Gas chromatography/mass spectrometry-based urine metabolome study in children for inborn errors of metabolism: An Indian experience

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A B S T R A C T

Objective: The present study highlights the feasibility of gas chromatography/mass spectrometry (GC/MS)-based analysis for simultaneous detection of ~200 marker metabolites in urine found in characteristic pattern in inborn errors of metabolism (IEM) in India.

Design and methods: During this retrospective study conducted from July 2013 to January 2016, we collected urine specimens on filter papers from Indian children across the country along with relevant demographic and clinical data. The laboratory technique involved urease pretreatment followed by deproteinization, derivatization, and subsequent computer-aided analysis of organic acids, amino acids, fatty acids, and sugars by GC/MS, which enable chemical diagnosis of IEM.

Results: Totally 23,140 patients were investigated for IEM with an estimated frequency of about 1.40%, that is, 323 positive cases. Most frequent disorders observed were of primary lactic acidemia (27.2%) and organic acidemia (methylmalonic aciduria, glutaric acidemia type I, propionic aciduria, etc.) followed by aminoacidopathies (maple syrup urine disease, phenylketonuria, tyrosinemia, etc.). Furthermore, alkaptonuria, canavan disease, and 4-hydroxybutyric aciduria were also diagnosed. Prompt treatment following diagnosis led to a better outcome in a considerable number of patients.

Conclusions: GC/MS with one-step metabolomics enables quick detection, accurate identification, and precise quantification of a wide range of urinary markers that may not be discovered using existing newborn screening programs. The technique is effective as a second-tier test to other established screening technologies, as well as one-step primary screening tool for a wide spectrum of IEM.

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1. Introduction

The term “inborn errors of metabolism” (IEM), also referred to as congenital metabolic diseases, is described as the hereditary deficiency of enzymes or alteration in protein structure and function resulting in metabolic derangement that may have pathologic consequences [1]. These disorders result in substrate accumulation causing minor to severe neurological and psychiatric manifestations resulting in lifelong disability or death [2]. Although IEM have usually been considered pediatric diseases, they can present at any age [3].

In many countries, including India, neonatal screening programs are becoming popular slowly and remained limited to few metabolic disorders only. There are limited published studies on newborn screening in India despite very high prevalence of IEM to the extent of 1 per 1000 newborns [4]. In the multicentric Indian Council of Medical Research (ICMR) study (1984), 4.9% of the genetic causes of mental retardation were due to metabolic disorders [5]. Screening of mentally retarded children in India revealed that 0.5% to 5.75% of children had amino acid disorders [6,7]. Another Indian study found 1.9% incidence of amino acid disorders [8]. Thus, there is a high prevalence of children suffering from IEM who are not diagnosed and left untreated due to unavailability of an adequate diagnostic setup. Several peer-reviewed data emphasized the necessity of nationwide, large-scale newborn screening in India [6–9].

Early diagnosis and treatment are critical for patients with IEM [10]. The universal newborn screening (NBS) started using simple bacterial inhibition assay to screen phenylketonuria (PKU) in the early 1960s is a success story of preventive medicine [11]. Nationwide NBS has been performed since then in several developed countries for the target diseases with the goal to reduce neonatal/infant morbidity and mortality [12]. Most of these NBS programs are based on blood analysis, and very few programs use urine as primary screening sample. In fact, human urine contains numerous metabolites whose concentrations provide clues to diagnose enzyme dysfunctions and assist chemical diagnosis of IEM [13]. Initial screening of IEM using urine started with nonspecific chemical tests, such as the ferric chloride test. However, accurate identification of metabolic fingerprint of any IEM needs more specific tests, and urine analysis using gas chromatography/mass spectrometry (GC/MS) has been initiated. However, many factors (low throughput, high cost,
2.2. Clinical sample collection

The study samples were divided into five age groups. Group I included patients between 0 and 14 days old; Group II, 15 days to 2 months old; Group III, from 2 months to 12 months old; Group IV, from 1 to 4 years old; and Group V consisted of patients older than 4 years of age.

