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ORIGINAL ARTICLE

Screening of diseases associated with abnormal metabolites for evaluation of HPLC in organic aciduria profiling

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KEYWORDS

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Acylcarnitine

Abstract *Background:* Organic acid disorders are a heterogeneous group of inborn errors of metabolism, in which organic acids accumulate in the body. They have high prevalence in Egypt because of a high rate of consanguineous marriages. Here we report our experience with the diagnostic evaluation of patients with organic acidemias as well as several other inborn errors of metabolism (IEMs) by liquid chromatography–tandem mass spectrometry (LC/MS–MS), gas-chromatography mass spectrometry (GC/MS) and by isocratic cation exchange "high-performance liquid-chromatography" (HPLC) to evaluate the use of HPLC method for disease-associated metabolite screening.

Patients and methods: In this study, we screened 86 suspected Egyptians patients with organic acid disorders by LC/MS–MS, GC/MS and by HPLC aged from 3 days to 12 years old. Data obtained from the three methods were statistically analyzed to evaluate the specificity and sensitivity of the HPLC method over the other two methods and to pursue its precision in the diagnosis of

Abbreviations: GC/MS, gas chromatography/mass spectrometry; LC–MS/MS, liquid chromatography mass spectrometry/mass spectrometry; HPLC, high performance liquid chromatography; MSUD, maple syrup urine disease; PA, propionic acidemia; IVA, isovaleric acidemia; CoA, coenzyme A; GA-1, glutaric acidemia type I; BKT, β-ketothiolase deficiency; MMA, methylmalonic aciduria; PGA, pyroglutamic aciduria; UCD, urea cycle defect; NKHG, non-ketotic hyperglycinemia; CD, creatine deficiency; Q.MTD, query mitochondrial disorder; TP, true positive; FP, false positive; NA, not applicable; TN, true negative; FN, false negative

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organic acid disorders. Moreover, 17 urine samples were collected from patients with several other IEMs to evaluate the efficiency of HPLC in detecting abnormal metabolites in urine samples.

Results: The screening results showed that diagnostic efficiencies were varied among the three methods, HPLC showing a higher sensitivity of detecting normal urine as well as a highly satisfactory extent for the detection of different metabolic disorders. In addition, some typical urinary HPLC chromatograms of different metabolic disorders were presented to help the investigator who is going to start an organic aciduria screening program by HPLC to be familiar with various patterns.

Conclusion: This study has indicated that HPLC is an easy applicable and useful technique for the initial screening of organic acid disorders and many other disease associated metabolites.

1. Introduction

Organic acids comprise key metabolites of almost all pathways of intermediary metabolism as well as exogenous compounds [1]. Organic acid disorders (acidurias/acidemias) are an important class of hereditary autosomal recessive diseases due to gene defects in coding specific enzymes that are involved in the metabolism of amino acids or organic acids. This results in accumulation of organic acids in urine and, to a lesser extent, in other body fluids [2,3]. Therefore, comprehensive analysis of organic acids in body fluids has the potential of yielding information on the pathophysiological status of affected individuals [1,4]. Patients with organic acidurias frequently present with acute symptoms early in life [5]. The range of clinical manifestations is diverse, involving multiple body systems with a predominance of the central nervous system [6,7]. Clinical and laboratory findings that suggest an organic acidemia include acidosis, ketosis, hyperammonemia, abnormal liver function tests, hypoglycemia, and neutropenia [8]. The diagnosis is usually made by detecting an abnormal pattern of organic acids in a urine sample by gas chromatography–mass spectrometry (GC/MS) [9–11].

In GC/MS screening, organic acids are separated on the basis of their polarity and volatility and then bombarded by an electron beam that fragments the eluting molecules in a pattern characteristic of each organic acid. However, the organic acids must first be extracted from the urine sample and then chemically modified to make the organic acids sufficiently volatile before GC/MS analysis [12].

Another technique for organic acid analysis in urine is the isocratic ''high-performance liquid chromatographic'' (HPLC) technique has been developed. This depends on spectrophotometric and amperometric detection after combined reversed-phase and cation-exchange chromatography [13]. Advantages of this technique over conventional gas chromatography include, ease of sample preparation and the simultaneous detection of non-volatile fatty acids. Limitations include; its lack of sensitive identification system comparable to that of mass spectrometry, the presence of non-ultraviolet absorbing fatty acids and the relatively long retention times of phenolic compounds [9,13].

The urinary organic acid profile is abnormal in the face of any illness with decompensation; however, in some disorders diagnostic analytes may be present only in small or barely detectable amounts when the affected individual is not acutely ill. A plasma or serum acylcarnitine profile by liquid chromatography–tandem mass spectrometry (LC–MS/MS) can also provide a rapid clue to the diagnosis [12]. Urine acylcarnitine profiling is more complex and interpretation can be difficult [14].

Depending on the specific disorder, plasma amino acid analysis using a quantitative method such as column chromatography, high-performance liquid chromatography (HPLC), or GC/MS can also be helpful. Confirmatory testing involves assay of the activity of the deficient enzyme in lymphocytes or cultured fibroblasts and/or molecular genetic testing may also be needed [15].

This study was aimed to evaluate the use of the HPLC method to screen for disease associated-metabolites and compare its specificity and sensitivity with GC/MS for patients with organic acidemias and several other IEMs by tandem mass spectrometry, gas chromatography–mass spectrometry, and isocratic high performance liquid chromatography.

2. Patients and specimens

A total of 103 Egyptian patients, aged from 3 days to 12 years old, who were admitted to the Medical Genetics Unit of the Ain Shams University, Pediatrics Hospital, Cairo, Egypt from June 15th 2010 to February 25th 2013 and suffering from any of inborn error metabolic disorder were subjected to the screening study. They were divided into two groups. Group 1 with 86 patients (47 males and 39 females) who were suspected of having an organic acid disorder; it was subsequently divided into sub-groups according to their final diagnosis as shown in (Table 1).

