CASE REPORT

ADVANCEMENT IN ANALYTICAL TECHNIQUES IN NEWBORN SCREENING FOR DETECTION OF METABOLIC DISORDERS, PHENYLKETONURIA

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ABSTRACT

Newborn screening programs play a vital role in improving the lifestyle of an individual by reducing the morbidity and mortality rate. In most developed countries, it is an essential program carried out on all neonates. Many measures have been taken to improve NBS programs due to advancement in many of the analytical techniques. In this review advancement in techniques to detect Phenylketonuria (PKU) is studied. Over the years many assays such as colorimetry, Guthrie's test, fluorometry, chromatography, mass spectrometry and tandem mass spectrometry (MS/MS) have been evolved. And those did prove to help detect Phe and Try. However, ESI-MS/MS was studied to be the most sensitive and specific technique when in comparison to the other mass spectrometric techniques. As it has a LOQ and LOD of phe and tyr of 0.0015 and 0.0045µmol/L; 0.0025 and 0.007 µmol/L respectively. In future, newer approaches will enhance the detection and treatment of newborns affected by PKU and other inborn errors of metabolism.

Key words: Phenylketonuria, Analytical techniques, Mass spectrometry, Fluorometry, Colorimetry.

INTRODUCTION

Phenylketonuria (PKU) is a metabolic error caused due to the deficiency of phenylalanine hydroxylase, which is an enzyme responsible to convert phenylalanine (Phe) to tyrosine (Tyr). Tyrosine is an important nucleic acid, responsible for protein synthesis. In the early 1940s the frequency of PKU among neonates was studied as 1 in every 10,000 - 15,000 live births, resulting in a global threat (Hardelid *et al.,* 2008). Hence, different analytical methods were developed to detect PKU, the detection of PKU was done by identifying the different concentration of the metabolome responsible for PKU. Metabolomics is the term used to define a complete set of metabolites. They play a vital role in biochemical reactions of a cell. Their levels could be identified by the enzymatic activities and other regulatory processes like allosteric regulation of enzymes, protein-protein interactions, transcription and translation by interacting with their respective metabolites (Millington *et al.,* 1991). Metabolome analysis depends on two aspects; target analysis and metabolite profiling (Figure 1) (Fiehn, 2002). Since the metabolome has many chemical compounds it is difficult todetermine a complete metabolome. Thus, a range of combination of different analytical methods is used to determine a metabolite profile (Hellerstein, 2004; Paul, 2008).

There are many approaches to improve the analytical techniques in order to detect a range of inborn errors of metabolism (IEM's) to improve by identifying and quantifying both intracellular and extracellular metabolites of lower molecular masses (Hellerstein, 2004; Wilcken and Wiley, 2008).

Introduction to Inborn Errors of Metabolism

The concept of IEM's was explained by Archibald Garrod, in the year 1908. He correlated his theory with albinism, alkaptonuria, cystinuria and pentosuriaby assuming it was caused by a recessively inherited disease specific chemical error (Pierce *et al.,* 2011). IEM are caused due to a defect in either structure, synthetic function or transport mechanism which disrupts the metabolic network. They are classified into two types; intoxification (intermediate molecule accumulation) and energy deficiency type. This condition could be treated, if detected at an early stage. However, if treatment is delayed, it could cause conditions like mental retardation, motor impairment and physical disability (Koch *et al.,* 1966). One such example of an inborn metabolic error is Phenylketonuria (PKU). It is an autosomal recessive - genetic condition which was first described by Abjorn Folling in 1934. It is a result of mutation in the 12q23.2 region of the chromosome In the Phenylalanine Hydrpxylase (PAH). gene (1.14.16.1) or deficiency of PAH (Figure 2) (Deng *et al.,* 2005). Figure 3 describes the metabolic pathway of Phe and how PKU is developed in infants.

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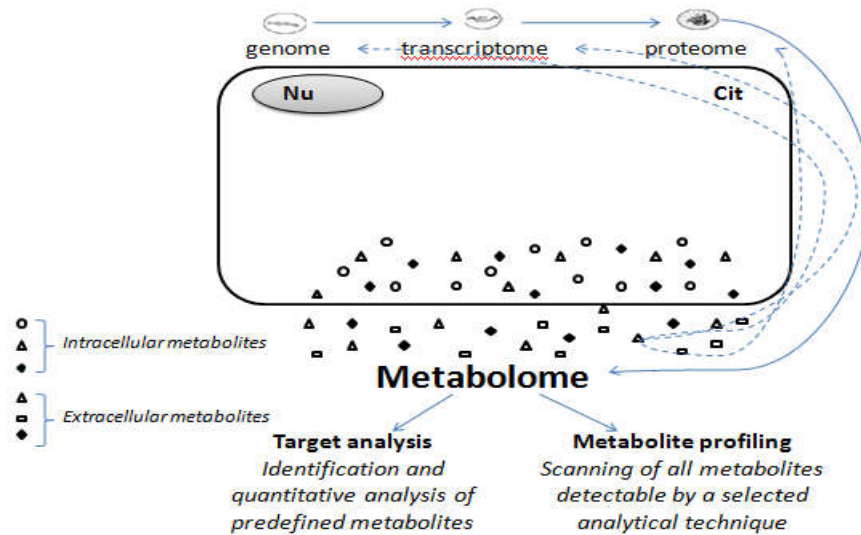
Figure 1. Metabolome analysis in the context of functional genomics. Nu, nucleus; Cit, cytoplasm. Metabolome consist of many chemical compounds like organic acids, carbohydrates, lipids, alcohols, ketones and other complex natural products
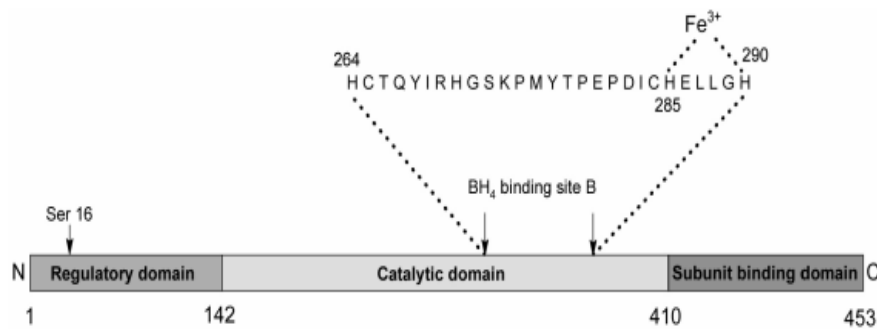

