Monitoring of Phenylalanine Levels in Patients with Phenylketonuria Using Dried Blood Spots: a Comparison of Two Methods

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Abstract

Phenylketonuria (PKU) is caused by deficient activity of phenylalanine hydroxylase (PAH), responsible for the conversion of phenylalanine (Phe) to tyrosine (Tyr). Monitoring of patients with PKU requires the measurement of Phe in plasma using high-performance liquid chromatography (HPLC) or in dried blood spots (DBS) using different techniques to adjust treatment strategy. The objective of this study was to evaluate Phe levels in DBS measured by two different methods and compare them with Phe levels measured in plasma by HPLC. We analyzed 89 blood samples from 47 PKU patients by two different methods: fluorometric method developed in-house (method A) and the commercially available PerkinElmer® Neonatal Phenylalanine Kit (method B) and in plasma by HPLC. The mean Phe levels by method A, method B, and HPLC were 430.4±39.9μmol/L, 439.3±35.4μmol/L, and 442.2±41.6μmol/L, respectively. The correlation values between HPLC and methods A and B were 0.990 and 0.974, respectively ($p < 0.001$ for both). Our data suggest that methods A and B are useful alternatives for monitoring Phe levels in patients with PKU, with method A being in closer agreement with the reference standard (HPLC).

Keywords

Phenylketonuria, treatment follow-up, fluorometric assay.

Introduction

Phenylketonuria (PKU) is an inborn error of metabolism caused by deficient activity of phenylalanine hydroxylase (PAH), which is responsible for the hydroxylation of phenylalanine (Phe) to tyrosine (Tyr) [1–3]. Excess levels of Phe have toxic effects on the central nervous system, leading to neurological manifestations such as cognitive impairment [4]. PKU is often diagnosed by neonatal screening, and treated with a Phe-restricted diet, and essential amino acid supplementation, in conjunction with laboratory and clinical monitoring [5]. The target Phe levels recommended by Brazilian clinical protocols and therapeutic guidelines for the treatment of PKU are 120 -360 μmol/L for patients aged 0-12 years and 120 -600 μmol/L for patients aged >12 years [6]. However, the European guidelines on PKU recommend target Phe levels of 120 -360 μmol/L for patients <12 years of age and 120 to 600 μmol/L for patients ≥12 years of age [7]. The American College of Medical Genetics and Genomics guideline recommends that Phe levels should be maintained in the range of 120-360 μmol/L in patients of all

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ages [8]. Nonetheless, Phe levels should be monitored throughout the patient's life as they reflect patient adherence to treatment.

The aim of the present study was to evaluate two different methods for monitoring Phe levels in patients with PKU, a fluorometric method developed in-house (adapted from Lubenow et al. 1994) [9] (method A) and the commercially available PerkinElmer® Neonatal Phenylalanine Kit (method B), both based on the quantitative determination of Phe in dried blood spots (DBS), by using Phe levels measured in plasma by high-performance liquid chromatography (HPLC) as the reference standard.

Materials and Methods

Currently the outpatient clinic of Hospital de Clínicas de Porto Alegre (HCPA), Southern Brazil, follows about 80 patients with PKU. Based on this number and using Fisher's Z-test for sample size calculation, a sample size of 46 patients was required for our tests. We were able to analyze 89 DBS samples from 47 patients with PKU (mean age 10.8 years) (Table 1).

DBS samples were obtained by spotting filter paper cards with heparinized whole blood. The cards were dried at room temperature for 4 h and then placed in plastic bags with silica gel for storage at –80°C until analysis. The whole blood samples were centrifuged, and the plasma obtained was subjected to HPLC analysis. HPLC analysis was made on the same or next day of the collection. DBS samples analyzes were made with samples stored in freezer -80°C for two weeks maximum.

This study was approved by the local research ethics committee and conducted in accordance with all applicable regulatory requirements.

Table 1. Sample description and assay results.