Group 2 was 17 urine samples (12 males and 5 females) from patients who were diagnosed with different other IEMs (Table 2). Moreover, a control group of 24 urine samples from healthy subjects, aged from 3 days to 10 years (7 samples from newborns <1 month old, 6 samples from infants between 1 and 12 months old, and 11 samples from healthy controls between 1 and 10 years old.), was tested by HPLC to determine the reference ranges for all organic acids found in normal urine samples. Organic acid calibrators were prepared in water at concentrations of 1000, 500 and 100 mg/dl for several standard organic acids (Sigma chemical Co. Ltd., Poole, Dorset, U.K.) that are possibly found in normal and metabolically disturbed urine samples (Table 3).

Urine specimens from all studied patients were collected in plastic laboratory containers and frozen immediately at -20 °C until analysis by HPLC. Another urine sample from the 86 patients suspected with organic acid disorders was collected for GC/MS analysis. Urine samples from neonates and

Table 1 Patient groups and subgroups.

Patients suspected with organic acid disorder ($N = 86$)				
	Subgroup	No. of Patients	Sex	
			Male	Female
1	Methylmalonic aciduria	15	8	7
2	Glutaric aciduria type 1	9	8	1
3	Isovaleric aciduria	2	0	2
4	Propionic aciduria	2	1	1
5	β-ketothiolase deficiency	1	1	0
6	Pyroglutamic aciduria	1	1	0
7	Maple syrup urine disease	2	2	0
8	Lactic acidosis	1	0	1
9	Questionable	9	6	3
10	Normal	44	20	24
Total		86	47	39

Table 2 Subgroups of patients with IEMs.

Patients with different IEMs ($N = 17$)				
	Subgroup	No. of Patients	Sex	
			Male	Female
1	Urea cycle defects	3	2	1
2	Mitochondrial disorders	9	6	3
3	Creatine deficiency	1	1	0
4	Non-ketotic hyperglycinemia	2	2	0
5	Dicarboxylic aciduria	1	0	1
6	Hyperoxaluria	1	1	0
Total		17	12	5

infants were collected in special sterile plastic bags then transferred into urine containers.

The work has been carried out in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The work was carried out after the acceptance of parents of the patients and acceptance of the Ethics Committee of the University.

3. Methods

3.1. Isocratic cation exchange "high-performance liquid-chromatography" (HPLC)

The technique was previously reported by Bannett and Bradey [13] for the determination of organic acids in urine.

3.1.1. Equipments and reagents

The HPLC equipment (supplied by Bio-Rad, Richmond, CA) consisted of a Model 2700T solvent delivery system, a D8I injector, a Model 450 wavelength detector and a Phenomenex® cation-exchange column maintained in a constant temperature at 37 °C, the solvent was H_2SO_4, 0.25 ml/L, with a flow rate 1 ml/min and the detecting wavelength was set at 210 nm.

3.1.2. Chromatography

Urine samples were filtered through 0.22 μm Millipore filter (Sartorius Stedim Biotech., Geottingen, Germany), related to creatinine concentration (injected volume was 10 μl if creatinine concentration < 0.02 mg/dl and a volume of 5 μl if creatinine concentration > 0.02 mg/dl) and mixed with 3-phenylpropionic acid as an internal standard before injecting it onto the column. The average chromatographic analysis time for urine was 1.5 h.

Table 3 Retention times of organic acids found in normal and abnormal urinary HPLC chromatograms.

ID	Organic acid	Mean RT (min) ± SD
1	Miscellaneous	5.40 ± 0.05
2	Oxalic acid	6.31 ± 0.06
3	**Methylcitric acid**	6.89 ± 0.06
4	**3-Hydroxyglutaric acid**	6.94 ± 0.18
5	**Pyruvic acid**	7.52 ± 0.11
6	**2-Keto-3-methylvaleric acid**	8.01 ± 0.02
7	Unknown	8.56 ± 0.08
8	**Propionylglycine**	8.58 ± 0.01
9	**Methylmalonic acid**	8.60 ± 0.06
10	**2-Ketoisocaproic acid**	8.68 ± 0.01
11	Succinic acid	9.83 ± 0.26
12	**3-Hydroxyproponic acid**	9.98 ± 0.21
13	**Lactic acid**	10.19 ± 0.09
14	3-hydroxybutyric acid	10.32 ± 0.10
15	**2-Methyl-3-hydroxybutyric acid**	10.32 ± 0.02
16	**3-Hydroxyisovaleric acid**	10.63 ± 0.04
17	**2-Hydroxyisovaleric acid**	10.74 ± 0.03
18	**2-Methylacetoacetic acid**	10.85 ± 0.06
19	**Glutaric acid**	11.16 ± 0.20
20	Uric acid	12.36 ± 0.07
21	**Propionic acid**	13.37 ± 0.26
22	**Isovalerylglycine**	13.75 ± 0.01
23	**Pyroglutamic acid**	13.96 ± 0.08
24	Unknown	15.51 ± 0.10
25	Unknown	16.62 ± 0.25
26	**Tiglylglycine**	18.49 ± 0.49
27	Unknown	21.94 ± 0.75
28	Unknown	24.88 ± 0.15
29	**Unknown**	28.30 ± 0.15
30	Unknown	30.63 ± 0.49
31	Hippuric acid	33.62 ± 0.19
32	Unknown	38.69 ± 0.21
33	Unknown	45.87 ± 0.11
3-phenylpropionic acid (internal standard)		75 ± 0.06

Peaks that are diagnostically significant were represented in bold, while many normal peaks remained unidentified.

3.2. Gas-chromatography mass spectrometry (GC/MS)

GC/MS was done in another laboratory to study urinary organic acid patterns by gas chromatography–mass spectrometry using GC/MS (Agilent Technologies Inc., QP2010). Sample preparation and detection procedures were based on methods reported previously by Kuhara [19].