Figure 2. Structural components of PAH. The catalytic domain of PAH contains motif of 26 or 27 amino acids which are responsible for ferric iron and cofactor (BH4) binding.
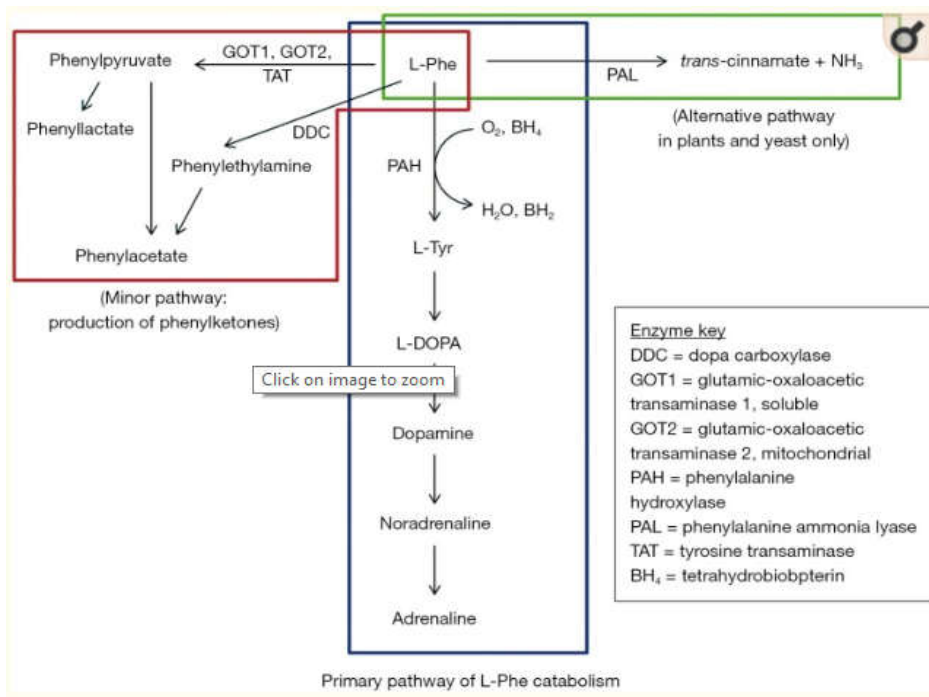

Figure 3. The metabolic pathway of Phenylalanine. The primary pathway (blue box) is the catalytic conversion of L-Phe to L-Tyr by phenylalanine hydroxylase (PAH), commonly in liver, as well as in the kidneys. In phenylketonuria (PKU), the deficiency of PAH enzyme leads to the production of phenyl ketones by an alternative pathway (red box), which leads to high phenylalanine (phe) (hyperphenylanemia) and low tyrosine levels in tissues. This leads to accumulation of toxic substances in the brain. Phenylalanine is acquired from either diet or proteolysis It is essential for protein synthesis, tyrosine and its derivatives like melanin, dopamine and nor epinephrine. The normal range of phe in blood is 1.3 to 2.0 mg/dL (80 to 120 μmol/ L). A third pathway (green box) can be found in plants and yeast involving the enzyme

If left untreated, it results in severe intellectual impairment along with microcephaly, autism, seizures, poor skin pigmentation and other symptoms related to development during growth of a child. However, children born with PKU lead relatively normal lives due to newborn screening (NBS) programs and with early dietary treatment programs (12, 13). Thus, over the decades there has been a significant development in the detection of PKU in neonates in order to identify any condition that may affect long term survival of the child. After which appropriate treatment and management strategies could be improvised (Hafid 2015).

Advancement in analytical methods to detect PKU in neonates

Several analytical techniques have been developed for the detection of various metabolites that are responsible for disease conditions. Hence over the past decade, many techniques have been developed and improved to overcome limitations such as cost effectiveness, accuracy, sensitivity and specificity (Rashed *et al.,* 1995). This review highlights the development of these techniques to screen for PKU in newborns (Chace and Hannon, 2016).

Newborn Screening for PKU began in the California in late 1940's, This technique utilized ferric chloride to observe a color change to blue green or grey green in the presence of phenylpyruvenic acid in urine. At first this was done as diaper or white filter paper test where reagents were added to the material containing urine, to observe color changes. Later it was advanced to a phenistic dipstick coated with buffered ferric chloride salt was used. This test can be performed in stored urine, however phenylpyruvenic acid was not identified in neonates until few weeks old. Additionally, it had low specificity since compounds with similar color can interfere with the test and produce false positive results (Wu *et al.,* 1979; Pierce *et al.,* 2011). Therefore, in 1963 bacterial inhibition assay was developed by Dr. Robert Guthrie. This assay detected elevated phenylalanine levels in blood. The samples were collected on a filter paper, and the dried sample was sent to central laboratories to be processed. The main principle of this assay was growth inhibition by an analogue (B2-thienylalanine) of the desired amino acid (phenylalanine) (Figure 5) (Wilcken and Wiley, 2008; Rashed *et al.,* 1999). However, this test was studied to show low sensitivity as it has a higher cut off values of 3.0mg/dL (180µmol/L) compared to mass spectrometric techniques (1.0 – 1.5mg/dL (60 - 90µmol/L)) (Borrajo, 2016). Therefore, quantitative techniques like gas chromatography, liquid chromatography and mass spectrometry were developed.

