DEVELOPMENT OF A GC-MS BIO-ANALYTICAL METHOD TO DETECT ORGANIC ACIDEMIA IN NEONATAL/ PAEDIATRIC URINE SAMPLE
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Abstract

There are some serious congenital disorders and Inborn Errors of Metabolism (IEM), which lead to disability and death of an infant. Most of these congenital disorders have safe and effective management, and if treated early can prevent significant mortality and morbidity. By the time symptoms are manifested, it is often too late and result in severe mental and physical disability in what could have been a completely normal child. There is insufficient epidemiological data to prove the number of cases of IEM in the country. The incidence also increases because of consanguinity prevalent in the country. Often the family suffers because, multiple progeny die from the same disease without proper diagnosis.

Analysis of urine organic acid provides information on the metabolism. In this research we have developed and validated a cost effective analytical method using GC-MS for analysis of three organic acids. RTX-5MS (5% phenyl) column was employed with programmed temperature from 50°C to 250°C with a total run time of 19.5 min. A simple extraction technique using ethyl acetate was employed and the derivatization was done in a single step with BSTFA mixture and the analyte in the ratio 1:1.

Linearity demonstrated was over a concentration range of 5µg to 100 µg with correlation coefficients ($r^2$) 0.994, 0.997, and 0.996 respectively for methyl malonic acid, glutaric acid, and adipic acid. Intra-day accuracy and precision were within the acceptable limits. The mean % recovery of methyl malonic acid, glutaric acid, adipic acid and tropic acid (ISTD) was 92.00, 72.73, 90.26 and 100.69 respectively. The developed method was used successfully to quantify the organic acids in urine samples of paediatric patients.

Key Words: Derivatization, GC-MS, IEM, Organic acidemia, Validation
1. Introduction

A genetic defect in any part of the major metabolic pathway is known as inborn errors of metabolism (IEM). Inborn errors of metabolism individually are rare but collectively are common. Many of the inborn errors of metabolism are treatable with diet or drug therapy. In IEMs single gene defects are responsible for the abnormalities in the synthesis or catabolism of proteins, carbohydrates or fats by way of defective enzymes or transport proteins, resulting in a block of metabolic pathway.

IEM disorders may manifest at any stage of life, from infancy to adulthood. A newborn affected with an organic acid disorder is usually well at birth and for the first few days of life. If the condition is not treated, babies usually develop vomiting, poor feeding, neurologic symptoms such as seizures abnormal muscle tone (floppy or stiff), and excessive sleepiness. This can progress to coma and death. Organic acidemia is caused due to some genetic defects, which results in deficiency of enzymes responsible for metabolism of aminoacids, carbohydrates and fatty acids. This results in increased levels of organic acids in our body. Early identification of IEM correlates with significant reduction in morbidity, mortality, and associated disabilities for those affected. Most of these disorders are treatable and require the emergency removal of the toxin by special diets, extracorporeal procedures, cleansing drugs or vitamin. To ensure rational treatment, correct genetic advice, and future antenatal diagnosis, it is very important to diagnose IEM accurately and early. In developed countries, metabolic disorders are detected through the newborn screening (NBS) programme using tandem mass spectrometry (TMS), whereas the developing countries have not implemented this programme yet, because of affordability issues. It is mandatory that any positive cases identified in TMS needs second level confirmation. Customised laboratory methods adequate to diagnose neonatal IEMs are scarce and lacking in many countries leading to delay diagnosis, treatment and poor survival rate in most cases. Eventhough there are several analytical techniques like NMR, LC-MS, Capillary Eleectrophoresis (CE) etc., available to an analyst, these methods has several constrains compared to GC-MS. Gas chromatography-mass spectrometry (GC-MS) is a first choice approach for the measurement of small molecule metabolites and in particular of primary metabolites such as organic acids and amino acids in clinical settings. This preference stems from the significant advantages offered by GC-MS, such as the existence of commercial spectral libraries, the potential for direct comparison and exchange of results from different laboratories, the unchallenged chromatographic resolution, accumulated experience in the field and affordability etc.
GC-MS is a method of choice in situations where it require highly sensitive and specific analysis such as in the case of analysis of trace amounts of biomarkers from biological matrix like urine and blood\textsuperscript{14,15}. GC-MS is an important analytical technique used in the diagnosis of metabolic disorders of newborn\textsuperscript{16}.

