Analytical Methods

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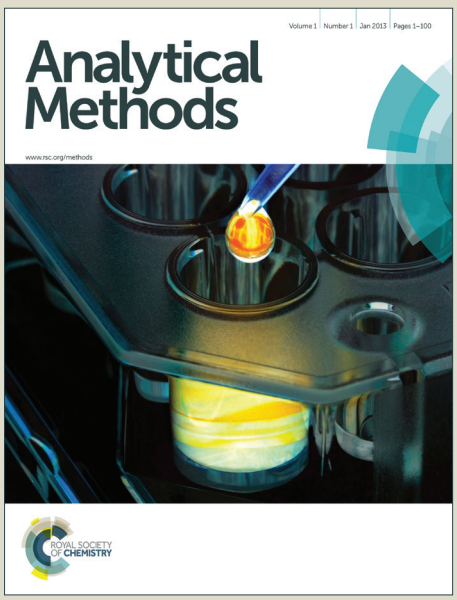

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A fast high performance liquid chromatographic (HPLC) analysis of amino acid phenylketonuria disorder in dried blood spots and serum samples employing C18 monolithic silica columns and photo diode array detection

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A gradient high-performance liquid chromatography- photo diode array detection (HPLC-PDA) method applying monolithic RP-C18 column was developed to facilitate the separation amino acid phenylketonuria disorder within 6 min. Phenylalanine (Phe) and tyrosine (Tyr) extracted from dried bloodspot (DBS) and serum protein precipitated by perchloric acid (5% v/v) were injected directly. The proposed method offers a sufficient chromatographic resolution (> 2), a wide range of linearity (0.1-3, 200 μM), good accuracy (98.3-103.5 for DBS sample and 88.2-104.2 for serum sample) and an acceptable precision (relative standard deviation of < 9.0 %). The statistical comparison of results, i.e. the obtained Phe and Tyr level in DBS and serum sample, did not show any significant difference. The applicability of the proposed method for screening newborns and monitoring of a diet therapy in PKU patients was examined by analysing 127 DBS and 22 serum samples and confirmed using liquid chromatography-mass spectrometry. Using of monolithic column with gradient elution proved to be more suitable for a simple and fast analysis in screening newborns and economical, green and patient friendly for regularly monitoring Phe and Tyr in PKU patients.

Introduction

Phenylketonuria (PKU) is one of the most common inborn errors of metabolism (IEMs) that was first recognized in 1934 by A. Fölling.[1, 2] This inborn error is caused by a defect in the phenylalanine hydroxylase (PAH) gene and is the main reason for inability to convert phenylalanine (Phe) into tyrosine (Tyr) leading to the increase of Phe level in the blood.[3, 4] Association of Phe in blood causes profound intellectual disability, neurological problems, eczematous rash, autism, seizures[3] and also mineral bone disease.[5] Positive PKU patients can manage the disorder with Phe-restricted and Tyr-supplemented diet [6], however other alternative therapies for PKU such as gene therapy, enzyme substitution therapy with phenylalanine ammonia lyase and etc. have been investigated.[7-12]

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Due to the above-mentioned reasons; it is vital to simultaneously determine Phe and Tyr in physiological fluidics of neonatal and screen them in order to regularly monitor and control the disorder. The obtained Phe level in blood (as the first biomarker) and the ratio of Phe to Tyr levels (as the secondary biomarker) can help to diagnose of PKU. Moreover it could be helpful to find the other IEMs if it is abnormal.[13, 14]

So far, many assays have been performed to describe the quantitation of Phe and Tyr levels with different analysis methods such as amino acid analyzers (AAA),[15] spectrometric methods,[16, 17] tandem mass spectrometry (MS/MS),[18-21] ion exchange chromatography with ultraviolet detection (IEC-UV),[22-24] gas chromatography- mass spectrometry (GC-MS),[25, 26] capillary electrophoresis-mass spectrometry (CE-MS)[27] and high performance liquid chromatography (HPLC) with ultraviolet (UV),[28-31] fluorescence (FL),[32-35] mass spectrometry (MS)[36, 37] and pulsed amperometric detection (PAD)[24, 38] on serum,[39] plasma,[28, 30] whole blood[29] and dried blood spots (DBS).[19, 27] It is noteworthy that the use of DBS specimens on filter paper (as patient friendly samples) has gained increasing attention in recent years due to its advantages such as requiring only a few