3.3. Acylcarnitines and amino acids profiling by liquid chromatography–tandem mass spectrometry (LC–MS/MS)

A rapid metabolic screening technique is the analysis of acylcarnitine profiles in dried blood spots by tandem quadrupole mass spectrometry (ACQUITY UPLC® System, Waters associates, Northwich, Cheshire, UK) [16]. Blood samples

were taken from the patients by heelstick, spotted on Whatman filter paper cards (Schleicher and Schuell 903; Dassel, Germany) and left to dry before screening by tandem mass spectrometry.

Sample preparation and detection procedures were based on methods reported previously [17,18]. Acylcarnitines were automatically calculated according to the assigned values of the internal standards using Math Lynx® software. Quality control samples were provided by the Center for Disease Control and Prevention, Atlanta, GA, USA.

3.3.1. Statistical analysis

Data acquired from the patients suspected with organic acid disorders ($N = 86$) were statistically analyzed by cross tabulation, chi square test and Z-test between two proportions [20]. Cross tabulation and chi square test were used to test the accuracy of HPLC, GC–MS and tandem mass spectrometry in the diagnosis of the patients suspected with organic acid disorder. Z-test between two proportions was used to compare between HPLC and GC–MS as screening tools for urinary organic acids. Scores were given to indicate the precision of testing as follows;

1 = Results are the same as the final diagnosis,
2 = Results are inconclusive (not diagnostic but support the final diagnosis),
3 = Questionable (unknown abnormality or no final diagnosis),
4 = Wrong diagnosis or false negative.

4. Results

Out of 86 cases, We had nine questionable cases: Two patients (11 and 12), a brother and his sister, had consistently elevated level of C4DC (C4-dicarboxylic or methylmalonic/succinyl-carnitine) upon LC–MS/MS screening, intermittently elevated methylmalonic acid in GC/MS screening and mildly elevated methylmalonic acid in HPLC screening and other HPLC urinary organic acids profile abnormalities. Differential diagnosis suggested methylmalonic aciduria (MMA) due to succinyl CoA ligase deficiency. However, no enzyme assay was available to prove the diagnosis. Other two patients were questionable upon GC/MS and HPLC screenings with elevated non-diagnostic urinary organic acids and were normal in metabolic screening by tandem mass spectrometry. We lost follow up of other five questionable cases and final diagnosis was not definitely determined made.

4.1. Normal urine pattern by HPLC

All chromatograms of normal control group showed that urea and uric acid usually were the major peaks (Fig. 1), other smaller peaks, all of them less than 20% of the size of uric acid peak, include; succinic, 3-hydroxybutyric, oxalic and hippuric acids which were detected in virtually all tested urine samples. Methylmalonic acid was detected in trace amount, which does not exceed 5 mmol/mol Cr, tiglylglycine and many other smaller unidentified peaks in normal urine samples.

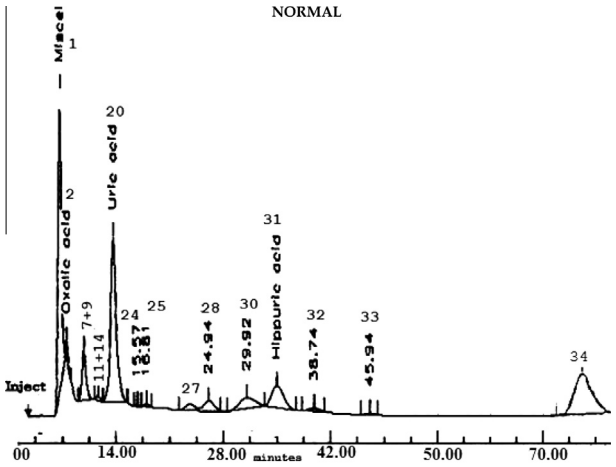

Figure 1 Typical chromatogram of urine from a normal neonate. Peak 26 represents tiglylglycine [18.49 ± 0.49] was also detected in normal chromatograms.

Table 4 Quantitative determination of urinary MMA level in five newly diagnosed mut-MMA patients by the HPLC technique.

Patient no.	MMA (mg/dl)	Urinary Cr[a] (mg/dl)	MMA mg/mg Cr
2	972	23	42,260
3	681.8	8	85,225
5	1119	33	33,909
8	104.5	23	4543
9	449	128	3507

[a] Urinary creatinine.

Retention times of organic acids detected in normal and abnormal urinary HPLC chromatograms are presented in Table 4. Organic acids are listed, starting from 1 (represents urea, creatinine, proprandiol and sulfate) to 34 (represents the internal standard), in order of their retention times [mean retention time (min) ± SD] to observe their recurrence in different samples.

4.2. Metabolic Profiling of samples with organic acid disorders

The initial screening of organic acids in urine, using HPLC method, has helped in many ways in the initial assessment and follow up of patients with different types of organic aciduria. The chromatograms of urine samples from several patients with organic acid disorders are presented in (Figs. 2–8) to help the investigator who is going to start an organic acid screening program by HPLC become familiar with various patterns. Peaks that are diagnostic for the diseases are very large compared with others.

Two different profiles of methylmalonic academia (MMA) patients (Fig. 2A) have shown an abnormal peak corresponding to MMA, in addition, smaller peaks of metabolites from isoleucine, such as tiglylglycine, and the secondary metabolites of propionate (3-hydroxypropionic and methylcitric acids). Concentration of urinary methylmalonic acid/creatinine (MMA/Cr) was quantitatively estimated by HPLC for patients