Patient	Sample ID	Sex	Age (Months / Years)	PKU Type	HPLC (μmol/L)	Method A (μmol/L)	Method B (μmol/L)
1	1	F	16y	Mild	345.8	349.6	389.0
2	2A	M	3y	Mild	24.2	29.3	73.9
	2B		4y		429.7	458.9	558.3
3	3A	F	5m	ND	212.6	240.4	186.6
	3B		5m		330.2	323.3	237.3
	3C		9m		146.2	130.3	146.0
4	4	M	10y	Mild	683.0	750.2	685.1
5	4A	F	9m	ND	355.7	344.1	384.7*
	5B		1y		627.3	524.4	407.6
	5C		1y		663.9	691.9	824.8
6	6	F	18y	Mild	419.3	453.4	539.7
7	7A	M	3y	Classic	242.2	248.8	200.0
	7B		3y		60.3	54.5	83.9
8	8	M	6y	Mild	109.4	92.8	143.2
9	9A	M	2y	ND	268.9	224	216.7
	9B		3y		52.9	52.4	82.8
10	10A	F	2y	Mild	36.0	53.7	79.1
	10B		2y		201.3	195.5	154.2
	10C		2y		207.6	202.1	166.0
11	11	F	20y	Classic	543.3	535.3	548.2
12	12	M	20y	ND	852.4	844.9	886.0
13	13A	F	5y	Mild	483.9	518.9	584.2
	13B		5y		17.1	34.2	70.5
	13C		5y		345.5	338.7	266.5
	13D		5y		423.4	401.9	384.5
	13E		5y		364.5	382.4	343.0*
14	14	M	22y	ND	1036.3	1092.5	984.8
15	15	M	23y	ND	729.2	626.4	777.0
16	16	M	18y	Classic	520.0	540.8	831.0*

Table 1. Cont.

Patient	Sample ID	Sex	Age (Months / Years)	PKU Type	HPLC (µmol/L)	Method A (µmol/L)	Method B (µmol/L)
17	17A	M	4y	Classic	18.2	34.5	56.8
	17B		4y		212.4	184.6	137.3
	17C		4y		373.8	355.1*	325.8*
18	18	M	37y	Classic	607.9	573.6*	673.3
19	19A	F	8y	Mild	375.8	371.5	661.0
	19B		8y		394.1	387.8	575.5
	19C		8y		297.3	244.4	273.7
	19D		8y		488.1	443.6	473.2
	19E		8y		236.5	211.8	176.3
	19F		8y		386.8	382.4	368.9
20	20A	F	5y	ND	483.7	442.5	467.6
	20B		5y		199.8	146.6	162.4
21	21A	F	1m	ND	2512.6	2316.1	1795.1
	21B		1m		883.6	917.7	799.9
	21C		1m		15.5	12.7	48.7
	21D		1m		2.9	16.4	43.7
	21E		1m		93.0	90.6	89.0
	21F		2m		99.4	63.3	97.9
	21G		2m		805.4	782.0	601.3
	21H		2m		208.9	211.8	148.9
	21I		3m		178.2	184.6	157.5
	21J		3m		349.2	304.1	439.4*
22	22A	F	16y	Classic	1188.0	1012.4	1027.7
	22B		17y		959.9	963.0	932.8
23	23A	M	9m	ND	25.6	23.9	57.3
	23B		9m		162.9	152.1	123.3
	23C		11m		22.4	27.4	74.1
	23D		11m		56.5	73.4	83.0
	23E		1y		88.1	71.0	104.8
24	24	M	19y	Classic	739.6	695.1	624.4
25	25	M	9y	Mild	253.8	271.5	247.6
26	26A	F	10y	Classic	404.8	374.7	458.2
	26B		10y		445.9	499.6	505.9
27	27A	M	6y	Classic	395.5	385.6	484.2
	27B		6y		489.4	373.0	491.4
28	28	F	22y	Mild	302.1	336.7	226.0
29	29	F	18y	Mild	397.0	309.5	532.2
30	30	M	17y	Classic	340.3	342.8	469.8
31	31	M	24y	Mild	740.7	753.1	676.4
32	32A	M	32y	Classic	1182.2	1180.3	782.4
	32B		32y		1045.0	1045.0	1213.3
33	33	F	5y	Classic	360.1	385.6	364.7

Table 1. Cont.