In this project we have developed a simultaneous method to detect and estimate the presence of organic acids such as glutaric acid, methyl malonic acid and adipic acid considering the fact that these disorders are treatable with special diets.

2. Materials and Methods

Chemicals such as adipic, glutaric, methyl malonic and tropic acid were purchased from Sigma–Aldrich. All chemicals and reagents used were of LC grade and were purchased from Merck Chemicals Mumbai, Biosolve Chemicals, Bangalore and Himedia Laboratories Pvt Ltd, Mumbai. N, O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) (with 1\%TMCS) were purchased from Spectrochem, Pvt.Ltd., Mumbai. The analysis of methyl malonic, glutaric, adipic acid was performed in GC-MS system (Shimadzu GC-17A Ver. 3) coupled to a mass selective detector (Shimadzu QP-5050A) using “GCMS real time analysis” software. Other instruments used in this study include bath sonicator (Serve well Instrument Pvt. Ltd.), analytical balance (SartoriusCP225D), vortex mixer(Spinix, India), centrifuge (REMI C24 centrifuge, India.), pH meter (Systronics\textcopyright pH system 361), micro-pipettes (Eppendorf), hot air oven (Osworld, India, nitrogen) evaporator (TurboVap LV, Caliper life sciences, San Jose) and auto analyser for determination of creatinine value (Cobas 6000, Roche, USA).

2.1. Preparation of standard solutions

For the construction of calibration curves, analyte stock solutions were prepared by dissolving the organic acids in methanol. The stock solution was further diluted in methanol to prepare eight working solutions of methyl malonic acid, glutaric acid and adipic acid with concentrations of 5, 10, 20, 30 40, 60, 80 and 100 µg for spiking neonatal/paediatric urine samples. Standard solutions were stored at 4°C. The internal standard stock solution was prepared by dissolving tropic acid in methanol to give concentration of 20 µg.

2.2. Collection of urine samples

Urine samples were collected from hospitalised children who were in the age group of 0-2 years admitted in the Kasturba Hospital, Manipal. Samples were pooled and were used for method development and validation. Urine samples were preserved by storing at -20°C.
2.3. Optimization of derivatization technique

Derivatization optimisation is very important especially in bioanalysis because the reaction conditions vary from molecule to molecule\textsuperscript{17, 18}. Organic acids are non-volatile in nature and need derivatization before analysis using gas chromatography. Derivatising reagents such as BSTFA (N,O-Bis(trimethylsilyl) trifluoroacetamide), BSA (Bistrimethylsilyl acetamide), and BSTFA+1\% TMCS (Trimethyl chlorosilane) mixture were tried based on information available in literature \textsuperscript{19,20}. Trials were made using different concentrations of 20, 50, 80, and 100\(\mu\)L of these reagents. Derivatization technique requires heat for the reaction to occur. So temperatures such as 50, 60, 70 and 80°C were employed and tried at different incubation periods of 10, 20, 30, 45, 60 and 120 min. In order to solubilise the derivatized acids, solvents such as pyridine and ethyl acetate were tried at a volume of 20\(\mu\)L.

2.4. Tuning the mass spectrometer

The mass spectrometer needs to be tuned to get the best signals. All quadrupole mass spectrometers are generally tuned with a compound of known mass spectra. Perfluorotributylamine (PFTBA) was used as the tuning agent. Tuning was done every day prior to initiation of a batch. The auto-tune programme adjusts the many parameters of the source, mass filter, and detector to give a consistent response to the tuning compound over the entire mass range, which in turn provides good sensitivity for accurate mass measurements. The auto-tune program uses three ions from the PFTBA spectrum for its tuning: m/z 69, 219 and 502. The program also uses the abundance of the naturally-occurring \textsuperscript{13}C isotopes at 70, 220, and 503 as a check for tuning.

