

## Chemical Diagnosis of Congenital Metabolic Disorders by Gas Chromatography / Mass Spectrometry (GC/MS) in India

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**KEY WORDS** GC/MS; developmental delay; IEM; genetic counselling.

**ABSTRACT** The present study reports the chemical diagnosis of 254 high-risk children by GC/MS using MILS method, which detects biochemical marker compounds specific for congenital metabolic disorders. Air-dried "urine filter paper" allows simple bedside collection, transportation and a link with other hospitals. Simultaneous analysis of 101 metabolic disorders has the advantage of a wide range of detection to the attending doctor having limited health infrastructure in small towns and rural areas. The definite diagnosis was done in 44 samples (17.3%), reflecting the vast unexplored population of varied genetic etiology. This higher incidence in comparison with the earlier reports (4-5%) from India, based on conventional methods indicated the urgency of latest technology. In critically-ill neonates, the metabolic abnormality was 24.3%. This emphasizes the crucial role of GC/MS in preventing mortality and morbidity. The high-risk genetic factors were consanguinity (13%), family history of mental retardation (13%), and stillbirth and deaths (33%), indicating the racial and ethnic diversity, as well as cultural and traditional impact. The 3 interesting cases among many are discussed where successful management and therapy was done. Accuracy of GC/MS analysis made genetic counselling more effective in evaluating the risk of Inborn Errors of Metabolism (IEM) in future pregnancy.

### INTRODUCTION

Since its first application by Tanaka (1966), GC/MS has been used worldwide in diagnosis of IEM because of its high accuracy, sensitivity and power of analyzing multiple compounds simultaneously. The attempt is made to avail the use of newer technologies like GC/MS for the diagnosis of congenital metabolic disorders (Dave 1998).

Health professionals in India are acutely aware of the need of genetic services in the patient care. Lack of accurate, sensitive and reliable diagnostic services even in big tertiary gov-

ernment or private hospitals deprive the clinicians of rapid and precise diagnosis in the clinically suspected metabolic disorders. Further, management and therapy gets delayed with the resultant morbidity and mortality. As a result, prevention of metabolic disorders and genetic counselling to the patient and family members is not achieved. Earlier hospital based studies reported 4-5% incidence of IEM using conventional methods like TLC (Bharucha 1998). Genetic epidemiological studies are lacking, and the data from the developed countries is empirically followed over here.

To the best of our knowledge, GC/MS diagnostic facility was introduced for the first time in India. Awareness is being created by seminars, and making the clinicians aware about the prevention and genetic counselling approach. A few interesting cases are illustrated.

### MATERIALS AND METHODS

#### Patients

Total 254 high-risk cases were referred by the neonatologists, pediatricians, pediatric neurologists and neurologists for GC/MS analysis during 1998-2000. There were 33 critically ill-neonates referred by NICU units.

#### Method

Matsumoto's method (Matsumoto and Kuhara 1996) was used for simultaneous analysis of 101 congenital metabolic disorders involving organic acids, amino acids, sugars, sugar acids, sugar alcohols, nucleic acids and nucleic acid bases. The 22 diseases are referred as the "target disorders" because of severity of illness and possible early treatment (Table 1). The MILS method is a rapid, practical and yet comprehen-

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sive from the metabolic point of view as it can be used for mass screening for all age groups ranging from neonates to the elderly (Kuhara et al. 1996). The method is ultra-sensitive, reliable, accurate and more specific as compared to the conventional screening methods (Kuhara and Matsumoto 1995) and used in newborn mass screening (Kuhara et al. 1998).