Patient	Sample ID	Sex	Age (Months / Years)	PKU Type	HPLC (μmol/L)	Method A (μmol/L)	Method B (μmol/L)
34	34	F	25y	Classic	1470.9	1310.6	1208.4
35	35A	F	7y	Classic	213.5	233.5	204.3
	35B		7y		259.0	217.2	190.8
	35C		7y		135.8	114.0	123.8
	35D		7y		333.3	316.8	321.5
36	36A	M	5y	Mild	366.4	396.4	446.6
	36B		5y		490.2	458.9	564.1
37	37	F	20y	Classic	1054.3	1071.6	801.7
38	38	F	65y	Classic	1028.6	1057.2	1002.1
39	39	M	64y	Classic	714.7	782.0	786.3
40	40	F	62y	Classic	613.2	658.9	598.4*
41	41	M	6y	Mild	149.1	107.4	111.5
42	42	F	2y	ND	30.8	23.7	60.3
43	43	F	5y	Classic	440.4	475.2	572.7
44	44	M	21y	Classic	1131.9	1099.8	1345.3
45	45	M	34y	ND	147.8	136.6	163.1
46	46	F	27y	Mild	797.4	742.9	819.1
47	47	M	31y	Mild	426.7	409.7	488.0

PKU: phenylketonuria; HPLC: high-performance liquid chromatography; F: Female; M: Male; m: month; y: year; ND: not defined. HPLC normal range: <1 month (38-137 μmol/L); >1 month and <2 years (31-75 μmol/L); >2 years and <18 years (26-91 μmol/L); >18 years (42-74 μmol/L). * Samples that did not agree; European treatment goals: <12 years (120-360 μmol/L); ≥12 years (120-600 μmol/L).

In-house Fluorometric Method (method A)

This method was adapted from the work done by Lubenow et al. 1994.

Reagents

All reagents were prepared with reagent water. Methanol was supplied by JT Baker. Acetone, copper sulfate and sodium carbonate were supplied by Synth. Succinic acid was supplied by Merck. Ninhydrin, L-leucyl-L-alanine and Phe were supplied by Sigma. Sodium potassium tartrate was supplied by Modern Chemistry.

Calibration Curve

A heparinized whole blood sample was collected and a 10mM Phe standard was prepared for the calibration curve. Concentrations of 60, 190, 320 and 670 μmol/L were used, and 30 μL of each standard concentration with whole blood was pipetted onto each spot of the filter paper cards. The cards with were dried at room temperature for 4 h, placed in plastic bags with silica gel, and stored at –80°C. The stability of the standards was tested by different assays. A piece of blank filter paper (3 mm in diameter without blood was used for blank determination

Assay

The blank filter paper and 3-mm spots containing standards were punched in duplicate, using a DBS Puncher (1296-071, DELFIA, PerkinElmer®) and placed into a 96-well V-bottom microplates. After the addition of 28 μL of protein precipitation solution (3.5 mL of methanol, 3.5 mL of acetone, and 1 mL of water for injection), the microplates were incubated for 30 min at 60°C (Trinest Incubator, PerkinElmer®) with shaking at 400 rpm . The microplates were left uncovered to allow complete evaporation. Then, 145 μL of water for injection was added and the microplates were covered with a plate sealer and incubated for an additional 20 min at 60°C. After incubation, 38 μL of the solution was pipetted and transferred to a black flat-bottom microplates, to which 31 μL of the solution for use (5 mL of 0.2 M succinic acid buffer [pH 5.9], 3 mL of ninhydrin reagent [2.4 μmol], and 1 mL of L-leucyl-L-alanine [0.098 μmol]) was added. The microplates were covered with a plate sealer, incubated for 100 min at 60°C with shaking at 400 rpm and cooled in a freezer for 5 min at –30°C. Subsequently, 195 μL of copper reagent solution for use (2 mL of reagent water and 1 mL of copper reagent stock solution [74 μmol copper sulfate, 5.8 μmol sodium potassium tartrate, and 960 μmol sodium carbonate]) was added with agitation for 15 min at room temperature. Fluorescence was measured using

a SpectraMax M2 Multi-Mode Microplate Reader (Molecular Devices) with excitation at 390 nm and emission at 486 nm. Concentrations between 200 and 600 μmol/L were diluted 1:3 and concentrations above 600 μmol/L were diluted 1:4 with copper reagent solution to maintain the linearity of the reaction.

