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Performance of laboratory tests used to measure blood phenylalanine for the monitoring of patients with phenylketonuria

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Abstract

Analysis of blood phenylalanine is central to the monitoring of patients with phenylketonuria (PKU) and age-related phenylalanine target treatment-ranges (0–12 years; 120–360 $\mu\text{mol/L}$, and >12 years; 120–600 $\mu\text{mol/L}$) are recommended in order to prevent adverse neurological outcomes. These target treatment-ranges are based upon plasma phenylalanine concentrations. However, patients are routinely monitored using dried bloodspot (DBS) specimens due to the convenience of collection. Significant differences exist between phenylalanine concentrations in plasma and DBS, with phenylalanine concentrations in DBS specimens analyzed by flow-injection analysis tandem mass spectrometry reported to be 18% to 28% lower than paired plasma concentrations analyzed using ion-exchange chromatography. DBS specimens with phenylalanine concentrations of 360 and 600 $\mu\text{mol/L}$, at the critical upper-target treatment-range thresholds would be plasma equivalents of 461 and 768 $\mu\text{mol/L}$, respectively, when a reported difference of 28% is taken into account. Furthermore, analytical test imprecision and bias in conjunction with pre-analytical factors such as volume and quality of blood applied to filter paper collection devices to produce

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AUTHOR CONTRIBUTIONS

S.J.M. was the lead author of the manuscript. All authors made contributions to the perspective/review, and/or acquisition of data, and/or analysis and interpretation of data. All authors participated in drafting the article and revising it critically for important intellectual content. All authors gave approval of the final version submitted.

ETHICS STATEMENT

This article does not contain any studies with human or animal subjects performed by the any of the authors. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention. Use of trade names is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention, the Public Health Service, or the U.S. Department of Health and Human Services.

DBS specimens affect the final test results. Reporting of inaccurate patient results when comparing DBS results to target treatment-ranges based on plasma concentrations, together with inter-laboratory imprecision could have a significant impact on patient management resulting in inappropriate dietary change and potentially adverse patient outcomes. This review is intended to provide perspective on the issues related to the measurement of phenylalanine in blood specimens and to provide direction for the future needs of PKU patients to ensure reliable monitoring of metabolic control using the target treatment-ranges.

Keywords

accuracy; bias; dried blood spots; monitoring; phenylketonuria; precision; treatment-ranges

1 | INTRODUCTION

Patients with phenylketonuria (PKU) (OMIM #261600) are managed by the use of a phenylalanine restricted diet (in conjunction with protein substitute supplements) to lower the blood phenylalanine concentrations and prevent the adverse neurological sequelae.¹ Several guidelines for the diagnosis and management of patients with PKU have been published over the years.²⁻⁶ One of the key recommendations is the monitoring of the phenylalanine restricted diet, using appropriate age-related phenylalanine target treatment-ranges to prevent the adverse neurological outcomes. In the latest set of guidelines,⁴ the following target treatment-ranges are recommended; 120 to 360 $\mu\text{mol/L}$ for individuals aged 0 to 12 years and 120 to 600 $\mu\text{mol/L}$ for individuals older than 12 years. It should be noted that the grade of recommendation for these target ranges is graded as category D (case series reports & expert opinions). Optimal target ranges have also been recommended for women trying to conceive and during pregnancy (120–360 $\mu\text{mol/L}$). An infant born to a PKU mother who is not achieving optimal control can result in neurological deficits, microcephaly and congenital heart defects. Accurate and reproducible monitoring of blood phenylalanine concentrations is therefore critical to the management of PKU. This review is intended to provide perspective on the issues related to the measurement of phenylalanine in blood specimens and to provide direction for the future needs of PKU patients in terms of accurate monitoring of metabolic control. With the evidence presented in this review, both laboratory scientists and clinicians should consider whether the analytical performance of the methods routinely used in their laboratory is adequate to support the use of the recommended patient target treatment-ranges in the management of patients with PKU.

2 | LABORATORY METHODS USED TO MEASURE BLOOD PHENYLALANINE

Traditionally, the standard method for the laboratory diagnosis and monitoring of PKU patients is by quantifying phenylalanine in deproteinized plasma samples, using ion exchange chromatography (IEC) with ninhydrin detection. While this method is reproducible, it has a long analytical run time (~2 hours per sample), low sample throughput and lacks specificity in comparison with liquid chromatography tandem mass spectrometry

(LC-MS/MS). These limitations are reflected in the number of laboratories moving away from IEC to LC-MS/MS in recent years.⁷

Irrespective of technique, the measurement of plasma amino acids can be performed with reasonable precision, with a typical intra-laboratory coefficient of variation (CV) for phenylalanine being approximately 5%. The CV is a measure of the variability of the test results and is the ratio of the SD to the mean of the results obtained multiplied by 100 (ie, $CV [\%] = SD/mean \times 100$). As expected, the inter-laboratory variation is greater, as shown by data from the European Research Network in Inherited Metabolic Diseases (ERNDIM) Quantitative Plasma Amino Acids External Quality Assessment (EQA) scheme, with an average CV of 9.5%, at a nominal concentration of 355 $\mu\text{mol/L}$ ($n = 282$ participants), and this can be attributed predominantly to the variation in the test standardization, compounded by differences in laboratory practice.⁸ Generally, an aqueous calibrator is used to standardize the test and these tend to be produced in-house, although an increasing number of laboratories now use a certified reference material (CRM), to standardize assays; the Sigma TraceCert solution and the National Institute of Standards & Technology SRM2389a solution, both of which are available commercially. It should be noted that these CRMs are aqueous solutions of amino acids and not matrix (plasma) matched.