Advancement in quantitative techniques

Many attempts were made for the development of mass screening programs. Although these techniques had a high throughput and was very precise, they had low sensitivity and specificity. Since the other amino acids could fluorescence at the same wavelength as the required amino acid, thus producing false positive results (Koch, 1999; Spronsen and Enns, 2010). Hence quantitative techniques like thin layer chromatography and gas chromatography which were based on the principle of separation of particles depending on volatility and polarity (Paul, 2008). However, these techniques alone were labor intensive, time consuming and not practicable. Therefore, these were modified and more often hyphenated with mass spectrometric assays; like gas chromatography mass spectrometry (GC/MS) (Sun *et al.,* 2015).

Gas chromatography mass spectrometry

Coupling of GC with MS has been able to bring about a high chromatographic resolution along with high reproducibility and a standard electron impact ionization. As a result, GC-MS is still known to be as the gold standard technique for metabolome analysis. Identification of metabolites uses retention time analysis. Since 1966, this technique has been common in detecting chemical disorders of organic acids. However, it is also useful in detecting N-acetyl derivatives in PKU, hence it is not specific for PKU (Table 1) (Deng *et al.,* 2002). Hence in 2005 Deng and his colleagues designed a microwave assisted derivatization (MAD) technique modified to analyze amino acids in blood samples. This technique was able to produce quick and specific results of amino acids by GC/MS in newborn blood samples (Figure 6). The principle of this is microwave-assisted sialylation followed by GC-MS analysis (Geng *et al.,* 2005). However, later that year Deng and his colleagues came up with another technique known as the HP6890 GC system coupled with HPMD5973 quadruple MS. This was an electron mode, where the samples where inserted into the mass spectrum directly, the target samples were separated using a HP5MS capillary column and the carrier gas used was helium and the analysis was done by full scan acquisition mode. Additionally, the derivatization step was cut down by using a sialyation BSTFA (*N, O*-bis-trimethylsilyl trifluoroacetamide) under microwave irradiation. Hence less human error and higher accuracy of test results (Figure 7). Limit of detection for Phe and Tyr were 0.48 and 0.16 µ*M*, respectively. This method has a good precision, good recovery, broad linear range and a low detection limit. The results obtained are shown in Table 2 (Deng *et al.,* 2005). The Advantage of this technique is that it can analyze volatile compounds; and has a high sensitivity. However, the major disadvantage of GC-MS is it has high efficiency only in high risk factors, cannot detect polar and non-volatile compounds and has gentle ionization; therefore fewer fragmentation. Due to this, liquid chromatography and tandem mass spectrometric methods are preferred over gas chromatography/mass spectrometric (GC-MS) methods.

Liquid chromatography

High performance liquid chromatography

Most commonly, reverse phase HPLC is used as a reliable technique to detect PKU due to its ability to determine amino acids simultaneously. Since 1996, material used as a HPLC column was monolithic silica. Since then, in 2004, C18 monolithic silica has been explained and modified to separate amino acids by many researchers. In 10 minutes, it separates 18 primary amino acids and naphthalene-2, 3-dicarboxaldehyde (NDA) for pre-column derivatization (Gustova, 2016). In 2007, Devall and his coworkers developed a method to separate 21 amino acids by 24 minutes; using HPLC column - Chromolith RP-18 and a derivatization reagent, ortho-phthaldialdehyde-3-mercaptopropionic acid (OPA-3-MPA). In 2011, Song and his coworkers separated 19 amino acids by pre-column derivatization by applying MonoClad C18-HS using 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), within 18 minutes (Figure 8) (Haghighi *et al.,* 2015).

Table 1. Results obtained for PKU positive patients using a GC-MS system Finnigan Voyager of EI. The samples were injected in splitless mode, analytes were separated using HP-5MS-capillary column and the carrier gas used was helium. Qualitative analysis was done by full scan acquisition mode and quantitative analysis was operated by SIM mode. The results obtained shows that the control group showed the phe: tyr ratio <1; whereas the PKU positive group showed elevated ratios. The limit of detection of Phe and Tyr were 1.5 and 0.8 µ*M*, respectively

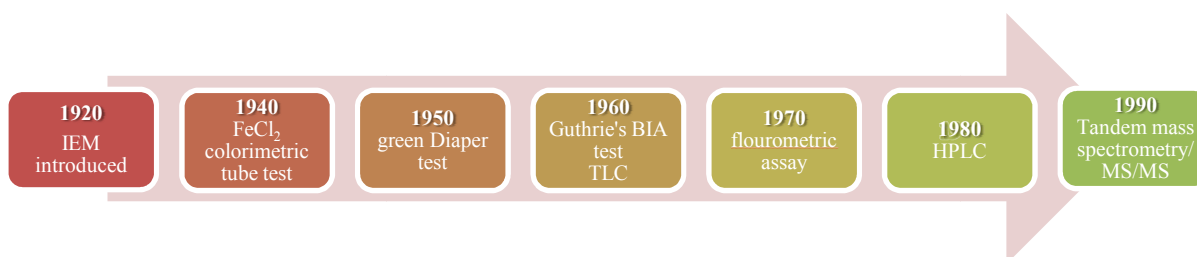| Sample	Phenylalanine (µ*M*)	Tyrosine (µ*M*)	Phe: Tyr
Control group			
1	209	261	0.80
2	78	199	0.39
3	177	209	0.84
4	156	224	0.69
5	107	219	0.49
PKU positive			
1	859	234	3.67
2	571	106	5.38
2	683	226	3.02
4	1023	208	4.92