drops of blood, stability of compounds in DBS and allowing storage and shipment at room temperature.[40-42] Furthermore, there is a low risk of DBS being infected with blood-borne viruses such as HIV, HBV and HCV. Therefore, DBS specimens on filter paper provide a fast and efficient way for collecting large numbers of blood samples for disease screening, diagnosis and monitoring of treatment efficacy in a public health setting.[43] Among the existing mentioned quantitative methods for analyzing biological fluidics, one of the reliable methods for simultaneous determination of PKU amino acids disorder is reversed phase high performance liquid chromatography (RP-HPLC) analysis with a UV or fluorescence detector, after pre- or post- column derivatization.[31, 34] All the existing derivatization methods present various analytical problems like derivative instability, reagent interference, long run time, relatively high cost per sample and long preparation times. Therefore, the ability to investigate amino acids, without derivatization step, eliminates these problems.[44]

In RP-HPLC methods, monolithic silica is one of the interested materials as stationary phase due to macropores and mesopores with an average diameter of 2 µm and 18 nm in its skeleton respectively. Using these materials as a HPLC column has been started since 1996. Compared with particle-packed silica C_{18} columns, Monolithic silica C_{18} has a low flow resistance which leads to a low column backpressure and fast analysis while providing a sufficient surface area for an efficient separation.[45] In 2004, several research groups including Dawsen and coworkers[46] used heretofore C_{18} monolithic silica to separate the amino acids with the aim of reducing analysis time. Mixture of 18 primary amino acids were separated based on napthalene-2.,3-dicarboxaldehyde (NDA) pre-column derivatization using monolithic C18 columns within 10 min., In 2007, Devall et al. developed a method for separating 21 amino acids within 24 min by using chromolith RP-18 as HPLC column and *ortho*-phthaldialdehyde- 3-mercaptopropionic acid (OPA- 3-MPA) as the derivatization reagent.[47] In 2011, Song et al. applied MonoClad C18-HS for separating 19 amino acids with pre-column derivatization of 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) within 18 min.[48]

The present study employed an economical, green and patient friendly RP-HPLC-PDA method while using a monolithic silica C18 column for rapid screening of the newborns and following the diet therapy of positive PKU patients without requiring any special and expensive instruments. This method was applied to 127 DBS specimens (including healthy newborns, healthy adult volunteers and PKU patients) and 22 serum samples (including healthy newborns, healthy adult volunteers and PKU patients) in which Phe and Tyr levels were measured directly without a pre- or post- column derivatization step. The obtained results were confirmed by liquid chromatography-mass spectrometry (LC-MS). Moreover, the Phe and Tyr contents in DBS and serum samples of the same people were studied comparatively. Finally, the correlation between results of the same samples was obtained while employing the ELISA methods and the method investigated in the present study.

Experimental

Materials and reagents

Phenylalanine (Phe), tyrosine (Tyr) and α-methyl phenylalanine as internal standard (I.S.) were obtained from Sigma- Aldrich (Steinheim, Germany). Perchloric acid was purchased from Merck (Darmstadt, Germany). HPLC-grade methanol (MeOH, 99.9%) and acetonitrile (ACN, 99.9%) were provided by Chem-Lab NV (Zedelgem, Belgium). Water was used after purification by Milli-Q system (Millipore, St. Quentin, France).

Instrument analysis

A HPLC system (Knauver, Berlin-Zehlendorf, Germany) consisting of a HPLC pump (Model 1000), photo diode array detector (Model 2800), a 5 µL loop and Chromgate software (Knauer, Berlin, Germany) was used. The separation was performed on a Chromolith® RP-18 column (4.6 mm i.d. × 100 mm length, Merck Millipore, Germany).

Mass spectra were acquired by a Thermofisher Scientific ion trap mass spectrometer (model: Finnigan TM LCQ TM DECA). The mass spectrometer was operated with electrospray ionization (ESI) interface in the positive ion mode with spray voltage of 4.5 kV, capillary voltage set to 46 V and cone voltage to -60 V. Also the capillary temperature was set at 200 ºC. Nitrogen was used as spray gas (60 mL min^{-1}) and make up gas (20 mL min^{-1}). Detection was carried out in full scan mode. Xcalibor 2SR.2 (copyright Thermo Electron Corporation 1998–2006) software was used to data processing and analysis.

