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Simple and sensitive high performance liquid chromatographic method for the simultaneous quantitation of the phenylalanine in human plasma

Hossein Danafar[1,2*], Mehrdad Hamidi[3]

[1] Zanjan Pharmaceutical Nanotechnology Research Center, Zanjan University of Medical Sciences, Zanjan, Iran
[2] Department of Medicinal Chemistry, School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran
[3] Department of Pharmaceutics, School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran

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Abstract

Phenylalanine (Phe) is the most reliable indicator for the diagnosis of phenylketonuria (PKU). The purpose of this study is to establish a reliable and quick method for the assignment of Phe in peripheral capillary blood from newborns and children by high performance liquid chromatography with ultraviolet detection (HPLC-UV). PKU is an inborn error of metabolism characterized by the inability of the body to use Phe. A rapid and sensitive high performance liquid chromatographic (HPLC) method has been developed for determination of Phe in plasma. The method uses a protein precipitation step with sulfosalicilic acid for sample preparation by separation on a Nova-pack C_{18} column using sodium acetate buffer and acetonitrile (94: 6 v/v) adjusted to pH 6.5 with glacial acetic acid. The eluted peaks detected by a UV detector was set at wavelength of 215 nm. The method was validated in the range of Phe concentrations from 0.1 to 20 µg/ml. The limits of detection (LOD) and quantitation (LOQ) of the method were 0.05 and 0.1 µg/ml, respectively. The average drug recovery from plasma was 88.60 percent throughout the linear concentration range., with the average within-run and between-run accuracy values of 103.3 and 115.350, respectively. The method is quick, easy, very steady and precise for the screen, assignment, and evaluation of Phe in human plasma by HPLC, which is particularly a useful way for screening and diagnosis of PKU and monitoring of a diet therapy.

Keywords: Phenylketonuria (PKU); phenylalanine (phe) assay, reversed-phase HPLC

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Introduction

Phenylketonuria (PKU) is an inborn error of metabolism characterized by the inability of the body to use phenylalanine (Phe) (Fig. 1). The inability to hydrolyze Phe to tyrosine (Tyr) may adversely affect the synthesis of tyrosine dependent neurotransmitter substances (1). Left untreated, severe mental retardation result (2). PKU is investigated by studied levels of Phe in the blood. The range levels were observed in classic PKU from 6 to 80 mg/dl in humans (3). PKU shows a spectrum of recessively inherited metabolic disorders where the transformation of the aromatic amino acid Phe to Tyr, which is the precursor of catecholamines, is phenylalanineired (4,5). Phe is known as essential aromatic acids in mammals. Another aromatic amino acid, Tyr is not important when sufficient Phe is available, because the amino acid is normally

* E-mail: danafar@zums.ac.ir

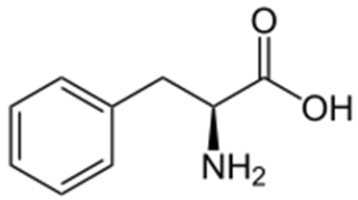

Figure 1 Chemical structure of phenylalanine

synthesized from Phe in the liver. Measurement of Phe levels in blood is an important way for the diagnosis and subsequent dietary management of PKU. Moreover simultaneous detection of blood Phe and Tyr concentrations and calculating the ratio of Phe/Tyr can reduce the false positive rate in PKU screening (6,7). Currently, high-performance liquid-chromatographic (HPLC) assay still roles an extremely important role in the assignment of Phe, and a variety of HPLC methods are used to the simultaneous determination of Phe in serum, plasma and dried blood-spot specimens (8-20)The evaluating and determination of PKU need to the measure of blood Phe levels using HPLC methods have been used for this measurement; however, part of these methods have shortages including instability of samples, cost of agents and rigidness encountered in sample preparation (21-26). In contrast, the HPLC methods used in this study is cheap, sensitive, quick, accurate and available. The objective of this study is to determine the blood Phe level in PKU humans using HPLC.

Materials and Methods

Materials

5-Sulfosalicylic acid (SSA), sodium acetate, glacial acetic acid, L-phenylalanine and HPLC-grade acetonitile and methanol were obtained from Merck (Darmstadt, Germany). Other chemicals and solvents were from chemical lab or HPLC purity grades, whenever needed, and were purchased locally.