The urine sample (25–50ml) is directly sent to the laboratory or transported on air-dried filter paper by mail with a detailed clinical history. The 100µl of eluate from urine filter paper was treated with 20µl urease solution at 37°C for 15 minutes. It was further deproteinized with ethanol, centrifuged, evaporated to dryness under reduced pressure. The organic compounds in urine were trimethylsilylated (TMS) by adding N,O-bis-trimethylsilyltrifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS), and heated at 90°C for 30 minutes. One or two µl of TMS derivatives of the organic compounds was injected into a Shimadzu QP-5050A GC/MS with Ultra Alloy capillary column (30m X 0.25 mm). The temperature of GC was kept at 60°C for 1 minute, and then increased up to 350°C at 17°C/min. Each 1µl sample was automatically injected in 20:1 split mode and mass spectrum was scanned with resolution mode from m/z 50 to m/z 650 every 0.25 seconds. The data was analyzed with computer-assisted program. Peaks in the TIC chromatogram that showed the profile of urinary metabolites were identified from each mass spectrum. Abnormal excretion of marker compounds for 101

metabolic disorders were checked for screening of inborn errors of metabolism.

## RESULTS

In 2 years, 44 metabolic abnormalities, out of 254 referral cases (17.3%) were detected (Table 2). A number of important congenital metabolic disorders like 2-amino adipic aciduria (2 cases), Canavan disease (3 cases), Fanconi syndrome (2 cases), Propionic acidemia (1 case), Glutaric aciduria (1 case), Methylmalonic acidemia (3 cases), Lactic aciduria (11 cases), Lesch-Nyhan (1 case), Ornithine Transcarbamylase deficiency (OTC) (2 cases), Fructose-1,6-diphosphatase deficiency (3 cases), Galactosemia (3 cases), Dicarboxylic aciduria with ketosis (4 cases) and others were detected. The 44 cases were confirmed to be IEM (Table 3). The 3 cases described below indicated the importance of GC/MS in accurate diagnosis and therapeutic management. These are Canavan disease, Lesch-Nyhan Syndrome and Ornithine Transcarbamylase Deficiency (OTC).

*Case 1 (GC/MS-88)*: One year old male child, born of consanguineous parents, presented with birth asphyxia, normal development till 3 months of age, and then showed regression of milestones, seizures and breath holding spasms 2–3 times/day (cyanotic spells while crying). He had sparse bright coloured hair, bradycardia, dystonic while crying and macrocrania. History of similar symptoms in the elder sib was found,

**Table 1: List of 22 Target Disorders for which Some Management and Therapy is Possible**

1. Methylmalonic acidemia	13. Lysinuria
2. Propionic acidemia	14. Cystinuria
3. Isovaleric acidemia	15. Tyrosinemia
4. Maple syrup urine disease	16. Glutaric aciduria type I
5. Galactosemia	17. β-hydroxy-β-methylglutaricaciduria
6. Phenylketonuria	18. β-methylcrotonylglycinuria
7. Hyperphenylalaninemia	19. β-ketothiolase deficiency
8. Homocystinuria	20. α-amino adipic-α-keto adipic aciduria
9. Alkaptonuria	21. Glutaric aciduria type II
10. Hyperglycinemia	22. *Neuroblastoma
11. Multiple carboxylase deficiency (Holocarboxylase synthase deficiency, Biotinidase deficiency)	
12. Urea cycle disorders Ornithine Transcarbamylase (OTC), Arginosuccinic Acid Synthetase (ASS), Arginosuccinic Acid Lyase (ASL), Arginase deficiencies (ARG), except Carbamyl Phosphate Synthetase (CPS), N-acetyl Glutamate Synthetase (NAGS).	

\* Not an IEM.

**Table 2: Chemical Diagnosis of Congenital Metabolic Disorders in High-Risk children by GC / MS.**

June 1998-June 2000			
Period	Total	Abnormal	Percentage
June 98–Sept. 98 (4 mths)	39	9	23 %
June 98–Dec. 98 (7 mths)	64	11	17 %
June 98–Aug. 99 (14 mths)	134	28	21 %
June 98–June 2000 (25 months)	254	44	17.32 %

who is confined to the bed since 10 years of age.

Increased excretion of N-acetylaspartic acid, a marker compound of Canavan disease was found by GC/MS analysis. The huge peak was confirmed as N-acetylaspartic acid by mass spectrum (Fig. 1). No other abnormality was found in the excretion of other amino acids, organic acids, sugars, sugar alcohols, sugar acids, nucleic acids and nucleic acid bases.

