# TWO YEARS EXPERIENCE OF ANALYTICAL AND DIAGNOSTIC CHALLENGES IN URINE ORGANIC ACID ANALYSIS ON GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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## ABSTRACT

***Objective:*** To evaluate the analytical and diagnostic challenges in interpreting the various organic acid results by gas-chromatography-mass spectrometry and to devise a protocol for analysis that is beneficial for prompt interpretation and diagnosis.

***Study Design:*** Retrospective study.

***Place and Duration of Study:*** Department of Chemical Pathology & Endocrinology, Armed Forces Institute of Pathology, Rawalpindi, from Apr 2015 to May 2017.

***Material and Methods:*** We reviewed clinical data, biochemical investigations and urine organic acid profiles of 110 patients received for evaluation of a suspected organic acid disorder. Urine organic acid analysis was carried out by gas chromatography – mass spectrometry using Mass Hunter software.

***Results:*** A total of 104 (99%) cases received were from the pediatric patients and 7 (6.3%) from adult patients. A total of 11 different organic acidurias were diagnosed. Other diseases (n=10) were also detected on the basis of their pathognomics metabolites and included tyrosinemia type 1 (n=4), alkaptonuria (n=5) and ornithine transcarbamoylase deficiency (n=1). Twenty-eight (25%) urine samples were either recalled or repeated for reasons like random urine sample yielding negative profiles in setting of a strong suspicion for organic aciduria (n=6), non-availability of clinical data (n=12) or delay in transportation >8 hours (n=10). Raised non-specific organic acid metabolites were seen in 23 (21%) cases. Lactic acid and ketones were detectable in 12 (11%) samples in the absence of raised plasma levels.

***Conclusion:*** Urine OA profiles must be interpreted in context of complete clinical, nutritional and biochemical findings. Each laboratory equipped with this facility should devise their analytical protocols for meaningful interpretation of results.

**Keywords:** Gas chromatography-Mass spectrometry, Inherited metabolic disorders, Organic aciduria.



## INTRODUCTION

Diagnosis of inherited metabolic disorders (IMDs) is challenging, time consuming and requires a great deal of expertise. Diagnostic modalities range from undertaking simple biochemical tests, to quantitation of special metabolites, to the more complex molecular genetic testing[1]. Several secondary/ pre-existing conditions in the children make the diagnosis even more challenging. Detection of various Organic acids (OA) and their metabolites is the key to the diagnosis of not only organic acidemias, but through testing of certain metabolites like homogentisic acid, orotic acid and succinyl acetone, a range of conditions like homocystinuria, ornithine transcarbamoylase deficiency, and tyrosinemia Type-1[2], can be detected on this panel. OA analysis by gas chromatography-mass spectrometry (GC-MS) is a state of the art technique for screening of OA in urine worldwide[3,4], and has a wide range of applications. However as appealing as it may seem, the interpretation certainly comes with its own challenges. Several pre-analytical issues including fasting, nutritional status[5], concurrent illnesses, certain types of diets, drugs like

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*Received: 20 Jul 2018; revised received: 14 Sep 2018; accepted: 26 Sep 2018*

valproic acid[6], timings of sample and adequate transport temperatures drastically affect the urine OA profiles. Furthermore, diagnosing any IMD involves a multi-disciplinary team approach[7]. A thorough clinical data and biochemical investigations are essential pre – requisites for diagnosis. Not many centers in Pakistan are currently performing this analysis; Rather most of the samples are being outsourced abroad. We report a two years experience of diagnostic and analytical challenges faced in interpreting OA profiles on GC-MS. This study will help us devise protocols for sample collection and workup of suspected IMDs that is beneficial for prompt interpretation and diagnosis of cases especially those demanding detection or exclusion thereof of organic acidemias.

