

New Enzymatic Colorimetric Method for the Quantitative Determination of Phenylalanine in Dry-Blood Spots

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Abstract

Phenylketonuria (PKU) is an autosomal recessive disorder, which is characterized by severe mental retardation, microcephaly and seizures. The symptoms of this disease can be prevented if detected soon after birth. Therefore, blood Phenylalanine (Phe) measurement is essential for the early diagnosis, treatment and dietary monitoring of PKU patients. The goal of this research was to introduce a rapid, precise and effective enzymatic colorimetric method using the recombinant *Bacillus badius* phenylalanine dehydrogenase (PheDH; EC 1.4.1.20) for the quantitative determination of Phe in dry-blood spots. This test was based on the enzymatic reaction of PheDH coupled with an artificial electron acceptor system composed of phenazine methosulfate (PMS) and iodinitro tetrazolium chloride (INT). This assay system contained PMS to transfer the electrons of NADH to INT, enabling the formation of formazan with an absorbance at 490 nm. Calibration curve was plotted and the experimental data were fitted by linear regression analyze. The regression equation and correlation coefficient (R^2) were $Y = 0.0109x + 0.032$ and $R^2 = 0.996$, respectively. This method showed a recovery in the range of 95.1%-102.6% and had the limit detection of 0.5 mg/dl for Phe. The between run coefficients of variation (CVs) mean was between 3.8% and 9.1%. The within-run CVs was between 8.5% and 18.6%. Furthermore, no interferences from other amino acids and Phe derivatives were observed. Altogether, we here presented a quick and reliable enzymatic colorimetric assay for application in newborn screening and monitoring of PKU patients.

Keywords: Enzymatic method; Phenylalanine (Phe); Phenylalanine dehydrogenase (PheDH); Phenylketonuria (PKU)

Introduction

Phenylketonuria (PKU) is an autosomal recessive disorder, caused by the deficiency of Phenylalanine

hydroxylase (PheOH) enzyme on the phenylalanine (Phe) metabolic pathway. It is one of the most frequent amino acids metabolic inborn disorders. This disease is characterized by the impaired conversion of Phe to

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tyrosine [1]. Therefore, the level of blood Phe is increased dramatically and results to the accumulation of this substance and its by-product in blood and tissues. Excess Phe and its by-product in the blood early in life impair the normal development of the brain and causes microcephaly, seizures and irreversible mental retardation. If the disease is diagnosed early in life and the patients are put under a Phe restricted diet, the symptoms can be prevented [2, 3]. For this reason, Phe measurement in blood is essential for the early diagnosis, treatment and dietary monitoring of PKU patients [4]. Many methods, including bacterial, fluorometric and tandem mass spectrometry (MS/MS) have been used for measuring Phe concentration in blood [5]. However, most of these methods are time-consuming and unsuitable for producing the rapid results, or they require specialized instruments and not available in every laboratory. Nonetheless, enzymatic methods are of particular interest because they are specific, rapid and affordable in most clinical laboratories [6, 7]. Besides that, some of the enzymatic methods such as phenylalanine amnio-lyase are lack of enough specificity for Phe as well [8, 9]. Considering the importance of objectiveness of the test result, it has therefore, been demanded to develop a method having simplicity, reliability as well as capacity to deal with a large number of samples. In this study, we developed an enzymatic endpoint assay using *Bacillus badius* phenylalanine dehydrogenase (PheDH; EC 1.4.1.20) coupled with an electron acceptor system for the rapid and accurate quantitative determination of Phe in dry-blood spots. The principle of this procedure was as follows. In the first step, PheDH catalyses, the NAD-dependent oxidative deamination of Phe to phenylpyruvate and NADH (Fig. 1). In the second step, phenazine methosulfate (PMS) transfers the electrons of NADH to iodinitro tetrazolium chloride (INT) as a reduction indicator, leading to the generation of formazan with an absorbance maximum at 490 nm. This enzymatic colorimetric assay may be possible to expand for other similar diseases such as maple syrup urine disease (MSUD) and homocystinuria (HCU).

Materials and Methods

Chemicals and Reagents

NAD⁺ was from Sigma-Aldrich (St. Louis, MO, USA) and L-phenylalanine was obtained from Merck (Darmstadt, Germany). INT (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride) and PMS (phenazine methosulfate) were purchased from Sigma-Aldrich Corp (USA). The specimen collection paper

used for blood spot samples was Schleicher and Schuell grade 903 (S&S 903). All other chemicals and biochemical reagents were of laboratory grade, and double-distilled water was used throughout the experiments.