Figure 4. Advancement in technology to screen for PKU in newborns

Figure 5. Diagrammatic representation of the mechanism of the "Guthrie" bacterial inhibition assay. In this representation the amino acid competes with the analogue at the level of cellular transport. This has low sensitivity due to the presence of complex biological matrices bacterial growth may be limited, hence resulting in false positive results. And the test results were reliable only after 2-3 days for better yield of phenylalanine
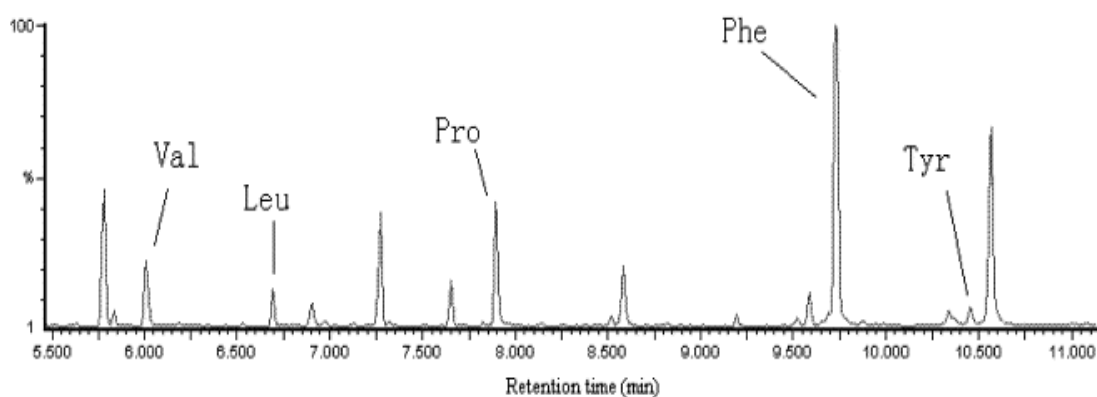

Figure 6. The SIM fragment ion *m/z* 91 $[C_6H_5CH_2]^+$ and *m/z* 203 $[CF_3COC_6H_4CH_2]^+$, characteristic ions of Phe and Tyr derivatives, respectively
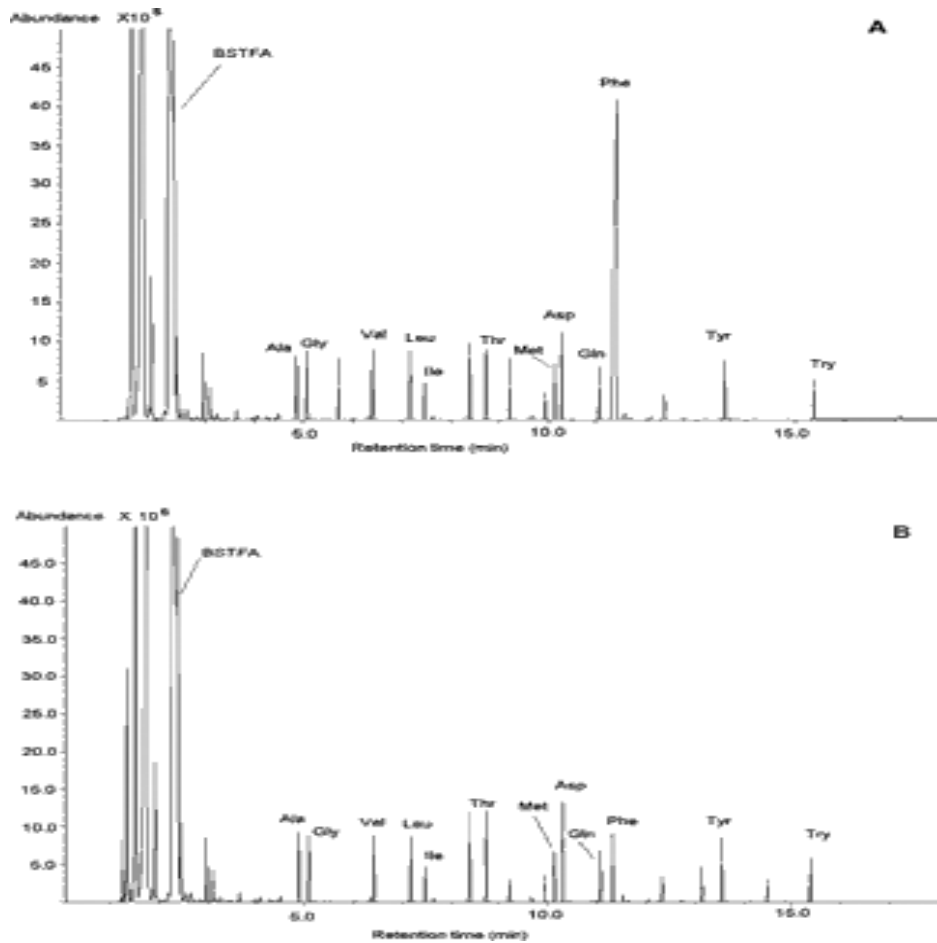
Figure 7. The GC/MS total ion chromatograms of a PKU-positive blood (a) and a control blood (b) after microwave-assisted derivatization
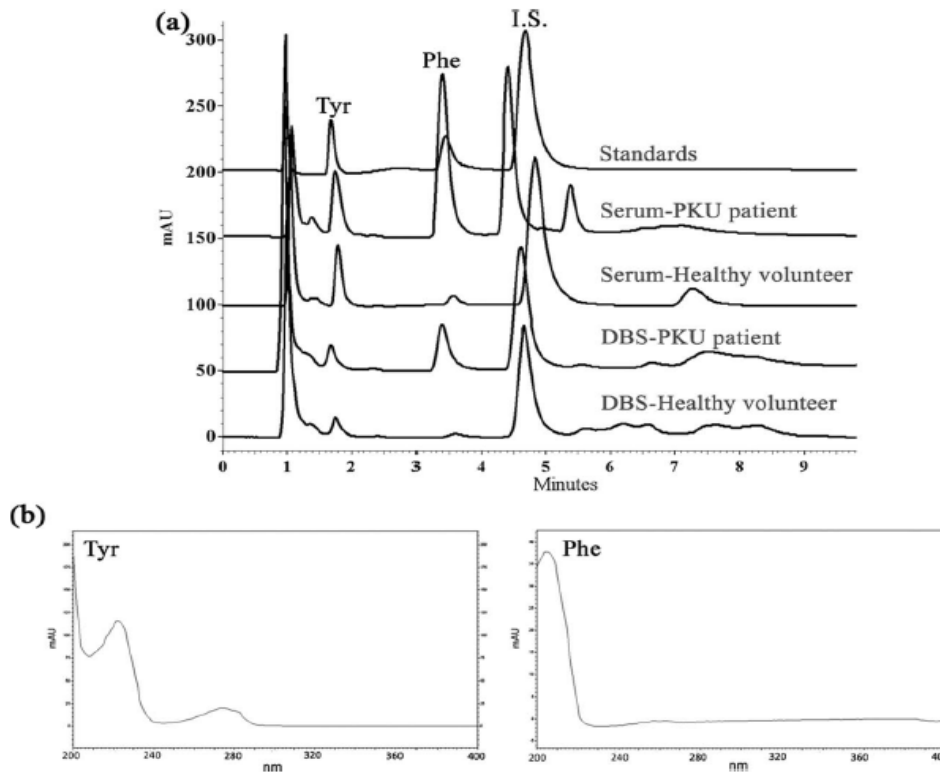

