

# A Description of Reference Ranges for Organic Acids in Urine Samples from A Pediatric Population in Iran

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## Abstract

**Background:** Organic acids refer to a family of compounds that are intermediates in a variety of metabolic pathways. Many organic acids are present in urine from clinically normal individuals. Elevated levels of urine organic acids cause to the organic acidurias, disorders in which some metabolic pathways in organic acid metabolism are blocked. The present work identified major and minor urinary acidic metabolites in normal subjects, and their quantitative ranges in a pediatric population of Iran.

**Methods:** Two hundred and fifty-one healthy subjects, including 132 males and 119 females, from 2 days to 15 years of age were enrolled. Urinary organic acids were extracted from urine with organic solvents and identified and quantified by gas chromatography-mass spectrometry.

**Results:** The results provide a foundation on which to check results for patients with potentially abnormal organic acidurias. By this method 98 organic acids were identified in a pediatric population of Iran.

**Conclusions:** The present work identifies and quantifies major and minor urinary metabolites excreted by normal subjects. We also analyzed urine from 30 patients with organic acid metabolism abnormalities and compared the concentrations of specific organic acids with those from urines of normal individuals.

**Keywords:** Gas chromatography/mass spectrometry, Iran population, Normal individuals, Urine organic acid analysis, Urine organic acids range

## Introduction

Approximately 50 diseases have been described in which an inherited single enzyme defect causes a high concentration of acidic metabolites in the blood or urine. Dysfunction in any protein complex that involves the absorption, transportation, activation, and/or application of a vitamin can result in an elevated urinary organic acid (1). With this in mind, organic acid concentrations in urine can serve as markers to diagnose the numerous genetic metabolic disorders known as organic acidurias (2). Organic acids refer to a family of compounds that are intermediates in a variety of metabolic pathways

including glycolysis and citric acid cycle metabolites, fatty acid oxidation, ketone metabolites and cofactors, and markers of detoxification (3, 4). Many organic acids are present in urine from clinically normal individuals (5). Elevated levels of urine organic acids cause to the organic acidurias, disorders in which some metabolic pathways in organic acid metabolism are blocked. An analysis of urinary organic acids is usually a key test in the assessment of patients with doubtful genetic disorders of organic acid metabolism and is frequently used in the diagnosis of persons with possible genetic disorders of

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mitochondrial fatty acid metabolism, amino acid disorders, and disorders of mitochondrial oxidative phosphorylation. Disorders of organic acid metabolism have various clinical presentations and ages of onset. Some of the common presentations include lethargy, fasting intolerance, myalgia, myopathy, exercise intolerance, cardiomyopathy, and various types of developmental or neurological disabilities such as seizures or vision deficits (6, 7).

Gas Chromatography/Mass Spectrometry (GC/MS) is the most reliable method for urine organic acid analysis (8). Its primary asset is that it allows precise and simultaneous quantification of different compounds in samples. It has contributed greatly to the understanding of many disease states. GC/MS was initially developed for qualitative purposes, and quantitative analytical criteria have seldom been extensively studied, if at all (9-11). Clearly, an encyclopedic analysis such as this compounds the potential information on the physiological and pathophysiological situation of different metabolic pathways and their interrelationships in the body, and may provide clinical relevant information for patient care (12, 13).

Knowledge of biological variation allows evaluation of population-based reference ranges. For example, it would be useful to know that a result found in a patient would likely differ from that of a healthy individual. The present work identified major and minor urinary acidic metabolites in normal subjects, and their quantitative ranges in a pediatric population of Iran. This could provide age-dependent reference intervals for laboratory staff and physicians using current methods and equipment, as currently available data is rather dated.

## Materials and Methods

### Subjects

Two hundred and fifty-one healthy subjects, including 132 males and 119 females, from 2 days to 15 years of age were enrolled. All of them were referred to our laboratory for organic acid analysis, and their results were normal. All procedures followed were in accordance with the ethical standards of the committee on human experimentation MUMS (Mashhad University of Medical Science). Informed consent was obtained from all patients for being included in the study.

### Chemical, Reagents and instrument

In this study chemicals and reagents with highest purity available were purchased. Pentadecanoic acid (PDA) and 0- (2,3,4,5,6-Pentafluorobenzyl) hydroxylamine hydrochloride (PFBH) were obtained from Sigma-Aldrich. Tetramethylsilane (TMS) Tri-Sil (BSA: pyridine) from Thermo Scientific was used as the derivatizing reagent. Ethyl acetate and n-Hexane was from Merck Millipore. We use a Clarus 500 GC-MS from PerkinElmer with an Agilent fused silica column 30 m x 0.25 mm x 0.25  $\mu$ m.

### Extraction, Derivatization, and Gas chromatography

The organic acids were extracted from urine by organic solvent extraction and then identified and quantified by GC/MS. Initially, the urinary pH was adjusted to 2 to 5, 50  $\mu$ l of 50 mg/ml pentafluoro benzyl hydroxylamine-hydrochloride solution were added, and the sample was incubated at room temperature for one hour. Subsequently, the pH was adjusted to 1 by dropwise addition of HCl. The acidic sample was extracted two times with 4 ml of ethyl acetate by vortexing the sample and centrifuging at 700 x g for 5 min. The organic supernatant layers from each extraction were transferred to a second vial, 25  $\mu$ l of pentadecanoic acid (PDA) used as an internal standard were added, and the solution was evaporated to dryness under nitrogen at 40 °C. Finally, the sample was silylated by adding 100  $\mu$ l of tetramethylsilane (TMS), vortexed, and incubated at 60 °C for 30 min. After the sample cooled to room temperature, 500  $\mu$ l of hexane were added and the sample was analyzed by GC/MS. The temperatures for the injectors and detectors were 250 and 300 °C, respectively. The GC temperature program was as follows: initial temperature was 70 °C, held for 4 min, increased to 180 °C at a rate of 20 °C/min, then to 200 °C at a rate of 4 °C/min, held for one min and finally to 275 °C at a rate of 3 °C/min and held for 10 min (8, 14).