Construction of Expression Plasmid for *B. badius* PheDH

PCR primers were designed based on the available nucleotide sequence of PheDH of the *B. badius* genome using DNASIS MAX software (DNASIS version 2.9, Hitachi Software Engineering Co., Ltd., Tokyo, Japan). A 1143-kb DNA fragment containing the *pdh* gene was amplified by PCR from the genomic DNA of *B. badius* with specific primers PDHFw (5'-TGGATCCAT GAGCTTAGTAGAAAA-3') and PDHRev (5'-GCGAAGCTTGTGCGAATATC-3'), which contained the restriction sites for *Bam*HI and *Hind*III, respectively. PCR amplification was performed in a 50 µl reaction mixture containing 20 pmol of each primer, 1X PCR buffer, 0.2 mM of each dNTP, 1.5 mM MgCl₂, 0.3 mg template DNA and 2.5 units of *pfu* DNA polymerase under amplification condition: preincubation at 95°C for 1 min and then 30 cycles of 95°C for 1 min, 61°C for 1 min and 72 °C for 2 min. The product of the PCR reaction was cut with *Bam*HI and *Hind*III, and then ligated into the pET23a (+) expression vector carrying a C-terminal His₆-tag previously digested with the same restriction enzymes. The resulting construct bearing the *pdh* gene was named pET23aPDH and transformed into the *E. coli* BL-21 (DE3). The correctness of the cloned gene was confirmed by nucleotide sequencing and no mutation was revealed.

Purification and Preparation of Recombinant Enzyme

For the production of target enzyme, *E. coli* BL21 (DE3) cells harboring plasmid pET23a with recombinant *B. badius* PheDH activity were cultivated in shake flasks containing Luria-Bertani (LB) medium supplemented with 0.1 mg /ml ampicillin at 37 °C and 140 rpm. After a further 8 h of cultivation, a 10 ml culture broth was transferred into 1 L of LB medium and incubated at 37 °C until an OD₆₀₀=1.0 was reached. The culture was then cooled to approximately 23 °C by stirring the flasks in an ice-water bath for 4 min. The T₇ promoter was induced by addition of 0.5 mM sterile isopropyl-β-D-thiogalactopyranoside (IPTG) and shaking at 23 °C for 5 h. After cultivation, cells were collected by centrifugation at 3500 rpm for 15 min and kept at -20 °C for further uses. The resulting pellets were dissolved in 0.1 M potassium phosphate buffer pH

7.0, 0.1 mM EDTA, and 1 mM dithiothreitol and then disrupted (20 min with a 9-KHz ultrasonic oscillator). This suspension was centrifuged at 10000 rpm at 4 °C for 20 min to clarity and dialyzed against the same buffer. Purification of recombinant enzyme in ATPS containing 9% (w/w) PEG and 16% (w/w) K_2HPO_4 has been described in our preceding paper [10, 11].

Controls and Standards

Stock solutions of 0, 2, 4, 8, 12, 16, 20, 24, 28 and 32 mg/dl L-Phe were prepared in deionized water. Standard containing various amounts of Phe was prepared by diluting the stock solutions with an aqueous solution of Bovin serum albumin (BSA). These samples were directly spotted on filter paper and dried at room temperature for at least 24 hours and then were kept in sealed plastic bags containing desiccant packages at -20 °C until used.

Blood Sample Preparation

The blood-spot samples for this study were obtained by finger prick from ten healthy, non-PKU volunteers and from PKU patients during our PKU screening program. From everyone, capillary blood was spotted directly onto filter paper and dried for DBS analysis. The DBS samples were also kept in sealed plastic bags with desiccant packages at -20 °C until used.

Enzymatic Colorimetric Method Design

The principle of this procedure (Fig. 1) was the quantitative determination of phenylalanine based on the use of *Bacillus badius* PheDH enzyme and its colorimetric measurement. In the assay procedure, 5-mm (diameter) dried blood spots (DBS) from calibrators, controls and patient specimens were punched into a 96-well microplate and left for 10 min at 95 °C in a bain-marie. At the end of this step, 150 μ L of 0.1 M Tris-HCl (pH 8.0) was added to each well and the plate was shaken for 60 min at 25 °C. The extracts were transferred to the corresponding test microplate, and the enzyme, coenzyme, PMS and INT reagents were then added to each well. After 60 min shaking, the absorbance was read bichromatically at 490/630 nm. Calibration curves were plotted and the experimental data were fitted by linear regression analyze.

Method Validation Tests

The analytical performance of the enzymatic method of interest was studied by the following parameters

[11, 12].

Precision: The intra-run precision was determined using data obtained from 20 replicates from three samples of 2, 32 mg/dl Phe concentration. The inter-run precision involved analysis of the above samples in 10 different assays.