Figure 8. Obtained chromatograms for dried blood spots and serum samples from a healthy volunteer and a PKU patient at optimum chromatographic conditions (monolithic silica C18: 4.6 mm _ 10 mm; mobile phase: a gradient profile of water and MeOH; flow rate: 1.5 mL min_1; wavelength: 210 nm, injection volume: 5 mL) and (b) photo diode array spectrum of Tyr and Phe

Table 2. Phe and Tyr concentrations in neonatal blood samples obtained by microwave-assisted derivatization followed by GC/MS. The PKU positive samples have a relatively higher phe: tyr ratio than the control groups

Control blood	1	163	206	0.79
	2	96	187	0.51
	3	164	235	0.70
	4	227	248	0.92
	5	241	265	0.91
	6	178	163	1.11
	7	126	193	0.65
PKU-positive blood	1	673	176	3.82
	2	776	204	3.80
	3	746	198	3.77

Table 3. The levels of abnormal amino acids for different amino acids disorders

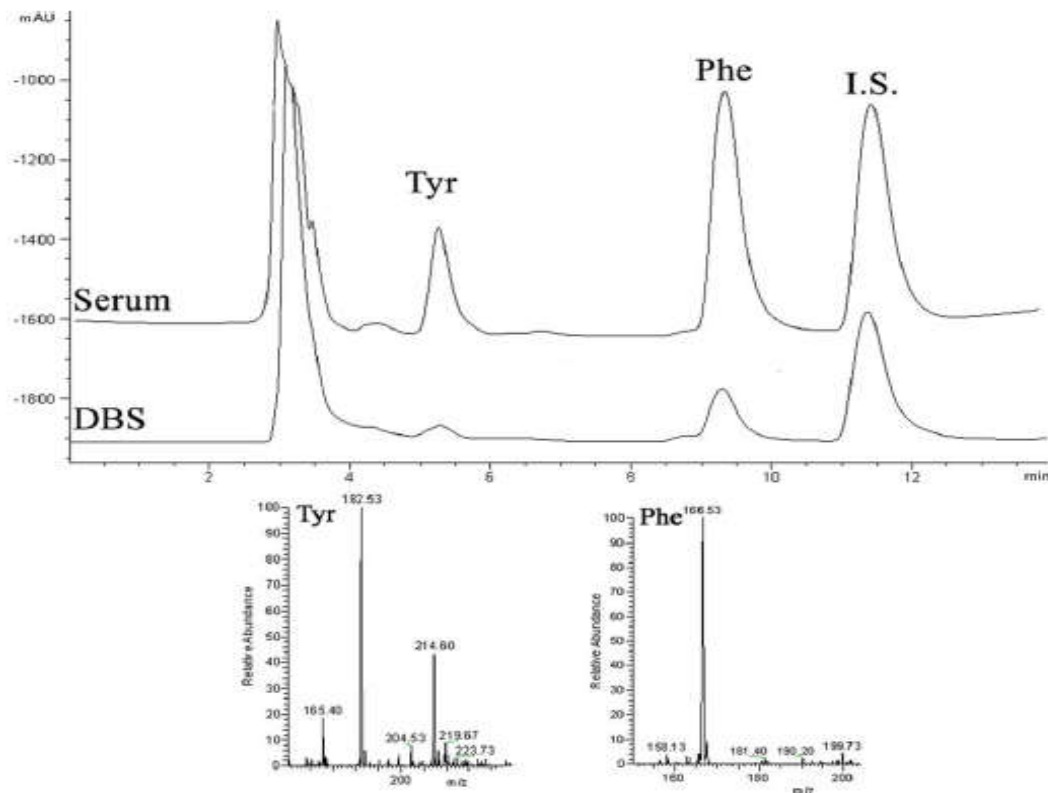| Disease	Abnormal Parameter	Concentration (µmol/L)
PKU	Phe	913.4 ± 372.5
	Phe/tyr	26.0 ± 11.8
Tyrosenemia	Tyr	341.9 ± 90.4
	Met	385.5 ± 279.3