### Statistical Analysis

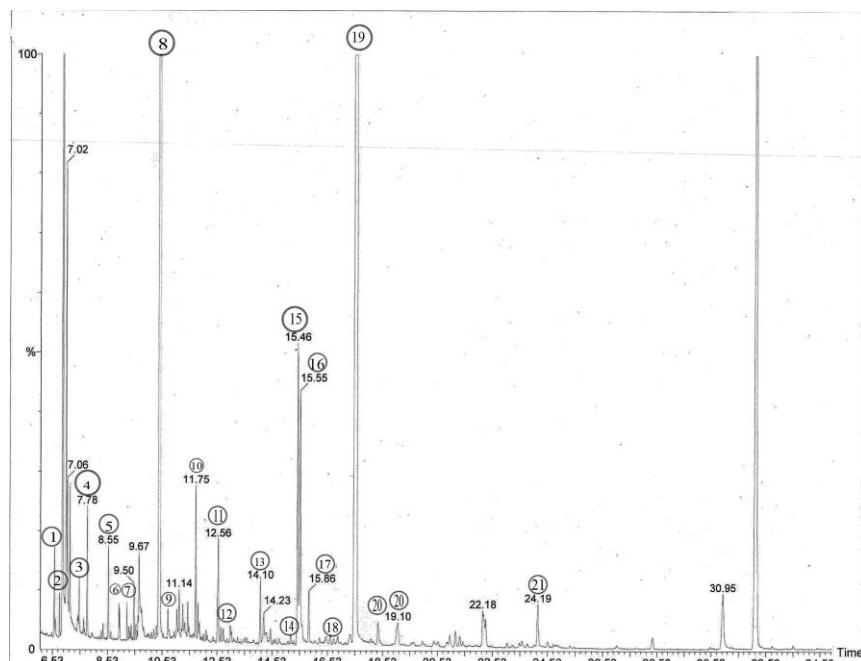
Statistical analysis was performed using SPSS version 16. The compounds were identified and their concentration ranges were determined for all individuals.

## Results

### Analysis of normal urines

We identified 98 compounds in urine samples from 251 healthy subjects. Compounds detected in 60-80% of the samples were Aconitic Acid, Adipic Acid, Succinic Acid, Citric Acid, 2,3- Dihydroxy Butyric Acid, 3,4-Dihydroxy Butyric Acid, Ethylmalonic Acid, Glycolic Acid, Hippuric Acid, Homovanillic Acid, 4-Hydroxy Benzoic Acid, 3-Hydroxy Isobutyric Acid, 2-Hydroxy Glutaric Acid, 4-Hydroxy Phenyl Acetic Acid, 3-Methyl Adipinic Acid, Methyl Succinic Acid, Methylmalonic Acid, 2-Methyl-3-Hydroxy Butyric Acid, Octendicarboxylic Acid, Oxalic Acid, 2-Oxoglutaric Acid, 5-Oxoproline, Pimelic Acid, Suberic Acid, VanillylMandelic Acid, Lactic Acid and Pyruvate. Compounds detected in 20-60% of the samples included Malic Acid, Benzoic Acid, Decadienic Dicarboxylic Acid, Azelaic Acid, 2,4-Dihydroxy Butyric Acid, 2-Ethyl-3-hydroxy Propionic Acid, Fumaric Acid, Glutaric Acid, Hydroxy Malic Acid, 3-Hydroxy Adipic Acid, 4-Hydroxy Hippuric Acid, HydroxyDecadicarboxylic Acid, 5-Hydroxy Hexanoic Acid, 3-Hydroxy-3-methyl Glutaric Acid, 3-Hydroxy Phenyl Acetic Acid, 4-Hydroxy Phenyl Lactic Acid, 3-Hydroxy Propionic Acid, (3-hydroxy phenyl)-3-Hydroxy Propionic Acid, 3-Hydroxy Isovaleric Acid, 2-Hydroxy Isovaleric Acid, Furan-2,5-Dicarboxylic Acid, 5-Hydroxymethyl-2-Furanoic Acid, 3-Methyl-4-Hydroxy Benzoic Acid, Phenyl Acetic Acid, PhenylLactic Acid and Phosphoric Acid.

Compounds detected in 0.5-20% of the samples included Carbamazepin, Cyclohexandiol, Decendicarboxylic Acid, Chloralhydrate Glucuronide, 4- Cresol, Erythronic Acid, 2-Furoylglycin, Hexenoic Dicarboxylic Acid, Homogentisic Acid, 2-Hydroxyadipinic Acid, 3-Hydroxy Benzoic Acid, 3-Hydroxy Butyric Acid, 4-Hydroxy Butyric Acid, 2-Hydroxy Butyric Acid, 3-Hydroxy Hippuric Acid, 5-Hydroxy Indole Acetic Acid, 3-Hydroxy Adipic Acid Lacton, 2-Hydroxy-3-methyl-Valeric Acid, 4-Hydroxy Phenyl Pyruvate, 4-Hydroxy Phenyl Propionic Acid, 2-Hydroxy Valeric Acid, Levulinic Acid, Mevalonic, N-acetylaspartate, 3-Methyl Glutaric Acid, 3-(3-methyl-4-hydroxyphenyl) 3-Hydroxy Propionic Acid, 3-Methyl-4-Hydroxy-Phenyl Lactic Acid, 2-Oxoadipinic Acid, 2-Oxo-3-Methylvaleric Acid, 2-Oxoisocapronic Acid, Palmitic Acid, Stearic Acid, Uracil, 4-Hydroxy Cyclohexyl Carbonic Acid, 3-Methyl Glutaconic Acid, Urate, 2-HydroxyIsocapronic Acid, HydroxyDecadicarboxylic Acid, Tricarballylic Acid, Tartaric Acid, Malonic Acid, Phenobarbital, Lauric Acid, 4-Hydroxy Cyclo Acetic, Mandelic Acid, and 2-Hydroxy Isobutyric Acid. Frequency, percentage (number of urines in which the compounds were detected), range of concentration, and pathogenetical area of these compounds are listed in Table 1. A typical chromatogram of urinary organic acids from a normal individual is shown in Figure 1(Fig.1)..



