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NEWBORN SCREENING FOR INBORN ERRORS OF METABOLISM- DRIED BLOOD SPOT TESTING AND TANDEM MASS SPECTROMETRIC TECHNIQUES

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Abstract
Inborn errors of metabolism (IEM) are a genetically disparate group of disorders arising out of defects in metabolic pathways leading to the excessive accumulation of metabolites. With development of the dried blood spot and tandem mass spectrometry (TMS), routinely assessing newborns for IEM's, also referred to as newborn screening (NBS) has been made largely possible. Several IEM's have a potential net benefit of screening, i.e. they are potentially treatable early in the course of disease before the onset of irreversible damage. NBS is a largely ignored facet in the pediatric health landscape of India and except for a minority of premier institutions, routine screenings incorporating testing for core disorders is not generally performed. It is only after accretions of obvious signs and symptoms are brought to the attention of a pediatrician that a high clinical suspicion in the direction of a metabolic disorder is finally elicited, which often results in delayed diagnosis. The single blood spot specimen detection kit is an inexpensive screening device which can detect with high and sensitivity and specificity several IEM's such as amino acid disorders and organic acidemias. Further confirmations are then made by techniques such as TMS. We plan to review the various screening tests such as dried blood spot and confirmatory testing by TMS for timely detection of major preventable IEM's.
Keywords: Tandem mass spectrometry, inborn error's of metabolism, newborn screening, dried blood spot, organic acidemia.

1.INTRODUCTION

Pre-symptomatic diagnosis of treatable inborn errors of metabolism (IEM) has always been a complex yet relatively ignored facet of neonatal care. Over 750 IEM's are documented, with possibly many variants of a milder or dormant nature tending to remain undetected or classified with non-hereditary diseases. Nonetheless, in many of the cases, prompt and early treatment may thwart establishment of permanent sequelae. Disorders arising due to underlying accumulation of metabolites, or 'progressive intoxication' from molecules which are otherwise unobtrusive in normal individuals form a large category of IEM's. These include the classic amino acid disorders such as phyenyl ketonuria (PKU), maple syrup urine disease (MSUD), homocystinuria, tyrosinemia, the organic acudurias such as propionic and methylmalonic aciduria , urea cycle disorders and glycogen storage disorders. No observable changes might be observed during the prenatal, natal and immediate postnatal periods. Symptoms may remain hidden for years and only triggered by stressors. Both acute as well as chronic clinical subsets may be seen, and a lack of remediation may result in progressive damage. Early diagnosis may result in dramatic improvements after relatively simple remedial measures such as dietary abstinence and detoxifying pharmacotherapy are adopte [1] The spectrum of pediatric diseases has been changing in India recently and the number of diagnosed inborn errors of metabolism's is steadily increasing. Special foods such as the foods for special medical purposes (FSMP) are now being imported into the country to treat early diagnosed IEM's after obtaining permission from the FSSAI (Food Safety and Standards Authority of India). Newborn screening is the need of the hour and the government must invest more resources in this direction. A study by the Indian council of medical research (ICMR) on 100,000 newborns revealed the much higher prevalence of IEM's such as congenital hypothyroidism (CH) (1 in 1130) and congenital adrenal hyperplasia (CAH) (1 in 5762) than was previously expected [2] The

Indian Pediatric experience suggests screening for common and easily treatable IEM's such as CH and glucose-6-phosphate dehydrogenase (G6PD) deficiency in India, because these are easy and inexpensive to treat. It is widely agreed that universal screening of newborns be instituted although no solid roadmap for the same has ever been executed in the country. Lal Path Labs took the initiative early on and National Institute of Mental Health and Neurosciences (NIMHANS) also started screening programs in the first decade of the 21st century. Presently several private NBS service providers, some participating in the Centers of Disease Control and Prevention (CDC) quality assurance programs have started diagnostics. The Recommended Universal Screening Panel (RUSP) as is adopted in the US is also available in some urban centers catering to the affluent sections. Only 3 Indian states/ Union territories, viz. Kerala, Goa and Chandigarh have instituted measures for NBS (for 3-6 IEM's) in designated hospitals on an official level. Unfortunately, certain IEM's which need Mass spectrometry (MS) instrumentation such as disorders of amino acids, organic acidemias and disorders of fatty acid oxidation are not included in these 3 states due to the high costs, lack of logistics and insufficient funds for treatment [3].