2.3. Urine sample processing by GC/MS

The laboratory technique involved urease pretreatment followed by deproteinization with alcohol and derivatization by silylation as described by Kuhara et al. with slight modification [15,16]. In brief, 100 μl was eluted using distilled water from dried urine filter paper and incubated with urease to remove urea followed by the addition of internal standard (heptadecanoic acid). Following deproteinization with ethanol and centrifugation, the supernatant was evaporated to dryness with mVac vacuum concentrator (SP Scientific, Genecad Ltd., Ipswich, Suffolk, UK). Residue was further derivatized by BSTFA/TMCS for 30 min and subsequent analysis of the derivatized extract was done by QP-2010 Plus GC/MS (Shimadzu, Tokyo, Japan) with Windows software GCMS Solution Version 2.5, and generated data was processed by computer-assisted algorithm. Urinary creatinine was measured by Jaffe’s kinetic method using an ARX-235 analyzer (Micro Lab Instruments, Ahmedabad, India) with intraday and interday CV% being 3.5% and 5.0%, respectively. Recovery standards were added along with internal standard during extraction process for QC check [17]. One retained sample was previously analyzed and controls were analyzed in each batch to assure reproducibility and internal quality check. Mixture of n-alkanes was run whenever required to check the retention time (RT) of every metabolite.

2.4. Computer-assisted GC/MS data interpretation

For GC/MS-generated data, compound annotation was done by comparing the mass fragments and retention time with the mass fragments of reference standards in spectral databases of NIST MS search 2.0 software using similarity of >80%. For each target, more than two ions are chosen because of the complexity of the metabolome. The markers were expressed as μmol/mol creatinine. After correction for creatinine, the values were compared with those obtained from age-matched reference ranges. At the start of our work, we used reference ranges from the literature [18–21]. The reference ranges were then refined for different age slots as per our own population-based cutoff values from a large number of normal samples recruited from an ongoing screening program. The mean and standard deviation (SD) of each metabolite were calculated from our sample database for specific age groups and the same were amended in the software. Periodic review of reference ranges were done on 6-monthly basis and amended in software for analysis of samples thereon. Excretion of metabolite in the range of mean + 2SD and mean + 3SD was considered mild elevation; more than mean + 3SD was considered as significant excretion. Significant excretion of any metabolite was reported in terms of multiples of standard deviation (xSD). Software was provided with metabolic fingerprint of every disorder in the scope. Positive flagged samples with typical fingerprint of any IEM were categorized as screened positive IEM and checked manually with two spectral libraries, namely NIST MS search Version 2.0 and AMDIS Version 2.7. Those samples with ambiguous metabolome pattern and abnormal profiles denoting disease were evaluated thoroughly and retesting was done if it seemed appropriate.

The diagnosis of primary lactic acidemia was established only after evidence of lactic aciduria for two or more occasions. All diagnoses were correlated with amino acid/acylcarnitines platform for expected metabolic fingerprint wherever feasible. Enzyme analysis was carried out for screened positive cases of biotinidase deficiency and galactosemia for confirmation. Our laboratory participated in the European Research Network for evaluation and improvement of screening, diagnosis, and treatment of inherited disorders of metabolism (ERNIMD) proficiency testing program with 100% satisfactory performance for last 3 consecutive years.

3. Results

From July 2013 to January 2016, we diagnosed 323 positive cases of IEM among the total 23,140 urine samples analyzed for IEM in our study with consistent (at least on two occasions) altered pattern of organic acid excretion characteristic of IEM (Table 1) [22]. Sex distribution of study population comprised more males (57.6%) as compared to females (42.4%). We found 167 (51.7%) male and 156 (48.3%) female IEM cases with insignificant difference in sex distribution of IEM. The distribution of all diagnosed IEM cases is represented in Fig. 1. Most common IEM found in our study group was primary lactic acidosis (27.2%) followed by methylmalonic acidemia (17.9%). Occurrence of organic acidemia was higher compared to aminoacidopathies. Fig. 2 represents the chromatographic fingerprint of a few common disorders diagnosed in our laboratory.