Instrument and HPLC method

The HPLC system to include of pump (KNAUER, model 1000, Germany), wavelength UV detector (KNAUER, model 2800 (DAD), Germany) used at a wavelength of 215 nm with the outputs to record and analyze using with a software (ChromGate, KNAUER, Germany). The drug analization was performed using a C_{18}analytical column (250mm×4.6mm, particle size 5µm; Perfectsill, MZ-Analysentechnik, Germany) equipped by a guard column of the same packing. The mobile phase sodium acetate buffer and acetonitrile (94: 6 v/v) adjusted to pH 6.5 with glacial acetic acid was with a flow rate of 1 ml/min. Sample injection to system (20 µL) was made by a loop.

Standard preparation

A stock solution of 10 µg/ml Phe in water was prepared, from which the concentrations of 20, 10, 5, 2.5, 1, 0.5 and 0.1 µg/ml were prepared by serially diluting this solution with the amount of water. A series of plasma samples with Phe concentrations of 20, 10, 5, 2.5, 1, 0.5 and 0.1 µg/ml were prepared by 1:10 dilution of the commended solutions with drug-free human plasma.

Assay procedure

To 500 µL plasma samples were added, to 400 µL water and 100 µL 30% sulfosalicylic acid. The mixture was vortexed for 30 s. After centrifuged at 12000 rpm for 15 min, the supernatant was isolated and a sample of 20 µL was injected into the HPLC immediately.

Analysis Validation Tests

a) Standard curve (Linear range)

The plasma samples with a series of known concentrations, prepared as commended, were analyzed in three runs and, in each case, the linear regression analysis was achieved on known concentrations of Phe against the corresponding peak heights and, then, the regression coefficient (r), slope, and y-intercept of the resulting calibration curves were calculated.

b) Within-run variations

In one run, three samples with concentrations of 10, 2.5, and 0.5 µg/ml (from high, middle, and low regions of the standard curve) were prepared in triplicate and analyzed by HPLC method. Then, the coefficient of variations (CV %) of the corresponding determined concentrations were determined in each case.

c) Between-run variations

On three different runs, samples from upper, intermediate, and lower concentration regions used for construction of standard curve (the same as within-run variations test) were prepared and analyzed by HPLC method. Then, the corresponding CV% values were determined.

d) Absolute recovery (accuracy)

For each sample tested for within- and between-run variations, the absolute recovery of the method was calculated as the percent ratio of the measured concentration (determined using standard curve) to the corresponding nominal added concentration.

e) Relative recovery (matrix effect)

Three samples with concentrations of 10, 2.5, and 0.5 µg/ml (from high, middle, and low regions of the standard curve) were prepared in triplicate and analyzed by HPLC method. Then, the ratio of the recorded peak heights to the peak heights calculated from the direct injection of the aqueous solutions of Phe with the same concentrations were determined as percentage in each case.

f) *Limits of detection and quantitation*

Limit of detection (LOD) was calculated at the lowest concentration to be detected, taking into investigation of a signal-to-baseline noise ratio of 3. Limit of quantitation (LOQ) was applied as the lowest concentration at which the precision to state by relative standard deviation (R.S.D.) is lower than 20% and accuracy to state by relative difference of the measured and true value (RE) is also lower than 20%. The LOQ was repeated five times for confirmation.

Results

Method development

To study of the complex biological matrix of the samples to be analyzed and the nature of the method to be applied for drug assay, the method development efforts were made in two different areas of sample preparation and analyte separation which are studied in detail in the following sections:

a) Sample preparation

Protein precipitation was need and important because this technique can not only purify but also concentrate the sample. sulfosalicylic acid, percholeric acid and water were all exanimate and water: sulfosalicylic acid (80:20 v/v) was finally selected because of its high extraction efficiency and less interference.

b) Analyte separation

In response to lack of an available, sensitive, and easy-to-use analysis method for Phe assay in plasma as an essential part of pharmacokinetic and bioequivalence evaluation projects on the drug we selected a simple and reliable HPLC method with UV detection based on the available equipments found in most pharmaceutical laboratories. Finally, initially a series of isocratic as well as gradient conditions using different usual mobile phase compositions, polarities, ionic strengths,

and pH values were examined a in order to determine the best condition for the analyte separation. Typical chromatograms produced from the developed method are shown in Fig. 2.

Method validation tests

a) Linearity

The method produced linear responses throughout the Phe concentration range of 0.1-20 µg/ml, which is suitable for intended purposes. A typical linear regression equation of the method was: y = 0.0215x+0.194, with x and y representing Phe concentration (in µg/ml) and peak height (in arbitrary units), respectively, and the regression coefficient (r^2) of 0.9915(Fig. 3).

