COMPARISON OF PLASMA PHENYLALANINE DETERMINATION BY DENSITOMETRY OF THIN-LAYER CHROMATOGRAMS AND BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN RELATION WITH THE SCREENING OF PHENYLKETONURIA

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Abstract

Objective. To compare two chromatographic methodologies for determination of plasma phenylalanine (Phe) and their usefulness for diagnosing hyperphenylalaninemia (HPA) and phenylketonuria (PKU).

Methods. The plasma amino acids were isolated and concentrated from blood collected from infants with HPA detected by newborn screening. The plasma Phe was determined in parallel by HPLC and by image-densitometry of 2D-TLC plates.

Results. Typical examples of 2D-TLC plates and HPLC chromatograms from infants with HPA and PKU are presented and evaluated. The Phe spot was visible on 2D - TLC plates at Phe concentrations higher than 300 $\mu\text{mol/L}$. The standard calibration curve traced after image-densitometry of the Phe spot presented high dispersion of values at each concentration of Phe, high SD values, the equation of the curve having a low R-squared value (0.862). In contrast, the standard calibration curve obtained by HPLC shows linearity on the range of concentrations from 100 - 16,000 $\mu\text{mol/L}$, extremely small SD values, the equation of the curve has a very high R-squared value (0.999).

Conclusions. The HPLC methodology is appropriate to confirm HPA detected by newborn or selective screening of PKU. The 2D - TLC methodology is adequate to detect patients with severe PKU.

Key words: phenylalanine, phenylketonuria, hyperphenylalaninemia, screening, densitometry, thin layer chromatography (TLC), high performance liquid chromatography (HPLC).

INTRODUCTION

Phenylketonuria (PKU) is the most prevalent inborn error of amino acid metabolism, about one case

per 10,000 livebirths in Europe, including Romania (1, 2). The main biochemical defect in PKU is the absence, or virtually absence of L-phenylalanine hydroxylase (PAH) enzyme activity (3 - 5). This enzyme (EC 1.14.16.1) normally converts phenylalanine (Phe) to tyrosine (Tyr) by hydroxylation; PAH requires the cofactor tetrahydrobiopterin (BH₄), molecular oxygen, and iron to do so (3, 5, 6). 98% of hyperphenylalaninemias are caused by mutations in the gene encoding PAH, the remaining 1%-2% of cases being attributable to defects in other enzymes involved in synthesis or regeneration of BH₄ (7).

Loss of PAH activity results in hyperphenylalaninemia (HPA), which is toxic to the brain (3 - 8). The mechanisms for HPA-induced damage to the brain have been extensively investigated (reviewed in ref. 5). Undetected and/or untreated PKU leads to severe cerebral damage, with progressive mental retardation, delays in motor development, seizures, autism and many other symptoms: characteristic urine odor, skin and hair depigmentation, eczematous rash (3 - 9), even endocrine abnormalities (10, 11).

The modern management of PKU is aimed to prevent, minimize (or even partially reverse) the cerebral damage due to HPA. This includes: a) detection of HPA by neonatal screening, performed in all developed (and many developing) countries, including Romania, in addition to screening for congenital hypothyroidism; b) confirmation of HPA within 10 - 30 days of life; c) the initiation of treatment by dietary means from the first weeks of life in cases of plasma Phe levels greater than 600 $\mu\text{mol/L}$ (or 360 $\mu\text{mol/L}$ recommended by some treatment centers) (4 -6, 12); d) monitoring the treatment of PKU for many years in order to maintain

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the plasma Phe level between 120 - 360 $\mu\text{mol/L}$ (1 - 6, 9), to avoid severe mental disability (1 - 6, 11, 12).

The newborn screening is performed on bloodspots within a few days of birth, using the Guthrie - Susi method (13) or more modern methods (14). In addition, there is a selective screening of PKU, in case of children who missed the neonatal screening (for various reasons) and are later consulted by family physicians, who suspect PKU (based on the symptoms mentioned above) and refer the subject to pediatricians or child neurologists for further investigations.

Determination of plasma Phe concentration is consequently of utmost importance to achieve all these goals. HPA detected by newborn screening should be confirmed by quantification of the level of blood Phe by another method before the treatment by dietary means is started (5, 6, 9). This is necessary to exclude cases with transient neonatal HPA or false positive results in determinations of Phe by routine newborn screening. HPA is defined by any plasma Phe concentration more than 120 $\mu\text{mol/L}$. It is now general agreement that maintaining the plasma Phe level within certain ranges (between 120 and 360 $\mu\text{mol/L}$, or equivalently 2 and 6 mg/dL) is the key point during treatment of PKU to avoid severe mental disability and the major public health problems linked to this rare disease, including the burden of institutionalized children (1 - 6, 15).

