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 then the metabolome has many chemical compounds it is difficult to determine 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 pentsosuriaby 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; intoxication (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 Hydpxylase (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.

*Corresponding author: Mathivathani Kandiah,*
Department of Biomedical Science, School of Science, BMS, 591, Galle Road, Colombo 06, Sri Lanka.
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 phenylpyruvic acid in urine. At first this was done as a qualitative 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 phenylpyruvic acid was not identified in neonates until few weeks old. Additionally, it had low specificity since compounds with similar color could 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-thiencylalanine) 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; Sprosen 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 sialylation 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 μM, 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-phthalaldehyde-3-mercaptopropionic acid (OPA-3- MPA). In 2011, Song and his coworkers separated 19 amino acids by pre-column derivatization by applying MonoClad C18-MS using 4-fluoro-7-nitro-2,1,3-benoxadiazoile (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 μM, respectively.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phenylalanine (μM)</th>
<th>Tyrosine (μM)</th>
<th>Phe: Tyr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>209</td>
<td>261</td>
<td>0.80</td>
</tr>
<tr>
<td>2</td>
<td>78</td>
<td>199</td>
<td>0.39</td>
</tr>
<tr>
<td>3</td>
<td>177</td>
<td>209</td>
<td>0.84</td>
</tr>
<tr>
<td>4</td>
<td>156</td>
<td>224</td>
<td>0.69</td>
</tr>
<tr>
<td>5</td>
<td>107</td>
<td>219</td>
<td>0.49</td>
</tr>
<tr>
<td>PKU positive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>859</td>
<td>234</td>
<td>3.67</td>
</tr>
<tr>
<td>2</td>
<td>571</td>
<td>106</td>
<td>5.38</td>
</tr>
<tr>
<td>3</td>
<td>683</td>
<td>226</td>
<td>3.02</td>
</tr>
<tr>
<td>4</td>
<td>1023</td>
<td>208</td>
<td>4.92</td>
</tr>
</tbody>
</table>

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

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 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 \([C_6H_{13}CH_2]^+\) 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

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

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

<table>
<thead>
<tr>
<th>Disease</th>
<th>Abnormal Parameter</th>
<th>Concentration (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKU</td>
<td>Phe</td>
<td>913.4 ± 372.5</td>
</tr>
<tr>
<td></td>
<td>Phe/tyr</td>
<td>26.0 ± 11.8</td>
</tr>
<tr>
<td>Tyrosenemia</td>
<td>Tyr</td>
<td>341.9 ± 90.4</td>
</tr>
<tr>
<td></td>
<td>Met</td>
<td>385.5 ± 279.3</td>
</tr>
</tbody>
</table>

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 μM for each; and the limit of quantification for the same were 0.1 μM. 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.
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 quadrupole 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.

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 12. Daughter ion spectra of [M + H]+ ions from selected amino 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 origmal 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

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 lineairties of the stable isotope labeling method were then evaluated. These were then analyzed by ESI-MS/MS
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.

Acknowledgement: Authors thank BMS for the financial aid and the support they gave in order, to make this article a success.

REFERENCES


Haghhighi, F., Talebpour, Z., Amini, V., Ahmadzadesh, A. and Farhadpour, M. 2015. A fast high performance liquid chromatographic (HPLC) analysis of amino acid phenylketonuria disorder in dried blood spots and serum samples, employing C18 monolithic silica columns and


******