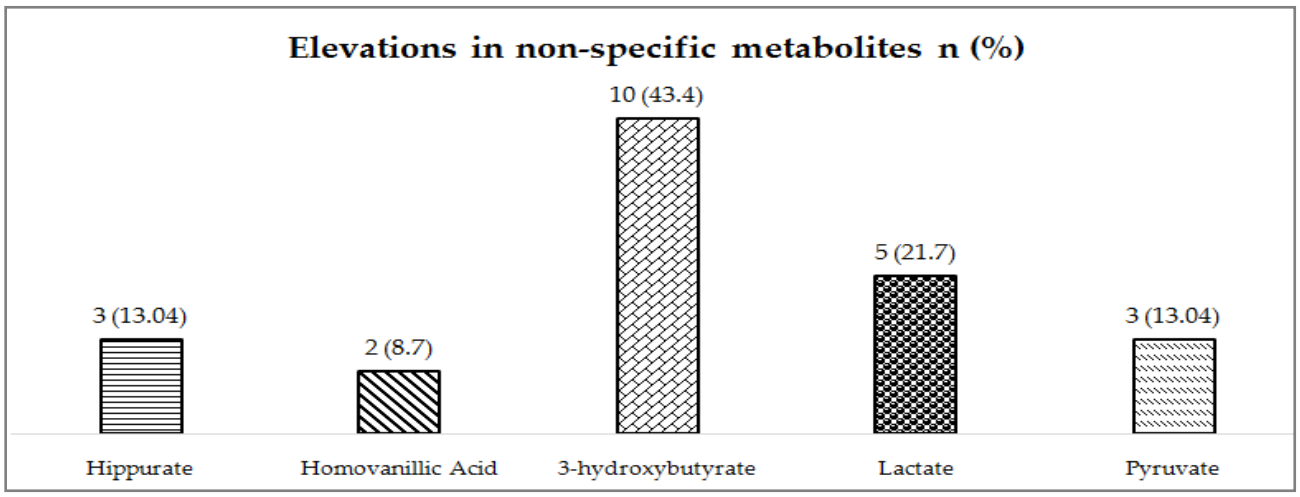


**Figure: Elevations in non-specific metabolites on organic acid analysis by GC-MS (n=23).**

## PATIENTS AND METHODS

This retrospective study was carried out in the department of Chemical Pathology & Endocrinology, Armed Forces Institute of Pathology, Rawalpindi, from April 2015 to May 2017, using non-probability convenience sampling. We reviewed clinical and biochemical data of 110 patients who had undergone OA analysis. Data included a wide range and age of patients from child out patient departments (OPD) (n=15), neonatal intensive care unit (NICU) (n=88), medical OPD (n=2), skin OPD (n=3) and orthopedic OPD (n=2). As per departmental protocol, an early morning spot urine sample was collected in a plain container (without preservative). The samples were transported immediately to the laboratory and were recommended to be kept refrigerated if there was anticipated delay of more than an hour in transportation. Urine OA screening was performed on GC-MS (Agilent Technologies 7890A GC system with 5975C inert Mass Selective Detector) with Mass Hunter software utilizing ORGASID library. Liquid-liquid extraction was utilized to extract OA from the samples followed by steps including oximation, extraction and derivatization with Methoxyamine hydrochloride (Sigma Aldrich), Ethylacetate (Merck), and BSTFA (Sigma Aldrich) respectively. Results were analyzed as mmol/mol of creatinine, to adjust the urine results for creatinine content. Advia 1800 clinical chemistry analyzer was used for determination of creatinine. The derivatization was achieved at a temperature of 50°C, and a reaction time of 30 minutes. The 3, 3 dimethyl glutaric acid (Sigma Aldrich) was utilized as internal standard (ISD). Results were accepted when the abundance of ISD in chromatogram was greater than 1.0 x $10^6$. Fifty-six OA and their metabolites were quantified based on specific ions in scanning mode. Patients' characteristics were enlisted in Microsoft excel – 2016 to calculate percentages and frequencies. Elevations in non-specific metabolites on organic acid analysis by GC-MS were represented by bar chart/graph.

## RESULTS

A total of 110 urine samples were analyzed. Among them 103 (94.5%) cases were from the pediatric patients whereas 7 (5.5%) cases were from adult patients. A total of 28 (25%) samples for OA analysis were either recalled or repeated for reasons such as random urine sample yielding negative profiles in setting of a strong suspicion for OA (n=6), non-availability of clinical data (n=12), delay in transportation of more than 8 hrs (n=10). Multiple raised non-specific organic acid metabolites were also observed (n=23) and depicted in figure. Lactic acid and ketones were detectable in 12 (11%) samples in the absence of raised plasma levels. The various organic acidurias diagnosed and their specific metabolites detected are shown in table-I. Other diseases detected on basis of elevations of their pathognomic metabolites on OA testing are shown in table-II.

**Table-I: Organic Acidurias diagnosed by Urine organic acid analysis on Gas Chromatography-Mass Spectrometry (n=11).**

| Organic Aciduria (n) | Metabolite Elevated |
|---|---|
| Methylmalonic Aciduria (4) | Methyl malonate, 2 Methyl gluconate, 3 hydroxy propionate |
| Propionic Acidemia (3) | 3-hydroxy propionate, 3-hydroxy butyrate, propionyl glycine, 3-keto, 2-methyl valerate |
| Glutaric Aciduria (3) | Glutaconate, 3 hydroxy glutarate, 3 hydroxy butyrate |
| Multiple Carboxylase deficiency (1) | 3-OH butyrate, 3-OH 3-methylglutarate, 3–OH Phenyllactic acid, 3-OH isovalerate |