The age of study population ranged from as young as 1 day to 13 years old. Mean age of diagnosis of IEM was 2.9 years and median age was close to 2 months. According to our data (Table 2), maximum frequency of IEM was observed in the age group of 15 days to 2 months (Group II), whereas Group I was the largest group referred for routine screening. The diagnosed cases within Group I were 15.6% of the total excretion of any metabolite was reported in terms of multiples of standard deviation (xSD). Software was provided with metabolic fingerprint of every disorder in the scope. Positive flagged samples with typical fingerprint of any IEM were categorized as screened positive IEM and checked manually with two spectral libraries, namely NIST MS search Version 2.0 and AMDIS Version 2.7. Those samples with ambiguous metabolome pattern and abnormal profiles denoting disease were evaluated thoroughly and retesting was done if it seemed appropriate.

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Table 1
Urine metabolic markers in Inborn Errors of Metabolism.

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Name of IEM</th>
<th>Primary markers</th>
<th>Secondary markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Primary lactic acidemia</td>
<td>Lactate</td>
<td>3-Hydroxybutyrate, 2- hydroxybutyrate, pyruvate, 2-methyl, 3-hydroxybutyrate, glycine, alanine, 4-hydroxy phenyl lactate, 2- hydroxyisovalerate.</td>
</tr>
<tr>
<td>2</td>
<td>Methylmalonic aciduria (MMA)</td>
<td>Methylmalonic acid</td>
<td>3-Hydroxypropionate, methyl citrate, propionyl glycine</td>
</tr>
<tr>
<td>3</td>
<td>Glutaric aciduria type I (GA1)</td>
<td>3-Hydroxyglutarate</td>
<td>Glutamate, glutaconate</td>
</tr>
<tr>
<td>4</td>
<td>Propionic aciduria (PROP)</td>
<td>Propionylglycine</td>
<td>3-Hydroxyglutarate, methyl citrate, tiglylglycerine, lactate, tiglylglycerine</td>
</tr>
<tr>
<td>5</td>
<td>Maple syrup urine disease (MSUD)</td>
<td>Leucine, isoleucine, valine, allo-iso leucine</td>
<td>Branched chain keto acids and Hydroxy-acids</td>
</tr>
<tr>
<td>6</td>
<td>Beta-ketothiolase deficiency (BKT)</td>
<td>2-Methyl 3-hydroxybutyrate, 2-methylacetocetic acid</td>
<td>Tigliylglycine</td>
</tr>
<tr>
<td>7</td>
<td>Ornithine transcarbamylase deficiency (OTC)</td>
<td>Ornithine, uracil</td>
<td>Glutamine, ornithine</td>
</tr>
<tr>
<td>8</td>
<td>Alkaptonuria (AKU)</td>
<td>Homogentisic acid</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>9</td>
<td>Phenylketonuria (PKU)</td>
<td>Phenylalanine, mandelate, 2-hydroxyphenylacetate, 3-Hydroxyphenylacetate, 2-Hydroxyglutarate, lactate, 2-Hydroxyacetate, lactate</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Glutaric aciduria type II (GA2)</td>
<td>Glutarate, even chain glycine conjugates (C4-C18)</td>
<td>3-Hydroxyisovalerate, methylcitrate, tiglylglycerine, lactate, tiglylglycerine</td>
</tr>
<tr>
<td>11</td>
<td>3-Methylcrotonyl CoA Carboxylase Deficiency (MCC)</td>
<td>Methylcrotonylglycine</td>
<td>3-Hydroxyisovalerate, methylcitrate, tiglylglycerine, lactate, tiglylglycerine</td>
</tr>
<tr>
<td>12</td>
<td>Galactosmia</td>
<td>Galactose</td>
<td>Galactitol, galactonate</td>
</tr>
<tr>
<td>13</td>
<td>Tyrosinemia (Tyr)</td>
<td>Tyrosine, succinylactone</td>