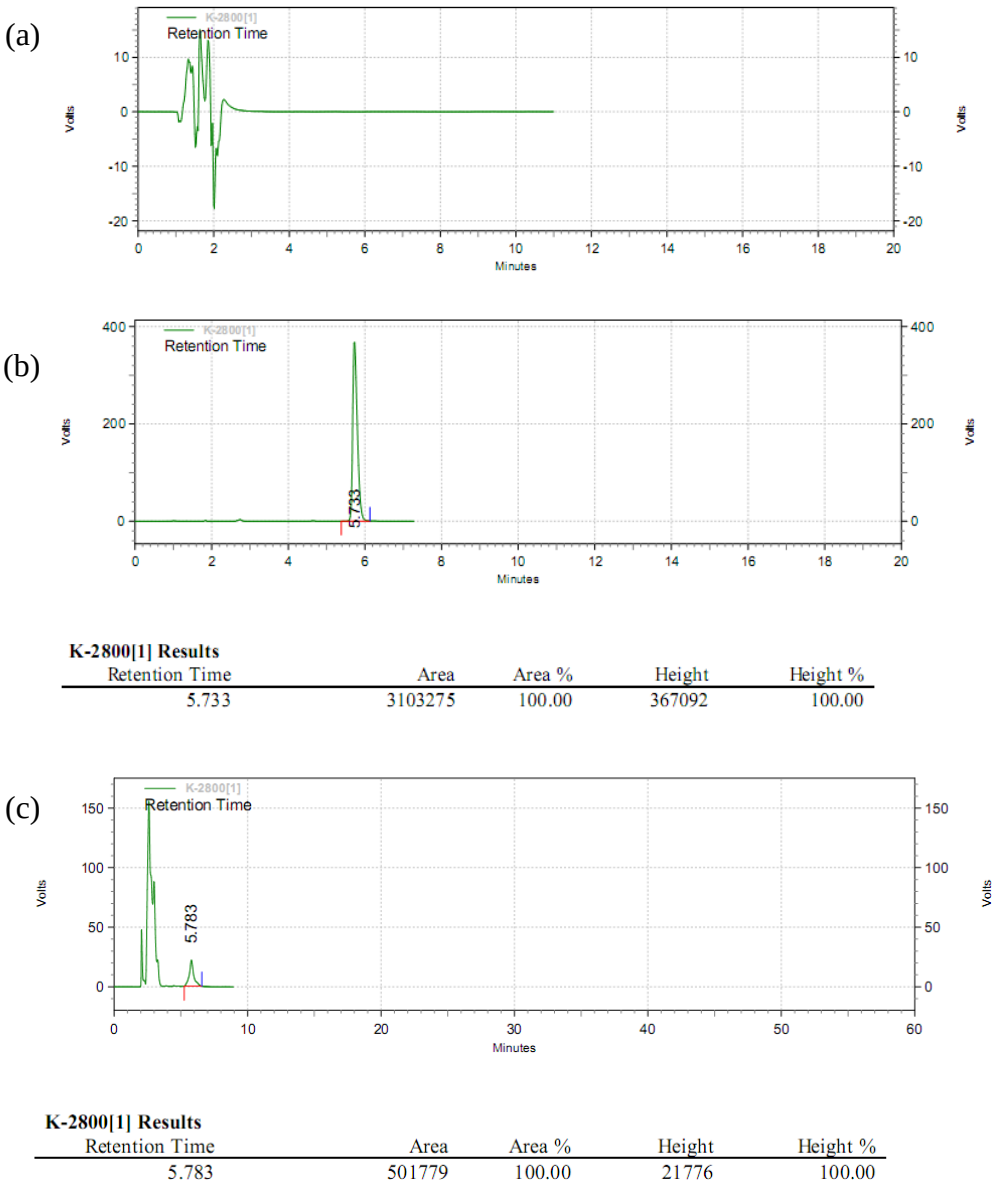

Figure 2 Typical chromatograms of the HPLC method developed for phenylalanine assay in human plasma: a); Chromatogram of stock solution b) human plasma spiked to a 5µg/ml drug concentration; C) human blank plasma