Plasma amino acid analysis is impractical for routine monitoring of metabolic control due to the logistics required in collecting a venous blood specimen twice weekly to monthly (depending on age and clinical need) from every patient, including very young children. Plasma amino acid analysis undertaken in this way has been used primarily for diagnosis and clinical situations, in which a full profile of amino acids is informative, for example, complete nutritional assessment. Instead, measurement of phenylalanine in dried bloodspot (DBS) specimens is widely favored due to the convenience of collecting blood from a finger-prick onto filter paper in the patient's home and mailing the sample directly to the laboratory. Monitoring patients using DBS has been routinely done by a variety of methods since the inception of screening for PKU. Methods used include: bacterial inhibition assay, fluorimetry, IEC, high performance liquid chromatography and enzymatic analysis.⁹⁻¹² During the last 25 years flow-injection analysis tandem mass-spectrometry (FIA-MS/MS) has been used to analyze DBS for patient monitoring, following the introduction of this technology for routine newborn screening of PKU.¹³ Measurement by FIA-MS/MS has a shorter analysis time and high sample throughput; results can therefore be communicated to patients in a timely manner allowing prompt dietary adjustment.

FIA-MS/MS is inherently limited by lack of specificity as analytes are not separated chromatographically. Instead, specificity is achieved by multiple reaction monitoring, which allows rapid and continuous monitoring of the specific daughter ions relating to the analyte of interest, hence any isobaric compound with a common daughter ion has the potential to interfere, for example, benzocaine (anesthetic agent found in some antiseptic wipes which may be used to clean skin prior to sample collection),¹⁴ lorazepam, and omeprazole (<https://massbank.eu/MassBank/>). When these assays were originally established, FIA-MS/MS proved to be a robust assay with rapid throughput (1.5 minutes per sample) and adequate sensitivity and specificity. We established a snapshot of current FIA-MS/MS analytical performance by circulating a commercially available DBS QC material to 16 UK

laboratories. The mean value obtained was 174 $\mu\text{mol/L}$ (range 100–256 $\mu\text{mol/L}$) with an inter-laboratory CV of 20.2%. The assigned target-value of the material (167 $\mu\text{mol/L}$) also highlighted the large biases displayed by individual laboratories, which ranged from –40% to 53%. Technology has evolved in recent years and modern mass-spectrometers now have the ability to scan faster. These changes, in conjunction with the introduction of ultra-performance liquid chromatography, mean that laboratories now have the capability to routinely analyze phenylalanine in DBS with superior specificity and precision, and with a comparable analysis time and robustness.

A further limitation to the utility of DBS specimens for monitoring patients is the lack of a commercially available CRM for DBS amino acids on which to standardize laboratory tests. As a result, DBS calibrators tend to be produced in-house by collecting blood from a healthy donor and adding an aqueous phenylalanine enrichment prior to application onto filter paper. The exact preparation of the DBS calibrator varies between laboratories, for example, volume of blood added to the filter paper, varying hematocrit of the specimen, or use of lysed blood specimens can all affect the measured concentration.^{15–18} More importantly, the method used to assign DBS calibrator values can influence the analytical result.

3 | DERIVATION OF THE TARGET TREATMENT-RANGES FOR “BLOOD” PHENYLALANINE AND THE DIFFERENCES BETWEEN DBS AND PLASMA PHENYLALANINE RESULTS

It should be recognized that the vast proportion of studies evaluating the neurological outcomes in patients with PKU, are mostly based upon plasma phenylalanine concentrations^{4,5,19} and these studies form the basis of the recommended target treatment-ranges. However, these guidelines refer to the monitoring of “blood” phenylalanine concentrations, but do not make any distinction between the two specimen types,^{2,3,5,6} or refer to the observed differences, but then do not provide guidance as to the most appropriate specimen type to be used.⁴ This is disconcerting in that there are significant differences between plasma phenylalanine measured by IEC and DBS phenylalanine measured by FIA-MS/MS, with concentrations in DBS being up to 18% to 28% lower (Table 1). The difference between DBS and plasma phenylalanine concentrations is reduced from –28% to –15% when both plasma and DBS are analyzed using IEC.¹⁹ Furthermore, the difference between liquid whole blood and plasma phenylalanine concentrations is ~8% when measured by the same laboratory technology (Table 1). These findings suggest that the reported differences between plasma and DBS specimens are due to several factors; distribution of phenylalanine between plasma and erythrocytes, extraction efficiency of phenylalanine from DBS and laboratory instrument test biases. Using DBS collected by trained healthcare professionals, we observed that DBS phenylalanine concentrations measured with FIA-MS/MS are 18% lower relative to the paired plasma concentrations measured by IEC (Figure 1), which is similar to those reported previously. The negative bias of 18% in our laboratory is reproducible and stable over a long period of time.

The differences between DBS and plasma phenylalanine concentrations could have a significant clinical impact in terms of management. For example; it is recommended that

during pregnancy “blood” phenylalanine concentrations should be maintained between 120 and 360 $\mu\text{mol/L}$. Assuming these target ranges are based upon plasma concentrations, but the patient is monitored using DBS concentrations, a DBS concentration of 360 $\mu\text{mol/L}$ is equivalent to a plasma concentration of 425 $\mu\text{mol/L}$ in our laboratory based upon current performance. Whereas, in a laboratory with a negative bias of 28%, DBS specimens with phenylalanine concentrations of 360 and 600 $\mu\text{mol/L}$, at the upper critical target treatment-range thresholds would be plasma equivalents of 461 and 768 $\mu\text{mol/L}$, respectively (Figure 2).