2.5. Optimization of GC-MS operating conditions

Trials were performed with available columns such as DB-1 and RTX-5MS (30m, 0.25 mm I.D., 0.25 \(\mu\)m film) to decide a suitable column for separation based on the polarity. Optimisation of GC-MS conditions require suitable injection port temperature, interface temperature, column oven temperature, rate of increase in temperature and hold time. Trials for injection port temperature and interface temperature were made from 250 to 300°C. Column oven temperature was programmed from 50°C to 290°C at various rates ranging from 1-20°C per min and at different hold times. Trials for deciding the injection volume were performed at 1 and 2\(\mu\)L respectively both with split and split-less modes. The mass range for scan acquisition was fixed at 50-400m/z with solvent cut off time at 6 min.

2.6. Optimization of sample extraction procedure (Urine sample preparation)

Initial trial for extraction of organic acid was performed as per the reported literature\textsuperscript{13, 21}. This reported procedure was optimised further to increase the recovery. Urine extraction technique requires organic acid conversion from
polar to non-polar form to get maximum extraction efficiency. In this regard trials were performed by changing the volume of 1N NaOH and 3N HCl added to the urine sample. Hydroxylamine hydrochloride was used to remove the ketoxime group from urine sample. Trials were performed to optimize the incubation time, temperature and the quantity of sodium chloride added. Ethyl acetate was used as the extraction solvent. The volume of ethyl acetate required and the rpm for maximum extraction efficiency were determined by various trials. Organic acids were spiked (10% spiking) to urine to achieve concentrations in the linearity range. Spiking concentration for the internal standard (ISTD) selected was 20µg.

2.7. Validation of the method

The developed method was validated for system suitability, linearity, accuracy, precision, recovery, carry over, sensitivity and stability as per the USFDA guidelines for bioanalytical method validation (Guidance for Industry: Bioanalytical method validation, 2001, Published by Food and Drug Administration Center for Drug Evaluation and Research (CDER), U.S. Department of Health and Human Services).

2.7.1. System suitability test:

Prior to analysis of samples each day, the operator must establish that the GC-MS system and the procedure are capable of providing the required and accurate data within the limits. The system suitability was measured by injecting six replicate injections of reference solution equivalent to MQC (Median Quality Check) standard and evaluated the retention time (RT) and peak area ratio.

2.7.2. Linearity:

Linearity of an analytical procedure is its ability to obtain test results which are proportional to the concentration of analyte in the sample. The linearity of the calibration curve in neonatal/paediatric urine was confirmed by plotting the peak-area ratio (drug/internal standard) for the corresponding acids using least-squares linear regression analysis. The calibration curve included blank sample (matrix sample processed without ISTD), zero sample (matrix sample processed with ISTD), and eight non-zero standards covering the range. Weighting factor was not used for determination of curve fitting using linear regression. Mean, SD, % CV and % nominal concentration of mean back calculated value at each calibration level was calculated as per formulas, to determine precision at each calibration level.

2.7.3. Selectivity/Specificity: Specificity evaluation defines any endogenous materials and/or degradation peaks appearing at the same retention time of methyl malonic, glutaric, adipic acid and IS in the GC-MS chromatograms. A
minimum of six different drug free matrix lots were extracted with and without ISTD to assess the specificity of the method. Selectivity was evaluated by injecting extracted blank urine and comparing with the response of extracted LOQ (Limit of Quantification) QC samples processed with ISTD.

2.7.4. **Sensitivity:** The sensitivity was established by injecting six different LOQ samples of the organic acids and determining the %CV at appropriate confidence intervals.

2.7.5. **Carry over check:** To evaluate the efficiency of rinsing solution, the carry over check is performed by injecting blank reconstitution solution after the ULOQ (Upper Limit of Quantification) sample. If the carry over check is not done properly then the estimated concentrations will become cumulative by each injection and it would eventually leads to reporting of false positive results.

2.7.6. **Precision and accuracy:**

The precision was measured by the within day percent coefficient of variation (%CV) over a concentration in the calibration range of methyl Malonic acid, Glutaric acid and Adipic acid in urine during the course of validation. Within day precision was determined by the analysis of six replicates of the calibration curve in the same day. The %CV of the measured concentrations was used to determine the precision. The accuracy was defined as the absolute mean value of the ratio of the back calculated mean values of the unknown samples and their nominal values, expressed as a percentage. The measured concentrations of the within day analysis were compared to the nominal concentrations to determine the accuracy.