Neonatal Phenylalanine Kit (method B)

The Neonatal Phenylalanine Kit was supplied by PerkinElmer®, and the assay was performed according to the manufacturer's instructions. Fluorescence was measured using a SpectraMax M2 Multi-Mode Microplate Reader (Molecular Devices) with excitation at 390 nm and emission at 486 nm. Concentrations between 400 and 600 μmol/L were diluted 1:3 and concentrations above 600 μmol/L were diluted 1:4 with the copper sulfate solution provided in the kit to maintain the linearity of the reaction.

High-Performance Liquid Chromatography

HPLC was performed on a Shimadzu RF-10AXL Fluorescence Detector using heparinized plasma samples. Deproteinization was performed in 50 μL of plasma by adding 200 μL of methanol, followed by centrifugation at 2000 rpm for 10 min. Then, 40 μL of the deproteinized supernatant was placed in a test tube and 10 μL of internal standard, 50 μL of 4% mercaptoethanol and 200 μL of ortho-phthaldialdehyde were added. This mixture was vortexed and poured into the apparatus. The total running time for each sample was 1 h.

Data Analysis

Phe levels of 120-360 μmol/L for patients <12 years of age and 120-600 μmol/L for patients ≥12 years of age were considered to be within the treatment target range. Spearman correlation was used to assess the relationship between quantitative variables, and Bland-Altman plots were used to assess agreement between methods. The slope, y-intercept and linear correlation coefficients were calculated in Excel 2007. Statistical analysis was performed using SPSS, version 18.0. A p-value ≤ 0.05 was considered significant for all analyses.

Results

All samples analyzed by HPLC, whether altered or normal (above or within reference values), showed similar results with the two test methods. The results given by the three methods are shown in Table 1.

The mean Phe levels were 430.4 ± 39.9 μmol/L by method A, 439.3 ± 35.4 μmol/L by method B, and 442.2 ± 41.6 μmol/L by HPLC. The correlation values between method A and HPLC, were 0.990, 1.0365, 3.8125, and 0.9873 according to Spearman, slope, y-intercept, and linear correlation ($p < 0.001$) (Figure 1, while the correlation values between method B and HPLC were 0.974, 1.0795, 31.98, and 0.895, respectively ($p < 0.001$) (Figure 2).

The samples were then divided according to their concentrations into Group 1 (< 360.0 μmol/L), Group 2(>360.0 to 600.0 μmol/L) and Group 3 (> 600.0 μmol/L). Bland-Altman