*Case 2 (GC/MS-193)* : A 3 year old male child presented with microcephaly, delayed developmental milestones, hypertonia, hypopigmented hair, hyperactive behaviour, mental retardation, and onset of generalized seizures at one and a half years of age. Karyotype revealed normal chromosomal pattern. On biochemical analysis, increased plasma ammonia (670  $\mu\text{Mol/L}$ ), serum uric acid (6.6 mgm%), and calcium (9.99mgm%) were found. Serum lactate (1.50 mmol/L) was within normal range.

The Total Ion Chromatogram (TIC) of TMS derivatives of the urinary organic compounds showed a huge peak of hypoxanthine at the retention time of 10 minutes. It was confirmed by its mass spectrum (Fig. 2) Hypoxanthine and Xanthine are the 2 marker compounds for Lesch-Nyhan syndrome. In our patient, Xanthine, the second marker compound was very low. The clinical symptoms and other biochemical parameters were correlated to diagnose this case as Lesch-Nyhan Syndrome. The follow-up was maintained with sodium benzoate therapy.

*Case 3 (GC/MS 187)* : A 14 day old female child having normal birth history, was admitted

to NICU for recurrent episodes of hypoglycemia, lethargy and poor feeding. Metabolic acidosis and seizures were absent. Biochemical analysis showed increased serum ammonia (168  $\mu\text{Mol/L}$ ) and lactate (3.3 mmol/L). The urine was sent for analysis on the 14<sup>th</sup> day after birth, suspecting congenital metabolic disorders.

The Total Ion Chromatogram (TIC) showed peaks of uracil and orotate, the marker compounds of Ornithine Transcarbamylase deficiency (OTC), at the retention time of 8 and 15 minutes respectively. The mass spectra confirmed the OTC deficiency (Figs. 3A & B) in this case.

## DISCUSSION

Urinary water-soluble compounds are the end-products of the catabolism of amino acids, organic acids, sugars, sugar acids, sugar alcohols, nucleic acids and nucleic acid bases. The IEM can be detected by abnormal excretory metabolites of these compounds by GC/MS. In many of the metabolic disorders, the consequences can be prevented by early treatment. Delay in treatment however lowers the therapeutic effect. Early treatment is therefore critical for prevention of mental retardation (irreversible brain damage).

The present method is simplified for use in multiple sample analysis by Matsumoto and Kuhara (1996). As a result, rapid, practical and simultaneous analysis of amino acids, sugars, sugar acids, sugar alcohols, nucleic acids and nucleic acid bases *in addition to organic acids is possible*. Thus, it is a highly comprehensive diagnostic tool for a wide range of metabolic disorders and helps in differential diagnosis, especially in a country like our's where advanced laboratory infrastructure and trained manpower resources are inadequate.

### Canavan Disease

*The Case 1* represents one of the 3 cases detected (Table 3) as Canavan disease (Mc Kusick MIM 271900). Canavan (1931) described an infant with prominent enlargement of head, cerebral and cerebellar spongy degeneration. The distinct clinical entity is credited to Van Bogaert and Bertrand (1967), who described the essential