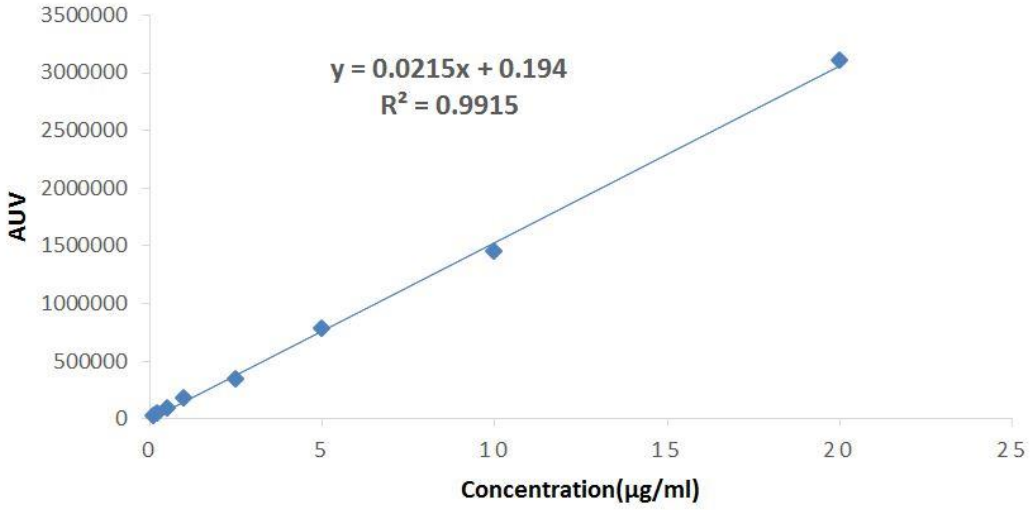

Figure 3 Standard curve of phenylalanine

b) Within-run variations and accuracy
The within-run variations of the HPLC method as well as the corresponding absolute recoveries are shown in Table1.

c) Between-run variations and accuracy
The between-run variations of the HPLC method as well as the corresponding absolute recoveries are shown in Table 2.

d) Relative recovery
The relative recovery of Phe using the assay method is shown in Table 3.

e) Limit tests
The limits of detection (LOD) and quantization (LOQ) of the method were 0.05 and 0.1µg/ml, respectively.
In general, the results of the validation tests showed that the method has a high degree of accuracy, repeatability, reproducibility, and recovery with application limits being in the desired range for routine applications.

Discussion
Untreated PKU patients may lead to mental retardation, microcap and other serious medical problems. However, if PKU patient is degassed early enough; they can grow up with normal brain development by treatment and monitoring Phe levels through mixture of diet and medication. Detection of Phe concentration in blood is the chosen method for diagnosis of PKU and treatment assignment. Methods for quantitative assignment of Phe in blood samples include HPLC method (8–20), fluorometric method (27), enzymatic method (28–30). Among these methods, HPLC is currently regarded as a simple, accurate, and rapid method to screen amino acid metabolic disorders in newborn. However, these methods declared above have some disadvantages, containing requirements of complex reprivatizing agents or sophisticated sample preparation (12-15). For example, phenyl isothiocyanate reagent was applied for the pre column derivatization of the amino acids (16). For the diagnosing of PKU or monitoring of in PKU patients, repeated Phe determinations are required. To achieve this goal, it is highly desired to have a single, simple and inexpensive analytical method. In this study, we established a HPLC-UV method for

Table 1 Within–run variations and accuracy of the HPLC method for quantitation of phenylalanine (n = 3)

Nominal Added Concentration (µg/ml)	Run Number	Measured Concentration (µg/ml)	Mean ± SD	CV%	Accuracy	Mean ± SD
0.5	1	0.45	0.53 ± 0.073	14	90.2	105 ± 14
	2	0.53			105.2	
	3	0.60			119.6	
2.5	1	2.83	25.62 ± 3.55	13.86	113.36	102.48 ± 14.18
	2	2.69			107.64	
	3	2.16			86.44	
10	1	11.34	10.29 ± 1.12	10.89	113.4	102.9 ± 11.2
	2	10.43			104.3	
	3	9.11			91.1	

Table 2 Between–run variations and accuracy of the HPLC method for quantitation of phenylalanine (n = 3)