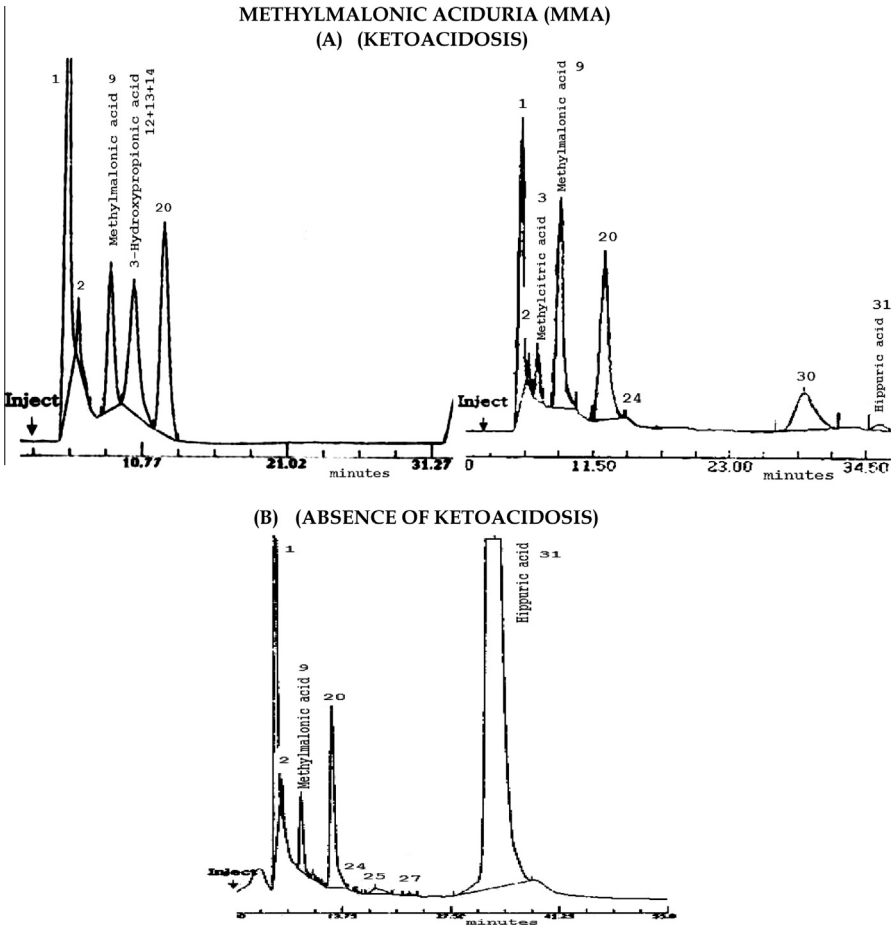

Figure 2 Urinary organic acids from patients with methylmalonic aciduria diagnostic peaks are: 3, methylcitric acid [6.89 ± 0.06]; 9, methylmalonic acid [8.60 ± 0.06]; 12, 3-hydroxyproponic acid [9.98 ± 0.21]. (A) Untreated methylmalonicaciduria patients showed highly elevated MMA, methylcitric acid and 3-hydroxypropionic acid. (B) Diet controlled MMA patient; An abnormal peak corresponding to hippuric acid can be seen, along with a smaller peak of methylmalonic acid.

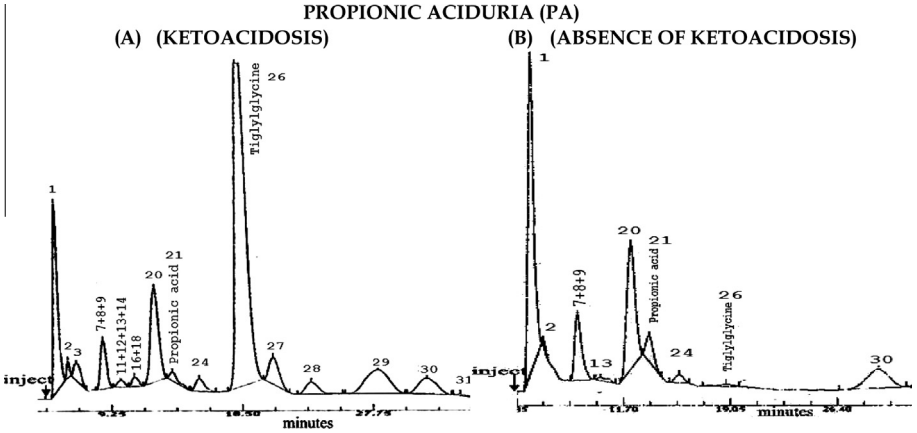

Figure 3 Urinary organic acids from a patient with propionic aciduria diagnostic peaks are: 3, methylcitric acid [6.89 ± 0.06]; 8, propionylglycine [8.58 ± 0.01]; 12, 3-hydroxyproponic acid [9.98 ± 0.21]; 13, lactic acid [10.19 ± 0.09]; 14, 3-hydroxybutyric acid [10.32 ± 0.10]; 16, 3-hydroxyisovaleric acid [10.63 ± 0.04]; 18, 2-methylacetoacetic acid [10.85 ± 0.06]; 21, propionic acid [13.37 ± 0.26]; 26, tiglylglycine [18.49 ± 0.49].

2, 3, 5, 8 and 9 at the onset of the symptoms, where MMA was significantly elevated (up to 2000 mg/mg of creatinine) and easily detected in urine (Table 4). MMA level in urine has been completely normalized with treatment as shown in Fig. 2B,

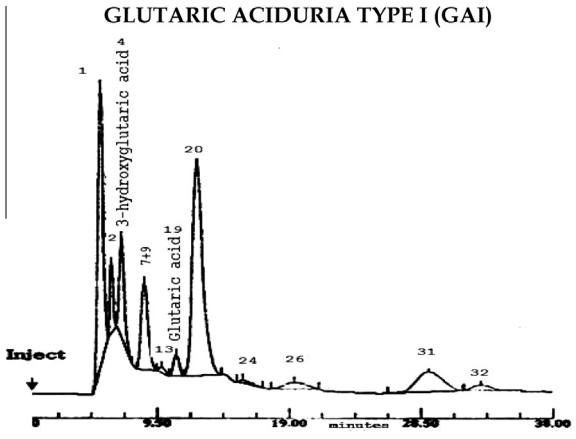

Figure 4 Urinary organic acids from a patient with glutaric aciduria type I diagnostic peaks are: 4, 3-hydroxyglutaric acid [6.94 ± 0.18]; 13, lactic acid [10.19 ± 0.19]; 19, glutaric acid [11.16 ± 0.20].

