PLASMA PHENYLALANINE DETERMINATION BY QUANTITATIVE DENSITOMETRY OF THIN LAYER CHROMATOGRAMS AND BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN RELATION WITH MODERN MANAGEMENT OF PHENYLKETONURIA

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Abstract

Background. The modern management of phenylketonuria (PKU) consists of generalized newborn screening (NBS) for hyperphenylalaninemia (HPA), confirmation of HPA in children detected in the NBS, introduction of dietary treatment in the first weeks of life, followed by monitoring the treatment of PKU for decades to maintain phenylalaninemia within the limits that will not affect the brain. The present study aimed to evaluate the usefulness of two chromatographic methodologies for determination of plasma Phe level in the routine management of PKU: the two dimensional thin layer chromatography (2D - TLC) and the high performance liquid chromatography (HPLC) procedures, respectively.

Material and Methods. Samples of blood from 23 children with HPA detected by neonatal screening or with confirmed PKU who received treatment by low-Phe diet were analyzed to estimate the plasma Phe level by the two chromatographic procedures.

Results. In case of three subjects the very low concentrations of plasma Phe could not be detected by the 2D - TLC methodology, since the spot was not visible on the chromatogram. In four patients the differences between the values of plasma Phe determined by the two methodologies are not statistically significant, while in fifteen subjects the differences are highly statistically significant. This is due to the greater errors that appear in the case of 2D - TLC methodology. In the range of concentrations of plasma Phe higher than 360 µmol/L (which is the cut-off value for HPA), although in four cases there were statistically significant differences in the level of plasma Phe determined by the two methodologies, the value obtained by the 2D - TLC methodology was high enough to influence the decision of changing the diet so that HPA is kept under control. In addition, the intense spot of Phe on the 2D - TLC chromatogram may be detected even by an unexperienced

laboratory specialist.

Conclusion. The HPLC procedure for measurement of plasma Phe level is very suitable to be used in the routine management of PKU. The 2D - TLC procedure may be accompanied by relatively high errors; however, it detects patients with severe PKU.

Key words: phenylalanine (Phe), phenylketonuria (PKU), hyperphenylalaninemia (HPA), high performance liquid chromatography (HPLC), thin layer chromatography (TLC).

INTRODUCTION

Phenylketonuria (PKU) is the most common inborn error of amino acid metabolism, about one case per 10,000 livebirths in Europe (1), caused by absent or virtually absent phenylalanine hydroxylase (PAH) enzyme activity (2 - 4). This enzyme (EC 1.14.16.1) normally converts phenylalanine (Phe) to tyrosine and the absence of PAH enzyme activity leads to abnormally high levels of Phe and its metabolites in the blood, which is toxic particularly to the brain. If not detected early and treated by dietary means (a low-Phe diet) hyperphenylalaninemia (HPA) causes irreversible mental retardation, delays in motor development, autism, seizures and other symptoms (5 - 7), even endocrine abnormalities (8). Fortunately, PKU may be detected by modern newborn screening programs (1, 9 - 12). Romania started the national program for screening of PKU and of congenital hypothyroidism in 1999. Both diseases can be detected and treated by specific procedures, while undetected will result in

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poor neuropsychological outcome, even irreversible mental retardation.

In case of PKU, phenylalaninemia can be maintained below the neurotoxic level by low-Phe diet. The cerebral damage can be largely eliminated only if the dietary treatment is started in the first weeks of life (preferable in the first 15 days) and the plasma Phe level is kept within certain ranges (between 2 and 6 mg/dL, or equivalently 120 and 360 $\mu\text{mol/L}$ (1-4, 9 - 14). PKU is a disease that has a special relevance from the public health perspective: if PKU is not detected by newborn screening and the low-Phe diet is not introduced early or the plasma Phe level is not kept within the above mentioned ranges, the irreversible mental retardation occurs, leading to major problems, for the patients and their family, but also for the society, including the burden of institutionalized children (15 - 18).