Nominal Added Concentration (µg/ml)	Run Number	Measured Concentration (µg/ml)	Mean ± SD	CV%	Accuracy	Mean ± SD
0.5	1	0.68	0.65 ± 1.01	15.61	136.02	129.40 ± 20.08
	2	0.73			145.02	
	3	0.53			106.80	
2.5	1	2.83	2.73 ± 0.25	9.34	113.36	109.16 ± 10.21
	2	2.44			97.52	
	3	2.92			116.60	
10	1	10.28	10.75 ± 0.44	4.12	102.8	107.5 ± 4.4
	2	11.16			111.6	
	3	10.81			108.1	

Table 3 Relative recovery of phenylalanine by the HPLC method (n = 3)

Nominal Added Concentration (µg/ml)	Recovery (%)	Mean ± SD
0.5	82.65 80.92 75.00	84.97 ± 1.25
2.5	81.89 70.51 85.09	84.17 ± 1.61
10	82.78 75.34 87.20	96.68 ± 9.99

simultaneously detecting Phe levels in human plasma. The outcomes showed that our HPLC-UV method fully satisfied these conditions as mentioned above. For this method, the plasma preparation for analysis consist of a protein precipitation method. The validation tests on the developed method showed acceptable degree of linearity, sensitivity, precision, accuracy and recovery for the method.

Conclusion

Briefly, we have successfully recognized a HPLC-UV method for simultaneously identifying Phe in human plasma. This method delivers a quick and a cheap alternative to those using HPLC with pre- or post-column derivatization. A simple HPLC method was established and indorsed for Phe assay in plasma. The method was used successfully for quantization of Phe in plasma samples. System suitability tests showed that the established method is of suitable separation efficiency and peak shape.

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Conflict of interest

Authors had declared no conflict of interest

References

1. Belmont-Martínez L, Fernández-Lainez C, Ibarra-González I, Guillén-López S, Monroy-Santoyo S, Vela-Amieva M. Evaluaciónbioquímica de la fenilcetonuria (PKU): del diagnóstico al tratamiento. Acta Pediatr Méx 2012;33:296–300. Spanish.
2. Mitchell JJ. Phenylalanine Hydroxylase Deficiency. In: Pagon RA, Adam MP, Bird TD, editors. GeneReviews™ [Internet]. 2010 Jan 10 [updated 2013 Jan 31; cited 2014 Jan]. Seattle (WA): University of Washington; 1993–2014. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1504.
3. Scriver C, Kaufman S. The hyperphenylalaninemias. In: Scriver C, Beaudet AL, Sly WS, Barton C, Valle D, Kinsler KW, et al, editors. The metabolic and molecular bases of inherited disease.8th ed. Vol II. New York: McGraw-Hill. 2001; p. 1667–724.
4. Smith I, Philip L. The hyperphenylalaninemias. In: Fernandes J, Saudubray JM, Van den Berghe G, editors. Inborn metabolic diseases, diagnosis and treatment. 3rd ed. Heidelberg (DE): Springer-Verlag. 2000; p. 171–83.
5. Guthrie R, Susi A. A simple phenylalanine method for detecting phenylketonuria in large