Figure 9. LC-MS analysis of real samples. (a) Chromatograms of a dried blood spot and a serum sample from a PKU patient, (b) mass spectrum of Tyr and Phe with 182.47 and 166.53m/z respectively. Limit of detection for Phe and Tyr were 0.04 µ*M* for each; and the limit of quantification for the same were 0.1 µ*M*. This method has wide linear range of 0.1–3200 µM and a good recovery
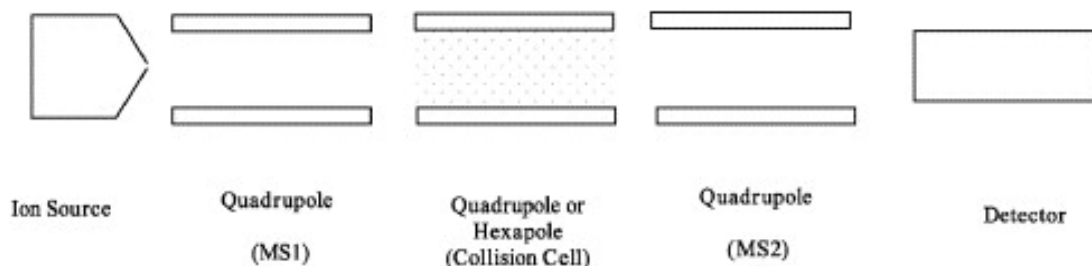

Figure 10. Schematic representation of a triple quadruple tandem mass spectrometer used to analyze 3070 children. Ions formed in the ion source are transferred to MS1, filtered and passed on to the collision cell where they are accelerated and collide with an inert gas causing collision-induced dissociation. The product ions are selectively filtered by MS2 before being passed to the detector
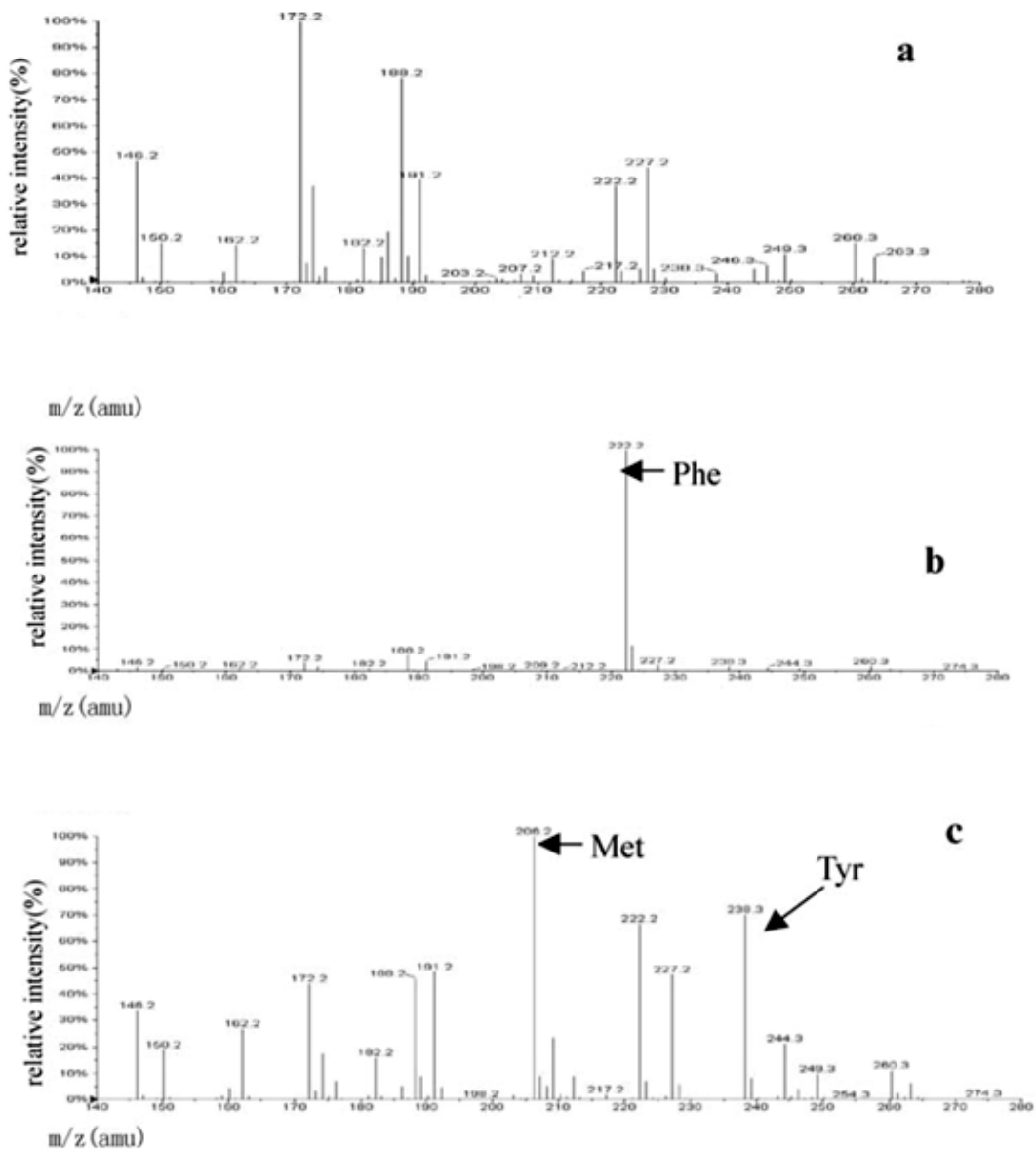
Figure 11. There were 212 cases out of which 48 were PKU and 7 were tyrosenemia type 1Amino acid profiles of amino acid diseases.
a: healthy child; b: Patient with phenylketonuria. c: Patient with tyrosinaemia type I

A study was conducted for the rapid screening of newborns in 2015, using RP-HPLC- PDA with monolithic silica in C18 column. The internal standard used was a-methyl phenylalanine obtained from Sigma-Aldrich. The separation was done on a Chromolith RP-18 column. HPLC system consisted of HPLC pump and a photo diode array detector. Phe and Tyr levels were directly analyzed without a pre or post-column derivatization. Results obtained were confirmed by liquid chromatography-mass spectrometry (LC-MS) (Figure 9) (Haghighi *et al.,* 2015).