4 | ADDITIONAL FACTORS THAT AFFECT PATIENT PHENYLALANINE TEST RESULTS

Although the published patient treatment and monitoring guidelines emphasize the importance of biochemical monitoring of phenylalanine concentrations, the impact of the following factors on the final result reported are not considered in any of the guidelines:

- The significance of the analytical test variation (imprecision) and analytical test bias (inaccuracy) in the measurement of phenylalanine.
- The total allowable error (TAE) for a phenylalanine test that can be tolerated before it has a significant impact upon clinical decision making.
- The impact of the pre-analytical factors such as the amount or volume of blood applied to the filter paper to produce DBS specimens on the phenylalanine results obtained.

5 | ANALYTICAL TEST VARIATION (IMPRECISION)

Laboratory tests are not perfect and it is imperative that healthcare professionals understand the factors that affect the phenylalanine test results that they receive and act on. A requirement for the accreditation of clinical laboratories according to the International Standards Organization (ISO) 15 189, is to provide the measurement of uncertainty (MU) for each test performed.²⁵ MU is defined as the value that is associated with the test result that describes the dispersion of values that could be obtained for the test result due to the uncertainties arising within the test procedure. MU is calculated using quality control (QC) specimens analyzed in every batch of patient samples over a period of time to encompass the combined effect of all the analytical factors that influence the test result.²⁶ In one author’s laboratory (S.J.M.) the mean and SD of a DBS QC specimen analyzed in 166 separate test batches over an 8-month period using FIA-MS/MS was 361 (19.3) $\mu\text{mol/L}$, corresponding to an analytical inter-assay CV of 5.3%. Using these data, the MU of the test can be calculated using the following equation:

$$MU = SD \times 1.96.$$

Using a coverage factor of 1.96, there is a 95% chance that the true result lies within a range covered by the result value \pm MU. The calculated MU for the DBS phenylalanine test is 37.8 $\mu\text{mol/L}$ or 10.5%. This means that for a DBS specimen with a phenylalanine concentration

at the critical upper treatment threshold of 360 $\mu\text{mol/L}$, we are 95% confident that the result is between 322.2 and 397.8 $\mu\text{mol/L}$ (ie, 360 $\mu\text{mol/L} \pm 10.5\%$). However, this concentration range does not include variability due to the pre-analytical factors such as difference in DBS specimen collection techniques or the inherent differences between different laboratory test methodologies. The inter-assay CV and MU for the plasma assay in our laboratory is 3% and 5.8% (ie, 1.96×3), respectively.

Understanding the variability of the test used to monitor patients serially in order to assess optimal dietary control is also important and the reference change value (RCV) can be used to determine this. The RCV refers to the minimum critical difference between two consecutive results, in the same patient, which needs to be exceeded in order for a significant change to have occurred.²⁷ As there are two sets of phenylalanine results to consider, we must take into consideration two sets of variations that must be combined to produce the RCV. This combined variation is represented by the following equation:

$$RCV = 2.77 \times \sqrt{CV_A^2 + CV_I^2}.$$

where CV_A = the analytical CV of the test and CV_I = within person biological variation. The CV_I of phenylalanine in healthy adult individuals is 9.5%²⁸ and the CV_A (SJM - laboratory) for a DBS phenylalanine QC is 5.3%. The two sets of results being compared need to be greater than 2.77 (ie, 2×1.96) times the analytical and within-person biological variations. The calculated RCV for phenylalanine using DBS specimens is 30.2%. Therefore, for a patient with a DBS phenylalanine concentration of 360 $\mu\text{mol/L}$, an increase or decrease of >109 $\mu\text{mol/L}$, would have to occur before the results are considered significantly different (95% confidence), that is, not due to the within-person biological variation or test variation.

It could be argued that including the effect of biological variation within the RCV calculation is not applicable as PKU patients are prescribed a specialist diet and supplements in a manner to maintain stable phenylalanine concentrations throughout the day.^{4,29} Furthermore, patients are advised to collect specimens at the same time of day, usually fasting in the morning, when the phenylalanine concentration is highest.²⁹ Therefore, assuming optimal compliance with this regimen, the use of the RCV calculation may not be an appropriate approach to assess serial changes in phenylalanine concentrations in a patient, as only large variations may be deemed as a significant change. An alternative approach to calculate the critical difference between two patient results would be to remove the contribution of the within-person biological variation. This can be calculated by multiplying the assay imprecision (5.3%) by 2.77 which equals 14.7%. Therefore at a concentration of 360 $\mu\text{mol/L}$, an increase or decrease of >52.9 $\mu\text{mol/L}$ in consecutive DBS specimens would be considered significant. However, it should be noted that the MU and RCV calculations do not take into account the test bias. The RCV value using plasma test results is 27.6% and 8.3% (ie, $2.77 \times 3\%$) when the contribution of biological variation is removed.