The determination of plasma Phe level is of utmost importance for the correct management of PKU. The “gold standard” method for measuring the level of Phe in plasma is tandem mass spectrometry (MS/MS), that is affordable only to the most developed countries (1, 13 - 15). In the Laboratory of Genetic Explorations of Cluj County Clinical Emergency Hospital headed by the corresponding author and, recently, in collaboration with researchers of the Institute of Life Sciences, “Vasile Goldiș” Western University of Arad, two less expensive methodologies for measuring the concentration of plasma Phe were developed and evaluated: the image-densitometry of two dimensional thin layer chromatograms (2D - TLC) and the high performance liquid chromatography (HPLC) procedures, respectively (19 - 21).

The current study aims to evaluate the usefulness of these two chromatographic methodologies in the routine management of PKU, which consists of generalized newborn screening for hyperphenylalaninemia (HPA), confirmation of HPA by determination of plasma Phe level in all newborn children who presented HPA in the screening, introduction of dietary treatment in the first weeks of life, followed by monitoring the treatment of PKU for decades to maintain phenylalaninemia within the limits that will not affect the brain.

MATERIALS AND METHODS

Subjects and study design

Samples of blood from children with HPA detected by newborn screening or with confirmed PKU who received treatment by low-Phe diet in various

clinics of the Cluj Children Clinical Emergency Hospital are routinely referred to the Laboratory of Genetic Explorations (LGE) of Cluj County Clinical Emergency Hospital for determination of plasma Phe. An informed consent was obtained from the parents of the children and the study was approved by the Local Ethics Committee of the institutions where the blood specimens were processed.

Out of the samples referred to the LGE we selected in this study those of twenty-three children to perform comparative analyses of plasma Phe by the HPLC and by the 2D - TLC procedures.

Blood specimen collection and separation of plasma

Blood was collected into heparinized Vacutainer tubes (Becton Dickinson, Basle, Switzerland) and plasma was prepared by centrifugation at 1600g and 22°C, within 60 min after the collection of blood.

Isolation and concentration of plasma amino acids

The isolation and concentration of plasma aminoacids was performed by the “desalting” procedure developed by Wadman and coworkers (22, 23) as described previously in detail (20). The concentrated solution of amino acids free of salts and other interfering substances (called the plasma extract) was used for the analysis of aminoacids by HPLC and another aliquot for the analysis of amino acids by 2D - TLC.

Chromatographic analysis of amino acids

HPLC analysis of Phe concentration was performed using a Dionex Ultimate 3000 instrument (low pressure gradient pump, Dionex 3000) equipped with a Ultimate 3000 diode array detector. The column used was a Thermo Scientific Acclaim 120, C18, 5 μm Analytic (4.6 x 250 mm) column, coupled with an Acclaim C18 guard column. The working conditions were as follows: the mobile phases were acetonitrile (solution A) and water at pH 6 with formic acid (solution B), isocratic elution with 30% solvent A and 70% solvent B, elution time 5 minutes, column temperature 30°C, injection volume 10 μL , detection wavelength 210 nm. Plasma concentration of Phe was calculated by using a calibration curve for the correspondence between the Phe concentration ($\mu\text{mol/L}$) and the arbitrary absorption units (mAU) (as described in ref. 20).

2D - TLC determination of plasma Phe concentration was performed as described previously

(20). The aminoacids from the plasma extract are separated on 5 x 5 cm plates of cellulose on glass using the procedure of Wadman and coworkers (22, 23). Phe spot has a specific position, separated from other aminoacids. A very intense spot of Phe is visible in case of HPA (including the PKU patients) and less intense spots are visible in case of lower concentrations of plasma Phe. Below a certain level of plasma Phe the spot is not visible (i.e. Phe is not detectable). Examples of such aspects of 2D-TLC chromatograms of plasma from children without or with HPA have been presented previously (21).

The final step is quantifying the level of plasma Phe by imaging-densitometric methodology applied on a Bio-Rad GS-700 imaging densitometer (Bio-Rad Laboratories, Molecular Bioscience Group (Hercules, CA, USA), coupled to a computer. The Molecular Analyst /PC Windows software for Bio-Rad's Image Analysis Systems Version 3.1 was used. The Phe spot volume (optic density x spot area) and the spot volume of a similar area of background are measured on the TLC plate. Then the parameter called Adjusted Volume is calculated as the difference between the Phe spot volume and the background spot volume. Plasma concentration of Phe is calculated by using a calibration curve for the correlation between the Phe concentration ($\mu\text{mol/L}$) and the Adjusted Volume (19, 20).

