



Research Paper

Diagnosis of Phenylketonuria by mass spectrometry: An overview

Accepted 10th July, 2022

ABSTRACT

Newborn screening of phenylketonuria (PKU) is an important aspect in the world since PKU is a serious worldwide inborn genetic disorder that causes different mental and physiological problems to human. Diagnosis of PKU at the earliest stage is the best circumstance because PKU is one of the few genetic diseases that can be controlled by dietary treatment. Therefore, newborn screening of PKU has been included in clinical newborn screening panel of most countries now with various detection techniques, e.g. bacterial inhibition assay (Guthrie test) and tandem mass spectrometry (MS/MS). This study would review and summarize different newborn screening technologies of PKU in order to have an overview of the history and development in molecular diagnosis of PKU. Diagnostic mechanisms, applications, advantages and limitations of different newborn screening techniques of PKU would also be studied at the same time.

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Key words: Mass spectrometry, Phenylketonuria, Phenylalanine, blood, diagnosis.

INTRODUCTION

Phenylketonuria (PKU) is an autosomal recessive inborn genetic disorder of phenylalanine (Phe) (Figure 1) metabolism resulting from mutations in phenylalanine hydroxylase (PAH) gene located on chromosome 12. PAH is a hepatic enzyme that metabolizes amino acid Phe to amino acid tyrosine (Tyr). PKU patients with the deficiency of PAH enzyme results in failure of the enzymatic conversion of Phe to Tyr in liver and leads to Phe accumulate and are converted into phenylketone (Williams et al., 2008) (Figure 2). Untreated PKU can cause seizures, autistic-like behaviors, microcephaly and mental retardation (Filiano, 2006; Koch et al., 2003; Pietz et al., 1999). Autosomal recessive genetic disorder means that two PKU alleles are needed in homozygote for an individual to have symptoms of the PKU disease while carriers of one PKU allele with a normal allele (heterozygote) do not have any symptoms of the PKU disease (Ten, 1978) (Figure 3). In 1934, PKU was discovered by a Norwegian physician called Dr. Ivar Asbjørn Følling and hence PKU is also known as Følling's disease in Norway (Folling, 1994). Newborn screening for PKU was first introduced in United States in 1963 and it has been

included in the newborn screening panel of most countries now, with various detection techniques, e.g. bacterial inhibition assay (Guthrie test) in the early 1960s (Guthrie and Susi, 1963), immunoassays using photometric or fluorometric detection in 1990's (Fekkes, 1996; Sarwar and Botting, 1993), amino acid measurement using chromatography in 1980's (Deyl et al., 1986; Dale et al., 2003) and tandem mass spectrometry (MS/MS) in 1990's (Chace et al., 1993; Dietzen and Weindel, 2010; Zytkevich et al., 1945). Most babies after birth 2 to 7 days are screened for PKU with the use of dried blood spot (DBS) samples obtained by neonatal heel prick (Figure 4).

The newborn screening test is repeated once at approximately two weeks of age to confirm the initial testing results and prevent any initially missed PKU cases. It is important to diagnose PKU at the earliest stage because PKU is one of the few genetic diseases that can be controlled by diet. By controlling Phe levels through diet, that is, low consumption of Phe or a combination of diet and medication, PKU affected newborns can grow up with normal development. Dietary treatment of PKU was

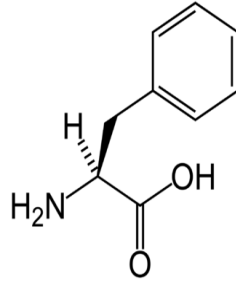


Figure 1: Chemical structure of phenylalanine (Phe).

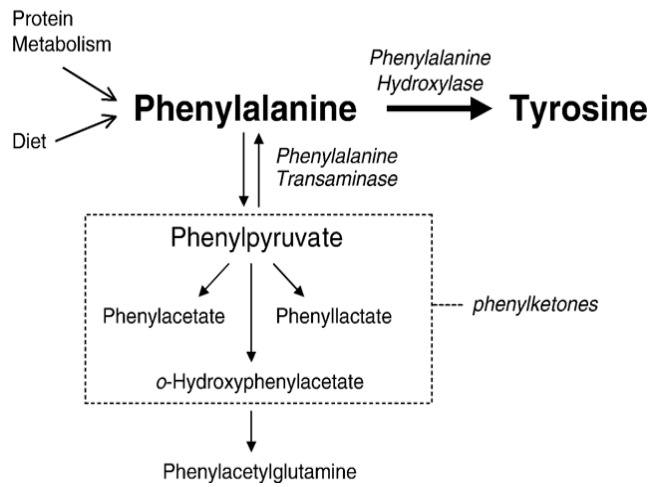


Figure 2: A diagram showing metabolism of phenylalanine (Phe) in human (Chace and Kalas, 2005).