2.7.7. **Extraction efficiency (Recovery study):** Recovery of organic acid was evaluated by comparing the mean peak areas of three extracted LQC, MQC and HQC samples to mean peak areas of three neat reference solutions (unextracted). Recovery of (ISTD) was evaluated by comparing the mean peak areas of extracted samples to mean peak areas of neat reference solutions (unextracted) of the same concentration.

2.8. **Stability Studies**

Stability studies such as stock solution stability, bench top stability and freeze and thaw stability of organic acids have been evaluated during validation. Stability was determined as mean % change in stability samples with respect to comparison samples.

2.8.1. **Stock solution stability:** Stock solution stability was performed at room temperature for 8.0 hours and at 2 to 8°C for 30 days. Stock solution stability was assessed by comparing freshly prepared samples of organic acid with that of stability samples at MQC level by performing five injections of each acid. Five injections each of comparison
and stability samples were performed and mean % change for Adipic acid, Glutaric acid, Methyl malonic acid and Tropic acid after 8.0 hours at room temperature and after 30 days at 2 to 8 °C were calculated.

**2.8.2. Stability in matrix (Bench top stability and Freez-thaw stability):**

Six replicates of LQC and HQC in biological matrix were withdrawn and thawed unassisted at room temperature and kept unprocessed for 7 hours (stability samples). After 7 hours fresh calibration was prepared with one set of low and high QC samples (comparison samples). Both comparison samples and stability samples were processed and analyzed in single run. Freeze thaw stability in plasma was assessed by analyzing six replicates of LQC and HQC samples after three freeze and thaw cycles. Samples are stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 12 to 24 hours under the same conditions. The freeze–thaw cycle was repeated two more times and then analyzed on the third cycle. If an analyte is unstable at the intended storage temperature, the stability sample is frozen at -70°C during the three freeze and thaw cycles. Calibration standards, quality control samples (comparison samples) and stability samples were processed and analyzed in single run.

**2.9. Application of the developed method for routine analysis of clinical samples**

The validated method was used for analysis of organic acids in urine samples obtained from hospitalised children. The children were in the age group of 0-2 years and were not on any special diet. 5 mL urine was collected in the early morning during the spontaneous micturition. Quantification of MMA, GA and AA in urine samples was done using the developed method and reports were given in millimol/mol of creatinine.

**3. Results and Discussion**

**3.1. Optimized GC-MS conditions and temperature programme**

Separation was accomplished with RESTEX RTX-5MS (30m, 0.25 mm I.D., 0.25 μm film) column. Helium was used as carrier gas at a linear velocity of 36.4 cm/s to achieve a flow rate of 1.2mL/ min and the injector split ratio was set to 1:4. The oven program was started at 50°C with initial hold time for 1 min and was increased at the rate of 10°C /min to 100°C with a hold for 1 min, and then increased at the rate of 20°C /min to 250°C, with a final hold for 5 min. The total run time was 19.5 min. The temperatures of the injection port and transfer line were both 250°C. The analyses were performed on a gas chromatograph coupled to a mass selective detector with electron impact (EI) ionisation and single quadrupole analyser. Acquisition of data was done in the Total Ion Current (TIC) scan mode with a scan speed of 1000. The scan range was set between m/z 50–400 and the solvent cut off was set at 6 min.
Chromatogram obtained for the optimized method is provided in Figure 1. Tuning of the mass spectrometer was performed using the auto-tune option from the real time analysis. After completion of the tuning, the tune file was saved for use with the batch, after checking and confirming that it passes the acceptance criteria.

![Chromatogram](image)

**Figure 1:** Chromatogram showing well resolved peaks of MMA (9.45), GA (11.27), AA (12.08) and TA (Internal standard) (12.75) after method optimization.

### 3.2. Optimized extraction method and derivatization technique

Urine sample equivalent to 0.25 mg of creatinine (creatinine value was determined previously using Jaffe’s method) was taken in a glass tube. Spiked the analytes and internal standard (50µl for 400µg/mL) to this sample and adjusted the pH to basic by adding approximately 0.5 mL of 1N NaOH. To this was added 500µL of 50g/L aqueous hydroxylamine hydrochloride, capped tightly and placed in the oven at 60°C for 30 min. The solution was then cooled for about 5 min and added NaCl until saturation (approximately 1 spatula). Adjusted the pH to acidic with 3N HCl (approximately 0.5mL) and vortex mixed for 1-2 min. 3 mL of ethyl acetate was added as the extraction solvent and mixed with vortex again for 1 min and then centrifuged at 2500 rpm for 8 min. The organic phase (ethyl acetate phase 2.7mL) was carefully withdrawn and transferred to nitrogen evaporation tube and evaporated to complete dryness at 50°C and added 80µL of BSTFA+1%TMCS, mixed and capped tightly with parafilm and incubated at 80°C for 30 min. Cooled the derivatized sample to room temperature and added 20µL ethyl acetate to solubilise the derivatized sample to prevent any precipitation. 1µL was injected to GC-MS for analysis.