Countries such as the US have adopted newborn screening (NBS) with much success, screening 4 million newborns every year for IEM's. From manually performed bacterial inhibition assays to tandem mass-spectrometry based techniques, NBS for these rare diseases has come a long way. The standard procedure involves adsorption of capillary blood obtained from a heel prick onto a cellulose based membrane, usually a Whatman filter card, which is subsequently allowed to dry. The dried blood spot (DBS)is subject to further analysis, the liquid chromatography- tandem mass spectrometry (LC-MS/MS) being a very efficacious and proven method. Some studies have also investigated the potential of dried plasma spots [4].

DBS testing has all the advantages of a convenient and hassle-free diagnostic procedure. Smaller volumes of sample are required, which is a constant concern in newborns, especially in those with low birth weight. The relative stability of dried samples over extended periods simplifies logistical efforts and drastically minimizes costs. Periods of storage at room temperature from 2 to 4 weeks has a negligible effect on most parameters measured on DBS, although storage at -20 ºC improves stability if stored for longer periods.[5] No complex gadgets and facilities are required to transport such specimens, with regular post also utilized for such purposes, these factors being all the more valuable in a developing country setup. The problem of inherent fragility of certain analytes in biological fluids is circumvented as no special procedures are required to maintain stability of such molecules, which remain largely unreactive. The issue of infectivity of specimens and other biohazards risks is also addressed. No specialized training and/or personnel are required to perform the relatively less invasive 'pricking' maneuver, further simplifying testing. Widespread adoption of NBS using this simplistic method of sample collection has paid dividends, as several countries with such programs have markedly reduced incidences of certain IEM's, an example being PKU, which is considered 'eradicated' in certain areas.[6]

2. THE UTILITY OF MS IN COMBINATION WITH DBS

DBS has been coupled with mass spectrometry (MS) since several decades and widely implemented by several governments the world over ever since the beginning of the 21st century. These relatively modern-era devices differentiate molecules based on the mass/charge ratio (m/z) and can analyze complex samples for target analyses with high accuracy. Prior to MS, the DBS samples need to be further processed and fractionated by other techniques such as liquid chromatography. Tandem mass spectrometry utilizes two consecutive mass spectrometers to further increase range of analyses and throughput. After the processing and ionization steps, the 'precursor' ions which enter the first MS system are 'sieved' for ions within a specified range of mass. This selected lot of ions enters the collision chamber for fragmentation, the products of this step are then again subjected to a second MS step. Finally, the signals generated are picked up by a detector, and the masses of the precursor ions are correlated with products ions, enabling identification of compounds. In Fig. 1, the setup can be programmed to scan multiple patterns of fragments, thus more than one IEM can also be detected in a single run from an original DBS specimen. [7]