Ultrasonic instrument (DT31H, 35 KHz, Bandelin electronic, Berlin, Germany) and Vortex mixer (WiseMix®, Vm-10, Witeg, Germany) were also used. Methanol and acetonitrile were employed as organic solvents in the aqueous mobile phase and the flow rate varied from 1 to 2 mL min^{-1}. The separation was performed at 30 ℃ temperature. The injection volume was 5 µL and the ultraviolet spectrometric detection was carried out at a wave length of 210 nm.

Preparation of standard solutions

20 mM stock solutions of each amino acid (Phe and Tyr) in addition to a mixture of both and 2.5 mM internal standard solution (α-methyl phenylalanine) were prepared by dissolving appropriate amounts of the amino acids in perchloric acid 5% in water (v/v). All stock solutions were kept at 4 ˚C and were stable for four months. Working solutions were prepared by successive dilution of the stock standard solutions.

Sample collection

Informed consent was obtained for all participant's parents prior to experiments and ethical approval was obtained from the local Committee of Mofid Children's Hospital (30 January 2014). The study was performed in accordance with the principles of the Declaration of Helsinki.

DBS SPECIMENS

The specimens were collected from 127 people including 18 newborns, 107 PKU patients under treatment (aged from 1

month to 30 years) and 2 healthy adult volunteers. Fasting venous blood samples were collected on the hatman 903® protein saver cards (Dassel, Germany) and stored at room temperature until the analysis was performed. Blood spots were taken by creating punctures with 6.18 mm diameter using a puncher and they were then transferred to glass vials and incubated at 92 ˚C for 15 min. 30 µL of perchloric acid 5% in water (v/v) was used as an extraction solvent containing the 900 µM of internal standard (α-methyl phenylalanine). Samples were sonicated for 20 minutes and then vortexed for 20 minutes. Finally, 5 µL of supernatants were injected into HPLC system for analysis.

SERUM SAMPLES

The serum sample of 9 healthy newborns, 11 PKU patients under treatment and 2 healthy adult volunteers was taken and kept at -20˚C. The samples were subjected to a protein precipitation step prior to injection. For this purpose, 30 µL of perchloric acid 5% in water (v/v) (as deproteinization solvent containing 900 µM internal standard) was added to 30 µL of sample. The mixture was first vortexed and then centrifuged at 16, 000 g for 10 min. Then 5 µL of the supernatants were injected into HPLC system for analysis.

Validation study

Calibration standards for the amino acids with different a concentrations of 100, 200, 400, 600, 900, 1300, 1900, 2500 and 3200 µM respectively, were prepared by diluting the stock solutions with ultrapure water. Each of these stock solutions contained 900 µM of the internal standard. In order to construct calibration curve for each analyte; ratios of the analyte peak area to internal standard amount were used from integrated chromatograms.

Linearity of each calibration curve was evaluated by the least-squares regression method which was used for calculating the regression coefficient (R) value, y-intercept, and slope of the regression line. The limit of detection (LOD) and limit of quantification (LOQ) were calculated as $3s_a/b$ and $10s_a/b$ respectively where s_a refers to the standard deviation of the intercept and b is the slope of the regression line.

The intra-day precision of the method was determined by running specimens of healthy volunteer five times consecutively while calculating the concentration of the analytes of interest based on the calibration equation. Inter-day precision was calculated by running specimens of healthy volunteer over five different days. In order to determine the recovery of the method, the specimens of healthy volunteer was spiked with standards at four different concentrations and repeated in triplicate for serum samples and duplicated for DBS samples.

Results and discussion

Chromatographic condition

Optimization of chromatographic resolution of two amino acids and internal standard was achieved by sequentially altering (a) isocratic or gradient elution, (b) content of organic solvents in the aqueous mobile phase and (c) flow rate. Firstly, in order to optimize the resolution at the monolithic silica column the separation was performed at the isocratic mode with aqueous mobile phases containing methanol (MeOH) and acetonitrile (ACN), respectively, from 3 to 10 % v/v. In real samples (DBS and serum), the best resolution was achieved by using MeOH as an organic solvent at highest analysis time.