**Fig 1A** A typical GC-MS chromatogram of urinary organic acids from a normal individual. The compounds noted in the figure are TMS-derivatives of the following compounds: 1: Lactic acid, 2: Glycolic acid, 3: Oxalic acid, 4: 3-hydroxy butyric acid, 5: Urea, 6: Phosphoric acid, 7: Succinic acid, 8: Pentafluorobenzyl hydroxyl amine, 9: Pyruvic acid, 10: 2-hydroxy glutaric acid, 11: 4-hydroxy phenyl acetic acid, 12: Tartaric acid, 13: Aconitic acid, 14: Homovanillic acid, 15: Citric acid, 16: Hippuric acid, 17: 3-(3-hydroxyphenyl)-3-hydroxyl propionic acid, 18: 3-methoxy-4-hydroxyl mandelic acid, 19: Internal standard, 20: 2-oxoglutaric acid, 21: 4-hydroxyl hippuric acid.

**Table 1.** Frequency, percentage and range of concentration of compounds in normal urine

|    |  | OA Name                          | A R | F               | %         | RR<br>( $\mu\text{mol}/\text{mmol Creat}$ ) | PA<br>( $\mu\text{mol}/\text{mmol Creat}$ ) |      |
|----|--|----------------------------------|-----|-----------------|-----------|---|---|------|
| 1  |  | Aconitic Acid                    | Yes | 2 D-1 Y<br>>1 Y | 110<br>86 | 44<br>34                                    | <25<br><17                                  | >60  |
|    |  |                                  | No  |                 | 55        | 22  |   |      |
| 2  |  | Adipic Acid                      | Yes | 2 D-1 Y<br>>1 Y | 120<br>63 | 48<br>25                                    | <19<br><9                                   | >60  |
|    |  |                                  | No  | -               | 68        | 27  |   |      |
| 3  |  | Malic Acid                       | Yes | 2 D-1 Y<br>>1 Y | 34<br>11  | 13.5<br>4.5                                 | <12<br><9                                   | >30  |
|    |  |                                  | No  |                 | 206       | 82  |   |      |
| 4  |  | Benzoic Acid                     | Yes | 2 D-1 Y<br>>1 Y | 31<br>15  | 12.35<br>5.98                               | <34<br><90                                  | >250 |
|    |  |                                  | No  |                 | 205       | 81.67                                       |   |      |
| 5  |  | Succinic Acid                    | Yes | 2 D-1 Y<br>>1 Y | 120<br>86 | 47.8<br>34.26                               | <200<br><83                                 | >500 |
|    |  |                                  | No  |                 | 45        | 17.93                                       |   |      |
| 6  |  | Carbamazepin                     | Yes | 2 D-1 Y<br>>1 Y | -<br>4    | -<br>1.59                                   | -<br><24                                    | >72  |
|    |  |                                  | No  |                 | 247       | 98.4  |   |      |
| 7  |  | Cyclohexandiol                   | Yes | 2 D-1 Y<br>>1 Y | 17<br>2   | 6.77<br>0.8                                 | <13<br><10                                  | >30  |
|    |  |                                  | No  |                 | 232       | 92.4  |   |      |
| 8  |  | Decadienic Dicarboxylac Acid     | Yes | 2 D-1 Y<br>>1 Y | 55<br>20  | 21.9<br>7.97                                | <10<br><8                                   | >25  |
|    |  |                                  | No  |                 | 176       | 70.12                                       |   |      |
| 9  |  | Decendicarboxylic Acid           | Yes | 2 D-1 Y<br>>1 Y | 8<br>2    | 3.19<br>0.8                                 | <12<br><8                                   | >25  |
|    |  |                                  | No  |                 | 241       | 96.01                                       |   |      |
| 10 |  | Chloralhydrate Glucuronide       | Yes | 2 D-1 Y<br>>1 Y | 2<br>7    | 0.8<br>2.79                                 | <80<br><236                                 | >400 |
|    |  |                                  | No  |                 | 242       | 96.41                                       |   |      |
| 11 |  | Citric Acid                      | Yes | 2 D-1 Y<br>>1 Y | 115<br>91 | 45.82<br>36.25                              | <87<br><72                                  | >250 |
|    |  |                                  | No  |                 | 45        | 17.93                                       |   |      |
| 12 |  | 4-Cresol                         | Yes | 2 D-1 Y<br>>1 Y | 5<br>31   | 1.99<br>12.35                               | <4<br><10                                   | >15  |
|    |  |                                  | No  |                 | 215       | 85.66                                       |   |      |
| 13 |  | Azelaic Acid                     | Yes | 2 D-1 Y<br>>1 Y | 41<br>38  | 16.33<br>15.14                              | <11<br><9                                   | >25  |
|    |  |                                  | No  |                 | 172       | 68.52                                       |   |      |
| 14 |  | 2,3-Dihydroxy Butyric Acid       | Yes | 2 D-1 Y<br>>1 Y | 92<br>76  | 36.65<br>30.28                              | <10<br><8                                   | >25  |
|    |  |                                  | No  |                 | 83        | 33.07                                       |   |      |
| 15 |  | 2,4-Dihydroxy Butyric Acid       | Yes | 2 D-1 Y<br>>1 Y | 58<br>36  | 23.11<br>14.34                              | <5<br><4                                    | >15  |
|    |  |                                  | No  |                 | 157       | 62.55                                       |   |      |
| 16 |  | 3,4-Dihydroxy Butyric Acid       | Yes | 2 D-1 Y<br>>1 Y | 104<br>52 | 41.43<br>20.72                              | <7<br><4                                    | >15  |
|    |  |                                  | No  |                 | 95        | 37.85                                       |   |      |
| 17 |  | Ethylmalonic Acid                | Yes | 2 D-1 Y<br>>1 Y | 111<br>81 | 44.22<br>32.27                              | <17<br><13                                  | >45  |
|    |  |                                  | No  |                 | 59        | 23.5  |   |      |
| 18 |  | Erythronic Acid                  | Yes | 2 D-1 Y<br>>1 Y | 26<br>4   | 10.36<br>1.59                               | <9<br><5                                    | >15  |
|    |  |                                  | No  |                 | 221       | 88.05                                       |   |      |
| 19 |  | 2-Ethyl-3-hydroxy Propionic Acid | Yes | 2 D-1 Y<br>>1 Y | 35<br>16  | 13.94<br>6.37                               | <12<br><10                                  | >30  |
|    |  |                                  | No  |                 | 200       | 79.68                                       |   |      |