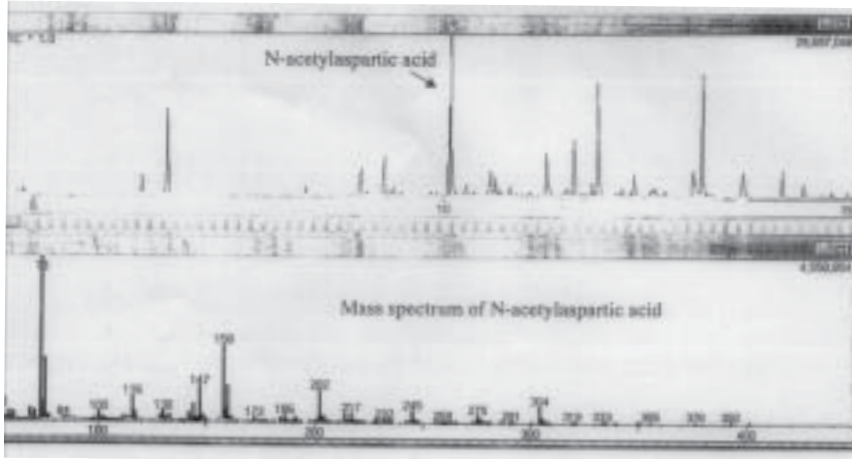


Fig. 1. Total Ion Chromatogram of urinary metabolites of a patient with Canavan Disease and Mass Spectrum of N-Acetylaspartic acid.

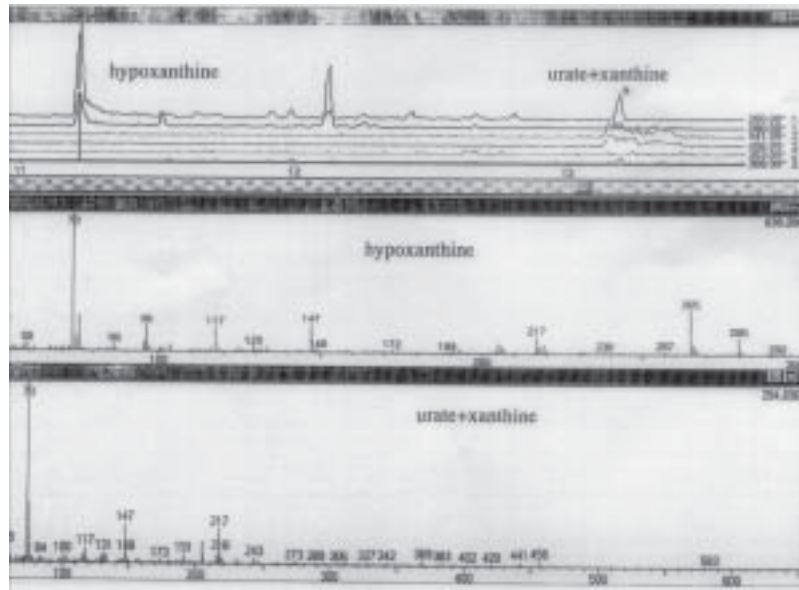
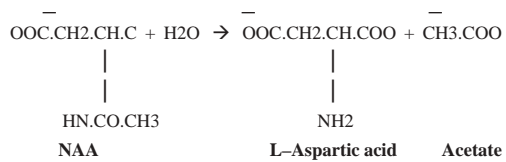


Fig. 2. Total Ion Chromatogram of urinary organic compounds of a patient with Lesch-Nyhan Syndrome and Mass Spectrum of hypoxanthine and urate + xanthine.

pathologic and clinical features and its occurrence in Ashkenazic infants.



#### Hydrolysis of NAA by aspartoacylase (ASPA)

The biochemical defect of this disorder is aspartoacylase deficiency which results into increased excretion of N-acetylaspartic acid (NAA) (Luo and Huang 1984; Shaag et al. 1995) (Fig. 1). The gene for this enzyme is identified on chromosome 17p13-pter region correlating with infantile spongy degeneration, and exploring the