Tandem Mass Spectrometry

Since 2000, due to the development of tandem mass spectrometry (MS/MS), tests for various diseases have been included in NBS programs. MS/MS was first initiated by Hunt and coworkers in metabolic profiling of carboxylic acids in urine, in 1982 (Pourfarzam and Zadhoush, 2013). Tandem mass spectrometer consists of a triple quadruple mass spectrometer that consists of two mass analyzers; MS1 and MS2, that are separated by a collision cell and 3 mass filters (HPLC columns) in tandem, and a photomultiplier detector (Figure 10).

This is a powerful technique which enables to generate greater fragments at a very short time and quantify them. Analysis depends on two aspects; precursor ion and neutral loss scan (Rashed *et al.,* 1995; Carpenter and Wiley, 2002; Banta-Wright *et al.,* 2004). In 2002, a 3 year study was conducted by Han and colleagues to screen for IEM by analyzing amino acid and acyl carnitine profiles using MS/MS. Agilent 1100 HPLC chromatograph along with API 2000 tandem mass spectrometer was used. The amino acids were analyzed in neutral scan loss mode (m/z 102) (Figure 11). The data obtained were automated by chemo view v1.2 software. Diagnosis depends on quantification of results. Elevated or decreased levels of amino acids, and their respective ratios are determined (Table 3) (Chace *et al.,* 1998; Garg and Dasouki, 2006; Han *et al.,* 2007). Due to the growing interest and advancement in technology, fast ion bombardment (FAB) techniques had been hyphenated with MS/MS. FAB-MS/MS is a soft ionization technique which eliminated the need of chromatography was being studied (Rashed *et al.,* 1995).

Fast atom bombardment MS/MS

FAB-MS/MS also known as liquid secondary ion mass spectrometry (LSIMS/MS) is abroad spectrum analytic

technique which uses soft ionization method; and eliminates the use of chromatography. Thus, shortening the analytical time up to one minute per sample. This technique has been expanded to detect amino acid profiles; and is proven to be a useful tool in diagnosing PKU (Rashed *et al.,* 1999; Matsumoto and Kuhara, 1996). A study was carried out by Millington and his team to diagnose IEM's using (LSIMS/MS) is shown in Figure 12 and 13 (Matsumoto and Kuhara, 1996). For this technique Samples were treated with methanol along with the internal standards. For the mass spectrometer, VG-TRIO-3 with an l-250 data system and a QUATTRO with a data system were the two triple quadruple mass spectrometers used. Photomultiplier diodes were used to analyze the samples. To obtain accurate results in order to detect PKU, butyl esters derivatives and a stable ionization procedure were used. Regardless of its advantage of elimination of chromatography and broad spectrum analysis, it is difficult to maintain sensitivity for more than 4 hours; as it requires frequent cleaning and maintenance.
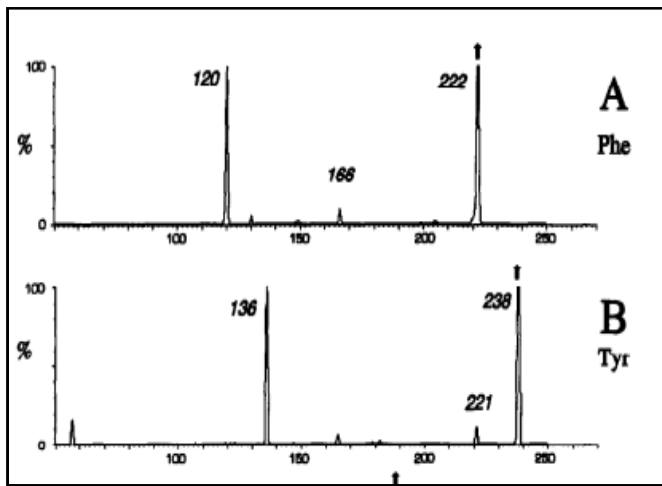

Figure 12. Daughter ion spectra of [M + H]+ ions from selected ammo acid butyl esters standard mixture obtained by FAB-MS-MS A, phenylalanine, B, tyrosine

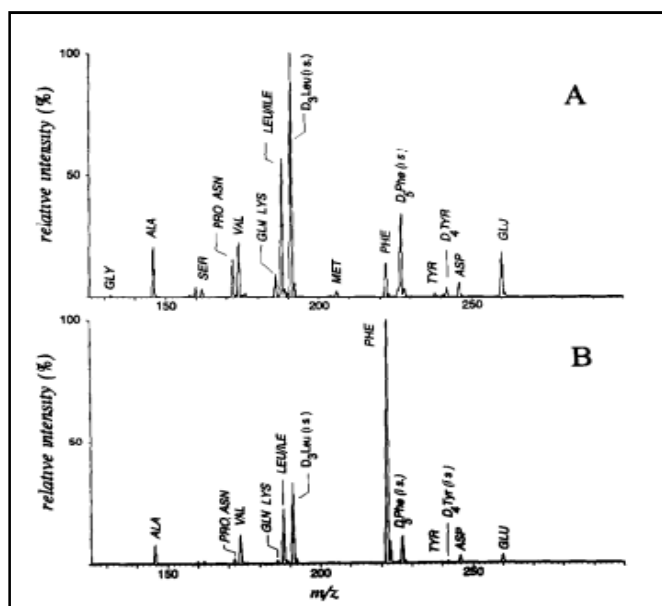
Figure 13. Amino acid profiles obtained by LSIMS-MS-MS analysis of 25~1 blood spots from orlgmal Guthrie cards used for neonatal screening A, normal individual, B, confirmed case of PKU. The scan function IS the neutral loss of 102 Da, the parent ions are displayed on the m/z axis

Also, it is unable to have a continuous flow, has a low sample throughput; as this system is unable to be automated. Therefore, electrospray tandem mass spectrometry (ESI-MS/MS) techniques are much preferred to be used (Rashed *et al.,* 1999).