Our group has previously described a simple, very sensitive and accurate high performance (HPLC) procedure for the simultaneous determination of Phe (and Tyr) plasma concentrations (16, 17) to be used in early detection of HPAs. On the other hand, the analyses of plasma aminoacids by two dimensional thin layer chromatography (2D - TLC) according to the procedures described by Wadman and coworkers (18, 19) have been used for decades by the group of the corresponding author in Cluj-Napoca, Romania to detect children with PKU (reviewed in ref. 20). Moreover, a method for determination of plasma Phe concentration by image-densitometry on 2D - TLC chromatograms has been developed (21).

The current study aims to compare the above mentioned two procedures (HPLC *versus* image-densitometry on 2D - TLC chromatograms) for the determination of plasma Phe concentration in relation to the routine screening of PKU in children.

MATERIALS AND METHODS

Reagents

Acetonitrile (HPLC grade), phenylalanine

and most of other reagents (analytical grade) were purchased from Merck (Darmstadt, Germany), except for t-butanol (purchased from Scharlab S.L., Sentmenat, Spain), methyl ethyl ketone (purchased from Lach-Ner, Neratovice, Czech Republic), ethanol and acetone (purchased from Chimopar, Bucharest, Romania). Dowex-50X8, 50-100 mesh was purchased from Bio-Rad Laboratories (Richmond, CA, USA).

Procedures

Blood specimen collection and separation of plasma

Blood from infants with HPA detected by neonatal screening for PKU performed in the Cluj County Clinical Emergency Hospital was collected into heparinized Vacutainer tubes (Becton Dickinson, Basle, Switzerland) and referred to Cluj County Emergency Hospital Laboratory of Genetic Explorations. Plasma was prepared by centrifugation R 1600 g and at 22°C within 90 min after the collection of blood.

Isolation and concentration of plasma amino acids

The isolation and concentration of amino acids was performed by the "desalting" procedure developed by Wadman and coworkers (18, 19), described previously in detail (16). Briefly, plasma is deproteinized with trichloroacetic acid, the sample is filtered and the filtrate is passed through a column of Dowex-50X8. The column is washed with distilled water and eluted with 2M ammonia. After evaporation in vacuum at 40°C, the residue is dissolved in a small volume of water; this is the concentrated solution of amino acids free of salts and other interfering substances. An aliquot of the solution was used for the analysis of amino acids by HPLC, another aliquot for the analysis of amino acids by 2D - TLC.

Chromatographic analysis of amino acids

HPLC analysis of plasma Phe was performed as previously described (16). 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).

2D - TLC determination of plasma Phe concentration was performed in several steps. First, the amino acids from the plasma extract (prepared by ion-exchange column chromatography as described above) are separated on 5 x 5 cm plates of cellulose on glass using the procedure of Wadman and coworkers (19). A small volume (2 - 4 μL) of the concentrated solution of amino acids is applied in the lower left corner of the chromatogram 9 mm from the edges. The development

RESULTS

of chromatograms is performed in small glass tanks (tightly closed with a cover). The solvents must be prepared shortly before use. The first run is performed in 96% ethanol:water (86:14, V/V) until the front reaches 5 mm from the edge of the plate. After drying in air the chromatogram is developed a second time in the same direction; duration: 2 x 30 min. After drying the chromatogram is turned at right angles and the second run is performed in t-butyl alcohol:methylethylketone:25% ammonia:water (5:3:1:1, V/V). The development is repeated after the chromatogram is dried; duration: 2 x 50 min. After drying at 100°C the chromatogram is stained by spraying with a solution of ninhydrin 0.42%, isatin 0.01%, lutidin 1% (in acetone) then dried again at 100°C. Maximum color is obtained after 120 min.

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 1.3. 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.

Figure 1. HPLC chromatograms of Phe standard solutions at five concentrations. The standards were prepared by appropriate dilution of a stock solution of Phe (1834.4 $\mu\text{mol/L}$) in deionized water. The concentrations of Phe were: I -1635,5 ($\mu\text{mol/L}$); II - 865.6 ($\mu\text{mol/L}$); III - 423.5 ($\mu\text{mol/L}$); IV - 221.5 ($\mu\text{mol/L}$); V - 107.2 ($\mu\text{mol/L}$). Absorption (at 210 nm) is given in arbitrary absorption units (mAU).