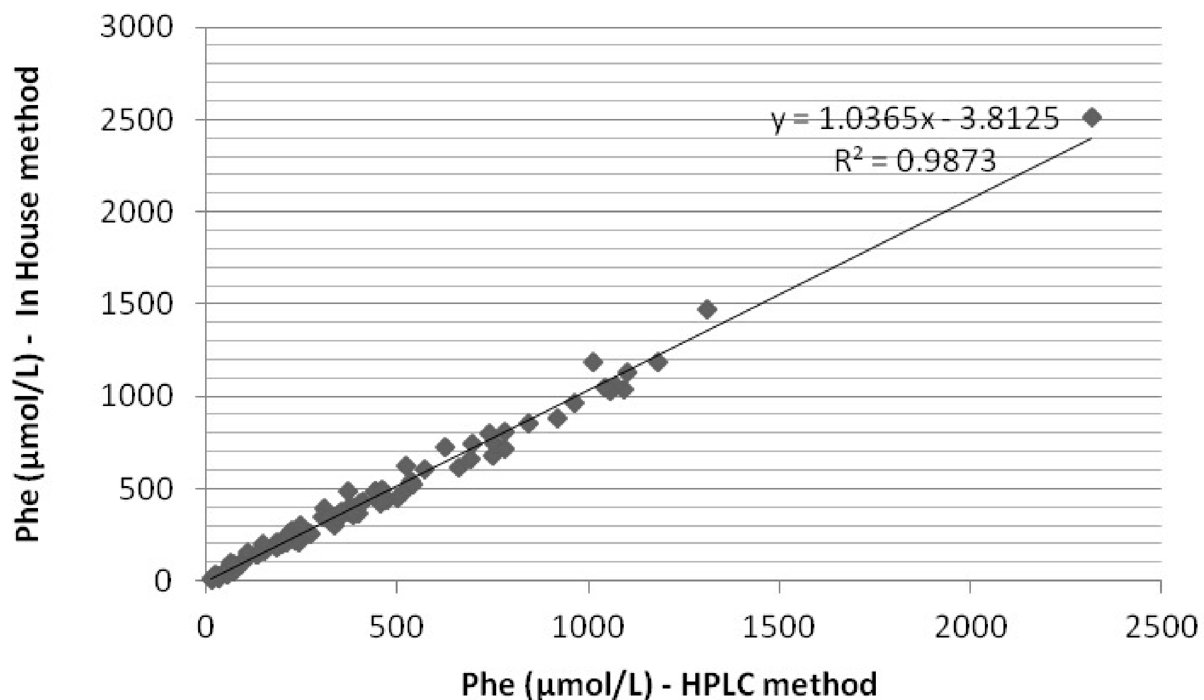

Figure 1. Correlation between phenylalanine (Phe) concentration in dried blood spots measured by the in-house method (method A) and by high-performance liquid chromatography (HPLC) (n = 89 blood samples).

analysis showed in method A, the lower the Phe concentration in the sample, the greater the error compared to HPLC (Figure 3). The comparison of HPLC with method B showed a much greater variability between these two methods (Figure 4). Method A had a sensitivity of 97% and a specificity of 100%, while method B had a sensitivity of 96% and a specificity of 80%.

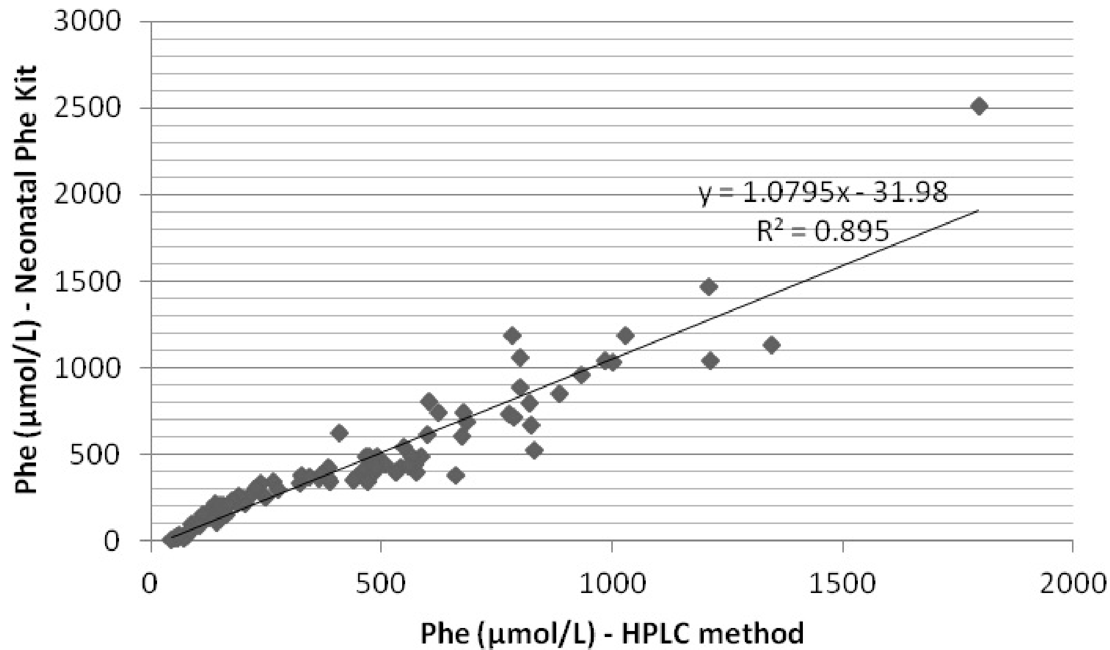

Figure 2. Correlation between phenylalanine (Phe) concentration in dried blood spots measured by the Neonatal Phe Kit (method B) and by high-performance liquid chromatography (HPLC) (n = 89 blood samples).