Recovery: recovery was measured by adding 100 μ L of stock Phe standard to 900 μ L normal whole blood specimens.

Linearity: the linearity was analyzed by making several dilutions of a plasma pool with added L-Phe (32 mg/ml).

Sensitivity: the detection limit of the assay was determined with different concentrations of Phe from 0-4 mg/dl after 10 determinations.

Interference: to investigate the specificity of the proposed method, enzyme activity towards amino acids of L-tyrosine and L-tryptophan as well as other metabolites such as phenylpyruvic and ascorbic acid were also assayed.

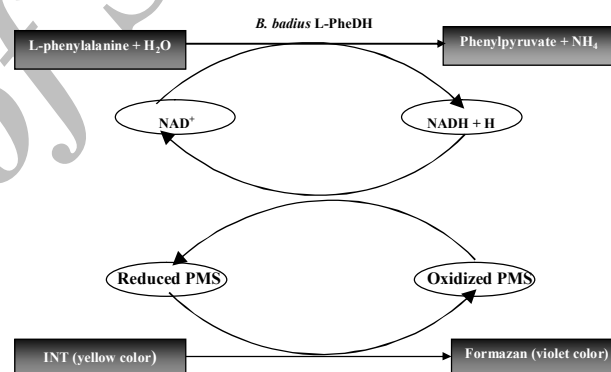


Figure 1. Enzymatic colorimetric method scheme for the quantitative determination of phenylalanine.

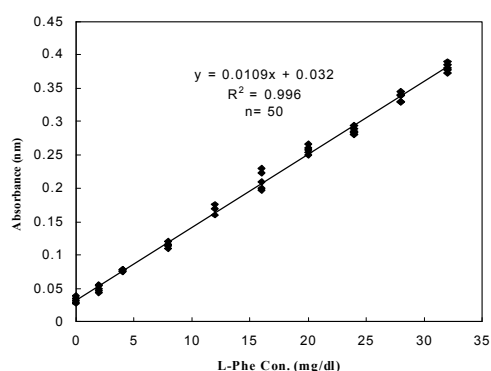


Figure 2. Calibration curve for the enzymatic colorimetric phenylalanine determination in DBS. Experiments for each concentration were performed in triplicate run.

Table 1. Precision data of the enzymatic assay method

Standrad	Concentration (mg/dl)	Wthin-run (n=10)		Between-run (n=10)	
		(mean±SD)	CV%	(mean±SD)	CV%
Phe	1	4.5± 0.2	3.9	27.3±5.1	18.6
	6	17.4±1.6	9.4	94.4±8.0	8.5
	12	153.5±5.8	3.8	166.8±18.9	11.3
	24	349.4±14.4	4.1	363.0±41.1	11.3

SD; Standard Deviation, CV; coefficient of variation

Statistical Analysis

Data are expressed as mean ±SD. Linear regression coefficients and correlation coefficient (R^2) were calculated using Sigma Plot 7.0 software.

Results

The linear calibration curve was determined by using standard solutions of L-phe in the concentration range from 0 to 32 mg/dl with the DBS filter papers (Fig. 2). Each plot was displayed as an average value of triplicate measurement. According to Figure 2, the regression equations and correlation coefficient (R^2) were $Y = 0.0109x + 0.032$ and $R^2 = 0.996$, respectively. Analysis of the standard curve for a Phe range of 0-32 mg/dl showed a nearly linear curve which confirms the linearity of the method in this range. Precision of the assay was defined by replicate analysis of intra- and inter-assay variations. As shown in the Table 1, the between run coefficients of variation (CVs) mean was between 3.8% and 9.1%. The within-run CV was between 8.5% and 18.6%. Furthermore, it can be found that the presented method was capable of measuring a relatively wide spectrum of possible concentrations of serum Phe from normal to highly elevated levels with suitable accuracy. The analytical recoveries for Phe added to blood spots was depicted in Table 2. As can be

seen, the enzymatic determination of Phe in DBS showed a recovery in the range of 95.1%-102.6%, thus fulfilling the requirement for a screening method. The sensitivity was 0.5 mg/dl for Phe, highlighting the limitation of the method in the lower range. Concentration differences of 0.5 mg/dl and 1 mg/dl Phe were detectable at the 95.6% confidence level. No interferences were observed with normal and elevated concentration of tyrosine, tryptophan, phenylpyruvic and ascorbic acid.