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|    |                              |     |         |     |       |       |       |
|----|------------------------------|-----|---------|-----|-------|-------|-------|
| 20 | Fumaric Acid                 | Yes | 2 D-1 Y | 56  | 22.31 | < 14  |       |
|    |                              |     | >1 Y    | 30  | 11.95 | < 7   | > 30  |
|    |                              | No  |         | 165 | 65.74 |       |       |
| 21 | 2-Furoylglycin               | Yes | 2 D-1 Y | 2   | 0.8   | < 4   |       |
|    |                              |     | >1 Y    | 3   | 1.19  | < 4   | > 12  |
|    |                              | No  |         | 246 | 98    |       |       |
| 22 | Glutaric Acid                | Yes | 2 D-1 Y | 76  | 30.28 | < 8   |       |
|    |                              |     | >1 Y    | 39  | 15.54 | < 5   | > 20  |
|    |                              | No  |         | 136 | 54.18 |       |       |
| 23 | Glycolic Acid                | Yes | 2 D-1 Y | 116 | 46.25 | < 14  |       |
|    |                              |     | >1 Y    | 92  | 36.65 | < 16  | > 45  |
|    |                              | No  |         | 43  | 17.13 |       |       |
| 24 | Hippuric Acid                | Yes | 2 D-1 Y | 53  | 21.11 | < 158 |       |
|    |                              |     | >1 Y    | 65  | 25.9  | < 253 | > 500 |
|    |                              | No  |         | 133 | 52.99 |       |       |
| 25 | Hexenoic Dicarboxylic Acid   | Yes | 2 D-1 Y | 47  | 18.72 | < 5   |       |
|    |                              |     | >1 Y    | 1   | 0.399 | -     | > 15  |
|    |                              | No  |         | 203 | 80.88 |       |       |
| 26 | Homogentisic Acid            | Yes | 2 D-1 Y | 1   | 0.399 | -     |       |
|    |                              |     | >1 Y    | -   | -     |       |       |
|    |                              | No  |         | 250 | 99.6  | -     |       |
| 27 | Homovanillic Acid            | Yes | 2 D-1 Y | 124 | 49.4  | < 14  |       |
|    |                              |     | >1 Y    | 91  | 36.25 | < 8   | > 25  |
|    |                              | No  |         | 34  | 13.55 |       |       |
| 28 | 2-Hydroxyadipinic Acid       | Yes | 2 D-1 Y | 6   | 2.39  | < 5   |       |
|    |                              |     | >1 Y    | -   | -     |       | > 5   |
|    |                              | No  |         | 245 | 97.6  |       |       |
| 29 | Hydroxy Malic Acid           | Yes | 2 D-1 Y | 34  | 13.55 | < 10  |       |
|    |                              |     | >1 Y    | 30  | 11.95 | < 7   | > 20  |
|    |                              | No  |         | 187 | 74.5  |       |       |
| 30 | 3-Hydroxy Adipic Acid        | Yes | 2 D-1 Y | 91  | 36.25 | < 10  |       |
|    |                              |     | >1 Y    | 14  | 5.58  | < 5   | > 15  |
|    |                              | No  |         | 146 | 58.17 |       |       |
| 31 | 3-Hydroxy Benzoic Acid       | Yes | 2 D-1 Y | 7   | 2.79  | < 6   |       |
|    |                              |     | >1 Y    | 21  | 8.37  | < 8   | > 30  |
|    |                              | No  |         | 223 | 88.84 |       |       |
| 32 | 4-Hydroxy Benzoic Acid       | Yes | 2 D-1 Y | 76  | 30.28 | < 12  |       |
|    |                              |     | >1 Y    | 58  | 23.11 | < 16  | > 50  |
|    |                              | No  |         | 117 | 46.61 |       |       |
| 33 | 3-Hydroxy Butyric Acid       | Yes | 2 D-1 Y | 28  | 11.15 | < 63  |       |
|    |                              |     | >1 Y    | 8   | 3.19  | < 55  | > 150 |
|    |                              | No  |         | 215 | 85.66 |       |       |
| 34 | 4-Hydroxy Butyric Acid       | Yes | 2 D-1 Y | 1   | 0.399 | -     |       |
|    |                              |     | >1 Y    | -   | -     |       |       |
|    |                              | No  |         | 250 | 99.6  |       |       |
| 35 | 2-Hydroxy Butyric Acid       | Yes | 2 D-1 Y | 14  | 5.58  | < 8   |       |
|    |                              |     | >1 Y    | 3   | 1.19  | < 8   | > 25  |
|    |                              | No  |         | 234 | 93.23 |       |       |
| 36 | 3-Hydroxy Isobutyric Acid    | Yes | 2 D-1 Y | 89  | 35.46 | < 28  |       |
|    |                              |     | >1 Y    | 72  | 28.68 | < 15  | > 60  |
|    |                              | No  |         | 90  | 35.86 |       |       |
| 37 | 3-Hydroxy Hippuric Acid      | Yes | 2 D-1 Y | 10  | 3.98  | < 11  |       |
|    |                              |     | >1 Y    | 31  | 12.35 | < 18  | > 45  |
|    |                              | No  |         | 210 | 83.66 |       |       |
| 38 | 4-Hydroxy Hippuric Acid      | Yes | 2 D-1 Y | 50  | 19.92 | < 15  |       |
|    |                              |     | >1 Y    | 45  | 17.93 | < 20  | > 50  |
|    |                              | No  |         | 156 | 62.15 |       |       |
| 39 | HydroxyDecadicarboxylic Acid | Yes | 2 D-1 Y | 63  | 25.1  | < 25  |       |
|    |                              |     | >1 Y    | 10  | 3.98  | < 10  | > 50  |
|    |                              | No  |         | 178 | 70.92 |       |       |