**Table-II: IMDs other than organic acidurias diagnosed by OA analysis on GC-MS (n=10).**

| Disease(n) | Metabolite Detected | Other Biochemical derangements |
|---|---|---|
| Tyrosinemia Type 1(4) | Succinyl acetone | Raised Phenylalanine and methionine on plasma amino acid analysis; deranged LFTs |
| Alkaptonuria (5) | Homogentisic Acid | - |
| Urea Cycle defect-OTC deficiency (1) | Orotic Acid | Raised plasma ammonia; Raised glutamine and alanine and decreased citrulline on plasma amino acid analysis |

## DISCUSSION

Several investigators have frequently discussed diagnostic experiences and challenges for IMDs[8,9]. Afroze *et al* have recently reported a five years experience of managing and diagnosing patients with suspected IMDs[10]. However, studies documenting the practical challenges in diagnosis of IMDs especially for organic acidemias are sparse. This test facility in our set up is amongst one offered by very few centers in Pakistan[11] and has created a way forward for prompt and effective diagnosis for organic acidurias and other related IMDs whose metabolites can be detected in urine, thus saving time and cost involved in out-sourcing the samples abroad. We employed liquid-liquid extraction which is a commonly employed approach for sample treatment[12].

Interpreting results of OA with multiple raised metabolites remained the biggest diagnostic challenge. OA profile of one of the cases referred by pediatricians with strong suspicion of an organic academia revealed multiple raised OAs like 3-hydroxy isovalerate, 3-hydroxybutyrate, lactate and pyruvate. However, no increase in isovaleryl glycine or 3-hydroxyisovaleric acid was noted even on a repeated OA testing so isovaleric aciduria was excluded. This was much in accordance to two cases reported in literature[13,14]. Two cases had incidental finding of markedly elevated homo-vanillic acid (HVA) excretion in the urine. HVA is a dopamine metabolite and is often elevated due to stress increasing catecholamine output from the adrenal

gland. Elevated HVA may also result from the intake of medications/ supplements like L DOPA, dopamine, phenylalanine, or tyrosine. However, both cases had a negative nutritional history and raised levels were contributed to acute metabolic stress. Three samples revealed markedly elevated hippurate levels without any contaminant increase in any other organic acids. Tracing the source of this contaminant revealed that most of these samples had been kept at room temperature for a period of >10 hrs before being dispatched to the laboratory. It is well known that hippuric acid in urine is derived from bacterial breakdown of chlorogenic acid and high levels indicate GI microbial over-growth rather than inherited organic acidemia[15], and may be seen by bacterial contamination of urine samples[16]. A repeat analysis ensuring timely transport to the laboratory revealed normalization of these levels. Amongst the non-specific metabolites, raised 3-hydroxybutyrate levels was the commonest finding (43%), in the absence of other specific metabolites. It is well known that 3-hydroxybutyric acid is the end-product of rapid or excessive fatty acid breakdown. Common causes of elevation are prolonged fasting, protein malnutrition or high fat diet. In the absence of other raised specific organic acids, these samples were reported as absent for any significant organic aciduria.

All 5 adult cases referred with strong suspicion of alkaptonuria, from medical OPD (n=1), orthopedic department (n=1) and dermatology departments (n=3) tested strongly positive for marked elevations of homogentisic acid. Reason for this excellent diagnostic yield (100%) in adults could be due to ease of control of pre-analytical factors like diet and early morning (first voided) sampling, in the setting of rational test referral owing to prominent clinical features like joint deformities and/or characteristic skin lesions. Pre-analytical control is a necessary requirement for organic acid testing. An early morning (first voided) sample is best for analysis of various OA metabolites, is more concentrated and defects of amino acid catabolism and fatty acid oxidationare more distinct. Hence, we recalled all random samples especially received in setting of a strong clinical suspicion of organic aciduria. One of the limitations of the study is that we still need to define and interpret population based reference ranges as the concentration of organic acids in urine varies from population to population due to genotype, food habits and other environmental influences[17].

## CONCLUSION

Urine OA profiles must be interpreted in context of complete clinical, nutritional and biochemical findings. Each laboratory equipped with this facility should devise their analytical protocols for meaningful interpretation of results.

### Author's Contributions

Ayesha Hafeez: Devised the project, Literature search, data collection, and manuscript drafting.

Asma Hayat: Design of work, manuscript drafting, and data interpretation.

Javeriahafeez: Data analysis, Literature search, article writing.

Saima Shakil Malik: Data analysis and contributed towards the interpretation.

Muhammad Tahir Khadim: Revision of important intellectual content, Final approval of manuscript.

Nisar Ahmed: Performed the analytical measurements and data analysis.

All authors read and approved the final manuscript.

## CONFLICT OF INTEREST

This study has no conflict of interest to declare by any author.

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