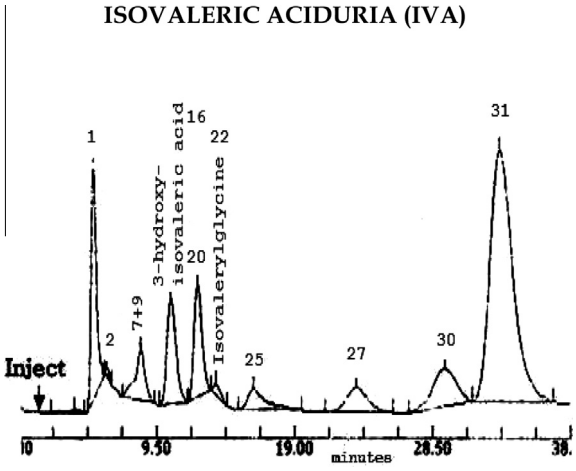

Figure 5 Urinary organic acids from a patient with isovaleric aciduria diagnostic peaks are: 16, 3-hydroxyisovaleric acid [10.63 ± 0.04]; 22, isovalerylglycine [13.75 ± 0.01].

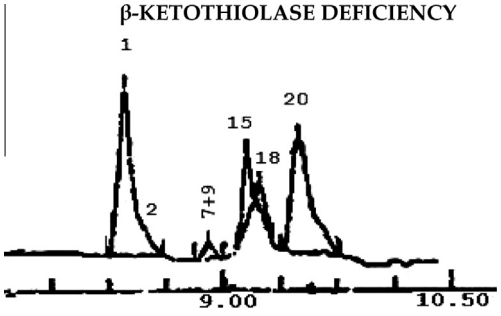

Figure 6 Urinary organic acids from a patient with β-ketothiolase deficiency diagnostic peaks are: 15, 2-methyl-3-hydroxybutyric acid [10.32 ± 0.02]; 18, 2-methylacetoacetic acid [10.85 ± 0.06].

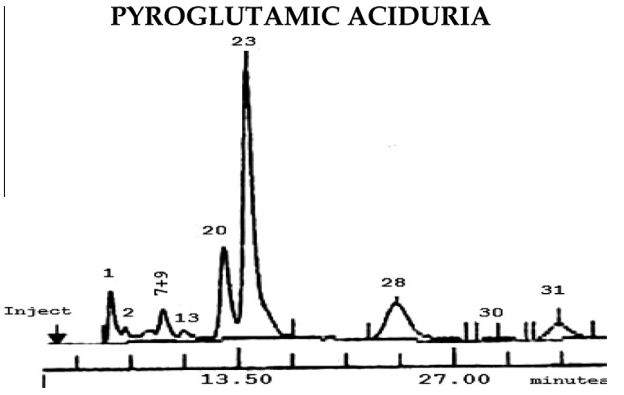

Figure 7 Urinary organic acids from a patient with pyroglutamic aciduria diagnostic peaks are: 13, lactic acid [10.19 ± 0.09]; 23, pyroglutamic acid [13.96 ± 0.08].

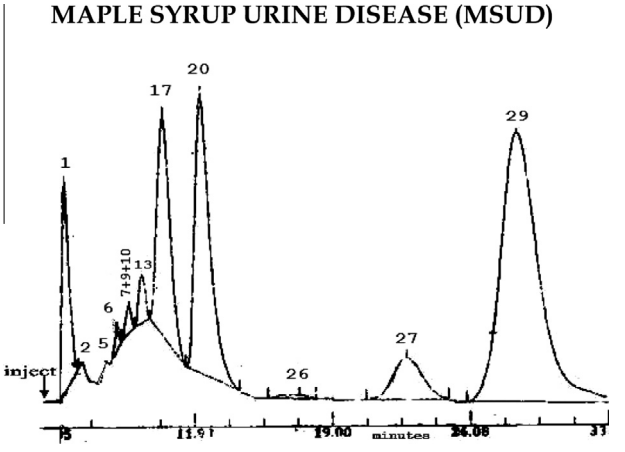

Figure 8 Urinary organic acids from a patient with maple syrup urine disease (MSUD) diagnostic peaks are: 5, pyruvic acid [7.52 ± 0.11]; 6, 2-keto-3-methylvaleric acid [8.01 ± 0.02]; 10, 2-ketoisocaproic acid [8.68 ± 1]; [16, lactic acid [10.19 ± 0.09]; 17, 2-hydroxyisovaleric acid [10.59 ± 0.02].

where hippuric acid can be seen along with a smaller peak of methylmalonic acid. We reported propionic academia (PA) (Fig. 3A before and B after management) in two patients, where the lower 3-hydroxypropionic acid level could characterize the ketoacidotic state profile of PA from multiple carboxylase deficiency (MCD). Elevated levels of specific organic acids were diagnostically significant in the initial urinary organic acids screening by HPLC, as glutaric and 3-hydroxy-glutaric acids in glutaric aciduria type I (GA1) (which were remarkably elevated in all patients) (Fig. 4), 3-hydroxyisovaleric acid in patients with isovaleric aciduria (IVA) (Fig. 5), 2-methyl-3-hydroxybutyric and 2-methylacetoacetic acids in patients with β-ketothiolase deficiency (BKT) (Fig. 6), and pyroglutamic acid in a patient with pyroglutamic aciduria (Fig. 7).

Multiple urinary organic acid abnormalities reported by HPLC for maple syrup urine disease (MSUD) patients, who were diagnosed by LC–MS/MS, included pyruvic, 2-keto-3-methylvaleric, 2-ketoisocaproic, lactic and 2-hydroxyisovaleric acids (Fig. 8).