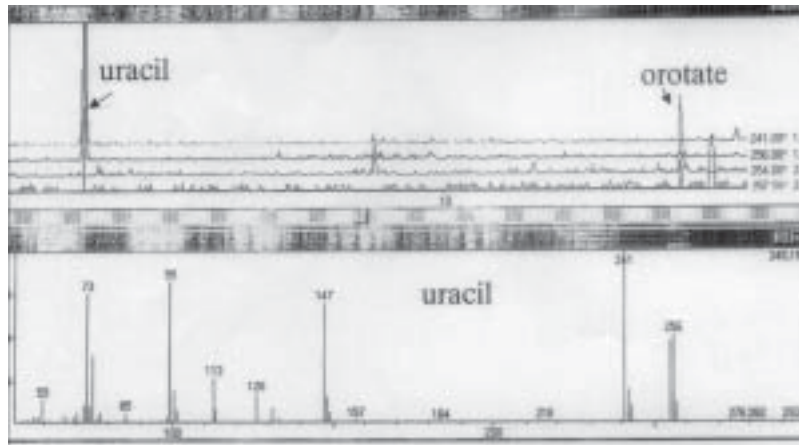


Fig. 3A. Total Ion Chromatogram of urinary metabolites of a patient with neonate onset of OTC deficiency.

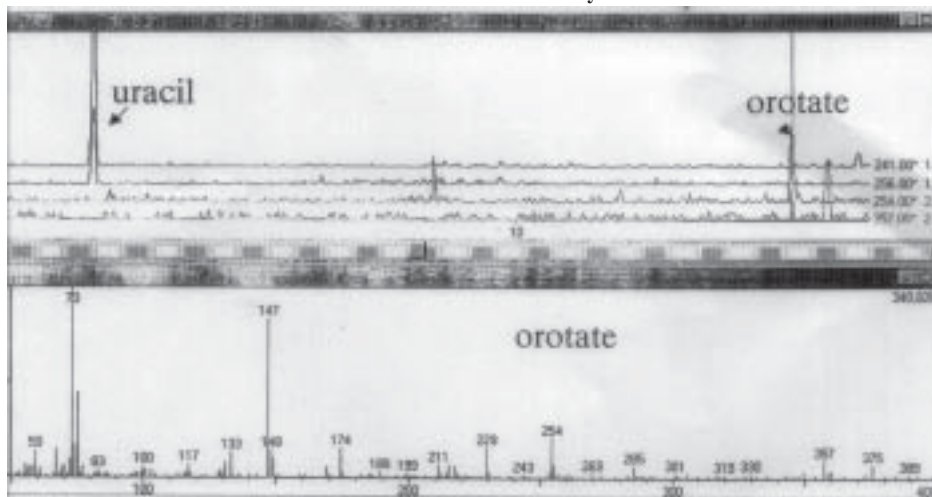


Fig. 3B. Mass Spectrum of urinary metabolites of a patient with neonate onset of OTC deficiency.

possibility for gene replacement therapy (Leone et al. 2000). Chemical diagnosis by GC/MS is more feasible as specific enzyme assays and DNA studies are time consuming, tedious and also needs appropriate laboratory infrastructure. Although, the brain CT and MRI show diffuse symmetrical degeneration of white matter, later involving gray matter, these changes are not always present.

The prenatal diagnosis is possible in cultured amniotic fluid cells or chorionic villus samples by measuring aspartoacylase activity (Keeley 1993). This is important in prevention of the

disease, as the genetic counselling is possible in the affected parents and family members.

### Lesch–Nyhan Syndrome

Since CREMERE offers healthcare and rehabilitation for the mentally retarded, this case was referred for mental retardation and behavioural problems. Here he was clinically diagnosed as Lesch–Nyhan syndrome (Lesch and Nyhan 1964). In this X-linked recessive disorder, the biochemical deficiency of the enzyme called hypoxanthine–guanine phos-

phoribosyltransferase (HPRT) may be partial. Our patient (*Case 2*), showed hypoxanthine in the urine, and characteristic self-mutilating behavioural problems, though not very aggressive. Unremarkable prenatal and birth history with delayed milestones in this case, was well correlated with the reported literature (Seegmiller et al. 1967). The child came to us mainly for mental retardation and hyperactivity, and was undiagnosed till the age of 3 years. He is now 5 years old and well under control with sodium benzoate therapy (Stout and Caskey 1989). The periodic plasma ammonia levels are monitored and the special educational training is being given for rehabilitation.