Discussion

PKU is an inherited metabolic disease that causes to mental retardation if not detected soon after birth. This inborn error is characterized by the elevated levels of Phe in blood. Therefore, measuring of blood Phe concentration is very important for the diagnosis and treatment [13]. Different methods have been reported for this purpose, including the Guthrie bacterial inhibition assay (BIA), fluorometric, spectrophotometric, chromatographic and enzymatic assay. Among them, enzymatic methods are of particular interest and widely used mainly due to their advantages such as selectivity, sensitivity and low cost [14]. As a matter of fact, the HPLC and tandem mass spectrometry (MS/MS) are not yet as widely available in every laboratory as are enzymatic techniques for the routine screening and monitoring of PKU. The extremely high cost equipment and the need for sample derivatization are their main disadvantages [15]. On the other hand, the established enzymatic methods that have been previously described for the determinations of phe are either not have enough selectivity toward this amino acid. Therefore, new methods that are selective, simple and inexpensive, while offering rapid and quantitative determination of phe are needed. Considering the improvement in sensitivity and specificity of enzymatic tests and the quality of care for PKU patients, we thus aimed to find a new technology, which is suitable for accurate assay of Phe in DBS. Our presented procedure

Table 2. Recover of Phe added to DBS

Phe Concentration (μmol/L)			Reovery (%)
Added	Expected	Observed	
0	—	12.3	—
43.7	56.0	64.0	114.3
80.1	92.4	88.3	95.6
110.9	123.2	118.6	96.3
136.9	149.2	136.9	91.7

Table 3. Comparison of the analytical performance of presented enzymatic test with the two used enzymatic methods for measurement of Phe in DBS

Method	Sensitivity	Analysis Time	Recovery (%)	Precision (%)	
				Within-run	Between-run
Presented in this study	0.5 mg/dl	90 min	114.3	3.8-9.4	8.5-18.6
Enzymatic, Cobas Bio centrifugal	1 mg/dl	240 min	89.4	4.0-9.5	9.8-19.0
Enzymatic, <i>B. sphæricus</i> PheDH and glutamine transaminase K	2 mg/dl	180 min	91.0	5.0-10.2	9.2-23.0

involves the coupling of *B. badius* PheDH reaction with the reduction of INT. An important feature in the design of this test was the use of *B. badius* PheDH which has the narrowest board specificity toward Phe [16]. In fact, with this Phe specific enzyme, it makes it possible to increase the specificity and the sensitivity of detection with virtually no interferences from other compounds as well [17]. As a result, this procedure was capable of measuring Phe in the low range which could be useful in the screening. Thus, it can be said that the presented enzymatic assay differed from the previously reported techniques. First, our assay was carried out in a microplate reader instead of a conventional spectrophotometer, thus allowing the simultaneous analysis of a large number of samples. Second, we increased the sensitivity and selectivity by using the *B. badius* PheDH. Third, we changed the assay to an endpoint assay at 490 nm wave length to produce the red formazan (Fig. 3) instead of 550 nm wavelength which was conventional for all routine clinical laboratories. The parameters of our method were also

comparable to other methods for determination of Phe. It had a wider linear range, good precision that correlated with those of the previously used technique [18, 19]. The material cost of the assay was estimated to be about 2 \$ per sample in triplicate, which was economical to use rather than the enzymatic commercial kits [6]. The short analysis time was another advantage of this method. Time required for each assay was 90 min which was favorably comparable than other enzymatic methods [6, 11]. Besides the above-mentioned advantages, to verify the validity of the described quantitative test, we compared the performance parameters of our method with the two used enzymatic methods to determine Phe concentrations in DBS (Table 3). As can be found, the analytical performance of the presented assay in this work were satisfactory than the previous assays. This comparison also reconfirmed the performance of our test for neonatal screening.

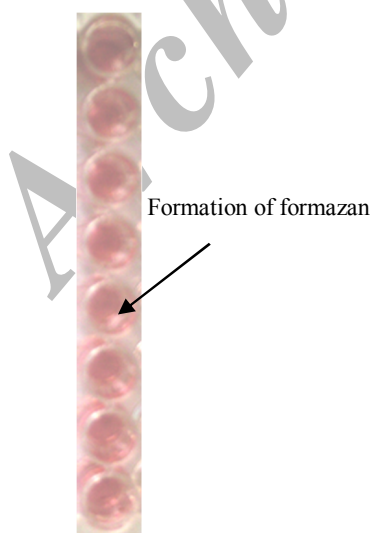
In conclusion, we developed a simple, reliable and quick enzymatic endpoint assay based on *B. badius* PheDH for the determination of Phe in DBS. Our results suggested that this novel method could serve for both screening and monitoring of plasma Phe concentrations in PKU patients who are being treated by diet. We hope that similar methods from this type can be used in the screening and motoring of diseases such as MSUD and HCU.

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**Figure 3.** Image of formazan formation in the reported enzymatic colorimetric method.

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