Statistical analyses were performed using the Microsoft Office, Excel statistical package version 2007. Descriptive statistics (mean, standard deviation) and Student t-test were used in comparative analyses. The statistical significance was denoted with a p value <0.05 .

RESULTS

The characteristics of the 23 children selected for this study, representative for the typical situations when determination of plasma Phe level is required, and the comparison of the plasma Phe level values determined by 2D - TLC and HPLC are presented in Table 1.

Figure 1 presents a graph with comparative plasma Phe level values of the 23 patients determined by 2D - TLC and HPLC. The values are represented in the increasing order of the plasma Phe level value determined by HPLC.

Several observations can be made analysing the data presented in Table 1 and Figure 1. First, in case of very low concentrations of plasma Phe (patients 1, 4 and 10) the 2D - TLC methodology may fail to detect Phe, since the spot is not visible on the chromatogram. In contrast, the concentration of Phe can be determined by HPLC. In a single case (patient 9) the level of plasma

Table 1. Characteristics of the study subjects and comparative descriptive statistics of the values of plasma phenylalanine concentrations determined by 2D-TLC and HPLC methodologies (N.S. - not statistically significant)

Patient Number	Sex	Age	Plasma phenylalanine concentrations ($\mu\text{mol/L}$) Determined by 2D-TLC	Determined by HPLC	p value of the difference between the two methods
1	M	8 years	Not detectable	7.8 $\pm$ 0.9	
2	M	10 years	74.0 $\pm$ 5.2	10.5 $\pm$ 0.1	P<0.0001
3	F	28 days	73.3 $\pm$ 1.7	25.2 $\pm$ 2.1	P<0.0001
4	F	5 days	Not detectable	26.7 $\pm$ 0.3	
5	F	10 days	4.6 $\pm$ 0.9	26.9 $\pm$ 0.4	P<0.0001
6	F	2.5 months	15.4 $\pm$ 3.2	27.3 $\pm$ 2.6	P=0.002
7	M	15 days	53.4 $\pm$ 2.8	43.1 $\pm$ 1.4	P=0.002
8	F	12 days	70.2 $\pm$ 3.3	63.9 $\pm$ 0.8	P=0.004
9	M	7 years	639.8 $\pm$ 6.1	70.6 $\pm$ 4.4	P<0.001
10	F	1 month	Not detectable	79.4 $\pm$ 0.1	
11	F	4 months	93.0 $\pm$ 7.0	87.0 $\pm$ 0.3	P=0.304
12	M	2.5 years	136.0 $\pm$ 5.0	91.0 $\pm$ 1.1	P=0.0007
13	M	1 month	112.5 $\pm$ 6.5	96.9 $\pm$ 0.6	P=0.019
14	F	7 months	121.9 $\pm$ 5.3	157.2 $\pm$ 26.4	P=0.064
15	F	1.5 months	50.2 $\pm$ 2.4	212.5 $\pm$ 0.2	P<0.0001
16	F	1.5 months	100.6 $\pm$ 7.1	309 $\pm$ 1.2	P<0.0001
17	F	9 months	270.7 $\pm$ 11.0	350.7 $\pm$ 5.6	P=0.0018
18	F	9 years	6372 $\pm$ 9.8	661.8 $\pm$ 13.6	P=0.062
19	F	9.5 years	969.6 $\pm$ 12.2	1513.4 $\pm$ 6.2	P<0.0001
20	F	11 months	1795.7 $\pm$ 34.0	2425.0 $\pm$ 9.0	P<0.0001
21	M	2.3 months	5013.4 $\pm$ 18.3	2587.7 $\pm$ 8.2	P<0.0001
22	F	2 months	2750.0 $\pm$ 53.1	2673.0 $\pm$ 33.4	P=0.174
23	M	2.5 months	3774.7 $\pm$ 17.2	3498.8 $\pm$ 115.9	P=0.022

Figure 1. Graph showing the comparative plasma Phe level values of the 23 patients determined by 2D - TLC and HPLC.

Phe determined by 2D - TLC methodology appeared to be much higher (~ 640 µmol/L) compared to ~71 µmol/L determined by HPLC.