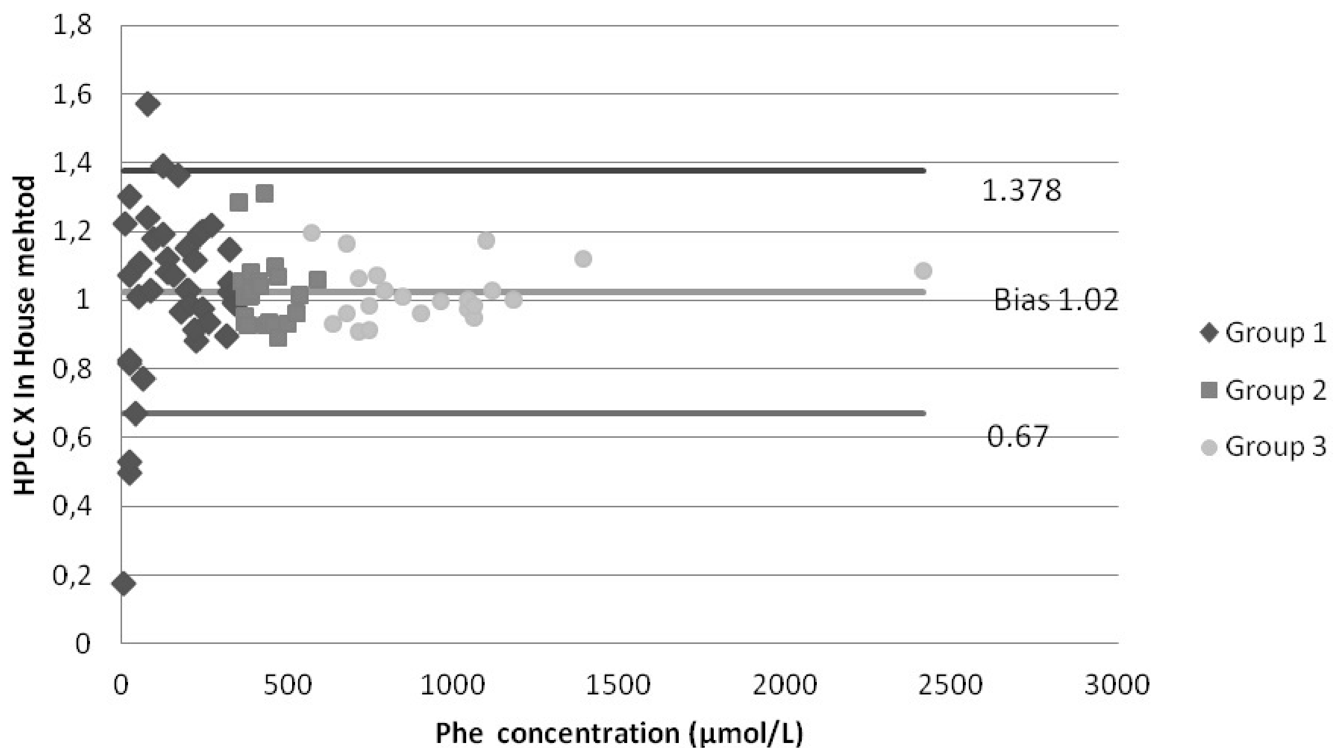

Figure 3. Ratio of phenylalanine (Phe) concentration in dried blood spots measured by the in-house method (method A) and by high-performance liquid chromatography (HPLC) (n = 89 blood samples). Group 1: <360.0μmol/L; Group 2: >360.0 μmol/L and <600.0 μmol/L; Group 3: > 600.0 μmol/L.