Electrospray Tandem Mass Spectrometry

Commonly ESI-MS/MS has been a useful technique in detecting components of acyl carnitines. Recently, it has been expanded to the use of the technique in analyzing organic and amino acid profiles. Compared to the FAB-MS/MS; this technique is able to have a completely automated sample introduction system. It enables to have a broad-spectrum analysis and is useful in NBS programs in detecting IEM's (Rashed *et al.,* 1999). Many new approaches of liquid chromatography mass spectrometric methods have been evolved due to the complexity of biological matrix in blood and to improve the quality of analysis. Initially, along with LC-MS an internal control was required for every target amino acid detected. Since it was not practicable, stable isotope labeling method was used as internal standard quantification. This eliminated the use of a different internal standard for each target (Figure 14) (Rashed *et al.,* 1999). A study was conducted to propose an appropriate to determine amino acids in serum samples. Amino acids were labeled with isotope-coded MASC and provided with isotopic variants which co eluted on reversed phase column. And the LOQ and LOD of phe and tyr were 0.0015 and 0.0045μmol/L; 0.0025 and 0.007 μmol/L respectively (Rashed *et al.,* 1999). A study was carried out by Rashed and his team presented the automated system. A VG Quattro triple quadruple mass spectrometer equipped with a Jasco model PU- 980 HPLC pump and an AS-950 auto sampler were used for ESI-MS/MS (Figure 15) (Rashed *et al.,* 1995).
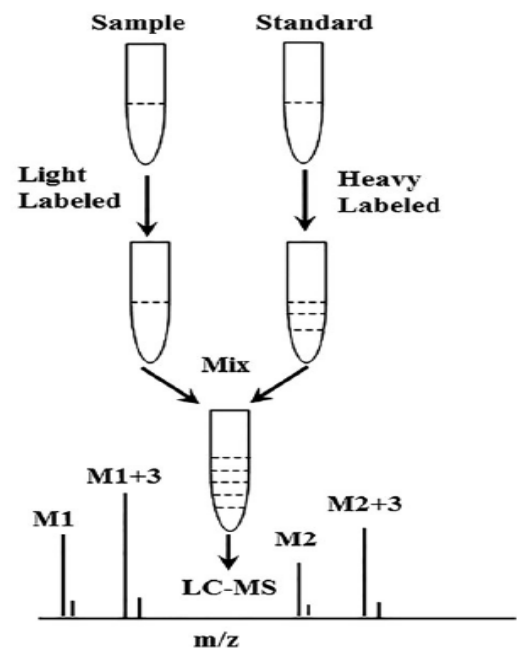

Figure 14. Quantification strategy of stable isotopic labeling. Agilent-1290 series HPLC system coupled with agilent-6460 triple quadrupole MS-MS system was equipped with an electrospray ionization (ESI) source; for HPLC–MS-MS analysis. Samples were separated using a SB C18 column. The linearities of the stable isotope labeling method were then evaluated. These were then analyzed by ESI-MS/MS
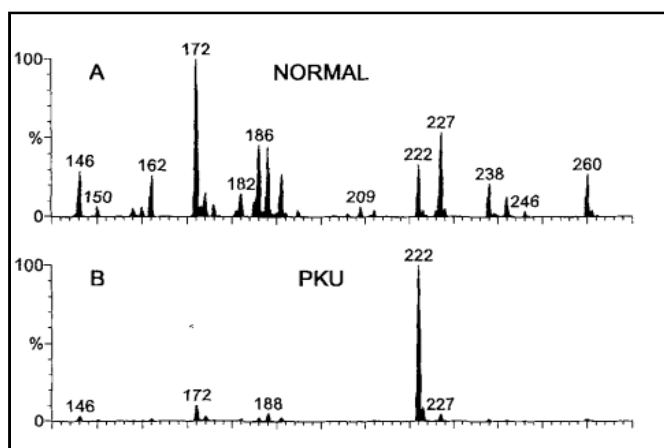
Figure 15. Blood amino acid profiles obtained by ESI-MS/MS analysis, scanning for a constant neutral loss of 102 D. (A) Normal; (B) a case of PKU. . The signals in the profile correspond to the protonated molecular ions (MH+) of amino acid butyl esters. , phenylalanine (222; d,-labeled isotope 227) ;), tyrosine (238; d4-labeled isotope 242. In this technique initially, the samples were treated with isotope-labeled internal standards and derivatized by butyl esters. Collision gas used was argon. For amino acid profiling, 2 neutral scan loss functions were used (102 D and 119D). Samples were directly inserted to injector port on the auto sampler connected directly to a fused silica tube. Calibration curves for phenylalanine were linear in the range of 5-800 nmol/pL

Summary

In summary, the detection of an abnormal result from an amino acid or acyl carnitine profile is essential in NBS programs. As explained in this review, detection of phenylalanine and tyrosine in newborns is very important due to the hazardous after effects that maybe caused if the IEM is present. Out of all the techniques, GC-MS is still known to be the gold standard technique even though the other techniques have a lower LOD and LOQ for phenylalanine and tyrosine. FAB/MS/MS is another technique that could be used as it is also a highly developed, sensitive technique which eliminates the use of chromatography and saves much time. But a more advanced ESI-MS/MS that is been used due to the capability of automating the whole process. It is one of the best technique compared the other techniques that have been described here. It has advantages of higher sensitivity, accuracy of mass and high resolution. However, research is currently undergoing to overcome disadvantages such as maintenance, false-positive results, labor consumption to screen for PKU with better understanding in the future.

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