6 | ANALYTICAL TEST BIAS (INACCURACY)

Test bias refers to the difference between the test result obtained and the reference or “true” value. Clinical laboratories compare their analytical performance with results from EQA

schemes (Table 2). In an attempt to define what the acceptable test bias of phenylalanine should be, the biological variation of phenylalanine can be used to derive the acceptable bias, and this has been calculated to be 10.4%.²⁸ Furthermore, the Horwitz equation can also be used to predict inter-laboratory variation on the basis of analyte concentration alone, as it is independent of method, specimen matrix and analyte. The Horwitz equation (%) = $2C^{-0.15}$, where C is the concentration of the analyte, expressed as a mass fraction.³⁰ For phenylalanine in the concentration range of 10 to 500 $\mu\text{mol/L}$, this would equate to a target inter-laboratory variation of approximately 10%. It is clear that the plasma tests conform to this calculated acceptable bias of 10%, whereas the DBS tests are highly variable depending on the EQA scheme (Table 2) and this is in part due to the fact that there is no CRM commercially available for DBS phenylalanine tests. Interestingly, the target test bias of 10.4% derived from biological variation is similar to that of 10% calculated using the Horwitz formulae:

The UK National External Quality Assessment Service and the Centers for Disease Control and Prevention (CDC) schemes have been available for many years and are intended to assess the performance of the newborn screening programs. More recently ERNDIM has introduced a DBS scheme targeted towards an assessment of control in patients receiving treatment. The pilot scheme was operational in 2017 and 2018 and supplied 79 laboratories with four DBS specimens (phenylalanine range 120–940 $\mu\text{mol/L}$) and a DBS phenylalanine calibrator (350 $\mu\text{mol/L}$). Laboratories were asked to submit results from both the DBS specimens and the DBS calibrator. Results from the four specimens were then virtually “calibrated” for each laboratory using the standard material. The inter-laboratory CVs from the “raw” and the “calibrated” results were calculated for the four specimens (Table 3). It can be seen that the mean inter-laboratory CV is improved by the use of this virtual standard from 20% using the “raw” data to 10% when the “calibrated” data is analyzed. This effect is evident at all concentrations from 120 to 940 $\mu\text{mol/L}$.³¹ Effectively, inter-laboratory variation could be reduced significantly by introducing a common DBS calibrator. In order to address the issues regarding the inter-laboratory biases observed between newborn screening laboratories, a European working calibrator for DBS phenylalanine (EWS-Phe-01) was created³² and this is now produced by the International Society for Newborn Screening. A multi-analyte DBS amino acid reference material has also been produced by the CDC in the United States.³³ However, these materials are only available in limited quantities to kit manufacturers and EQA scheme organizers.

Reporting inaccurate monitoring results could have profound effects in that patients may be falsely reassured with lower results, where laboratories have a negative bias for DBS phenylalanine and conversely, those laboratories with a positive bias providing falsely elevated results which may prompt a stricter dietary regimen, which may lead to non-compliance issues. Therefore, with such large and variable biases for DBS phenylalanine results being observed between different laboratories, it is evident that consideration should be given to test bias when utilizing the treatment-ranges.

7 | ESTABLISHING OPTIMAL ANALYTICAL PERFORMANCE TARGETS FOR PHENYLALANINE TESTS

Unfortunately, there is not a single simple approach to establishing optimal analytical targets for analytes measured in the clinical laboratory and several strategies exist.³⁴ The TAE of a test can be used to establish performance targets for a test. The TAE of a test is the maximum error that can be tolerated for a test before it has a significant impact upon clinical decision-making. TAE is not based on the performance of the analytical test being used, but is dependent on the clinical use of the test result and the inherent biological variability of the analyte.³⁵ TAE should be derived objectively from an analysis of studies assessing the clinical impact of the test performance, although this is often difficult to achieve.

Other strategies include professional recommendations by expert groups or those derived from data obtained from biological variation. The TAE of 18.2% and a bias of 10.4% for phenylalanine have been calculated using data from the biological variation of amino acids in plasma from healthy adult subjects.²⁸ The total error (TE) of the phenylalanine tests can be calculated using the following formulae:

$$TE = \text{Test bias} + (1.96 \times \text{test CV})$$

Table 2 shows the TE of the plasma and DBS tests in our laboratories. On comparing these to the TAE of 18.2%, it would suggest that the plasma phenylalanine tests are clinically fit for purpose to monitor patients. Conversely, the performance of the DBS tests is less consistent, depending on which EQA results are used to calculate the test bias (Table 2).

To date no study has derived an analytical goal from studies that have assessed the clinical impact of test performance on clinical outcomes in patients with PKU. However, it is possible to derive a TAE using clinical outcome studies. Data from a meta-analysis of 17 studies (n = 432 patients), demonstrated a threshold effect of a phenylalanine concentration > 400 µmol/L, that was associated with an IQ of less than 85 in patients aged <6 years.³⁶ Furthermore, it was also shown that those patients (age range 8–13 years) with a lifetime phenylalanine >400 µmol/L, did worse than those with a phenylalanine <400 µmol/L in all tests assessing executive function.³⁷ It is therefore imperative that laboratory tests must be able to distinguish with confidence between a test result of 360 µmol/L (upper-limit of the target treatment-range) and one of 400 µmol/L, which is potentially damaging. Therefore the measurement error of the test must not exceed the difference between the two test results (ie, 40 µmol/L), which relates to a TAE for the test of 11.1% (ie, [40/360] × 100). This is significantly lower than the TAE of 18.1% derived by biological variation. However, it is recognized that using biological variation to derive TAE can lead to over estimation.³⁸ If we look at whether the test is able to confidently detect a change between 360 and 300 µmol/L, the TAE would be 16.7% (ie, [60/360] × 100), which is closer to the TAE of 18.2% derived by biological variation. Therefore, when comparing patient phenylalanine test results to target treatment-ranges we must understand the TE of the test and whether or not it is fit for purpose, that is, the TE of the test used should ideally be less than the TAE of the test.