Organic acids were identical to those detected with GC/MS for all organic acid disorders. Acylcarnitines and amino acids profiling by tandem mass spectrometry would be more applicable for rapid diagnosis of MSUD (by elevated Leu:Ile, Leu:Ala, Leu:Phe and Valine), IVA (by elevated Isovalerylcarnitine C5), GA1 (by elevated Glutarylcarnitine C5-DC), Inconclusive for MMA, PA (with elevation of both Propionylcarnitine C3 and propionylcarnitine/acetylcarnitine C3:C2), BKT (with elevated 2-methyl, and 3-hydroxybutyrylcarnitine C5-OH) and not diagnostic in cases with pyroglutamic aciduria and lactic acidosis (with falsely negative results).

4.3. Urine samples from patients with IEMs other than organic acidemia

Among the 17 patients with IEMs other than organic acidemia (Table 1), HPLC has detected abnormal urinary organic acid profiles in patients 1–3 with urea cycle defects (where lactic and pyruvic acids were significantly elevated) (Fig. 9) and in mitochondrial disorders cases, patients from 9 to 17, (where elevated lactic and/or pyruvic acids, and either glutaric or 3-hydroxyglutaric acid has been detected (Fig. 10)). Abnormal profiles have been detected by HPLC in cases of creatine deficiency, hyperoxaluria (Fig. 11) and dicarboxylicaciduria (Fig. 12), while abnormalities have not been detected by HPLC in cases of non-ketotic hyperglycinemia.

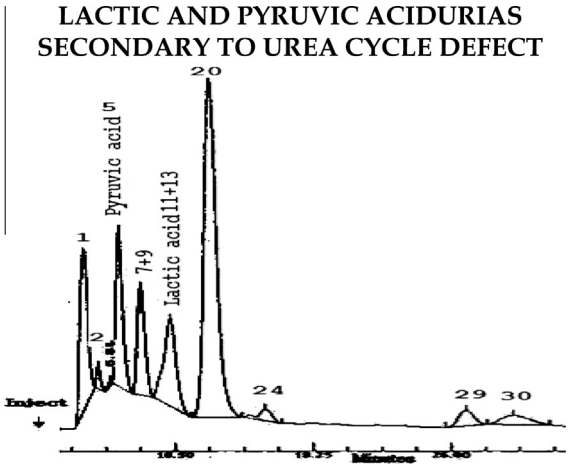

Figure 9 Urinary organic acids from a patient with lactic and pyruvic acidurias diagnostic peaks are: 5, pyruvic acid [7.52 ± 0.11]; 13, lactic acid [10.19 ± 0.09].

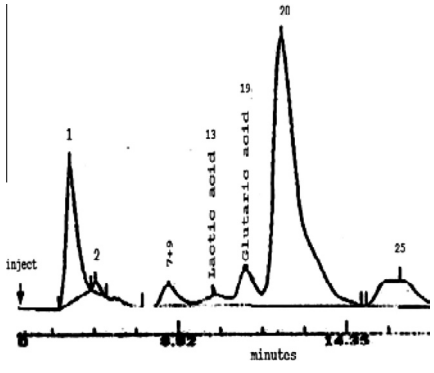

Figure 10 Query mitochondrial disorder diagnostic peaks are: 13, lactic acid [10.19 ± 0.09], 19, glutaric acid [11.16 ± 0.20].

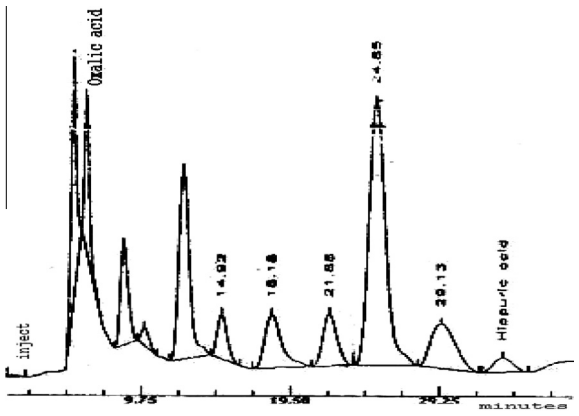

Figure 11 Hyperoxaluria diagnostic peak is oxalic acid [6.31 ± 0.06].

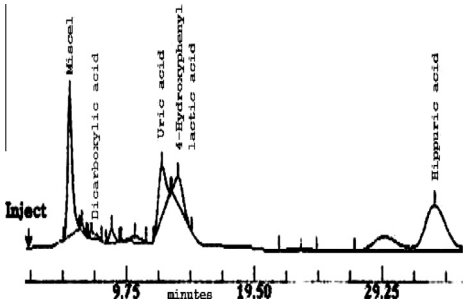

Figure 12 Dicarboxylicaciduria diagnostic peaks are dicarboxylic acid [7.02] and 4-hydroxyphenyllactic acid.

4.3.1. Statistical analysis

Table 5 presents the results of comparison between the three methods by cross tabulation and the chi square test. There is a statistically significant difference between the three methods ($X2 = 54.206$, $p < 0.001$). The highest and best accuracy was reported with GC–MS (88.5%) in the diagnosis of organic acidurias compared to the other two methods. HPLC had the highest questionable results (18.1%) while LC–MS/MS gave the highest inconclusive results (22.4%).

Table 6 presents the comparison between HPLC and GC/MS in group 1 screening results. It showed higher sensitivity (92%) and predictive power for negative results (92%) of HPLC compared to GC–MS that showed higher specificity (100%) and predictive value of positive results (100%).

5. Discussion

This study highlights some important aspects of testing, developing and validating an isocratic cation exchange HPLC method for qualitative analyses of urine samples from neonates and infants suspected of having organic aciduria and other metabolic disorders. We subjected a group of 86 urine samples of

Table 5 Accuracy of the result in the three methods in specimens of patients suspected with organic acid disorders.