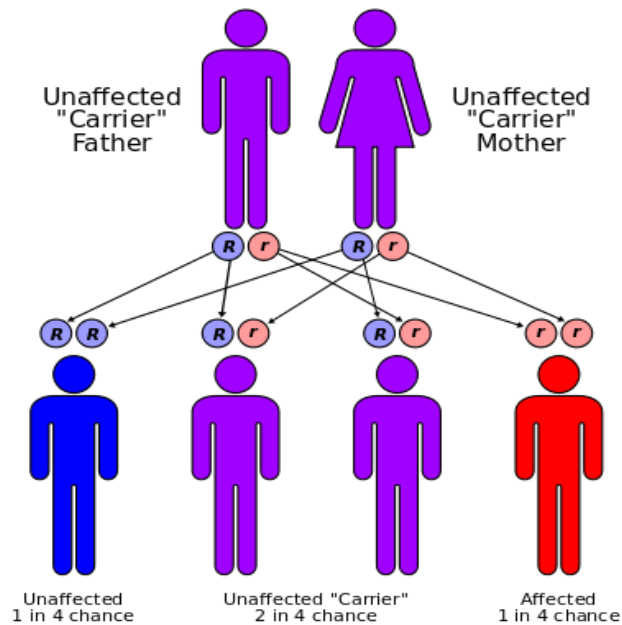


Figure 3: A diagram showing autosomal recessive genetic disorder.



Figure 4: Photograph showing the use of neonatal heel prick method to obtain dried blood spot (DBS) samples from newborn.

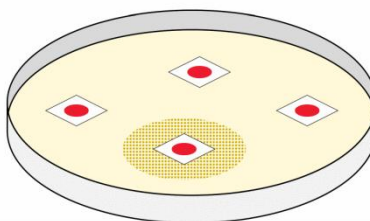


Figure 5: A diagram showing Guthrie test analysis of PKU.

proposed in 1930's and became available in 1950's, it showed a great improvement in youngest patients. The mean incidence of PKU differs broadly in various countries. For examples, frequency of PKU in United States and China determined at a rate of about 1 in 15,000 and frequency of PKU in Turkey determined at a rate of about 1 in 2,600 (Jones and Bennett, 2002; Ozalp et al., 2001). PKU is a worldwide inborn genetic disorder problem therefore more rapid and accurate molecular diagnostic techniques for newborn screening of PKU are necessary. For dietary treatment, special diet that is low in phenylalanine is suitable for all PKU patients for normal health and development. Foods high in Phe such as meat, eggs, cheese, fish, nuts, legumes, dairy products and milk, should be strictly avoided or restricted. Diet foods and soft drinks that contain sweetener aspartame should also be avoided since aspartame consists of Phe. For medical treatment, oral administration of tetrahydrobiopterin (BH₄) tablet can lower blood Phe levels to normal ranges since BH₄ is a coenzyme for proper activity of the enzyme PAH and a cofactor oxidation of phenylalanine (Surtees and Blau, 2000; Michals-Matalon, 2008; Burton et al., 2007).

Bacterial inhibition assay (Guthrie test)

Bacterial inhibition assay (Guthrie test) is the first newborn screening technique for PKU. The mechanism is that the test uses a specifically prepared agar plate with a strain of bacteria *Bacillus subtilis* (ATCC 6051) grows on it as a marker for the presence of high levels of phenylalanine

(Phe) and phenylpyruvate. With the use of a chemical called B-2-thienylalanine which would inhibit the growth of the bacteria on the culture media. Growth of the bacteria can be restored by adding Phe and phenylpyruvate to the culture media. For newborn patients with PKU, their blood (obtained from heel puncture) or urine (obtained from diaper) would have high levels of Phe and phenylpyruvate, therefore if their blood or urine are added to the agar plate and incubated overnight, growth of the bacteria in the agar plate would be restored, showing a positive result of PKU diagnosis. This test can also perform semi-quantitation to determine the concentration of Phe and phenylpyruvate in the sample by using the growth of the bacterial colony as an indicator (Guthrie and Susi, 1963; Husband, 1965). The experimental details of the Guthrie test are using neonatal heel prick method to collect a drop of blood (~50 μ L) on a piece of filter paper of a newborn. A small portion of the filter paper (~1/8" blood dot) is punched out and placed on a specifically prepared agar plate containing B-2-thienylalanine and *Bacillus subtilis*, then incubated overnight (Figure 5). After that, bacterial growth would be observed surrounding the filter paper of the PKU patients due to the blood Phe and phenylpyruvate in filter paper diffused into the agar plate, overcome the inhibition and hence the bacteria grow.