The aim of this study was to compare two chromatographic methodologies for determination of the level of plasma phenylalanine (Phe) in order to evaluate their usefulness for the diagnosis of hyperphenylalaninemia (HPA) and phenylketonuria (PKU) in relation with the screening of this most prevalent inborn error of amino acid metabolism.

The HPLC chromatograms of Phe standard solutions at different concentrations ranging from very high (1635.5 $\mu\text{mol/L}$, peak V) to very small (107.5 $\mu\text{mol/L}$, peak I) are presented in Figure 1. The peaks are very well defined at all concentrations. The ranges of concentrations have been chosen to correspond from the normal Phe concentrations (below 120 $\mu\text{mol/L}$) to the very high concentrations (over 1200 $\mu\text{mol/L}$), as found in the most severe cases of PKU, non-treated or badly treated by dietary means.

The standard calibration curve obtained by HPLC (Fig. 2) shows linearity on the range of concentrations chosen and it appears to start in the origin of the two axes. Moreover, a large number of measurements have been performed at each concentration of the standard solutions (12 - 21 measurements); under these circumstances extremely small sample to sample differences and very small values of the standard deviation were found. The equation of the curve has a very high R-squared value (0.999). This indicates that the procedure described here is very accurate.

Figure 2. Graph showing the Phe concentration vs. Phe peak area given arbitrary absorption units (mAU) multiplied by time (min). HPLC calibration curve was obtained with five concentrations of Phe standard solutions (the same concentrations as in Fig. 1). The means and standard deviations of 12 - 21 measurements/standard solutions are indicated. The equation of the line is: $Y = 0.0685x + 0.2208$; the R-squared value is 0.999. Regression analysis was performed using the Microsoft Excel Software.

Figure 3. Aspects of 2D - TLC chromatograms of plasma from two boys who were found to have HPA by newborn screening. There is no Phe spot visible in the case of the CONTROL subject; in contrast, a very intense Phe spot is visible in case of the PKU patient.

Figure 5. HPLC chromatogram of Phe from plasma of the CONTROL subject. Absorption (at 210 nm) is given in arbitrary absorption units (mAU).

Figure 7. HPLC chromatogram of Phe from plasma (diluted 1:1 in water) of the PKU subject. Absorption (at 210 nm) is given in arbitrary absorption units (mAU).

Figure 4. Graph showing the values of the Adjusted Volume vs. Phe concentration. This 2D - TLC calibration curve was obtained with Phe standard solutions having the following concentrations (in $\mu\text{mol/L}$): 1200, 900, 600, 300. The means and standard deviations of 6 measurements/standard solutions are indicated. The equation of the line is: $Y = 0.012x + 0.2525$; the R-squared value is 0.8625. Regression analysis was performed using the Microsoft Excel Software.

Figure 6. HPLC chromatogram of Phe from undiluted plasma of the PKU subject. Absorption (at 210 nm) is given in arbitrary absorption units (mAU).

The 2D thin layer chromatograms of amino acids from plasma of two boys who were detected by newborn screening to have HPA are presented in Figure 3. The blood from both boys has been referred for confirmation of HPA. In case of one boy, 14 days old, no Phe spot could be seen on the 2D - thin layer chromatogram, so that HPA was excluded, therefore the subject is presented as CONTROL. In contrast, on the 2D - thin layer chromatogram of the second boy (30 days old) an intense spot corresponding to Phe may be seen, corresponding to a severe form of PKU.

The determination of plasma Phe concentration of these two illustrative cases was performed comparatively by 2D - TLC and HPLC methodologies.

In order to evaluate the possibility of quantification of Phe plasma concentration by 2D -

TLC a standard curve was traced (Fig. 4). In contrast with the standard curve obtained by HPLC, in case of the curve traced after measurements of the Adjusted Volume by 2D - TLC, a rather high dispersion of values at each concentration of standard Phe solution was found. Very high values of the standard deviation were calculated (see values in the Legend of Fig. 4). In addition, the equation of the curve has a low R-squared value (0.862). This indicates that quantification of Phe plasma concentration by the densitometric procedure is accompanied by relatively high errors. In addition, the lowest concentration of a standard solution that could be clearly seen (and consequently quantified) on the chromatogram was 300 $\mu\text{mol/L}$, which is above the cut-off value of the normal plasma Phe concentration (120 $\mu\text{mol/L}$).