Precision in group 1	Metabolic Screen		GC/MS		HPLC		Chi square	*p*-Value
	No.	%	No	%	No	%		
Results are the same as the final diagnosis	52	61.2	69	88.5	63	75.9	54.206	< 0.001
Inconclusive (not sufficient for final diagnosis)	19	22.4	0	0	0	0		
Questionable (abnormal but unknown diagnosis)	7	8.2	2	2.6	15	18.1		
False negative or wrong diagnosis	7	8.2	7	9	5	6		
Total	85	100	78	100	83	100		

Regarding accuracy, there is a statistical significance between the three methods, GC/MS had the highest accurate results compared to other methods. Inconclusive results were more with metabolic screening while HPLC had the highest questionable results.

Table 6 HPLC versus GC–MS in organic acid disorder patients.

Group 1: Organic acidurias		HPLC			GC/MS					
		Positive	Negative	Total	Positive	Negative	Total			
Criterion	Positive	36	3	39	32	6	38			
	Negative	8	36	44	0	42	42			
	Total	44	39	83	32	48	80			
Index	HPLC				GC/MS				*z*	*P*-Value
	Estimate	SE	Lower 95% CI	Upper 95% CI	Estimate	SE	Lower 95% CI	Upper 95% CI		
Sensitivity	0.9231	0.0427	0.7913	0.9838	0.8421	0.0592	0.6875	0.9398	1.5727	> 0.05
Specificity	0.8182	0.0581	0.6729	0.9181	1	0	0.9159	1	4.7088	< 0.01
Predictive value of positive test	0.8182	0.0581	0.6729	0.9181	1	0	0.8911	1	5.0015	< 0.01
Predictive value of negative test	0.9231	0.0427	0.7913	0.9838	0.875	0.0477	0.7475	0.9527	0.969	> 0.05

There are statistically significant differences between the results of the two methods regarding specificity and predictive value of positive test. GC/MS had higher specificity and predictive power for positive test compared to HPLC method.

Egyptian patients suspected with organic acid disorders, aged from 1 day to 12 years of life, to the screening programs by liquid chromatography–tandem mass spectrometry (LC–MS/MS), gas chromatography–mass spectrometry (GC–MS) and cation exchange isocratic ''high-performance liquid-chromatography'' (HPLC).

In all of the normal chromatograms we obtained by HPLC in this study, the major identifiable peak was uric acid, which matches with the finding reported by Bannett and Bradey [13]. Other peaks identified in normal urine samples, all of them less than 20% of the size of uric acid, were hippuric and oxalic acids, which were detected in virtually all tested urine samples (Fig. 1). A flattened peak of succinic acid and 3-hydroxybutyric acid has also been detected in normal urine samples that may be misinterpreted as lactic acid [13].

Methylmalonic acid detected in trace amount due to bacterial growth does not exceed 5 mmol/mol Cr, but in cases of urinary tract infection it was confusing to characterize the normally from abnormally elevated MMA in urine [21]. Therefore, an approach for the quantitative detection of methylmalonic acid in urine has been done in this study for patients 2, 3, 5, 8 and 9 at the onset of the symptoms, where MMA was significantly elevated (up to 2000 mg/mg of creatinine) and easily detected in urine. Tiglylglycine was also reported in the majority of normal chromatograms as reported by Bennett et al. [22].

We presented some typical urinary HPLC chromatograms of different metabolic disorders to help the investigator who is going to start an organic aciduria screening program by HPLC become familiar with various patterns. The results showed that this method is useful as an initial screening tool of organic acids in urine samples of neonates and infants, where it can detect any abnormalities of organic acids in urine. Although the identity of metabolites in these urine samples was later confirmed by GC/MS analysis, the initial identification was done by HPLC alone, with the use of the table of retention time values (Table 3) of recalibrated organic acids of those reported by Bannett and Bradey [13].

Patients with methylmalonic aciduria showed elevated methylmalnic, methylcitric and 3-hydroxypropionic acids (which lies in the same retention times of lactic and 3-hydroxybutyric acids). Sometimes propionic, fumaric and lactic acids were also detected. Elevated C4-dicarboxylic carnitine may contribute to the diagnosis of methylamalonic aciduria due to succinyl CoA ligase deficiency as reported in this study in patients 11 and 12. We detected the same pattern in patients with propionic acidemia except normal MMA level which could be distinguished in propionic acidemia patients. Patients with glutaric aciduria type 1 have shown elevated glutaric acid and 3-hydroxyglutaric acid. Diagnosis of isovaleric aciduria was not conclusive with the detection of 3-hydroxyisovaleric

acid and isovalerylglycine and absence of isovaleric acid in most cases. Abnormalities were reported by Bannett and Bradey [13] in patients with β-ketothiolase deficiency as unidentified peaks, which were identified in this study as 2-methyl-3-hydroxybutyric and 2-methylacetoacetic acids. We also identified other diagnostic peaks for different metabolic disorders as pyroglutamic acid in pyroglutamic aciduria which tested normal via acylcarnitines profiling, pyruvic acid, 2-keto-3-methylvaleric acid, 2-ketoisocaproic acid, lactic acid and 2-hydroxyisovaleric acid in maple syrup urine disease (MSUD) but screening by LC–MS/MS would be more reliable for this disorder.

Data obtained from the three methods, LC–MS/MS, GC–MS and HPLC, showed varied diagnostic power among the two tested groups. Statistical analyses showed higher sensitivity (92%) and positive power in detecting negative results (92%) to HPLC compared to GC–MS. It also showed the lowest accuracy in diagnosis (75%) and the highest score in detecting urinary organic acids profile abnormalities (76.5%). This means that we can rely on HPLC in detecting urinary organic acid abnormalities but the results will not be conclusive in the diagnosis.

In this study also, we tested about 17 patients with different IEMs to test the power of HPLC to detect normal urine samples. Although a chromatogram from a patient with gross dicarboxylic aciduria did not appear to be as abnormal as the corresponding GC/MS profile with glycerol detection, results were abnormal to a great extent in most cases as the detection of lactic and pyruvic acids in urea cycle defect (UCD) (adipic and orotic acids only detected GC/MS) and different mitochondrial disorders, glutaric, 3-hydroxyglutaric and lactic acids in some mitochondrial disorders, oxalic acid in the case of hyperoxaluria, while abnormalities have not been detected by HPLC in cases of non-ketotic hyperglycinemia. This means that, in most cases of metabolic disorders, HPLC can exclude normal and abnormal urinary organic acid profiles effectively.