The amount of Phe in blood is approximately proportional to the amount of the bacteria growth which is measured as the diameter of the colony. Therefore, the concentration of Phe in blood can be semi-quantified by comparing the diameter of the colony measured to the diameter of reference colonies which are prepared by

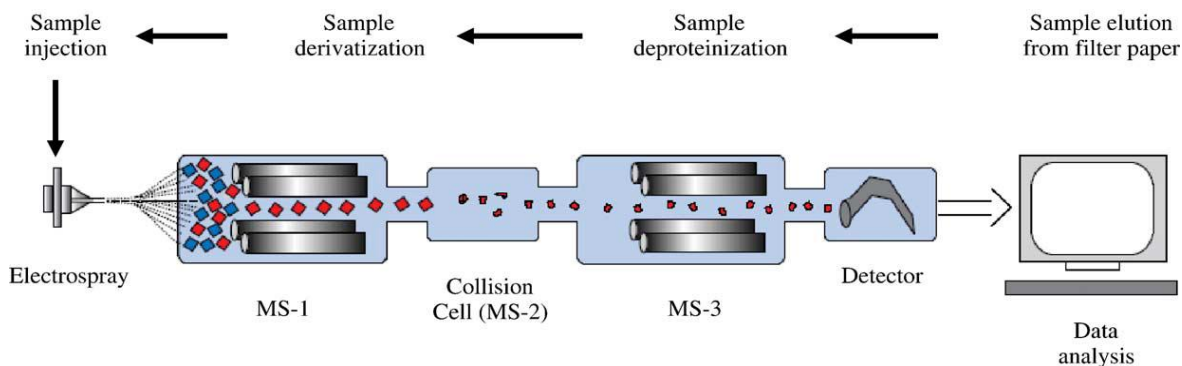


Figure 6: Schematic diagram showing the design of a triple quadrupole mass spectrometer (Chace and Kalas, 2005).

incubating a series of different concentration of phenylalanine standard in the specifically prepared agar plates. For normal healthy people without PKU, Phe levels are generally less 120 $\mu\text{mol/L}$ (<2 mg/dL), and the detection limit of Phe levels by Guthrie test is 180 to 240 $\mu\text{mol/L}$ (3 to 4 mg/dL) (Kennedy et al., 1973; Francis, 1971). Guthrie test has been used since the late 1960s as a major newborn screening test of PKU and is widely used in North America and Europe. However, this test is gradually being replaced in many countries by tandem mass spectrometry which is a simpler and more rapid diagnostic technique for a wide variety of diseases. Since Guthrie test is relatively slow and low throughput, it involves overnight incubation, it is more labour intensive, less specific, less sensitive and less accurate compared to tandem mass spectrometry.

Tandem mass spectrometry (MS/MS)

Mass spectrometry (MS) is a powerful analytical tool for qualitative and quantitative analysis of metabolites based on its high sensitivity and specificity. Qualitative identification of chemical compounds and metabolites can be easily done based on their specific mass-to-charge ratio (m/z value) of the molecular ion. And quantitative determination of concentration of chemical compounds and metabolites can also be easily done based on the ion intensity of the molecular ion measured by either absolute quantitation or relative quantitation using external standard method or internal standard method (Hardy et al., 2002; Ceglarek et al., 2002). Chromatography including gas chromatography and liquid chromatography are usually coupled with MS to perform separation of chemical compounds and metabolites in complex biological matrix or mixture, e.g. serum and urine, before detecting by MS detector. If the chromatography technology applied without coupled with MS detector, the accuracy and results of the analysis would be very limited because the identification of compounds base on the retention time only, unknown compounds co-elute at the same retention time can never

be determined and hence it would affect the accuracy of qualitative and quantitative analysis (Jones and Bennett, 2002). Tandem mass spectrometry (MS/MS) was first used as a clinical diagnostic tool for detecting acylcarnitines abnormal of fatty acid oxidation disorders in 1984 (Vreken et al., 1999). It was then applied to dried blood spots test and used to analyze amino acids in order to detect over thirty metabolic diseases in a single analysis in 1990 (Chace et al., 1993; Chace et al., 1995; Chace et al., 1996). In 1994, Dr. Edwin sponsored a commercial laboratory called Neogen to motivate research studies in elaboration of newborn screening using tandem MS technology. And in 1997, collaboration between North Carolina State Laboratory and Neogen lead to first statewide extended newborn screening using tandem MS (Yu and Gu, 2006).