populations of newborn infants. Pediatrics 1963;32:338–43.
6. Levy PA, Miller JB, Shapira E. The advantage of phenylalanine to tyrosine ratio for the early detection of phenylketonuria. Clin Chim Acta 1998;270:177–81.
7. Eastman JW, Sherwin JE,Wong R, Liao CL, Currier RJ, Lorey F, et al. Use of the pheylalanine: tyrosine ratio to test newborns for phenylketonuria in a large public health screening programme. J Med Screen 2000;7:131–5.
8. Hilton MA. Liquid-chromatographic direct determination of phenylalanine andtyrosine in serum or plasma, with application to patients with phenylketonuria. Clin Chem 1982;28:1215–8.
9. Roesel AR, Blankenship PR, Hommes F. HPLC assay of phenylalanine and tyrosinein blood spots on filter paper. Clin Chim Acta 1986;156:91–6.
10. Rudy JL, Rutledge JC, Lewis SL. Phenylalanine and tyrosine in serum and eluatesfrom dried blood spots as determined by reversed-phase liquid chromatography. Clin Chem 1987;33:1152–4.
11. Atherton ND, Green A. HPLC measurement of phenylalanine in plasma. Clin Chem1988;34:2241–4.
12. Vollmer DW, Jinks DC, Guthrie R. Isocratic reverse-phase liquid chromatographyassay for amino acid metabolic disorders using eluates of dried blood spots. Anal Biochem 1990;189:115–21.
13. Qu Y, Miller JB, Slocum RH, Shapira E. Rapid automated quantitation of isoleucine,leucine, tyrosine, and phenylalanine from dried blood filter paper specimens. Clin Chim Acta 1991;203:191–8.
14. Reilly AA, Bellisario R, Pass KA. Multivariate discrimination for phenylketonuria(PKU) and non-PKU hyperphenylalaninemia after analysis of newborns' driedblood-spot specimens for six amino acids by ion-exchange chromatography.Clin Chem 1998;44:317–26.
15. Allen KR, Degg TJ, Rushworth PA, Smith M, Henderson MJ. Measurement of phenyl lanine and tyrosine in plasma by high-performance liquid chromatography using theinherent fluorescence of aromatic amino acids. Ann Clin Biochem 1999;36:207–11.
16. Dale Y, Mackey V, Mushi R, Nyanda A, Maleque M, Ike J. Simultaneous measurement of phenylalanine and tyrosine in phenylketonuric plasma and dried bloodby high-performance liquid chromatography. J Chromatogr B Analyt Technol Biomed Life Sci 2003;788:1–8.
17. Hong M, Tang AG. Determination of phenylalanine and tyrosine in serum byhigh-performance liquid chromatography with fluorescent detection. Zhong NanDa Xue Xue Bao Yi Xue Ban 2004;29:67–71.
18. Jeong JS, SimHJ, Lee YM, Yoon HR, Lee DH, Hong SP. Determination of phenylalaninein blood by high-performance anion-exchange chromatography-pulsed ampere metric detection to diagnose phenylketonuria. J Chromatogr A 2009;1216:5709–14.
19. Kand'ár R, Záková P. Determination of phenylalanine and tyrosine in plasma anddried blood samples using HPLC with fluorescence detection. J Chromatogr B Analyt Technol Biomed Life Sci 2009;877:3926–9.
20. Kim NH, Jeong JS, Kwon HJ, Lee YM, Yoon HR, Lee KR, et al. Simultaneous diagnostic method for phenylketonuria and galactosemia from dried blood spots usinghigh-performance liquid chromatography-pulsed amperometric detection. J Chromatogr B Analyt Technol Biomed Life Sci 2010;878:1860–4.
21. Madira WM, Xavier F, Stern J, Wilcox AH, Barron JL. Determination and assessment of the stability of phenylalanine and tyrosine in blood spots by HPLC. Clin Chem 1992;38:2162–3.
22. Kok WT, Brinkman UA, Frei RW. Rapid determination of phenylalanine and tyrosine in urine and serum by HPLC with electrochemical detection. J Pharm Biomed Anal 1983;1:369–72.
23. Qu Y, Slocum R, Fu J, Rasmussen WE, Rector HD, Miller JB, et al. Quantitative amino acid analysis using a Beckman system gold HPLC 126AA analyzer. Clin Chim Acta. 2001; 312(1–2):153–62.
24. Bartolomeo MP, Maisano F. Validation of a reversed-phase HPLC method for quantitative amino acid analysis. J Biomol Tech. 2006;17:131–7.
25. Gatti R, Gioia MG. Liquid chromatographic fluorescence determination of amino acids in plasma and urine after derivatization with phanquinone. Biomed Chromatogr. 2008;22:207–13.
26. Kandar R, Zakova P. Determination of phenylalanine and tyrosine in plasma and dried blood samples using HPLC with fluorescence detection. J Chromatography B AnalytTechnol Biomed Life Sci 2009 15;877:3926–9.
27. Gerasimova NS, Steklova IV, Tuuminen T. Fluorometric method for phenylalanine microplate assay adapted for phenylketonuria screening. Clin Chem 1989;35:2112–5.
28. Wendel U, Koppelkamm M, Hummel W, Sander J, Ulrich L. A new approach to the newborn screening for hyperphenylalaninemias: use of L-phenylalanine dehydrogenase and microtiter plates. Clin Chim Acta 1990;192:165–70.
29. Wibrand F. Amicroplate-based enzymatic assay for the simultaneous determination of

phenylalanine and tyrosine in serum. Clin Chim Acta 2004;347:89–96.

30. De Silva V, Oldham CD, May SW. L-Phenylalanine concentration in blood of phenylketonuria patients: a modified enzyme colorimetric assay compared with amino acid analysis, tandem mass spectrometry, and HPLC methods. Clin Chem Lab Med 2010;48:1271–9.