On the basis of the results the gradient profile employed consisted of two components including deionized water and methanol. The gradient profiles of the eluent were optimized with respect to optimum peak separation at the minimum analysis time. The best gradient profile at a flow rate of 1.5 mL $min^{-1}$was obtained by increasing the MeOH percentage up to 3%, during first five min of analysis and then decreasing to 100% water (i.e. at t = 0 min; 100% water, t = 5 min; 97: 3 v/v water: MeOH and t = 8 min, 100% water) at a column temperature of 30 °C.

Phe, Tyr and I.S were separated well (chromatographic resolution >2) in real specimens (DBS and serum) under the optimum conditions (Fig. 1). The retention time was about 1.8, 3.6 and 4.7 min for Tyr, Phe and I.S., respectively. This retention times were consistent from run to run with a RSD of < 2.8 % between runs.

To confirm the results, the LC-MS was investigated with the following conditions; the percentage of MeOH increased up to 4% during the first 8 min of analysis with the flow rate of 0.5 mL min^{-1}. LC-MS chromatograms of DBS and serum of a PKU patient are shown in Fig. 2a. As the Fig. 2b illustrates, the mass spectrums of peaks at 5.3 and 9.5 min confirmed the presence of Tyr and Phe in real sample respectively.

Method validation

Under the chromatographic conditions described, the linearity of the method for analysing samples was tested using a set of amino acid standards with concentration levels in the range of 100 to 3200 µM containing 900 µM internal standard. The calibration graphs were constructed by plotting peak areas of amino acid/ internal standard ratio vs. concentrations of amino acid/ internal standard ratio. The value of slope, the linear range, the regression coefficient (R), the limits of detection (LOD) and quantitation (LOQ) of two amino acids for both specimens are summarized in Table 1.

A wide range linearity and good regression coefficient (R) were achieved for the two compounds. The LOQ was as low as 0.1 µM which cover the lowest reported alert value for Phe levels in blood. The intra-day repeatability of the methodology was obtained by the same day analysis of five replicates of DBS and serum samples of healthy volunteer. To assess inter-day reproducibility, five replicates of the same DBS and serum samples were analysed in five different days. The results are shown in Table 2. The relative standard deviation (RSD) values below 9.0% show that the method has a good precision.

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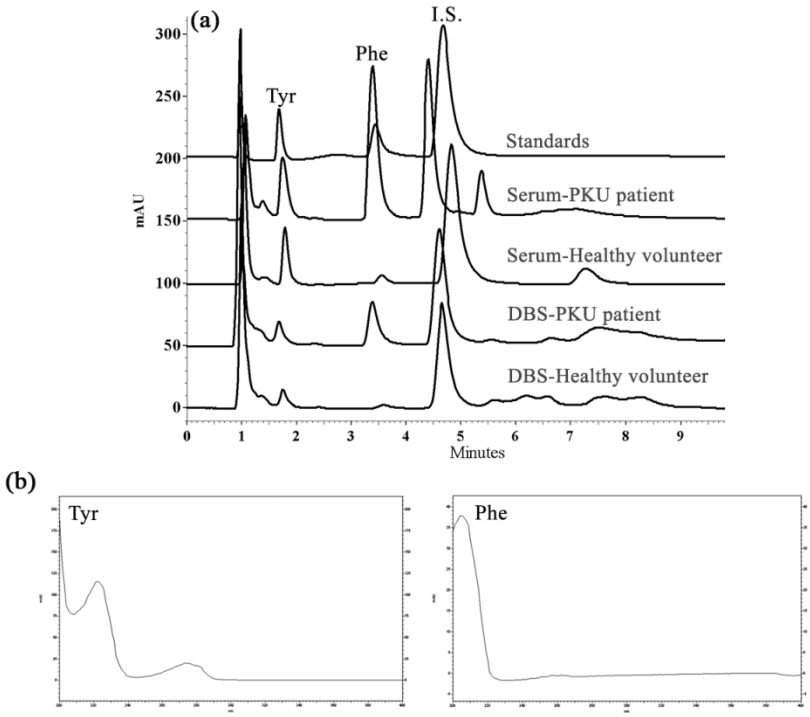

Fig. 1 (a) Obtained chromatograms for dried blood spots and serum samples of healthy volunteer and PKU patient at optimum chromatogr aphic conditions (monolithic silica C_{18}: 4.6 mm × 10 mm; mobile phase: gradient profile of water and MeOH; flow rate: 1.5 mL min^{-1}; wavelength: 210 nm, injection volume: 5 µL) and (b) photo diode array spectrum of Tyr and Phe.