|    |   |     |                 |            |                |               |       |
|----|---|-----|-----------------|------------|----------------|---------------|-------|
| 40 | 2-Hydroxy Glutaric Acid                       | Yes | 2 D-1 Y<br>>1 Y | 114<br>76  | 45.42<br>30.28 | < 19<br>< 8   | > 50  |
|    |   | No  |                 | 61         | 24.3           |               |       |
| 41 | 5-Hydroxy Hexanoic Acid                       | Yes | 2 D-1 Y<br>>1 Y | 44<br>16   | 17.53<br>6.37  | < 6<br>< 3    | > 15  |
|    |   | No  |                 | 191        | 76.09          |               |       |
| 42 | 5-Hydroxy Indole Acetic Acid                  | Yes | 2 D-1 Y<br>>1 Y | 11<br>4    | 4.38<br>1.59   | < 8<br>< 6    | > 8   |
|    |   | No  |                 | 236        | 94.02          |               |       |
| 43 | 3-Hydroxy Adipic Acid Lacton                  | Yes | 2 D-1 Y<br>>1 Y | 39<br>3    | 15.54<br>1.19  | < 15<br>< 7   | > 40  |
|    |   | No  |                 | 209        | 83.27          |               |       |
| 44 | 3-Hydroxy-3-methyl Glutaric Acid              | Yes | 2 D-1 Y<br>>1 Y | 91<br>33   | 36.25<br>13.15 | < 16<br>< 6   | > 40  |
|    |   | No  |                 | 127        | 50.6           |               |       |
| 45 | 3-Hydroxy Phenyl Acetic Acid                  | Yes | 2 D-1 Y<br>>1 Y | 6<br>58    | 2.39<br>23.11  | < 5<br>< 11   | > 30  |
|    |   | No  |                 | 187        | 74.5           |               |       |
| 46 | 4-Hydroxy Phenyl Acetic Acid                  | Yes | 2 D-1 Y<br>>1 Y | 133<br>100 | 52.99<br>39.84 | < 104<br>< 87 | > 300 |
|    |   | No  |                 | 18         | 7.17           |               |       |
| 47 | 4-Hydroxy Phenyl Lactic Acid                  | Yes | 2 D-1 Y<br>>1 Y | 68<br>21   | 27.09<br>8.37  | < 28<br>< 8   | > 100 |
|    |   | No  |                 | 162        | 64.54          |               |       |
| 48 | 4-Hydroxy Phenyl Pyruvate                     | Yes | 2 D-1 Y<br>>1 Y | 5<br>-     | 1.99           | < 15          | > 60  |
|    |   | No  |                 | 246        | 98.01          |               |       |
| 49 | 3-Hydroxy Propionic Acid                      | Yes | 2 D-1 Y<br>>1 Y | 27<br>21   | 10.76<br>8.37  | < 5<br>< 3    | > 15  |
|    |   | No  |                 | 203        | 80.88          |               |       |
| 50 | 3-(3-hydroxy phenyl)-3-Hydroxy Propionic Acid | Yes | 2 D-1 Y<br>>1 Y | 27<br>70   | 10.76<br>27.89 | < 16<br>< 31  | > 100 |
|    |   | No  |                 | 154        | 61.35          |               |       |
| 51 | 4-Hydroxy Phenyl Propionic Acid               | Yes | 2 D-1 Y<br>>1 Y | 1<br>-     | 0.399          | -             |       |
|    |   | No  |                 | 250        | 99.6           | -             |       |
| 52 | 3-Hydroxy Isovaleric Acid                     | Yes | 2 D-1 Y<br>>1 Y | 44<br>28   | 17.53<br>11.15 | < 24<br>< 12  | > 80  |
|    |   | No  |                 | 179        | 71.31          |               |       |
| 53 | 2-Hydroxy Isovaleric Acid                     | Yes | 2 D-1 Y<br>>1 Y | 44<br>18   | 17.53<br>7.17  | < 6<br>< 5    | > 20  |
|    |   | No  |                 | 189        | 75.3           |               |       |
| 54 | 2-Hydroxy Valeric Acid                        | Yes | 2 D-1 Y<br>>1 Y | 1<br>-     | 0.399          | -             |       |
|    |   | No  |                 | 250        | 99.6           | -             |       |
| 55 | Furan-2,5-Dicarboxylic Acid                   | Yes | 2 D-1 Y<br>>1 Y | 31<br>46   | 12.35<br>18.33 | < 11<br>< 11  | > 50  |
|    |   | No  |                 | 174        | 69.32          |               |       |
| 56 | 5-Hydroxymethyl-2-Furanoic Acid               | Yes | 2 D-1 Y<br>>1 Y | 40<br>56   | 15.94<br>22.31 | < 24<br>< 21  | > 80  |
|    |   | No  |                 | 155        | 61.75          |               |       |
| 57 | Levulinic Acid                                | Yes | 2 D-1 Y<br>>1 Y | 2<br>-     | 0.8            | < 7           | > 7   |
|    |   | No  |                 | 249        | 99.2           | -             |       |
| 58 | 3-Methyl Adipinic Acid                        | Yes | 2 D-1 Y<br>>1 Y | 110<br>82  | 43.82<br>32.67 | < 8<br>< 5    | > 25  |
|    |   | No  |                 | 59         | 23.5           |               |       |
| 59 | Methyl Succinic Acid                          | Yes | 2 D-1 Y<br>>1 Y | 76<br>69   | 30.28<br>27.49 | < 6<br>< 6    | > 18  |
|    |   | No  |                 | 106        | 42.23          |               |       |