8 | PRE-ANALYTICAL FACTORS AFFECTING THE MEASUREMENT OF PHENYLALANINE IN DBS SPECIMENS

The size (ie, volume of blood applied to the filter paper) and quality of the bloodspot has a significant impact on the results obtained. Phenylalanine concentrations are significantly lower in smaller DBS relative to larger DBS specimens.¹⁵⁻¹⁷ If a liquid blood specimen, containing phenylalanine at a concentration of 360 $\mu\text{mol/L}$, is applied to filter paper in different volumes (10–100 μL) the mean concentration of the actual measured phenylalanine in the DBS samples (using a standard 3.2-mm sub-punch for analysis) would vary from 300 $\mu\text{mol/L}$ in the 10 μL DBS to 400 $\mu\text{mol/L}$ in the 100 μL DBS (Figure 3). If the effect of DBS size and the MU of the test are combined, then the range of results that could be reasonably observed for a specimen with a concentration of 360 $\mu\text{mol/L}$ is anything between 269 and 442 $\mu\text{mol/L}$. However, this does not take into consideration the effect of the variable biases observed for the phenylalanine results, which may further compound these pre-analytical errors. A comparable effect is also seen for tyrosine concentrations,¹⁵ which can be simultaneously measured in the FIA-MS/MS DBS phenylalanine assay, and is used to monitor the nutritional supplementation of tyrosine.

Following the reported evidence that small and poor quality DBS specimens produce falsely low and erroneous results for the metabolites used for newborn screening,^{15,17} UK newborn screening guidelines for DBS specimen rejection/acceptance were developed. Several UK metabolic laboratories also adopted these specimen acceptance/rejection criteria for DBS specimens received for monitoring of PKU patients. Prior to implementation, an audit was undertaken to assess the impact on the DBS monitoring service. It was demonstrated in one of the author's laboratory (R.S.C.) that if these criteria were applied to specimens received for PKU monitoring over a 4-week period, 143 out of the 300 specimens (47.7%) received would have been rejected. The vast majority of these specimens were too small and would have produced significant negative biases. This finding highlights the fact that the negative bias observed due to small DBS and the lower concentration in DBS vs plasma specimens, would result in falsely lower results, thereby giving false reassurances as to optimal metabolic control in patients. Following patient / parent training for the collection of DBS specimens the number rejected due to being too small or poor quality reduced to 26.2%.

Filter paper collection devices for capillary blood collection from heel or finger pricks are Class II Medical Devices (21 CFR 862.1675), and should meet international criteria for performance by the Clinical & Laboratory Standards Institute.³⁹ The Newborn Screening Quality Assurance Program at CDC conducts voluntary evaluations of all lots of Food and Drug Administration-registered collection devices before they are released to the user community for newborn screening and other applications.^{18,40} In general, all filter paper lots comply with Clinical & Laboratory Standards Institute (CLSI) performance criteria.⁴⁰ The CLSI provides the framework for filter paper performance. Because filter paper is a natural product produced from cotton linters, defining how the matrix influences blood collection is important so that precision and reproducibility can be achieved from lot-to-lot.⁴⁰ These international standardization efforts ensure uniformity of specimen collection, calibrators, QC and reference materials for newborn screening assays. Using DBS specimens for patient

monitoring adds additional requirements for the precision and accuracy of analyte recovery. The type of matrix used for calibration and QC materials will influence the analyte recovery. Ideally, methods testing patient DBS specimens should also use DBS calibration and QC to correct for the filter paper matrix.

9 | DISCUSSION AND RECOMMENDATIONS

Although the difference between DBS and plasma phenylalanine concentrations in paired specimens has previously been reported, the clinical impact on patient management has not been assessed. A DBS result compared with a plasma target treatment-range could be falsely reassuring and potentially damaging, particularly in those laboratories where large negative biases are observed for DBS concentrations. To provide comparable results for patient monitoring, a calibration factor could be used to report DBS results as plasma equivalents to ensure meaningful comparison of results to the recommended target treatment-ranges. This is preferable to reporting patient results against different target treatment-ranges as this may cause confusion for both patients and clinicians. However, it is essential that the patient DBS specimens collected are of adequate size and quality to ensure accurate results, because the differences between DBS and plasma specimens are more variable when patients apply their own blood onto the filter paper collection devices compared to those applied by trained healthcare professionals.¹⁹ Improvement in DBS specimen quality could be potentially achieved by the use of blood collection devices, that collect defined volumes of liquid blood for sampling^{41–44} and such devices should be evaluated in order to improve the biochemical monitoring of patients. It has been highlighted that the development of point-of-care devices to measure liquid whole blood phenylalanine concentrations in the patient's home would optimize outcomes as a result of shorter turnaround times for results.^{4,45} However, until the issue of test calibration and alignment between plasma and DBS specimens has been addressed, caution should be taken before adopting such technology.