### 3.3. Results of validation of the method

#### 3.3.1. System suitability test:

The % CV for peak area ratio for methyl malonic acid, glutaric acid and adipic acid were 0.11, 0.09 and 0.45 respectively. These were within the acceptance limits of ≤2.0%. The %CV for retention times for the above organic acids and ISTD 0.0099, 0.0041, 0.0041 and 0.0029 respectively were also within the acceptance limits of ≤2.0%.
3.3.2. Linearity: The degree of response from the detector with respect to the injected concentration was found to be accurate and precise over the concentration range 5 - 100μg for all selected organic acids. The coefficient of determination ($r^2$) was found to be 0.994, 0.997, and 0.996 for methyl malonic acid, glutaric acid and adipic acid respectively. The linearity plots for these acids are provided in the Figure 2. The calibration range, calibration equation, retention time and the m/z of the ions identified in the mass spectra of derivatized acids are provided in Table 1.

![Linearity plot for the organic acids](image)

**Figure 2:** Linearity plot for the organic acids. MMA ($r^2=0.9943$), GA ($r^2=0.9974$), and AA ($r^2=0.9966$).

**Table-1:** Retention time, calibration range and the m/z of ions identified in the mass spectra of derivatized acids.

<table>
<thead>
<tr>
<th>Derivatized organic acid</th>
<th>Ions identified in mass spectra</th>
<th>Retention Time (min)</th>
<th>Calibration range</th>
<th>$r^2$</th>
<th>Calibration curve equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl malonic acid-diTMS</td>
<td>247 218</td>
<td>9.42</td>
<td>19.26-342.42</td>
<td>0.9943</td>
<td>$y=0.0177x + 0.0494$</td>
</tr>
<tr>
<td>Glutaric acid-diTMS</td>
<td>276 261</td>
<td>11.25</td>
<td>16.72-383.05</td>
<td>0.9974</td>
<td>$y=0.0137x + 0.0428$</td>
</tr>
<tr>
<td>Adipic acid-diTMS</td>
<td>275 271</td>
<td>12.07</td>
<td>15.56-309.58</td>
<td>0.9966</td>
<td>$y=0.0136x - 0.0028$</td>
</tr>
<tr>
<td>Tropic acid-diTMS</td>
<td>280 218</td>
<td>12.76</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

n= Calibration range expressed on milli moles, seven concentration levels were analysed, NA= Not Applicable.

3.3.3. Specificity and Selectivity: The peaks were of good shape, completely resolved from urine components and from each other under the optimized chromatographic condition at retention times of 9.45, 11.27, 12.08, and 12.75 min respectively for Methyl malonic acid, Glutaric acid, Adipic acid and Tropic acid (ISTD). At the same time, the
presence of organic acids already present in the urine, which gave a response >20% of the mean extracted LOQ QC sample is a deviation from the acceptance criteria for selectivity.

3.3.4. Sensitivity: The % CV determined at LOQ QC level for methyl malonic acid, glutaric acid and adipic acid 10.55, 3.77 and 3.63 respectively were within the acceptance criteria of not more than 20%. The calculated mean % nominal concentrations 101, 85.16 and 97% respectively were also within the acceptance limits of 80-120%.

3.3.5. Carry over check: There was no carryover after injecting the aqueous ULOQ followed by blank reconstitution solution. The same result was observed when extracted ULOQ was injected followed by blank urine.

3.3.6. Accuracy and Precision: The % nominal concentrations for quality control samples were within acceptance criteria of 85-115% for LQC, MQC and HQC samples, and 80-120% for LOQ QC. The %CV for quality control samples were within acceptance criteria of ≤ 15% for LQC, MQC and HQC samples and ≤ 20% for LOQ QC. The intra-day precision and accuracy of the method is given in Table 2.