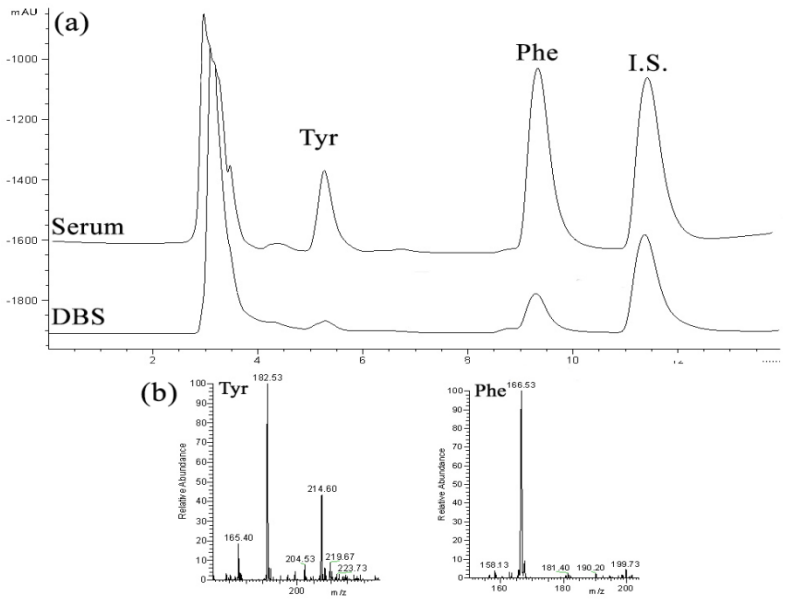

Fig. 2 LC-MS analysis of real samples. (a) chromatograms of dried blood spot and serum samples of PKU patient, (b) mass spectrum of Tyr and Phe with 182.47 and 166.53 m/z respectively. (note: since the working flow rate of LC-MS was set at 0.5 mL min^{-1}, the retention times were increased).

Table 1 Validation parameters for quantitative analysis of amino acids PKU disorder using the proposed method

Amino acid	Slope ± Standard error	Linear range (μM)	R[a]	LOD[b] (μM)	LOQ[c] (μM)
Phe	0.7316 ± 0.0083	0.1- 3200	0.998	0.04	0.1
Tyr	0.6118 ± 0.0066	0.1- 3200	0.998	0.04	0.1

[a]Regression coefficient, [b]Limits of detection, [C]Limits of quantification

Table 2 also presents recoveries data and their repeatability for the two amino acids analysed at four different spiking levels. The percentage of recoveries and RSD% ranged from 98.3% to 103.9% and 1.9% to 9.0%, for DBS samples and 88.2% to 104.2% and 0.5% to 5.5% for serum samples. All points were repeated in duplicate and triplicate for DBS serum samples respectively. Finally, in order to compare our proposed method with other reported methods, a compendium of the several reported HPLC and MS methods for measuring PKU metabolites are collected in Table 3 As shown, our proposed method not only showed wide range linearity and short analysis time but also it proved to be simple. Despite the fact that the use of MS as detection exhibited a lower LOD (0.001 μM) in comparison with the PDA detection (0.04 μM), the results of the study clearly show that mass spectrometry as detection method is not required.