<td>N-acetyl tyrosine, 4-hydroxyphenylacetate, 4-hydroxyphenylpyruvate, 4-hydroxyphenylacetate, phenylacetal acid, methylcitrate, tiglylglycerine</td>
</tr>
<tr>
<td>14</td>
<td>3-Hydroxy 3-methylglutaryl CoA lyase deficiency (HMGC)</td>
<td>3-Hydroxy 3-methyl glutarate</td>
<td>3-Methylglutaconate, 3-methylglutarate, 3-hydroxyisovalerate, adipate</td>
</tr>
<tr>
<td>15</td>
<td>Biotinidase deficiency (BTD)</td>
<td>Propionylglycine, methylcrotonylglycine</td>
<td>3-Hydroxypropionate, methyl citrate, tiglylglycerine</td>
</tr>
<tr>
<td>16</td>
<td>Isovaleric aciduria (IVA)</td>
<td>Isovalerylglycine</td>
<td>3-Hydroxyisovalerate, methylcitrate, tiglylglycerine, lactate, tiglylglycerine</td>
</tr>
<tr>
<td>17</td>
<td>Canavan disease</td>
<td>N-acetyl aspartate</td>
<td>3,4-Dihydroxy butyric acid, 2,4-dihydroxy butyric acid, 4,5-dihydroxyhexanoic acid, Low 3-hydroxybutyrate</td>
</tr>
<tr>
<td>18</td>
<td>4-Hydroxybutyric aciduria</td>
<td>4-Hydroxybutyrate</td>
<td>3-Hydroxypropionate, methylcitrate, tiglylglycerine, lactate, tiglylglycerine</td>
</tr>
<tr>
<td>19</td>
<td>Nonketotic hyperglycemiaemia (NKHG)</td>
<td>Glycine</td>
<td>Hydroxy-derivatives and glycine conjugates of medium chain fatty acids</td>
</tr>
<tr>
<td>20</td>
<td>Multiple carboxylase deficiency (MCD)</td>
<td>Propionylglycine, methylcrotonylglycine</td>
<td>Hydroxy-derivatives and glycine conjugates of medium chain fatty acids</td>
</tr>
<tr>
<td>21</td>
<td>Orotic aciduria</td>
<td>Orotic acid</td>
<td>Hydroxy-derivatives and glycine conjugates of medium chain fatty acids</td>
</tr>
<tr>
<td>22</td>
<td>Medium chain Acyl CoA Dehydrogenase deficiency (MCAD)</td>
<td>Adipate, suberate, sebacate</td>
<td>Hydroxy-derivatives and glycine conjugates of medium chain fatty acids</td>
</tr>
<tr>
<td>23</td>
<td>Pyroglutamic aciduria</td>
<td>5-Oxoproline</td>
<td>Lactate, pyruvate, glycine, 3-hydroxybutyrate, 3-Methylglutaconate, methylcitrate, tiglylglycerine</td>
</tr>
<tr>
<td>24</td>
<td>3-Methylglutatonic aciduria (MGA)</td>
<td>Methylglutaconic acid</td>
<td>Methylsuccinyl acid</td>
</tr>
<tr>
<td>25</td>
<td>Ethyl malonic aciduria (EMA)</td>
<td>Ethyl malonic acid</td>
<td>Glutaric acid</td>
</tr>
<tr>
<td>26</td>
<td>2-Hydroxyglutaric Aciduria</td>
<td>Methylcrotonylglycine</td>
<td>Glutaric acid</td>
</tr>
<tr>
<td>27</td>
<td>Thymine-Uraciluria (DPD)</td>
<td>Tigliylglycine</td>
<td>Glutaric acid</td>
</tr>
</tbody>
</table>

delayed referral and/or poor screening practices. Table 2 also suggests that organic acidemias, especially MMA, presented in almost all pediatric age groups.

The most common symptoms in the diagnosed case population were neurological (78%) and failure to thrive (77%), followed by dehydration (63%) and developmental retardation (56%). Neurological manifestations encompass seizures, ataxia, encephalopathy, dystonia, altered sensorium, and hypo/hyperreflexia. Neurological manifestations predominantly seen in late infancy (Group III and above) helped the clinicians to refer those cases to delve deeper for any metabolic cause. Lactic and/or ketoacidosis, hypoglycemia, and hyperammonemia were the major biochemical abnormalities found in babies confirmed for IEM. We found that increase in unmeasurable anions (>20 mmol/l) was more common in established organic acidemia with negative evidence of sepsis.