Table 2: Intra-day precision and accuracy of the method for the estimation of MMA, GA and AA.

<table>
<thead>
<tr>
<th>Nominal concentrations (µg)</th>
<th>Methyl malonic acid (MMA)</th>
<th>Glutaric acid (GA)</th>
<th>Adipic acid (AA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration found (µg)</td>
<td>Precision (%CV)</td>
<td>Accuracy (%)</td>
</tr>
<tr>
<td></td>
<td>(mean±SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 (LLOQC)</td>
<td>5.09±0.54</td>
<td>10.50</td>
<td>101.56</td>
</tr>
<tr>
<td>15 (LQC)</td>
<td>14.17±1.01</td>
<td>7.09</td>
<td>94.24</td>
</tr>
<tr>
<td>50 (MQC)</td>
<td>47.83±1.11</td>
<td>2.31</td>
<td>87.21</td>
</tr>
<tr>
<td>90 (HQC)</td>
<td>87.21±5.59</td>
<td>6.41</td>
<td>96.81</td>
</tr>
</tbody>
</table>

LLOQC= Lower limit of quality check point, LQC= Lowest quality check point, MQC= Median quality check point, HQC= Highest quality check point, n= 6, for each concentration.

3.3.7. Extraction efficiency or Recovery: Recovery was performed by analysing six replicates of extracted quality control samples along with post extracted quality control samples (prepared by spiking aqueous solutions into extracted blank urine samples) at LQC, MQC and HQC standards. The percentage recovery was determined by comparing the area of the extracted QC samples against equivalent post-extracted QC samples. Mean extraction recovery of Methyl malonic acid, Glutaric acid, Adipic acids and Tropic acid was found to be 92.00, 72.73, 90.26 and 100.69 respectively and this is within the acceptance limits of ≤ 20. Results of recovery study are provided in Table 3.
Table-3: Results of recovery study.

<table>
<thead>
<tr>
<th>Organic acid</th>
<th>Stock solution stability</th>
<th>Bench top stability at 30 days at 2-8°C</th>
<th>Freez- thaw stability (Three cycles)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean % Recovery (% CV)</td>
<td>Mean % Recovery (% CV)</td>
<td>Mean % Recovery (% CV)</td>
</tr>
<tr>
<td></td>
<td>LQC (15µg)</td>
<td>MQC (50 µg)</td>
<td>HQC (90 µg)</td>
</tr>
<tr>
<td>Methyl malonic acid n</td>
<td>87.43 (11.51)</td>
<td>96.89 (0.91)</td>
<td>94 (7.38)</td>
</tr>
<tr>
<td>Glutaric acid n</td>
<td>69.15 (6.50)</td>
<td>76.31 (3.15)</td>
<td>80.38 (1.68)</td>
</tr>
<tr>
<td>Adipic acid n</td>
<td>80.06 (8.36)</td>
<td>97.52 (3.75)</td>
<td>93.21 (3.12)</td>
</tr>
<tr>
<td>Tropic acid n</td>
<td>92.82 (3.99)</td>
<td>102.76 (2.91)</td>
<td>106.50 (4.13)</td>
</tr>
</tbody>
</table>

Mean Recovery ± SD     | 92 (4.26) ± 3.95         | 72.73 (4.08) ± 3.58                    | 90.26 (8.22) ± 7.42                 |
|                       |                         | 100.69 (5.73) ± 5.77                   |                                     |

LQC= Lowest quality check point, MQC= Median quality check point, HQC= Highest quality check point

N= Average of six determinations

3.4. Stability Study results

3.4.1. Stock solution stability: % change of methyl malonic acid, glutaric acid, adipic acid and tropic acid after 8 hours at room temperature was found to be in the range of 3.6 to 9.52. The % change after 30 days storage at 2 to 8°C was found to be in the range of 5.20 to 9.98. Both these results were well within the acceptance criteria of ± 10%.

3.4.2. Bench top stability: There were no significant changes in concentration and it can be concluded that MMA, GA, and AA is stable when stored on bench for at least 7.0 hours at room temperature. The % changes when compared with comparison QCs were in the range of 1.41 to 7.40. This was within the acceptance range of ±15%.