The HPLC determination of plasma Phe concentration was possible even for concentrations that did not result in a Phe spot visible on the 2D - thin layer chromatogram, as was the case of the CONTROL subject (Fig. 3). The peak of Phe appeared on the HPLC chromatogram (Fig. 5). The concentration of plasma Phe was calculated and appeared to be 43 $\mu\text{mol/L}$. This confirms that the HPLC method is very sensitive in evaluating the plasma Phe concentration.

The peak of Phe on the HPLC chromatogram of plasma extract in case of the PKU patient is presented in Figure 6. The concentration of Phe appeared to be very high, 2496 $\mu\text{mol/L}$, i.e. higher than the highest concentration of the standard solution (1600 $\mu\text{mol/L}$). When the plasma extract from PKU patient was diluted 1:1 with water the HPLC chromatogram (Fig. 7) shows the peak of Phe suggestive for half the concentration of Phe from Figure 6. Indeed, the value of plasma Phe for the diluted sample appeared to be 1242 $\mu\text{mol/L}$, i.e. extremely close to half of the concentration of the undiluted sample (2496 $\mu\text{mol/L}$). This confirms again the very high precision of the HPLC method.

DISCUSSION

The determination of plasma Phe concentration plays the key role in the modern management of PKU, aimed to prevent or minimize the intellectual impairment of affected children throughout the world. However, implementation of the management guidelines (6, 9) requires not only a robust infrastructure for the newborn screening, but also well equipped laboratories and expert personnel to confirm the HPA. These may be problems that cannot be solved in poor or developing countries in the world, so that PKU newborn screening is “a great

success” only for developed countries (22). Moreover, tandem mass spectrometry, the “gold standard” method for measuring the level of Phe in plasma is affordable only to the most developed countries in the world.

The methodology presented in this study is affordable to developing or even underdeveloped countries. On the one hand the HPLC method (16, 17) presents several advantages: is much less expensive compared to tandem mass spectrometry, is rather simple, very sensitive and highly accurate and it does not require special columns for amino acid analysis. Consequently, any laboratory having a HPLC instrument may set up the procedure. We recommend it as a good method to confirm HPA in subjects with HPA detected by newborn or selective screening. It should be mentioned that other authors found HPLC suitable for the determination of plasma Phe in patients with HPA (7) and the values of plasma Phe concentration determined by HPLC and tandem mass spectrometry were not significantly different (23).

On the other hand, the 2D - TLC methodology for quantification of Phe plasma concentration by the imaging-densitometric methodology using the Bio-Rad GS-700 imaging-densitometer is accompanied by relatively high errors. In addition, the lowest concentration of a standard solution that could be clearly seen (and consequently quantified) on the chromatogram was 300 $\mu\text{mol/L}$, which is above the cut-off value for the normal plasma Phe concentration (120 $\mu\text{mol/L}$). Consequently, the methodology cannot be used for detecting “mild” HPAs (between 120 - 300 $\mu\text{mol/L}$). It is possible that by using a densitometer dedicated to quantitative TLC the precision and sensitivity of the precision to be improved. However, the 2D - TLC is certainly adequate to detect patients with severe PKU, either in case of newborn screening or in case of selective screening, since the intense spot of Phe on the 2D-TLC chromatogram may be detected even by an unexperienced laboratory specialist. The patients with severe PKU are those who require dietary treatment as soon as the increased level of blood Phe is detected, in order to prevent (or at least minimize) the severe mental disability of affected children.

In conclusion, for determination of plasma Phe concentrations both chromatographic methodologies described in this study are useful for confirming the diagnosis of HPA and PKU in infants detected by neonatal or selective screening of PKU. The methodologies are particularly useful in countries that could not afford the high cost of more sophisticated methods. In this way a contribution may be made to

solve the major public health problems linked to PKU throughout the world, that of a significant number of children with preventable mental retardation not being detected in the first weeks of life, which is tragic for them and their families and represents a burden for the society when these children become institutionalized.

Conflict of interest

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

Acknowledgment

The authors thank Dr. F. C. Ladaşiu Ciolacu (Institute of Physical Chemistry, Justus-Liebig-University Giessen, D-35392 Giessen, Germany) who developed the HPLC procedure for assessment of plasma Phe by HPLC at Institute of Life Sciences, “Vasile Goldiș” Western University from Arad, Romania.

The corresponding author is grateful to his former coworkers with whom the methods of analysis of amino acids were first introduced in the medical laboratories in Romania (24, 25) and to Dr. L. Frunţescu (“Iuliu Haţieganu” University of Medicine and Pharmacy of Cluj-Napoca - Discipline of Cell and Molecular Biology) for help given in performing some densitometric analysis of plasma Phe. The corresponding author is also 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.

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