Table 2 Precision and accuracy of the proposed method to quantify Phe and Tyr in DBS and serum samples

DBS				Serum			
	Mean (μmol L^{-1})	RSD%	Recovery %		Mean (μmol L^{-1})	RSD%	Recovery %
Spiked level of Phe							
0	56.13	3.5[a] & 4.2[b]	-	0	52.66	5.5[a] & 8.2[b]	-
333.33	393.61	9.0	100.1	500	583.29	5.5	99.9
1000	1095.43	1.9	103.9	833.33	924.79	2.2	98.9
1670	1701.43	0.2	98.5	1500	1431.29	2.3	96.7
2670	2689.07	2.6	98.6	2833.33	2548.29	0.5	92.5
Spiked level of Tyr							
0	91.35	5.5[a] & 6.5[b]	-	0	120.28	6.2[a] & 8.6[b]	-
333.33	437.15	3.2	103.5	500	650.55	5.3	104.2
1000	1074.57	3.6	98.3	833.33	957.92	1.0	99.3
1670	1655.07	3.6	98.5	1500	1527.69	4.5	94.9
2670	2930.81	2.4	99.3	2833.33	2601.16	1.1	88.2

[a] Inter-day assay (n=5), [b] Intra-day assay (n=5)

Table 3 Comparison of the proposed method with several reported HPLC and MS studies on quantification of amino acids PKU metabolites

Method			Matrix	Linear range		LOD[a] (μM)		Analysis time (min)	Reference
				Phe[b]	Tyr[c]	Phe	Tyr		
High Performance Liquid Chromatography									
Derivatization reagent	Column	Detector							
-	Ultrasphere ODS	UV[d]	Serum and plasma	85-370 pmol	75-338 pmol	-	-	10	[28]
OPA-2MCE[e]	Dionex ion-exchange resin DC-4A	Flu[f]	DBS[g]	20-2000 pmol		-	-	20	[32]
-	Hypersil ODS	UV	Plasma	10-3500 μM	-	1	-	10	[30]
-	Hypersil C-18	MS[h]	DBS	-		0.001		10	[36]
PITC[i]	RP-C18	UV	DBS and serum	Up to 10000 μM		5000		10	[31]
-	Purospher STAR RP-18e	Flu	DBS and plasma	10-1500 μM	5-750 μM	10	5	8	[33]
-	Hypersil C8	UV	Plasma, serum and whole blood	6-1512 μM	5.5-1250 μM	1.5	1	10	[29]
OPA-3MPA[j]	Zorbax Eclipse XDB-C18	Flu	DBS and serum	12-1200 μM		0.1	0.5	10	[34]
-	Chromolith C18	PDA	DBS and serum	0.1-3200 μM		0.04	0.04	6	Presented study
Mass Spectrometry									
DART-TQ-MS/MS[k]			DBS	3.0–156.25 μM	-	1.0	-	~3.3 sample. min^{-1}	[21]
MS/MS			DBS	-	-	2	1	-	[19]

[a] Limit of detection, [b] Phenylalanine, [c] tyrosine, [d] Ultraviolet, [e] *ortho*- Phthaldialdehyde- 2- Mercaptoethanol, [f] Fluorescence, [g] Dried blood spots, [h] Mass spectrometry, [i] Phenyl isothiocyanate, [j] *ortho*- Phthaldialdehyde- 3-mercaptopropionic acid, [k] Direct analysis in real time ionization coupled with triple-quadrupole tandem mass spectrometry

Table 4 Statistical comparison of the obtained amino acids PKU metabolites levels in DBS and serum using the proposed method.

Person	Phe (DBS)	Phe (Serum)	Tyr (DBS)	Tyr (Serum)
	Observed (µM)	Observed (µM)	Observed (µM)	Observed (µM)
Healthy newborn	298.4	243.2	197.7	181.7
PKU positive newborn	498.6	482.7	129.9	107.8
Healthy adult	197.5	180.6	75.5	61.0
PKU positive adult	365.7	352.3	89.0	117.9
	t Critical	2.54	t Critical	0.50
	t Stat at $\alpha = 0.05$	3.18	t Stat at $\alpha = 0.05$	3.18

Statistical study of obtained DBS and serum sample results

To find out whether there is any differences between measured levels of Phe and Tyr in DBS and serum samples or not, four DBS and serum samples (belonging to a healthy newborn, a PKU positive newborn, a healthy adult and a PKU positive adult) have been analyzed with the proposed method in this study. The results are shown in Table 4. The t-paired statistical test for two sets of data (DBS and serum samples) has been obtained using Excel software (version 2010). Since the t statistical for Phe (2.5) and Tyr (0.5) is smaller than the t critical (3.18) at a significant level of 95 %, there is no significant difference between these two amino acids in the DBS and serum sample.