Recently, Sapropterin dihydrochloride (Kuvan), a synthetic form of the tetrahydrobiopterin cofactor for phenylalanine hydroxylase, has been used successfully to lower blood phenylalanine concentrations. A 30% reduction is defined as being responsive to therapy.⁴⁶ Furthermore, lesser reductions of 10% to 20% may represent clinically meaningful outcomes.⁴⁶ However, detecting changes in phenylalanine concentrations of 10% to 20% using DBS samples will be challenging due to the issues outlined. It is advisable that plasma phenylalanine measurements are used to determine the response to Sapropterin therapy.

The impact of the differences in phenylalanine results obtained from different specimen types and laboratory instruments on patient management using the recommended target treatment-ranges needs to be reviewed and guidance provided in future recommendations. In addition, future guidelines should include criteria for phenylalanine test performance and ensure traceability of the method/calibration used, including the use of the DBS calibration and control materials. No study to date has derived an analytical goal by assessing the clinical impact of test performance on clinical outcomes in patients with PKU. The TAE of the test is a useful goal, as this indicates the maximum error that can be tolerated before it has a significant effect on patient management. However, the use of accurate data from published studies to derive a TAE will be limited as many state that “blood” phenylalanine

was measured and do not state whether plasma or DBS specimens were used. Furthermore, reference to the laboratory instrumentation used to quantify the “blood” phenylalanine is rarely provided. This lack of detail of specimen type, test methodology and calibrator traceability used will impede and potentially weaken future clinical studies aimed at deriving TAE and meta-analyses assessing optimal target ranges (both upper and lower) to prevent adverse outcomes.

The results from the ERNDIM EQA pilot scheme in 2017 and 2018 indicate that there are significant problems with assay calibration resulting in an unwanted level of inter-laboratory variation and an inherent results bias in some laboratories. This makes European/International recommendations relating to target treatment-ranges difficult to implement. The development of a commercially available CRM to standardize DBS phenylalanine tests is essential to address these issues. An international effort between professional societies, expert scientific advisory groups, PKU patient advocacy groups and organizations that have the expertise and capabilities to produce CRM material is required, in order to standardize tests.

Clinicians should take into consideration the effect of the test variability and bias (ie, TE of the test), DBS size and quality in order to prevent over-interpretation of changes in phenylalanine concentrations, thereby preventing false reassurances as to optimal dietary compliance. Clinical laboratories undertaking the analysis of DBS for monitoring of PKU patients should ensure that: (a) standardized criteria for the acceptance/rejection of specimens is implemented with the aim of improving the quality of DBS for monitoring; (b) FIA-MS/MS methods are replaced with LC-MS/MS methods to improve analytical performance; and (c) a rigorous evaluation of the bias between plasma and DBS phenylalanine results is undertaken in laboratories to derive a calibration factor in order to report DBS results as plasma equivalents (ideally on an individual patient basis), thereby ensuring meaningful comparison of patient results to the recommended target treatment-ranges. This is of paramount importance especially in the context of those infants that may be the subject of safeguarding measures as a result of phenylalanine concentrations that are persistently outside the target treatment-ranges, and the MU of the phenylalanine test result should be provided to guide clinicians and dieticians to allow a more objective interpretation of the monitoring of serial results. Finally, patients and parents/carers should receive regular training on blood collection techniques to ensure that more accurate and less variable results are obtained in order to achieve optimal dietary control thereby reducing adverse neurological outcomes.

Abbreviations:

CDC	Centers for Disease Control and Prevention
CLSI	Clinical & Laboratory Standards Institute
CRM	certified reference material
CV_A	analytical coefficient of variation
CV_I	within person biological variation

DBS	dried blood spot
EQA	external quality assessment
ERNDIM	European Research Network in Inherited Metabolic Diseases
FIA-MS/MS	flow-injection analysis tandem mass-spectrometry
HPLC	high-performance liquid chromatography
IEC	ion exchange chromatography
MU	measurement of uncertainty
NIST	National Institute of Standards & Technology
PKU	phenylketonuria
RCV	reference change value
TAE	total allowable error
TE	total error

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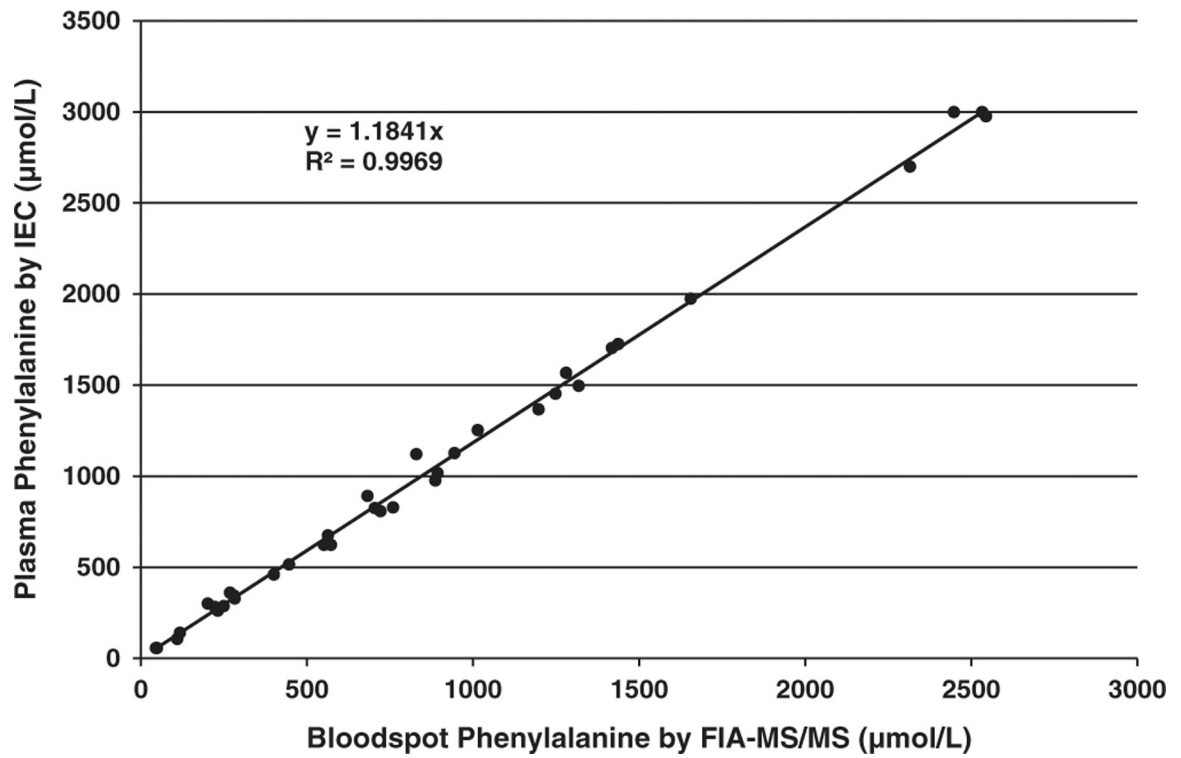