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|    |  |     |                 |           |                |               |       |
|----|--|-----|-----------------|-----------|----------------|---------------|-------|
| 60 | Methylmalonic Acid                                       | Yes | 2 D-1 Y<br>>1 Y | 79<br>50  | 31.47<br>19.92 | < 11<br>< 6   | > 40  |
| 61 | Mevalonic  | No  |                 | 122       | 48.6           | -             |       |
| 62 | N-acetylaspartate  | Yes | 2 D-1 Y<br>>1 Y | 1<br>250  | 0.399<br>99.61 | -<br>-< 5     | > 50  |
| 63 | 3-Methyl Glutaric Acid                                   | Yes | 2 D-1 Y<br>>1 Y | 2<br>11   | 0.797<br>4.38  | < 16<br>< 4   | > 12  |
| 64 | 3-(3-methyl-4-hydroxyphenyl)<br>3-Hydroxy Propionic Acid | Yes | 2 D-1 Y<br>>1 Y | 4<br>24   | 1.59<br>9.56   | < 13<br>< 9   | > 50  |
| 65 | 2-Methyl-3-Hydroxy Butyric<br>Acid                       | No  |                 | 223       | 88.85          |               |       |
| 66 | 3-Methyl-4-Hydroxy Benzoic<br>Acid                       | Yes | 2 D-1 Y<br>>1 Y | 81<br>29  | 32.27<br>11.55 | < 7<br>< 5    | > 20  |
| 67 | 3-Methyl-4-Hydroxy-Phenyl<br>Lactic Acid                 | No  |                 | 111       | 44.23          |               |       |
| 68 | Octendicarboxylic Acid                                   | Yes | 2 D-1 Y<br>>1 Y | 14<br>52  | 5.58<br>20.72  | < 29<br>< 10  | > 100 |
| 69 | Oxalic Acid  | No  |                 | 208       | 82.87          |               |       |
| 70 | 2-Oxoadipinic Acid                                       | Yes | 2 D-1 Y<br>>1 Y | 6<br>-    | 2.39           | < 5           | > 8   |
| 71 | 2-Oxo-3-Methylvaleric Acid                               | No  |                 | 245       | 97.61          |               |       |
| 72 | 2-Oxoglutaric Acid                                       | Yes | 2 D-1 Y<br>>1 Y | 116<br>52 | 46.21<br>20.72 | < 14<br>< 7   | > 40  |
| 73 | 2-Oxoisocapronic Acid                                    | No  |                 | 83        | 33.07          |               |       |
| 74 | 5-Oxoproline   | Yes | 2 D-1 Y<br>>1 Y | 114<br>57 | 45.42<br>22.71 | < 30<br>< 136 | > 80  |
| 75 | Palmitic Acid  | No  |                 | 92        | 36.65          | < 146         | > 500 |
| 76 | Phenyl Acetic Acid                                       | Yes | 2 D-1 Y<br>>1 Y | 2<br>21   | 1.19<br>8.37   | < 5<br>< 9    | > 25  |
| 77 | Phenyl Lactic Acid                                       | No  |                 | 203       | 80.88          |               |       |
| 78 | Pimelic Acid   | Yes | 2 D-1 Y<br>>1 Y | 61<br>17  | 24.31<br>6.77  | < 6<br>< 4    | > 20  |
| 79 | Stearic Acid   | No  |                 | 173       | 68.92          |               |       |
|    |  | Yes | 2 D-1 Y<br>>1 Y | 76<br>49  | 30.28<br>19.52 | < 5<br>< 5    | > 15  |
|    |  | No  |                 | 126       | 50.2           |               |       |
|    |  | Yes | 2 D-1 Y<br>>1 Y | 12<br>12  | 4.78<br>4.78   | < 7<br>< 8    | > 30  |
|    |  | No  |                 | 227       | 90.44          |               |       |

