phe level ($z = -3.34, p = 0.001, r = -.53$) and also 57 µmol/L (9%) lower than the Phe level measured in the capillary blood spots ($z = -2.80, p = 0.005, r = -.44$).

**Figure 1a** Phe level: plasma versus capillary blood spot
Differences between blood spot and plasma Phe analysis

**DISCUSSION**

In this study we evaluated differences between plasma Phe measurements measured by AAA and capillary and venous blood spots by tandem MS. We established a significant difference between mean plasma Phe level and the mean Phe level, measured in blood spots from capillary blood as well as in blood spots from venous blood. As both analyses are carefully monitored in quality control programs and yield (within the small methodical variation for both techniques) the same Phe concentrations, the observed differences are likely due to the type of blood sample, rather than to the type of analysis (tandem MS versus AAA).

Our findings correspond with previously published studies which showed that Phe levels in blood spots from venous blood were lower than plasma Phe values, measured by AAA (4,6). Our observation that the mean Phe levels in capillary blood spots are also significantly lower than the mean plasma Phe is probably even more important, as the procedure of taking capillary blood samples by finger prick at home is used in many centers as the method of choice for follow-up of PKU patients. This result is in agreement with a previous study, in which a mean difference of 73 µmol/L was found between plasma Phe values measured with AAA and capillary.
blood spots with limits of agreement from 216 to -363 µmol/L (7). Although there is a general underestimation of Phe concentrations from capillary blood spots, the Bland Altman plots demonstrate that the differences between the blood spot and plasma samples are variable and therefore it is not possible to apply a standard correction factor that can be used in clinical practice.

Home capillary blood sampling is currently the most convenient, relatively easy, and least time consuming method for the regular follow-up of PKU patients, and thus used as a basis for frequent adjustment of dietary therapy. In many centers, including ours, however, Phe levels measured at the regular visits to the outpatient clinic are performed in plasma (8).

The observed mean difference in Phe levels of 40 µmol/L between finger prick capillary blood and plasma can be clinically relevant, especially during periods when very strict control is paramount, such as during the first years of life and, in females, during pregnancy. Not taking these differences into account may result in unnecessary and confusing changes in daily protein intake. We conclude that it is strongly advised to adjust the dietary therapy only to Phe levels determined with one method; either from plasma samples, analyzed with the AAA, or from capillary blood spots, analyzed by tandem MS.
REFERENCES