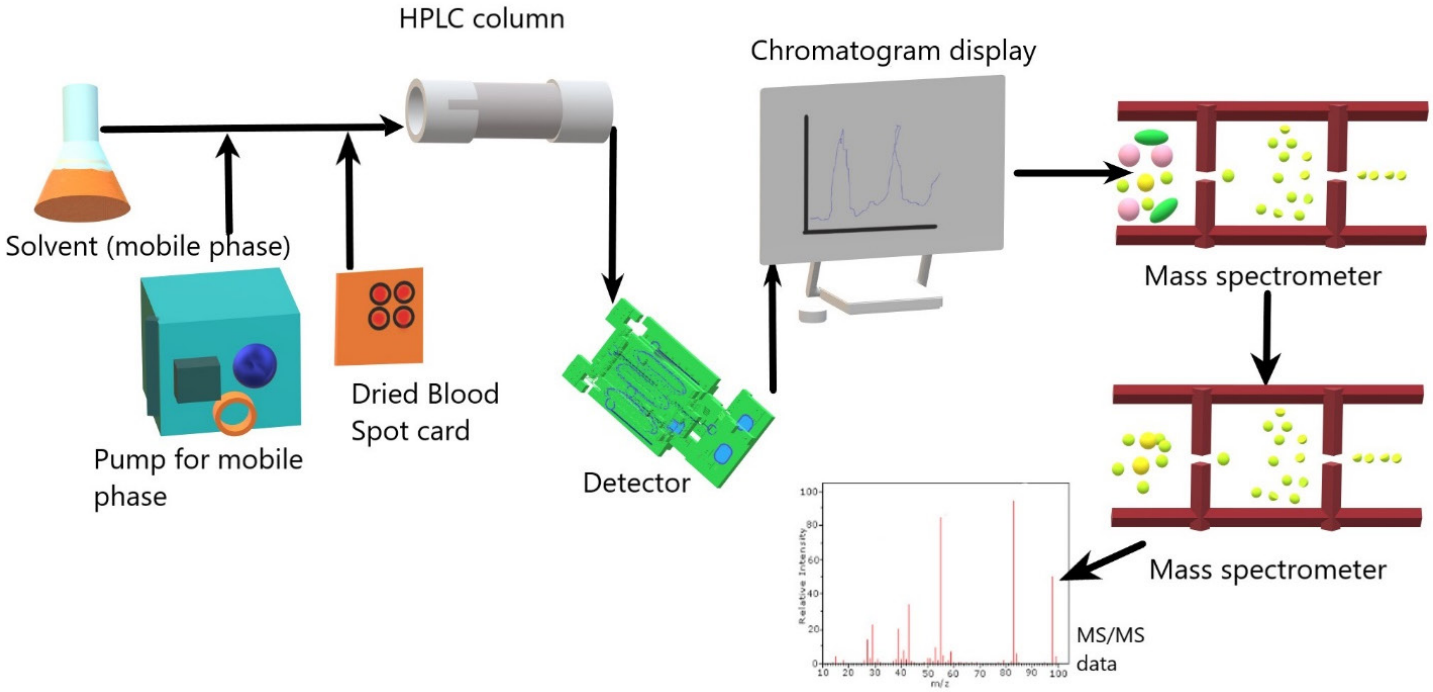

Fig. 1. A simplified depiction of the LC-MS/MS flowchart for screening of analytes on DBS samples.

Improved detection powers of modern triple quadrupole mass spectrometers has resulted in higher utility and feasibility of routine newborn screening with minimal chances of missing out on detection of rare IEM's. To screen DBS, immunoassays employing monoclonal antibodies are still used to confirm diagnosis of certain common IEM's such as congenital hypothyroidism and congenital adrenal hyperplasia. However, issues such as cross-reactivity with other background biomolecules, cost and time constraints in developing and using new assays has allowed renewed focus on MS based techniques. First used on DBS samples in 1976, MS evolved to harness better ionization techniques. [6] Initial hiccups in implementing DBS analysis were due to technical difficulties in punching equipment design and issues with cumbersome solvent-based extraction methods which made plasma analysis more feasible. Various types of detectors were also experimented upon in the last decades of the 20th century. Liquid chromatographic techniques coupled with Ultraviolet (UV), photo diode array (PDA) and fluorescence detectors were tried on DBS samples. Gas chromatography was also combined with MS but the procedure was time-consuming. Derivatization complexities reduced throughput. Therapeutic drug monitoring and pharmacokinetic study applications nevertheless provided impetus to research better and quicker techniques. Direct analysis of DBS (without preparatory techniques) in real-time MS (DART) has yielded good results. Electrospray ionization and atmospheric pressure chemical ionization have shown promising results. However, the lack of the chromatographic extraction reduces sensitivity and shows high interference from compounds present in matrix. LC coupled with MS/MS (Tandem MS) possesses sensitivity, selectivity as well as quantifying power required for DBS screening. The high throughput and lower costs make it perfect for mass screening of all DBS specimens for IEM's on a routine basis. The combination of LC- MS/MS has good linearities, excellent limit of detection (LOD), and eliminates interference of non-analytes [8] This has resulted in a paradigm shift from the 'one test-one disorder' model to the " one-test multiple disorders" model. Multiple IEM's can be detected from a single DBS sample. The typical workflow for a standard sample analysis involves punching of the DBS card and extraction with methanol (for amino acids or acylcarnitines), which may be followed by derivatization (butylation). This known as solid-liquid extraction (SLE). These initial steps constitute the 'offline workflow'. This is followed by