|    |                                    |     |         |     |       |      |       |
|----|------------------------------------|-----|---------|-----|-------|------|-------|
| 80 | Suberic Acid                       | Yes | 2 D-1 Y | 103 | 41.03 | < 12 | > 30  |
|    |                                    | No  | >1 Y    | 30  | 11.95 | < 10 |       |
|    |                                    |     |         | 118 | 47.02 |      |       |
| 81 | VanillylMandelic Acid              | Yes | 2 D-1 Y | 122 | 48.6  | < 12 | > 30  |
|    |                                    | No  | >1 Y    | 69  | 27.49 | < 5  |       |
|    |                                    |     |         | 60  | 23.91 |      |       |
| 82 | Lactic Acid                        | Yes | 2 D-1 Y | 90  | 35.86 | < 60 | > 200 |
|    |                                    | No  | >1 Y    | 82  | 32.67 | < 50 |       |
|    |                                    |     |         | 79  | 31.47 |      |       |
| 83 | Phosphoric Acid                    | Yes | 2 D-1 Y | 38  | 15.14 | < 31 | > 60  |
|    |                                    | No  | >1 Y    | 44  | 17.53 | < 14 |       |
|    |                                    |     |         | 169 | 67.33 |      |       |
| 84 | Pyruvate                           | Yes | 2 D-1 Y | 82  | 32.67 | < 32 | > 100 |
|    |                                    | No  | >1 Y    | 49  | 19.52 | < 15 |       |
|    |                                    |     |         | 120 | 47.81 |      |       |
| 85 | Uracil                             | Yes | 2 D-1 Y | 19  | 7.57  | < 20 | > 50  |
|    |                                    | No  | >1 Y    | 6   | 2.39  | < 10 |       |
|    |                                    |     |         | 226 | 90.04 |      |       |
| 86 | 4-Hydroxy Cyclohexyl Carbonic Acid | Yes | 2 D-1 Y | 6   | 2.39  | < 20 | > 30  |
|    |                                    | No  | >1 Y    | 15  | 5.98  | < 18 |       |
|    |                                    |     |         | 230 | 91.63 |      |       |
| 87 | 3-Methyl Glutaconic Acid           | Yes | 2 D-1 Y | 29  | 11.55 | < 9  | > 30  |
|    |                                    | No  | >1 Y    | 15  | 5.98  | < 14 |       |
|    |                                    |     |         | 207 | 82.47 |      |       |
| 88 | Urate                              | Yes | 2 D-1 Y | 7   | 2.79  | < 4  | > 8   |
|    |                                    | No  | >1 Y    | 1   | 0.4   | -    |       |
|    |                                    |     |         | 243 | 96.81 |      |       |
| 89 | 2- Hydroxy Isocapronic Acid        | Yes | 2 D-1 Y | 2   | 0.8   | < 3  | > 8   |
|    |                                    | No  | >1 Y    | -   |       |      |       |
|    |                                    |     |         | 249 | 99.2  |      |       |
| 90 | HydroxyDecadicarboxylic Acid       | Yes | 2 D-1 Y | 3   | 1.19  | < 10 | > 10  |
|    |                                    | No  | >1 Y    | -   |       |      |       |
|    |                                    |     |         | 248 | 98.81 |      |       |
| 91 | Tricarballylic Acid                | Yes | 2 D-1 Y | 11  | 4.38  | < 12 | > 30  |
|    |                                    | No  | >1 Y    | 7   | 2.79  | < 4  |       |
|    |                                    |     |         | 233 | 92.83 |      |       |
| 92 | Tartaric Acid                      | Yes | 2 D-1 Y | 13  | 5.18  | < 4  | > 20  |
|    |                                    | No  | >1 Y    | 7   | 2.79  | < 4  |       |
|    |                                    |     |         | 231 | 92.03 |      |       |
| 93 | Malonic Acid                       | Yes | 2 D-1 Y | 2   | 0.8   | < 14 | > 14  |
|    |                                    | No  | >1 Y    | -   |       |      |       |
|    |                                    |     |         | 249 | 99.2  |      |       |
| 94 | Phenobarbital                      | Yes | 2 D-1 Y | 1   | 0.4   | -    |       |
|    |                                    | No  | >1 Y    | -   |       |      |       |
|    |                                    |     |         | 250 | 99.6  |      |       |
| 95 | Lauric Acid                        | Yes | 2 D-1 Y | 1   | 0.4   | -    |       |
|    |                                    | No  | >1 Y    | -   |       |      |       |
|    |                                    |     |         | 250 | 99.6  |      |       |
| 96 | 4-Hydroxy Cyclo Acetic acid        | Yes | 2 D-1 Y | -   |       | < 3  | > 3   |
|    |                                    | No  | >1 Y    | 2   |       |      |       |
|    |                                    |     |         | 249 |       |      |       |
| 97 | Mandelic Acid                      | Yes | 2 D-1 Y | 6   |       | < 74 | > 80  |
|    |                                    | No  | >1 Y    | 2   |       | < 64 |       |
|    |                                    |     |         | 243 |       |      |       |
| 98 | 2-Hydroxy Isobutyric Acid          | Yes | 2 D-1 Y | 7   |       | < 9  | > 20  |
|    |                                    | No  | >1 Y    | 1   |       | -    |       |
|    |                                    |     |         | 243 |       |      |       |

OA Name=Organic Acids Name, AR=Age Range, F=Frequency, % =Percent, RR=Reference Range, PA=Pathgenical Area, Creat=Creatinine, D=Day, Y=Year,

### ***Analysis of abnormal urines***

In addition to the urines from healthy individuals, we also analyzed 30 abnormal urines from patients with methylmalonic aciduria, propionic aciduria, ethylmalonic aciduria, glutaric aciduria, maple syrup urine disease (MSUD), and isovaleric aciduria as described above.

Urine from 30 patients with methylmalonic aciduria (MMA, OMIM 251000) contained 35-3717  $\mu\text{mol}/\text{mmol}$  Creatinine of methylmalonic acid, which were 3-330 folds greater than the normal levels found in our study. Also methylcitrate, which was not present in the urine of normal individuals, was found in the MMA patients.

