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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 ofMedical 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 µm.

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 µl 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 µl 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 µl of tetramethylsilane (TMS), vortexed, and incubated at 60 °C for 30 min. After the sample cooled to room temperature, 500 µl of hexane were added and the sample was analyzed by GC/MS. The temperatures for the injectors and detectors were 250 and 300 ºC, 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.

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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, AdipicAcid, Succinic Acid, Citric Acid, 2,3- Dihydroxy Butyric Acid, 3,4-Dihdroxy 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-FuranoicAcid, 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 pathogenical 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)..

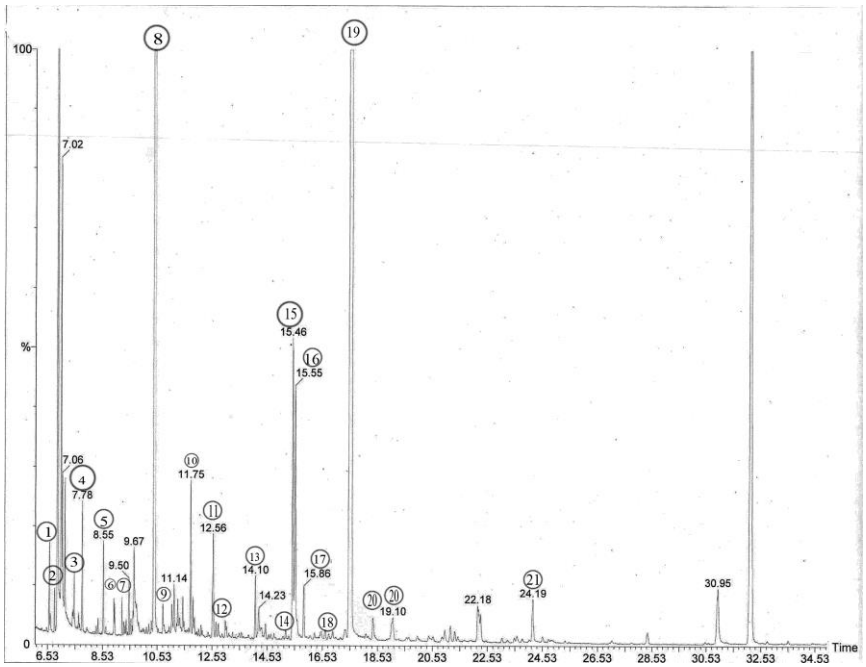

Fig 1.A typical GC-MS chromatogram of urinary organic acids from a normal individual. The compounds noted in the figure are TMS-derivatives of the following compounds: 1: Lactic acid, 2: Glycolic acid, 3: Oxalic acid, 4: 3-hydroxy butyric acid, 5: Urea, 6:Phosphoric acid, 7: Succinic acid, 8: Pentafluorobenzyl hydroxyl amine, 9: Pyruvic acid, 10: 2-hydroxy glutaric acid, 11: 4-hydroxy phenyl acetic acid, 12: Tartaric acid, 13: Aconitic acid, 14: Homovanilic 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.

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

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

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

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

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60	Methylmalonic Acid	Yes	2 D-1 Y	79	31.47	< 11	> 40
			>1 Y	50	19.92	< 6	
		No		122	48.6		
61	Mevalonic	Yes	2 D-1 Y	1	0.399	-	
			>1 Y	-		-	
		No		250	99.61	-	
62	N-acetylaspartate	Yes	2 D-1 Y	25	9.96	< 5	> 50
			>1 Y	2	0.797	< 16	
		No		224	89.24		
63	3-Methyl Glutaric Acid	Yes	2 D-1 Y	11	4.38	< 4	> 12
			>1 Y	9	3.58	< 3	
		No		231	92.04		
64	3-(3-methyl-4-hydroxyphenyl) 3-Hydroxy Propionic Acid	Yes	2 D-1 Y	4	1.59	< 13	> 50
			>1 Y	24	9.56	< 9	
		No		223	88.85		
65	2-Methyl-3-Hydroxy Butyric Acid	Yes	2 D-1 Y	81	32.27	< 7	> 20
			>1 Y	59	23.5	< 5	
		No		111	44.23		
66	3-Methyl-4-Hydroxy Benzoic Acid	Yes	2 D-1 Y	14	5.58	< 29	> 100
			>1 Y	29	11.55	< 10	
		No		208	82.87		
67	3-Methyl-4-Hydroxy-Phenyl Lactic Acid	Yes	2 D-1 Y	6	2.39	< 5	> 8
			>1 Y	-			
		No		245	97.61		
68	Octendicarboxylic Acid	Yes	2 D-1 Y	116	46.21	< 14	> 40
			>1 Y	52	20.72	< 7	
		No		83	33.07		
69	Oxalic Acid	Yes	2 D-1 Y	114	45.42	< 30	> 80
			>1 Y	81	32.27	< 18	
		No		56	22.31		
70	2-Oxoadipinic Acid	Yes	2 D-1 Y	3	1.19	< 5	> 25
			>1 Y	3	1.19	< 5	
		No		245	97.6		
71	2-Oxo-3-Methylvaleric Acid	Yes	2 D-1 Y	3	1.19	< 2	> 5
			>1 Y	2	0.8	< 5	
		No		246	98.01		
72	2-Oxoglutaric Acid	Yes	2 D-1 Y	96	38.25	< 136	> 500
			>1 Y	63	25.1	< 46	
		No		92	36.65		
73	2-Oxoisocapronic Acid	Yes	2 D-1 Y	2	0.8	< 2	> 2
			>1 Y	-			
		No		249	99.2		
74	5-Oxoproline	Yes	2 D-1 Y	80	31.87	< 9	> 20
			>1 Y	57	22.71	< 5	
		No		114	45.42		
75	Palmitic Acid	Yes	2 D-1 Y	6	2.39	< 5	> 5
			>1 Y	1	0.4		
		No		244	97.21		
76	Phenyl Acetic Acid	Yes	2 D-1 Y	27	10.76	< 11	> 50
			>1 Y	21	8.37	< 10	
		No		203	80.88		
77	Phenyl Lactic Acid	Yes	2 D-1 Y	61	24.31	< 6	> 20
			>1 Y	17	6.77	< 4	
		No		173	68.92		
78	Pimelic Acid	Yes	2 D-1 Y	76	30.28	< 5	> 15
			>1 Y	49	19.52	< 5	
		No		126	50.2		
79	Stearic Acid	Yes	2 D-1 Y	12	4.78	< 7	> 30
			>1 Y	12	4.78	< 8	
		No		227	90.44		

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

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

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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 µmol/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 µmol/mmol Creatinine of 3-hydroxy propionic acid, which was 5-23 fold greater than that in normal levels.

Urine from three cases with ethylmalonic acidurea contained 65-197 µmol/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 µmol/mmol Creatinine glutaric acid and 7-116 µmol/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 µmol/mmol Creatinine of isovaleryl glycine and 162 µmol/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 µmol/mmol Creatinine of 2-hydroxy isovaleric acid, 10-80 µmol/mmol Creatinine of 2-hydroxy isocapronic acid, and 190-1905 µmol/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-oeoxytetronic acid, 2-deoxytetronic acid, 5-hydroxymethyl-2-furoic acid, tetronlc acids, 2-oxoglutaric acid, 4-hydroxyphenylacetic acid, tartaric acid, 2-deoxypentonic acid, aconitic acid, hippuric acid, citric acid, glucono-1,5-Iactone, 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

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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 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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All authors declare they have no conflicts of interest.

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