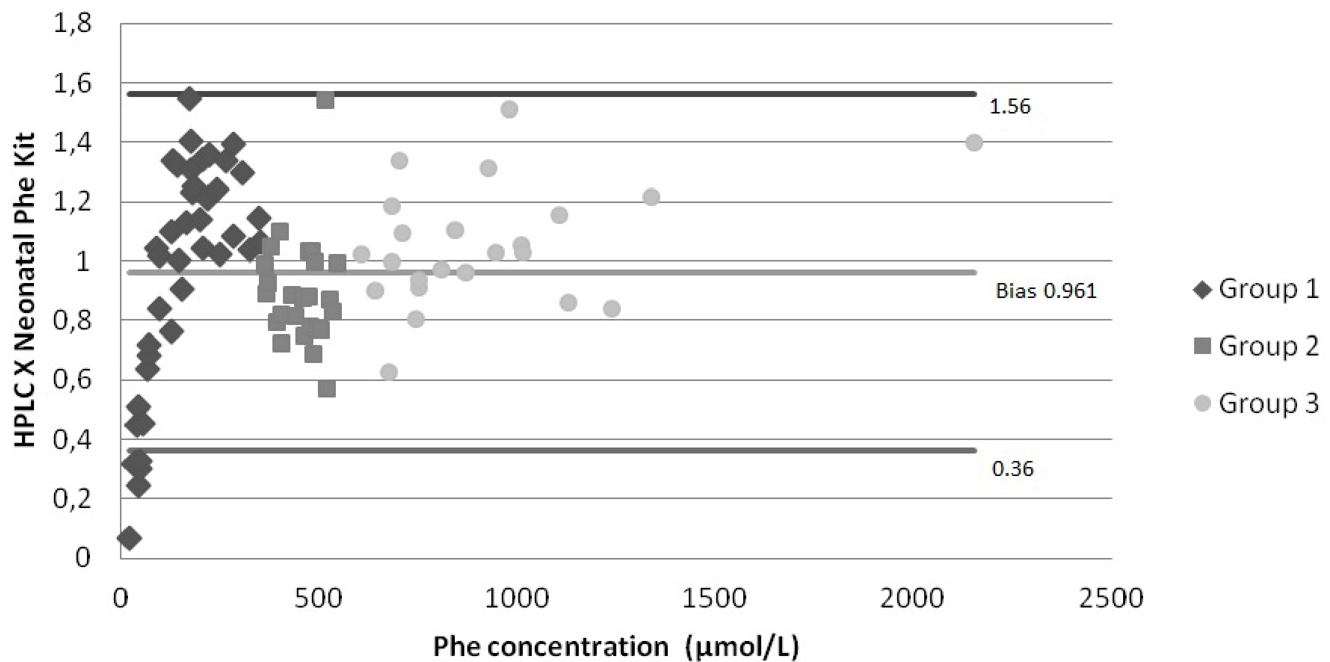

Figure 4. Ratio of phenylalanine (Phe) concentration in dried blood spots measured by the Neonatal Phe Kit (method B) and by high-performance liquid chromatography (HPLC) (n = 89 blood samples). Group 1: <360.0µmol/L; Group 2: >360.0 µmol/L and <600.0 µmol/L; Group 3: > 600.0 µmol/L.

Discussion

The present study showed a strong correlation between Phe measurements in plasma by HPLC and in DBS by a fluorometric method developed in-house (adapted from Lubenow et al 1994) [9] (method A) and by PerkinElmer® Neonatal Phenylalanine Kit (method B), with better agreement between method A and HPLC than between method B and HPLC.

In many countries, tandem mass spectrometry is used to measure Phe concentrations. Although this technology has a higher analytical sensitivity than fluorometric assays and also has the advantage of analyzing several amino acids at the same time, it is expensive and not available in most laboratories in low and middle-income countries. This highlights the importance of studies like the present one, since less expensive alternatives should be evaluated and introduced for the appropriate management of patients with PKU worldwide.

Phe measurements in DBS using HPLC, ion exchange chromatography, and tandem mass spectrometry have been widely reported for both screening and monitoring of patients with PKU [10–13]. However, only a few studies have used fluorometric methods for this purpose.