4. Discussion

4.1. Challenges of GC/MS as screening tool

The first basis of laboratory diagnosis of IEM is its typical metabolic fingerprint in specific matrix (e.g., urine, blood, or cerebrospinal fluid (CSF)) [23]. However, several diagnostic metabolites pose a problem for their poor recovery or identification by routine methods. Second, prematurity, physiology, medications, and drugs severely interfere with metabolic patterns, leading to ambiguous results [24]. Third, GC/MS is highly sensitive and produces immense, complicated data that demand interpretation from experienced biochemists. Fourth, lack of studies on the cutoff values of metabolites based on age or ethnicity adds to a more complex situation. Pattern recognition is therefore not easy by manual integration unless automated algorithms are customized for IEM to allow high sample throughput. The tedious sample processing and long sample run time result in low throughput not suitable for population screening [14]. Finally, a study is warranted to distinguish between primary genetic and secondary acquired complications.
4.2. GC/MS as population screening tool

One solution to address the above issues is an ever-growing library specifically meant for metabolic diseases. With more and more samples analyzed for IEM, new findings come in place and are added to the repository of information. Currently, the mass spectral library devised by NIST is the world’s most widely used reference library for spectrum analysis. Our approach has been a continually updating repository of information in the existing library, which becomes richer with every sample analysis. We accordingly developed our own database of urine metabolome, which enabled us to screen IEM in all age groups ranging from neonates to elderly and to figure out several confounding variables (drugs, physiological, and environmental influences) for accurate diagnosis.

GC/MS analysis has always been challenged for its low throughput and poor extraction using historical methods [23]. To tackle this issue, we used a simplified urease digestion method with derivatization by silylation with only 17 min runtime. Our method made interpretation easier with a computerized, automated diagnostic algorithm compared to conventional manual peak measurement with flagging of only few samples with ambiguous pattern, which required expert intervention. Periodic review of cutoff and provision of database for confounding variables, such as prematurity, drugs, etc., helped to detect false elevation of few metabolites, thereby improving the specificity of the test and quality of screening. Moreover, the computerized algorithm simplified the interpretation of samples with typical metabolic pattern of IEM (Fig. 2) and reduced the manual work of expert biochemists.

![Image](image_url)

**Fig. 2.** Urine total ion chromatogram of some positive cases. A) Glutaric aciduria type II (GA type II) B) 3-Hydroxy 3-methyl glutaryl CoA lyase deficiency (HMG) C) Isovaleric aciduria (IVA) D) Medium chain Acyl CoA dehydrogenase deficiency (MCAD).

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Age group of studied cases.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age group</td>
<td>Total number of cases screened (23140)</td>
</tr>
<tr>
<td>Group I</td>
<td>0–14 days</td>
</tr>
<tr>
<td>Group II</td>
<td>15 days–2 months</td>
</tr>
<tr>
<td>Group III</td>
<td>2 months–12 months</td>
</tr>
<tr>
<td>Group IV</td>
<td>1 year–4 years</td>
</tr>
<tr>
<td>Group V</td>
<td>&gt;4 years</td>
</tr>
</tbody>
</table>

IEM: Inborn errors of metabolism; PROP: Propionic aciduria; MSUD: Maple syrup urine disease; BKT: Beta ketothiolase; MMA: Methylmalonic acidemia; GA I: Glutaric acidemia type I.
such an analytical method, we could analyze 70 samples in a day using one instrument, and have the potential to analyze about 21,000 samples/year/machine, based on 300 working days in a year. The cost of our GC/MS setup is low compared to tandem mass spectrometry (TMS), and simplified sample preparation can encourage stakeholders to develop NBS facilities in remote places.