### Ornithine Transcarbamylase (OTC) Deficiency

OTC is an X-linked disorder; in which the mitochondrial matrix enzyme, catalyzes the biosynthesis of citrulline from ornithine and carbamyl phosphate (CP). Carbamyl phosphate synthetase (CPS) and OTC can be distinguished from each other by the level of urinary orotate. High levels occur in OTC deficiency as a consequence of diversion of accumulated mitochondrial CP to the cytosolic pyrimidines, including uracil, uridine, and pseudouridine which have been found in the urine of OTC deficiency (Valle and Simell 1995).

In this case, the recurrent episodes of hypoglycemia, lethargy and poor feeding made the pediatrician to consider the screening for organic acids. The GC/MS analysis could precisely detect OTC deficiency, and thereby the therapy. Protein restriction diet of 1.5 gm/kg/day supplemented with glucose intake; Sodium benzoate (100 mg/kg/day) was intermittently given to control ammonia. Plasma ammonia level was monitored monthly. This therapy was given only during infections. The investigations like EEG, CT and BERA test were normal. The baby is now 9 months old with no seizures or developmental delay, and is followed up regularly.

We diagnosed 2 cases of OTC deficiency (Table 3). The second case came for diagnosis at 7 years of age and is a classic presentation of irreversible brain damage due to delay in diagnosis resulting into severe mental retardation and seizures. Although the high plasma ammonia

**Table 3: Metabolic Abnormalities by GC/MS Analysis**

<i>S. Category of metabolic disorders No.</i>	<i>Total = 44</i>
1. Amino acidopathies and organic acidemias: 11	
2-aminoacidipic aciduria	2
Propionic acidemia	1
Glutaric aciduria	1
Methylmalonic acidemia	3
3-hydroxy-3-methylglutaryl-CoA-lyase deficiency	1
Alcaptonuria	1
Ornithine transcarbamylase deficiency	2
2. Lactic acidemia, hyperpyruvic acidemia: 11	
Lactic aciduria	10
Lactic aciduria with ketosis	1
3. Other disorders of amino acid and related compounds: 11	
Dicarboxylic aciduria with ketosis	4
Ketonuria	4
Canavan disease	3
4. Disorders of sugar metabolism: 6	
Fructose-1,6-diphosphatase deficiency	3
Galactosemia	3
5. Disorders of Purine, Pyrimidine metabolism: 1	
Lesch-Nyhan syndrome	1
6. Others: 4	
Fanconi Syndrome	2
Glycosuria	2

levels were monitored with protein restriction diet, the impact of therapy is less evident in comparison with the earlier case. This signifies the role of advanced diagnostic tool like GC/MS and MS/MS (Sweetman 1996) in neonates or newborn screening for prevention of the disabilities (Shoemaker and Elliott 1991; Kuhara et al. 1998).

### CONCLUSION

In the absence of cost-effective studies of newborn screening programmes or their inclusion in the government health policies, the use of present GC/MS methodology has proved its role in early detection and therapeutic management in high-risk neonates and children. Among the few multicentric studies, the largest number of patients with IEM seen in government hospital in Mumbai, over a period of 19 years (1978–mid 1997) is reported to be 781 (5.21%) out of the recorded 15,253 patients (Bharucha 1998). However, the present chemical diagnosis by GC/

MS has consistently demonstrated it as 17–23 %. The 8 critically ill neonates, out of 33 were diagnosed as IEM (24.3%).

The present study thus indicates that we are just looking at the tip of the iceberg of the whole situation considering the untapped rural, tribal and slum population of India. The 70% of India's population which live in villages need to be made aware, educated and motivated for detection of genetic disorders. The genetic epidemiological data, presently not available can be generated by upgrading the tertiary diagnostic centres with advanced state-of-the-art technology like GC/MS, LC/MS, MS/MS or FAB/MS. The national metabolic laboratory with peripheral network is the need of today to achieve the cost-effective screening programmes for the common genetic disorders in India. The great scope and potentiality for chemical diagnosis by biomedical mass spectrometry exists, considering the population of India to be more than one billion.

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