FIGURE 1.

Association between bloodspot phenylalanine concentrations measured by flow-injection analysis tandem mass-spectrometry (FIA-MS/MS) and plasma phenylalanine concentrations measured by ion exchange chromatography (IEC) in 38 paired plasma and bloodspot specimens (all samples collected were part of routine monitoring)

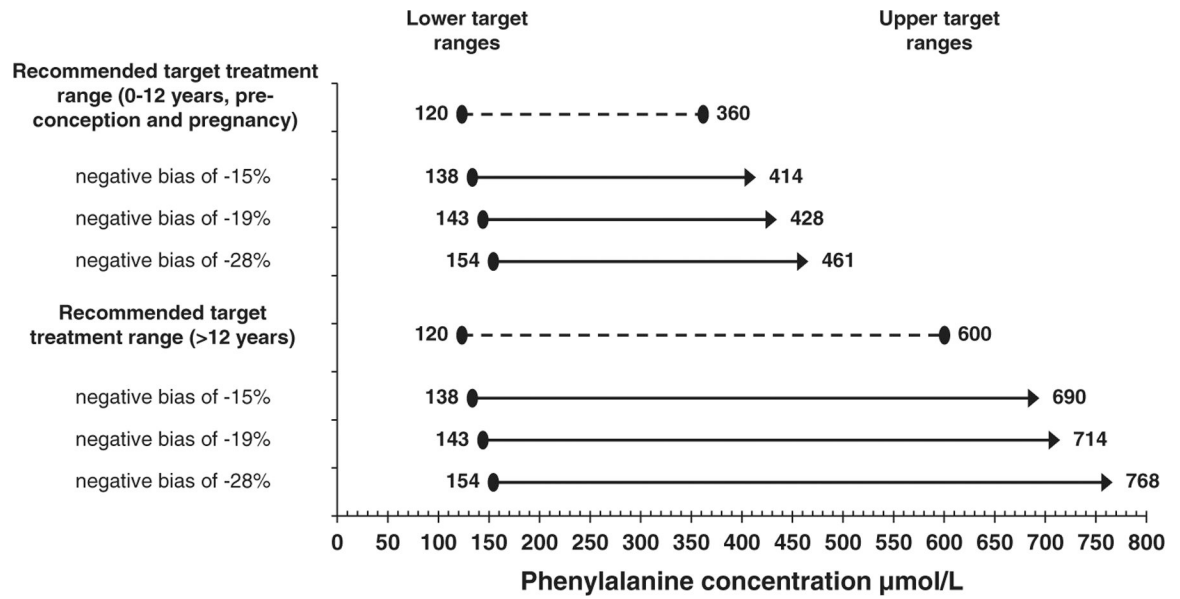


FIGURE 2. Diagram to demonstrate the impact of the observed negative bias between bloodspot and plasma phenylalanine concentrations on the recommended target treatment ranges