On the other hand, considering the remaining nineteen subjects, in patients 11, 14, 18, 22, the differences between the values of plasma Phe determined by the two methodologies are not statistically significant ($p > 0.05$), while in fifteen subjects the differences are highly significant. This is due to the greater errors that appear in the case of 2D - TLC methodology, as we have reported previously (21).

Another important observation is that in the range of concentrations of plasma Phe higher than 360 µmol/L (which is the cut-off value for HPA), although in four cases (patients 19, 20, 21, 23) there are statistically significant differences in the level of plasma Phe determined by the two methods, the value of plasma Phe obtained by the 2D - TLC method is high enough for clinicians (whom the results of determinations are sent) to take the decision to change the diet so that HPA is kept under control, to avoid as much as possible the mental retardation. In addition, the intense spot of Phe on the 2D - TLC chromatogram may be detected even by an unexperienced laboratory specialist.

DISCUSSION

The present study aimed to evaluate the usefulness of two chromatographic methods (the 2D

- TLC and the HPLC, respectively) for determination of plasma Phe level in the routine management of PKU. The children included in the study group were infants with HPA detected by neonatal screening (and the analyses were necessary to confirm or exclude PKU), while others were children with confirmed PKU and the plasma Phe level was determined as part of the monitoring management, i.e. to control that phenylalaninemia is maintained within the ranges (between 2 and 6 mg/dL, or equivalently 120 and 360 µmol/L) considered to be low enough to avoid the mental retardation, as well as other abnormalities mentioned above.

Since a very high number of analyses of plasma Phe level should be performed in the routine management of PKU (and the cost of analyses is not covered by the public health budget in most countries), it is essential to consider which methods should be used.

The “gold standard” method for measuring the level of Phe in plasma is tandem mass spectrometry (MS/MS), the technique recognized for its sensitivity and specificity in the diagnosis of PKU and other 30 inborn errors of metabolism (26). However, MS/MS is very expensive being affordable only to the most developed countries (1, 15, 16, 24). In the present study we compared two less expensive methodologies: the HPLC procedure and the 2D - TLC procedure.

It should be mentioned that HPLC is used for decades (25) to measure the blood concentration

of Phe and other amino acids for diagnosis and monitoring of patients with PKU. There are advantages of using HPLC for measuring the blood Phe levels by HPLC: the values did not differ statistically from the determinations performed by MS/MS (26). On the other hand a variety of HPLC approaches have been developed, including the use of special columns, or performing pre- or post-column derivatization, which are considered by Neureter *et al.* (27) disadvantages of the HPLC methods, as they may be rather expensive and time consuming. Consequently, MS/MS and some HPLC methods are affordable only to the developed countries in the world (2 - 7, 13, 32). Neureter *et al.* (27) also mentioned that “for specific studies of neuropsychiatric diseases, often only concentrations of Phe and Tyr, probably in addition to tryptophan but not of all the other proteinogenic amino acids, are of interest”.

The HPLC method used by us is rather simple, very sensitive and highly accurate and it does not require special columns for amino acid analysis.

In conclusion, the HPLC procedure for measurement of plasma Phe level is very suitable to be used in the routine management of PKU. The 2D - TLC procedure may be accompanied by relatively high errors and should be carefully used in monitoring the management of PKU, i.e. in estimation of phenylalaninemia within the ranges considered to be low enough to avoid the mental retardation (between 2 and 6 mg/dL, or equivalently 120 and 360 $\mu\text{mol/L}$). However, the 2D - TLC procedure is adequate to detect patients with severe PKU. In addition to the very high value of phenylalaninemia determined by video-densitometry, the intense spot of Phe on the 2D - TLC chromatogram may be detected even by unexperienced laboratory specialist. The patients could then be referred to clinicians who will monitor the dietary treatment.

Conflict of interest

The authors declare that they have no conflict of interest concerning this article.

Acknowledgements

The corresponding author is grateful to the late Professor S.K. Wadman (Laboratory of the Wilhelmina Kinderziekenhuis, Utrecht, The Netherlands) and his coworkers for their kindness to teach him the methods for analyses of amino acids for performing the diagnosis of amino acid metabolism disturbances. He is also grateful to his former co-workers with whom the methods of analysis of amino acids were first introduced in the medical laboratories in Romania (33).

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