either further processing such as centrifugation, dilution etc., or directly by analysis with LC, electrospray ionization (ESI) and tandem MS (LC-ESI-MS/MS). An alternate workflow is flow-injection analysis (FIA) coupled with ESI and MS/MS (FIA-ESI-MS/MS) which involves minimal sample preparation and the DBS extracts are directly diverted in the mobile phase towards the ESI apparatus. Analysis times are usually under 4 minutes. An exceedingly high sensitivity of this combination to detect abnormal metabolites seen in IEM's such as phenylalanine in phenylketonuria has surpassed other reference methods such as fluorometry by up to two orders of magnitude. The capability to screen not one IEM, but up to 40 diseases in a single assay on a single DBS is an added bonus. Often, ratios of different metabolites (eg.Phenyalanine/Tyrosine ratio in PKU) are calculated from the analyses for added sensitivity. Nonetheless, the LC-ESI-MS/MS workflow is widely used for DBS analysis. LC utilizes the reverse-phase mode, which is ideal for hydrophobic analytes. Confirmatory or 'second-tier' tests for IEM's are usually performed by LC-ESI-MS/MS.[9]

3. SCREENING FOR IEM'S

The DBS has been used to diagnose the disorders of CH and CAH, the former being usually further analysed by estimating thyroxine and thyrotropin by chemiluminescence and flouroimmunoassay methods. [9] 17-hydroxyprogesterone (17-OHP), the elevated metabolite in CAH, can also be confirmed from DBS by enzyme and radioimmunoassays. LC-MS/MS has been effectively used in CAH confirmation as a 'second-tier' test. Only 33% CAH cases are confirmed by clinical observation, while NBS accounts for around 67% of cases. Lysosomal storage disorders (LSD) which encompass abnormal accumulations of myelin, muscle, vascular endothelium and the mucopolysaccharidoses have been usually estimated by enzyme assays. Multiplexed MS/MS assays to estimate activities of enzymes implicated in the pathogenesis of Pompe's, Krabbe's, Gaucher's, Fabry's and Niemann-Pick disease have been developed and tandem MS assays for several other disorders are also being developed. Several institutions have been screening for LSD's based on the above MS/MS format. DBS subjected to MS/MS have been able to detect heterozygotes of Pompe's diseases with better accuracy and have larger analytical ranges than traditional fluorometric techniques. [10] For diagnosing Gaucher's disease, analysis of glucosylsphingosine from DBS samples using a ultra-performance liquid chromatography (UPLC) coupled with tandem MS has proved highly sensitive. [11] In the case of mucopolysaccharidoses, analysis of glycasaminoglycans by LC-MS/MS has proven successful as well. [12] A study analysing DBS samples for various lysosphingolipids by LC-MS/MS successfully helped in diagnosing for Gaucher's, Fabry's, Prosaposon deficiency and Nieman-Pick Type A and B diseases [13]. Quantitative LC-MS/MS Assays have also been designed successfully for organic acidurias, homocystinuria and cobalamin metabolism defects which have shown potential to reduce number of false-positives and increase positive predictive values dramatically [14] Inborn errors of amino acid metabolism can also be conveniently detected from DBS without the hassles of derivatization. 20 or more amino acids can be quantified in around 10 minutes by using a high-performance liquid chromatography (HPLC) and tandem MS setup. It has been recommended to initially screen for IEM's using HPLC and follow up with MS/MS in resource constrained settings [15].

4. CONCLUSIONS

In order to avoid the implications of missing out on preventable complications of IEM's, it is imperative from the both the humanitarian as well as sociological point of view to institute mechanisms for early detection. The DBS is cost-efficient, easy to analyse and store and LC-MS/MS technologies are becoming more sensitive and economical compared to the cost of a lifetime of morbidity. The Kerala model for NBS screening can be incorporated in the health programs on a state-wise basis keeping in mind the preponderance of specific IEM's in those areas. Particularly nodal facilities should be setup where DBS samples under suspicion maybe referred for second line LC-MS/MS screening. This will go a long way in improving neonatal and early childhood standards of living.

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