Tandem mass spectrometry (MS/MS) generally combines two mass analyzers with a collision cell in the middle to perform different scanning, (Figure 6) such as product ion scan, precursor ion scan and neutral loss scan (Figure 7). Triple quadrupole mass spectrometer (QqQ) is usually used to perform the tandem MS. It combines three quadrupole mass analyzers and the middle one functioned as collision cell. It allow further confirmation of the compounds detected by selecting the product ion in the first mass analyze (Q1), and then fragment the product ion in the collision cell (q2) by collision gas, the fragments of the product ion are finally detected in last mass analyze (Q3). By studying the fragmentation pattern of the product ion, chemical compounds or metabolites can be identified undoubtedly because different molecules gives different fragmentation patterns, the specific fragmentation patterns are unique fingerprints of each molecules and hence false positive results can be eliminated and avoided (Filiano et al., 2002; Chace, 2001) (Figure 8). Tandem MS can also be coupled with chromatography for molecular separation, and the run times for tandem MS coupled with chromatography for a single detection are significantly faster than the run times for HPLC or GC/MS analysis. For example, an ion-selective chromatographic tandem MS takes only about 30 min for a single phenylalanine (Phe) detection, while normal GC/MS takes about 60 min for

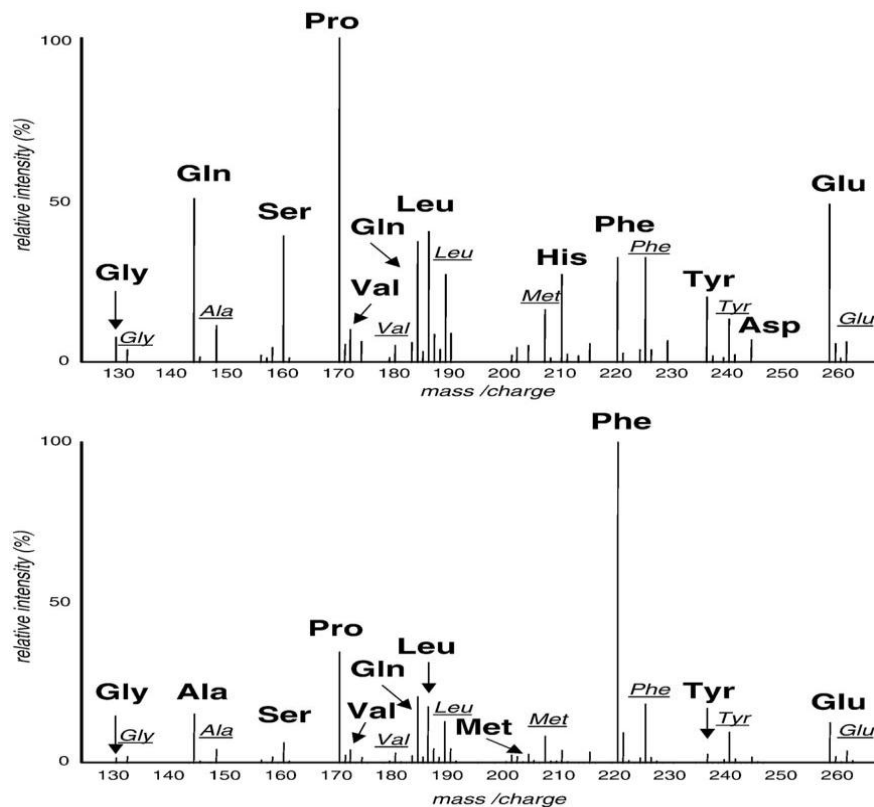


Figure 7: Neutral loss scan m/z 102 mass spectra of: (top) blood spot of a normal newborn, (bottom) blood spot of a newborn with phenylketonuria (Chace and Kalas, 2005).