Screening newborns and PKU patient follow up

127 DBS samples belonging to 18 newborns, 107 PKU patients under treatment (aged from 1 month to 30 years) and two healthy volunteers were analyzed. The obtained Phe and Tyr concentration for all samples are illustrated in Fig. 3. Among 18 newborns, 16 of them were considered healthy with Phe and Tyr concentration and Phe/ Tyr ratio of between 61.2-197.0 µM, 22-186 µM and 0.8-1.7. One of them with 541.34 µM Phe concentration and Phe/ Tyr ratio of less than 2.5 had PKU while another with 221.0 µM Phe concentration and Phe/ Tyr ratio of more than 2.5 seemed to have another IEM.

After screening PKU positive newborns, they should be treated with suitable diet and the Phe concentration should be regularly monitored to prevent irreversible physical and mental damages and it should continue throughout their whole life.[8] The most frequently recommended Phe concentration for children below and above 10 years of age is between 120-360 µM and 120- 600 µM, respectively [49, 50]. As seen in Fig 3 the concentration of Phe and Tyr in the samples of 30 out of 107 PKU patients who do not follow the diet properly is between 619.5-1898.6 µM and 9-150 µM, respectively. To investigate the application of our proposed method for diet therapy monitoring, the Phe blood levels of five children below two years of age have been measured using DBS samples. Ergo 120 µM and 360 µM were considered as upper and lower target cut off respectively. Fig 3 displays the Phe concentration changes between seven successive weeks just for two patients.

As it is evident, the Phe concentration of patient 1 has increased in two weeks because of some reasons while the Phe concentration in patient 2 was within the specified range.

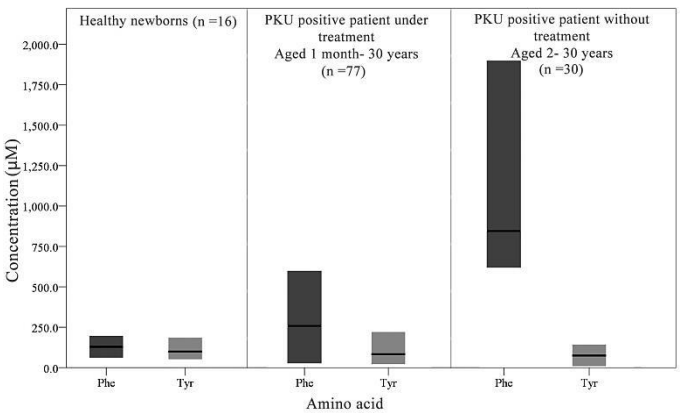

Fig. 3 Comparative diagram of Phe and Tyr obtained by the proposed method for healthy newborne, PKU positive patient under and without treatment.

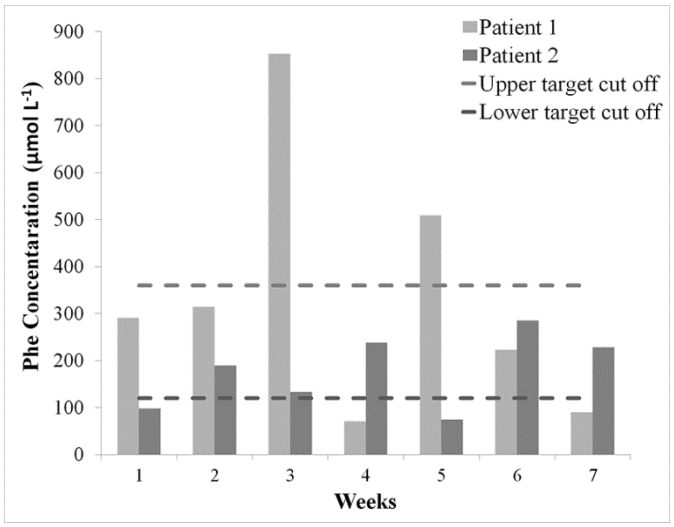

Fig. 4 Phe level of two PKU positive patients under diet treatment during seven successive weeks obtained by the proposed method.