4.3. Advantages of urine analysis for metabolic screening

Transportation of liquid urine samples is very challenging, posing several problems, such as spillage, stability, and bacterial contamination. The analytical method we adopted has the added advantage of using dried urine sample versus liquid urine. Since there can be dramatic degradation of certain diagnostic metabolites in liquid urine, such as succinylacetone and 4-hydroxybutyrate, such key metabolites can be preserved for their sensitive detection on filter paper [25].

The present study demonstrated that urine metabolome analysis using a computer-based algorithm can be an effective tool for population screening, especially in countries where advanced laboratory infrastructure and trained manpower resources are inadequate, such as India. Urine analysis has also been recommended by expert panel of American College of Medical Genetics (ACMG) for second-tier confirmatory testing of several core panel disorders. Expert opinion and peer-reviewed data strongly supported follow-up protocol that includes urine organic acid analysis for differential diagnoses as an important tool for screening [26]. Most importantly, primary urine metabolomic screening helped us to accurately diagnose cases of alkaptonuria, canavan disease, pyroglutamic aciduria, and orotic aciduria, which have been missed by routine screening. GC/MS thus proved more comprehensive than other NBS protocols picking up previously missed IEM. We experienced that the accuracy of GC/MS analysis has made genetic counseling of couples more effective for evaluating risk of IEM in future pregnancy [27]. Urine metabolome analysis immensely helped in our study for prognosis and treatment of established IEM and assisted for metabolic fingerprint in rare and undefined conditions.

4.4. Pattern of disorders in study population

The present report is a continuation of our previous study on the diagnosis of primary disorders of organic acid metabolism in routine and high-risk screening in Indian babies [28]. The most frequent disorder diagnosed in our study population was primary lactic acidemia (27.24%). We correctly identified patients with mitochondrial defects which had been missed using other technologies. The second–most common diagnosis found in the study population was MMA, followed by propionic academia (PROP). This finding re-established the fact that MMA is more commonly found in Asian population than PROP as described by Hori D [29].

Table 2 shows that only 19.5% of positive cases have been diagnosed before 15 days of age (Group I), through either routine NBS or high-risk screening. More toxic cases with early presentation of few organic academia (PROP, MSUD, etc.) have been referred in the early neonatal period. Nearly 65% of cases have been missed in the early neonatal phase and diagnosed in the age ranging from 15 days to 1 year (Groups II and III), the reasons of which could be lack of awareness in Indian medical fraternity toward NBS and the adoption of high-risk screening strategy. Other reasons for late diagnosis may be non-availability of technology, early death due to IEM, or a combination of these. It was surprising to find almost 15% cases were carrying IEM in the second year of life and beyond (Groups IV and V). Most of these cases have been referred for evaluation of neurological deficit and mental retardation. Age-specific IEM data thus demonstrated the poor screening initiative in India, practicing high-risk screening, and the need for better screening protocols. The current data might carry bias since there could be several cases with undiagnosed neonatal deaths.

IEM are commonly found in babies born to consanguineous parents, and we found several patients having a positive family history or previous unexplained deaths in the family [30]. These findings are consistent with the autosomal recessive inheritance of metabolic disorders.

Our results clearly depicted organic acidemia as more common IEM than aminoacidopathies and fatty acid oxidation disorders. Therefore, the NBS panel of disorders designed for other countries cannot be followed blindly in India. There is a need for addressing the core panel of disorders in India, which includes common conditions prevalent in the country.

Our data has limitations in regard to semiquantitative measurement of metabolites. Also, the exact data about the deaths and trends of complications occurred after diagnosis of IEM is lacking. Although the incidence of IEM cannot be drawn from the current study, we attempted to establish the prevalence of organic acidemias in high-risk cases to create awareness about the importance of nationwide newborn screening and prevention program.

5. Conclusion

Urine analysis using GC/MS has significantly improved the efficacy of neonatal screening programs, demonstrating the need for more comprehensive screening and improved throughput with advancement in computerized MS data handling. With the use of noninvasive urine sample collection on dry matrix, easy sample transport, and precise metabolome profiling, GC/MS has provided an efficient platform for routine and high-risk screening of IEM. The present data also necessitated nationwide selection of core panel of disorders for implementing NBS appropriate for the national requirements.

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References