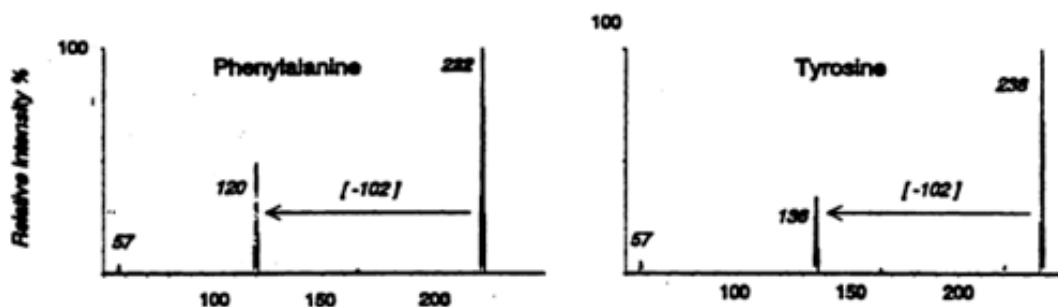


Figure 8: Product ion scan mass spectra of phenylalanine and tyrosine (Chace et al., 1993).

organic acids detection. Without coupling with the chromatography, samples can be directly injected into tandem MS for analysis when separation is not necessary, it takes less than 2 min for a Phe detected by tandem MS detection (Chace et al., 1995; Roe and Roe, 2000; Chace et al., 2001).

More than 20 inborn errors of metabolism now are capable to be detected in a single blood test using tandem MS (Rashed et al., 1995; Johnson et al., 1996). A recent study analyzed 3000 control specimens obtained from 4 different models of tandem mass spectrometers from a single vendor in two different laboratories, that is, Applied

Biosystems MDS-Sciex models 300, 365, 2000 and 300. The results showed an average Phe concentration of 49 Amol/L (0.8 mg/dL) which is consistent with the published values of Phe in the range of 40 -130 Amol/L (0.7 to 2.2 mg/dL) (Chace and Kalas, 2005). The detection limit of tandem MS is much lower than that of Guthrie test, and hence tandem MS provides more sensitive and accurate quantitation of Phe for newborn screening. Another study compared tandem mass spectrometry with fluorometry, the results indicated that tandem MS has a better performance and gives less false results compared to fluorometry when the median concentration of Phe is lower. The fluorometric

analysis results showed an increased concentration of Phe detected in all cases, that is, over a threshold of 180 Amol/L (3 mg/dL). While the tandem MS results showed the concentration of Phe detected was within the normal range (Chace et al., 1998). Therefore, tandem MS gives a more accurate measurement of the concentration of Phe compared to fluorometry. Moreover, tyrosine (Tyr) is also measured by tandem MS in some screening laboratories. It is because according to the metabolism of PKU, deficiency of phenylalanine hydroxylase (PAH) enzyme would cause a decrease in the conversion of Phe to Tyr.

Blood tyrosine level would decrease with time since Tyr is obtained mainly from the diet while blood phenylalanine (Phe) level would increase with time. Hence, a better indicator of PKU can be determined by using the ratio of the concentration of a metabolite that increases (Phe) to the concentration of a metabolite that decreases (Tyr). This indicator uses molar ratios to enhance the accuracy of the analysis results by eliminating the matrix interference and has been studied in a lot of research publications. By using the Phe/Tyr concentration ratio, 2 of 3 false positive results can be resolved (Chace et al., 1998; Schulze et al., 1999; Reilly et al., 1998). Therefore, molecular diagnosis of the concentration of Phe together with Phe/Tyr ratio gives a more accurate and indicative measurement of PKU. Disadvantages of tandem MS includes the equipments cost are relative high and certain level of expertise is needed to analyze the data generated by tandem MS since a lot of metabolites detected by MS/MS are involved in multiple varieties of disorders, and hence proficiency in data interpretation is required (Jones and Bennett, 2002). However, tandem MS provides very rapid, specific and sensitive molecular diagnosis. It is not only applied in newborn screening, but also be utilized in a wide variety of diseases diagnosis nowadays (Chace et al., 1998; Kushnir et al., 2001; Turner et al., 2009; Montine et al., 2006).

CONCLUSIONS

Newborn screening of phenylketonuria is undergoing a revolution over 50 years due to advances in molecular diagnostic technology. Tandem mass spectrometry is a key molecular diagnostic technique for newborn screening over the last decade. Although the cost of the mass spectrometer is high, it provides rapid and accurate results for both qualitative and quantitative analysis of a very wide range of different metabolites and chemicals from a single sample. Apart from these, more simple, rapid, accurate, reproducible and low-cost screening technologies for newborn screening of phenylketonuria are being developed and may be applied in future clinical diagnosis.

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Cite this article as:

Wong HY (2022). Diagnosis of Phenylketonuria by mass spectrometry: An overview. *Med. Med. Sci.* 10(8): 058-064.

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