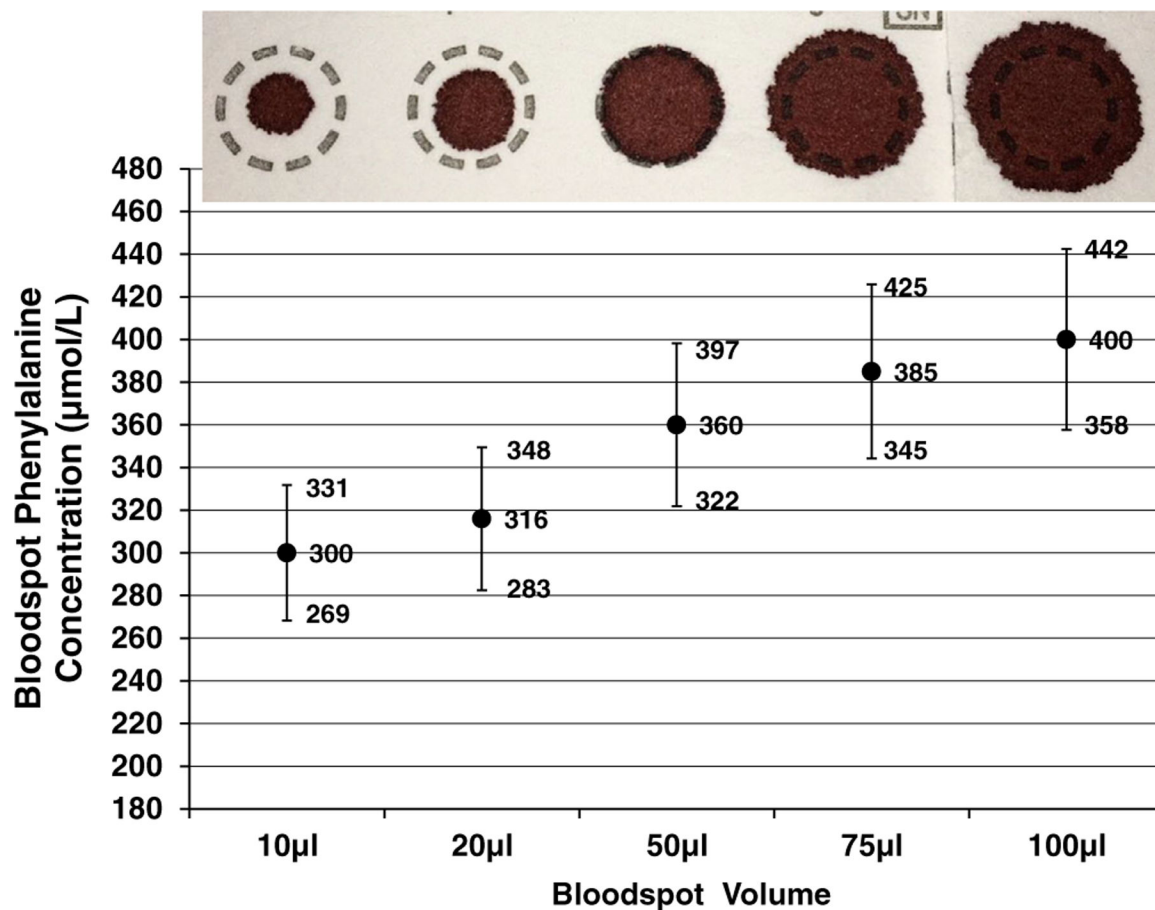


FIGURE 3. Combined effect of analytical measurement of uncertainty (MU) and bloodspot size/volume on phenylalanine concentrations. Blood was applied at the various volume to the filter paper (PerkinElmer 226) which contains 10-mm-diameter printed circles. Results are shown as the mean of results obtained for each of the bloodspot diameters and the MU (10.5%) applied to these results that is, the range of results that could be obtained

TABLE 1

Studies assessing the differences between plasma, DBS and whole blood phenylalanine concentrations using a variety of laboratory technologies

Plasma assay	DBS assay	Bias DBS vs plasma	Source
IEC	FIA-MS/MS	-19%	20
IEC	FIA-MS/MS	-28%	21
IEC	FIA-MS/MS	-26%	22
IEC	FIA-MS/MS	-28%	19
IEC	FIA-MS/MS	-18%	Present study
IEC	HPLC	-19%	20
IEC	IEC	-15%	19
Plasma assay	Whole blood assay	Bias – whole blood vs plasm	Source
IEC	IEC	-7.5%	23
HPLC	HPLC	-8	24

Abbreviations: DBS, dried blood spot; FIA-MS/MS, flow-injection analysis tandem mass-spectrometry; HPLC, high-performance liquid chromatography; IEC, ion exchange chromatography.

TABLE 2

Analytical performance of the plasma and DBS tests in our laboratories against the various EQA schemes

Sample type	Test imprecision (CV _A) %	Test bias %	TE of test %
Laboratory 1			
Plasma	3.0	1.6 ^a	7.5
Plasma	3.0	3.7 ^a	9.6
DBS	5.3	8 ^c	18.4
DBS	5.3	11.1 ^d	21.5
DBS	5.3	16 ^e	26.4
Laboratory 2			
Plasma	2.0	4.6 ^a	8.5
Plasma	2.0	2.1 ^b	6.0
DBS	5.1	2.9 ^c	12.9
DBS	5.1	18.2 ^d	28.2
DBS	5.1	2.7 ^e	12.7

Note: Test bias was calculated using the all laboratory mean for the participants using various EQA schemes. DBS phenylalanine measured by FIA-MS/MS in both laboratories. Plasma phenylalanine measured by IEC in both laboratories. TE is the total error of the test and is calculated as TE = Test bias + (1.96 × test imprecision (CV_A)).

Abbreviations: DBS, dried blood spot; EQA, external quality assessment.

^aUK NEQAS quantitative amino acids.

^bERNDIM plasma amino acids.

^cUK NEQAS DBS newborn screening.

^dERNDIM – DBS monitoring (Pilot scheme).

^eCDC – DBS Newborn Screening. DBS phenylalanine measured by FIA-MS/MS in both laboratories.

Inter-laboratory variation (expressed as CV%) with and without calibration using a DBS standard in 79 laboratories

TABLE 3

Parameter	Sample 1	Sample 2	Sample 3	Sample 4	Mean
Phenylalanine concentration (µmol/L)	120	330	420	940	452
Inter-laboratory CV without calibration	23%	20%	18%	20%	20%
Inter-laboratory CV with calibration	14%	9%	8%	9%	10%

Abbreviation: CV, coefficient of variation; DBS, dried blood spot.