Urine from five patients with propionic aciduria (PA, OMIM 606054) contained 28-115  $\mu\text{mol}/\text{mmol}$  Creatinine of 3-hydroxy propionic acid, which was 5-23 fold greater than that in normal levels.

Urine from three cases with ethylmalonic aciduria contained 65-197  $\mu\text{mol}/\text{mmol}$  Creatinine ethylmalonic acid, which were 3-12 folds greater than normal levels.

Urine from three patients with glutaric aciduria (OMIM 231670) contained 415-2800  $\mu\text{mol}/\text{mmol}$  Creatinine glutaric acid and 7-116  $\mu\text{mol}/\text{mmol}$  Creatinine of 3-Hydroxy glutaric acid. The glutaric acid concentrations 51-350 fold greater than normal levels. 3-hydroxy glutaric acid is not seen in normal individuals.

Urine from the patient with isovaleric aciduria (IVA, OMIM 243500) contained 2556  $\mu\text{mol}/\text{mmol}$  Creatinine of isovaleryl glycine and 162  $\mu\text{mol}/\text{mmol}$  Creatinine of 3-hydroxy isovaleric acid. The concentration of 3-Hydroxy isovaleric acid was 6-12 folds greater than normal. Isovaleryl glycine is not seen in normal individuals.

Urine from three patients with MSUD (OMIM 248600) contained 20-2600  $\mu\text{mol}/\text{mmol}$  Creatinine of 2-hydroxy isovaleric acid, 10-80  $\mu\text{mol}/\text{mmol}$  Creatinine of 2-hydroxy isocapronic acid, and 190-1905  $\mu\text{mol}/\text{mmol}$  Creatinine of 3-hydroxy butyric acid. These values were 3-30 folds greater than normal.

### ***Discussion***

Most often, laboratories do not quantify organic acids, or they express semi-quantitative results equivalents of an internal standard. Sweetmann considered that errors in quantitative results as great

as 50% would be acceptable for the diagnosis of inherited disorders but the error for organic acids should be <20%. In our opinion, lower analytical errors are desirable for some differential diagnoses, and for the diagnoses of patients having moderate hyper excretions. Moreover, essential biological variables such as the normal excretion concentrations and their variations according to age, genetic factors, and nutritional status have yet to be unequivocally established (12).

Dagleish et al. proposed the use of gas chromatography for the study of the trimethylsilyl and methyl esters of organic acids extracted from physiological fluids, and reported data on a wide range of synthetic reference compounds including butyric acid; di- and tri-carboxylic acids; hydroxy acids and keto acids; polyhydroxy and alicyclic compounds such as glycerol, inositol, quinic acid, shikimic acid, ascorbic acid, and sugar alcohols; aromatic hydroxy and acidic compounds, both benzenoid and indolic; sesquiterpenes; steroids; glycine conjugates; mercapturic acids; and glucuronides (15).

Tanaka et al. analyzed samples from 50 normal subjects and showed that compounds detectable in essentially all urines are succinic, adipic, p-hydroxyphenylacetic, hippuric, and citric acids (8). They used PDA as a qualitative and quantitative internal standard, as described in this study. The benefit of using pentadecanoic acid as an internal standard is that it elutes later than the organic acids; therefore, it does not overlap with them in the GC/MS elution.

In a similar study, Lawson AM et al. was studied the qualitative pattern of urinary organic acids in normal persons. Oxalic acid, sulfate, benzoic acid, phosphate, succinic acid, 4-deoxytetronic acid, 3-oxoeytetric acid, 2-deoxytetronic acid, 5-hydroxymethyl-2-furoic acid, tetronic acids, 2-oxoglutaric acid, 4-hydroxyphenylacetic acid, tartaric acid, 2-deoxypentonic acid, aconitic acid, hippuric acid, citric acid, glucono-1,5-lactone, glucuronic acid and gluconic plus glucaric acids were identified in urine from normal persons. In addition, small amounts of lactic, pyruvic, methylmalonic, oxalic, 3-hydroxybutyric, and furan-2,5-dicarboxylic acids were also found (16). This finding is consistent with our study, but we

quantify major and minor urinary metabolites excreted by normal subjects.

Chalmers et al. assessed variations of urinary organic acids excretion in normal persons and the effects of alterations in dietary combination on these metabolites. They found that dietary alterations produced small changes in the excretion patterns of these metabolites. Alteration in ranges of compounds excreted in urine for the normal population depend more on individual metabolic variations. These findings provide a basis for determination of the normal ranges (17).

Christou et al. proposed the use of gas chromatography for the determination of ten free organic acids by GC-MS with the aim to creation a method for organic acid profiling in human urine. This method was used to the quantitative analysis of urinary organic acids in hospitalized children (18).

## Conclusion

Routine GC-MS is acceptable for quantifying urinary organic acids. With the extraction method described here, urinary organic acids were analyzed both qualitatively and quantitatively. This method also made it possible to establish a more accurate concentration range of organic acids in urine than was

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previously available. From the above results, we suggest that this efficient method will be suitable for the quantitative analysis of organic acids for diagnoses and follow-up studies of patients with new or ill-defined disorders in most clinical laboratories. For successful quantification, one must ensure a constant fragmentation by use of adequate internal standards and perform external calibration. Generally, isotope standards would be preferable for an exact quantitation but this is not feasible for all acids (19, 20). Therefore, a single standard for diagnostic purposes and follow-up is acceptable. Standardization of this method would allow comparisons of values from different labs.

Our studies showed a diverse range of compounds; in addition to the previously recognized urinary organic acids, most of the compounds identified in our study had not been previously reported in a pediatric population of Iran.

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