Comparison of proposed method with ELISA

All of the DBS samples obtained from Mofid Children's Hospital (Iran, Tehran) were considered by the PKU-NEO ELISA KIT instruction (Pishtazteb company, Tehran, Iran) for measuring Phe levels and in this study, HPLC method was applied to determine Phe and Tyr levels in the same DBS and serum samples, the two methods could be compared by analyzing 108 DBS and 22 serum samples. The difference between results of ELISA and the proposed HPLC method and also the standard deviation (Sd) of differences was calculated and is presented in Fig. 5. A comparison of Phe concentration analyzed with different methods showed no significant bias (mean difference ± 2 Sd) and the few points that are upper than mean difference ± 2 Sd in plots could be considered as ELISA method errors at very high and low concentrations.

Although there are no significant differences between the results yielded by the two methods for measuring the concentration of Phe, it was not possible to measure the concentration of Tyr with ELISA method. Hence, the second biomarker of PKU disease for screening the neonatal (i.e. Phe/ Tyr) cannot be calculated with ELISA method which leads to deficiency of this method to confirm the PKU disorder.

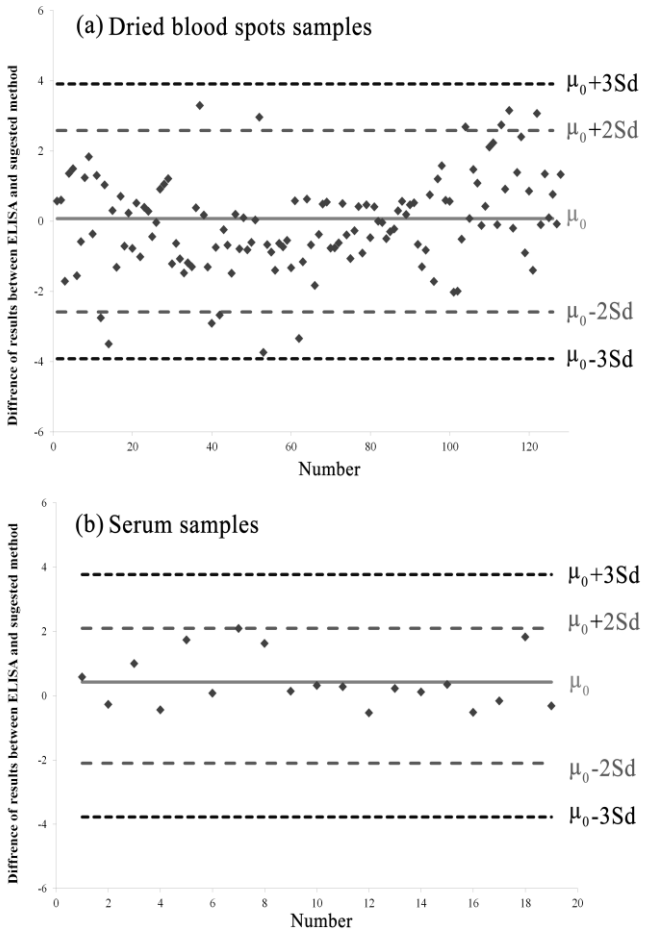

Fig. 5 Difference Phe levels (µM) between ELISA and the proposed method for (a) dried blood spots sample and (b) serum samples.

Conclusions

The present study addressed a validated HPLC–PDA assay using a monolithic silica C18 column for simultaneous determination of Phe and Tyr without derivatization in DBS and serum samples. Gradient profile was used and Phe, Tyr and internal standard were separated well in optimum conditions and the results were confirmed by LC-MS. The entire chromatographic analysis could be completed in less than 6 minutes. According to the results the advantages of this new method are: (1) simple sample preparation steps; (2) direct injection without derivatization; (3) complete separation of Phe, Tyr and internal standard in dried blood spot and serum samples without requiring any special and expensive instruments; (4) rapid, economical and green assay with respect to the high water content of the eluent and (5) highly accurate newborn screening and follow up for PKU. The developed method is very simple exhibiting an acceptable selectivity, linearity, accuracy and precision and has been reliably proven for screening newborns in real samples and for regular use to monitor the Phe and Tyr concentration in PKU patients.

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