3.4.3. Freeze and thaw stability: It was found that MMA, GA, and AA are stable when frozen and thawed for three cycles. % changes when compared with comparison QCs were in the range of 1.44 to 7.40. This result were within the acceptance range of ±15%. Results of stability studies are provided in the Table 4.

Table-4: Results of stability study.

<table>
<thead>
<tr>
<th>Organic acid</th>
<th>Stock solution stability</th>
<th>Bench top stability at 30 days at 2-8°C</th>
<th>Freez- thaw stability (Three cycles)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8 hours at room temp</td>
<td>30 days at 2-8°C</td>
<td></td>
</tr>
<tr>
<td>Methyl Malonic Acid n</td>
<td>3.6 (8.84)</td>
<td>7.4 (8.84)</td>
<td>3.33 (5.23)</td>
</tr>
<tr>
<td>Glutaric acid n</td>
<td>3.33 (5.23)</td>
<td>1.41 (3.32)</td>
<td>3.22 (5.23)</td>
</tr>
<tr>
<td>Adipic acid n</td>
<td>1.44 (3.32)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Glutaric Acid

<table>
<thead>
<tr>
<th>Concentration (millimol/mol of creatinine)</th>
<th>Normal range*</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMA</td>
<td>10.50-36.98</td>
<td>1.85</td>
<td>17.85</td>
<td>9.44</td>
<td>2.36</td>
<td>24.71</td>
<td>22.62</td>
<td>21.56</td>
</tr>
<tr>
<td>GA</td>
<td>12.66-29.87</td>
<td>3.33</td>
<td>23.00</td>
<td>10.35</td>
<td>6.59</td>
<td>10.98</td>
<td>22.24</td>
<td>11.56</td>
</tr>
<tr>
<td>AA</td>
<td>0.00-49.82</td>
<td>10.84</td>
<td>12.36</td>
<td>6.50</td>
<td>1.22</td>
<td>7.59</td>
<td>6.45</td>
<td>6.78</td>
</tr>
</tbody>
</table>

*Normal range vary with ethnicity and food habits.

NA= Not Applicable, ISTD= Internal standard, n= 6 for each concentration.

### 3.5. Application of the method for quantification of organic acids in hospitalised children

The results of analysis of organic acid levels in urine samples obtained from seven hospitalised children are provided in the Table 5. None of the sample showed elevated levels of organic acids compared to the normal range. The results were suggestive of the absence of any such metabolic disorders. Figure 3 shows a typical chromatogram obtained from one of the urine sample collected for analysis.

![Typical chromatogram obtained from the patient urine sample.](image)

**Figure 3:** Typical chromatogram obtained from the patient urine sample.

**Table-5:** Creatinine corrected urine excretion levels of organic acids measured in the samples collected from children in the age group of 0-2 years.

<table>
<thead>
<tr>
<th>Concentration of organic acids (millimol/mol of creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal range*</td>
</tr>
<tr>
<td>MMA</td>
</tr>
<tr>
<td>GA</td>
</tr>
<tr>
<td>AA</td>
</tr>
</tbody>
</table>

MMA: Methyl Malonic Acid, GA: Glutaric Acid, AA: Adipic Acid
4. Conclusions

The analytical conditions for the estimation of organic acids by GC-MS were studied with an aim to establish a method for organic acid profiling in neonatal urine to be used as a tool for the detection of metabolic disorders. A simple, specific and faster gas chromatography-mass spectrometry (GC-MS) method was developed for the quantitative determination of methyl malonic acid, glutaric acid and adipic acid in neonatal and paediatric urine sample. Linearity demonstrated was over a concentration rage of 5 to 100 µg with a correlation coefficient ($r^2$) 0.994, 0.997, and 0.996 respectively for these acids. Derivatization was achieved by employing a simple reaction with BSTFA+1%TMCS. Several parameters, which affected the yield of the derivatization reaction, such as reaction temperature and time were optimized. Organic acid extraction from urine was optimized for maximum recovery by varying the conditions. The developed method was successfully applied to the quantitative analysis of free organic acids in urine samples obtained from hospitalized children. Creatinine-corrected excretion rates of all analysed organic acids were within reference intervals.

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References


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