In a comparison of Phe concentrations measured in serum by a fluorometric method with Phe concentrations measured in DBS by a colorimetric method for monitoring of patients with PKU, a high correlation was found between the two methods ($r = 0.970$) [14], similar to that found in the present study between HPLC and method B ($r = 0.974$) but lower than that between HPLC and method A ($r = 0.990$). The comparison of a fluorometric test developed for neonatal screening in Cuba with the PerkinElmer® commercial kit used in the present study showed a good correlation between the two methods [15] consistent with the present results. However, because the test was developed only for neonatal screening, there was no comparison with HPLC as performed in our study. A comparison of Phe measured only by HPLC in serum vs DBS showed excellent correlation between the two matrices [16].

Based on the reference values given in the European guidelines on PKU (120 - 360 µmol/L for age <12 years; 120 - 600 µmol/L for age ≥12 years (Van Wegberg et al 2017), 89 samples tested in the present study, 37 (41.6%) were altered according to all three methods, which would prompt a recommendation for dietary management In only one sample HPLC values (373.8 µmol/L) would require dietary changes, while method A and method B values would not (355.1 and 325.8 µmol/L, respectively). Three samples were above and three were below the recommended Phe levels according to method B compared to HPLC and method A. Other two samples were above the desired Phe level as measured by both HPLC and method A, while only one was above the desired Phe level as measured by both HPLC and method B.

DBS samples are increasingly used in a wide range of analyses, such as screening, diagnosis, and therapeutic drug monitoring [17–19]. Advantages of this type of matrix include less invasive collection and need for a minimum amount of blood, which are particularly interesting when treating infants and children, and easy transportation of the samples to other facilities, such as reference laboratories [20–21].

However, commercial Kits for DBS samples are usually made for screening, e.g. for differentiating individuals showing

normal Phe levels from those ones showing higher Phe levels, and not for monitoring. Therefore, our hypothesis is that the commercial Kit is not sensitive enough to detect slight variations in Phe levels, as observed in our paper. This finding is very important since a mild variation onthe Phe levels may lead to a change in clinical management during monitoring. Although we have not performed a formal economic analysis, method A appears to be more cost-effective than method B, because for method A: a) reagents can be made in the laboratory; b) a smaller number of samples can be analyzed per time; and, c) the expiration date is shorter.

Conclusions

Phe levels in DBS samples from patients with PKU measured by a fluorometric method developed in-house (adapted from Lubenow et al. 1994) (method A) and by PerkinElmer® Neonatal Phenylalanine Kit (method B) were strongly correlated with Phe levels in plasma measured by the reference standard (HPLC). However, approximately 8% of the samples analyzed by method B were not in agreement with the values obtained with HPLC, which may influence the clinical management of patients. Method A appears to be an effective tool for monitoring PKU patients.

The commercial Kit is made for screening thus the main goal would be to differentiate individuals with normal Phe levels from individual with higher Phe levels (probably PKU patients) therefore, our hypothesis is that the commercial Kit is not sensitive enough to detect slight variations in Phe levels as observed in our work, wich method A shown a higher sensitivity and specificity than method B (commercial Kit) and this is very important once a slight variation on Phe levels may lead to a change in clinical management during patient monitoring. The execution time of the assays is almost the same. When we think about costs, although we have not performed a formal economic analysis, method A appear to be more cost-effective than method B, because the reagents can be made in the laboratory itself and also because we can analyze a smaller number of samples (for method B it would be worth having more samples to analyze), and the commercial Kit has a expiration date is also shorter than method A. For all these reasons we believe that method A its a advantage for laboratory practice, a great tool for monitoring PKU patients and a easy way to help clinicians for better treatment managing.

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Author Contributions

FMS – Data Curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – Review and editing;
MB – Methodology, Supervision, Validation, Writing – Review and editing;
GC – Methodology, Writing – Review and editing;
KMT - Methodology, Writing – Review and editing;
FHB - Writing – original draft, Writing – Review and editing;
AS – Formal Analysis
DMC - Formal Analysis
CRV - Writing – Review and editing;
MW - Writing – Review and editing;
RG - Writing – Review and editing;
IVDS – Conceptualization, Funding acquisition; Project administration; Supervision, Writing – original draft, Writing – Review and editing;

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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