However, some abnormalities detected by HPLC chromatograms may be an indicative for a pathophysiological illness as 4-hydroxyphenyllactic acid showed in patients with liver dysfunction.

Limitations include the inability to distinguish between isomers such as lactic acid, succinic acid and 3-hydroxypropionic acid and the lack of an identification system since one peak may indicate more than one organic acid which lies within closely the same retention time.

To conclude HPLC method is fast, inexpensive and easier in sample preparations compared to GC/MS [19] and LC–MS/MS [14,16]. It is also more easily applicable to routine analysis in non-specialized laboratories and a useful technique not only for initial screening of organic acid disorders but also of many other metabolic disorders.

Conflict of interest

The authors declare no conflict of interests.

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References

[1] Hoffman GF, Feyh P. Organic acid analysis. In: Blau N, Duran M, Blaskovics ME, Gibson KM, editors. Physician's guide to the laboratory diagnosis of metabolic diseases. Heidelberg: Springer-Verlag; 2003. p. 27–44.
[2] Rinaldo P. Organic acids. In: Blau N, Duran M, Gibson KM, editors. Laboratory guide to the methods in biochemical genetics. Heidelberg: Springer-Verlag; 2005. p. 171–206.
[3] Hoffman GF, Zschocke J, Nyhan WL. Inherited metabolic diseases; A clinical approach. Heidelberg: Springer-Verlag; 2010.
[4] Pang CCP. Laboratory investigation of inherited metabolic diseases. HKMJ 1996;3:264–73 [Link].
[5] Narayanan MP, Kannan V, Vinayan KP, Vasudevan DM. Diagnosis of major organic acidurias in children: two years experience at a tertiary care centre. Indian J Clin Biochem 2011;26:347–53 [PubMed].
[6] Dionisi-Vici C, Deodato F, Röschinger W, Rhead W, Wilcken B. 'Classical' organic acidurias, propionic aciduria, methylmalonic aciduria and isovaleric aciduria: long-term outcome and effects of expanded newborn screening using tandem mass spectrometry. J Inherit Metab Dis 2006;29:383–9 [PubMed].
[7] Saudubray JM, Charpentier C. Clinical phenotypes: diagnosis/algorithms. In: Scriver C, Beaudet A, Sly W, Valle D, editors. The metabolic and molecular bases of inherited disease. New York: McGraw-Hill; 2001. p. 1327–403.
[8] Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Kinzler KW, et al. The metabolic and molecular bases of inherited disease. 8th ed. New York: McGraw-Hill, Inc.; 2001 [Link].
[9] Tanaka K, Hine DG, West-Dull A, Lynn TB. Gas chromatographic method of analysis of urinary organic acids. I. Retention indices of 155 metabolically important compounds. Clin Chem 1980;26:1841–6 [PubMed].
[10] Tanaka K, West-Dull A, Hine DO, Lynn TB, Lowe T. Gas-chromatographic method of analysis of urinaryorganic acids. II. Description of the procedure, and its application to diagnosis of patients with organic acidurias. Clin Chem 1980;26:1847–53 [PubMed].
[11] Chalmers RA, Lawson AM. Organic acids in man. Analytical chemistry, biochemistry and diagnosis of the organic acidurias. London: Chapman and Hall; 1982 [PubMed].
[12] Yinghua DU. Methods for screening urine for organic acids.US 2010/0126254 A1 2010;12:621–671. [Link].
[13] Bannett JM, Bradey CE. Simpler liquid-chromatographic screening for organic acid disorders. Clin Chem 1984;30:542–6 [PubMed].
[14] Lewis-Stanislaus AE, Li L. A method for comprehensive analysis of urinary acylglycines by using ultra-performance liquid chromatography quadrupole linear ion trap mass spectrometry. J Am Soc Mass Spectrom 2010;21:2105–16.
[15] Seashore MR. The organic acidemias: an overview. In: Pagon RA, Adam MP, Bird TD, editors. GeneReviews™ [Internet]. University of Washington, Seattle: Seattle (WA); 2001. p. 1993–2013 [Updated 2009 Dec 22, PubMed].
[16] Han LS, Ye J, Qiu WJ, Gao XL, Wang Y, Gu XF. Selective screening for inborn errors of metabolism on clinical patients using tandem mass spectrometry in China: a four-year report. J Inherit Metab Dis 2007;30:507–14 [PubMed].

[17] Han LS, Gao XL, Ye J, Qiu WJ, Gu XF. Application of tandem mass spectrometry in diagnosis of organic acidemias. Zhonghua Er Ke Za Zhi 2005;43:325–30 [PubMed].

[18] Zytkovicz TH, Fitzgerald EF, Marsden D, Larson CA, Shih VE, Johnson DM, et al. Tandem mass spectrometry analysis for amino, organic, and fatty acid disorders in newborn dried blood spots: a two-year summary from the New England newborn screening program. Clin Chem 2001;47:1945–55 [PubMed].

[19] Kuhara T. Diagnosis and monitoring of inborn errors of metabolism using urease-pretreatment of urine, isotope dilution, and gas chromatography-mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2002;781:497–517 [PubMed].

[20] Zhou XH, Obuchowski NA, McClish DK. Statistical methods in diagnostic medicine. 2nd ed. New York: Wiley-Interscience; 2011.

[21] Kumps A, Duez P, Mardens Y. Metabolic, nutritional, iatrogenic, and artifactual sources of urinary organic acids: a comprehensive table. Clin Chem 2002;48:708 [Pubmed, Link].

[22] Bennett JM, Powell S, Swartling JM, Gibson KM. Tiglylglycine excreted in urine in disorders of isoleucine metabolism and the respiratory chain measured by stable isotope dilution GC–MS. Clin Chem 1994;40(10):1879–83 [PubMed].