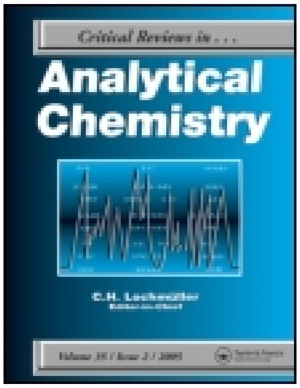

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Current Applications of Gas Chromatography/Mass Spectrometry in the Study of Organic Acids in Urine

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Current Applications of Gas Chromatography/Mass Spectrometry in the Study of Organic Acids in Urine

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Gas chromatography/mass spectrometry (GC/MS) is the most suitable technique for the diagnosis of disorders of organic acid metabolism, known as organic acidurias. They are inherited metabolic disorders that are characterized by an excessive amount of organic acids and their metabolites. Now in many clinical and diagnostic laboratories, automated, quantitative, and qualitative procedures for complex mixture analysis of urinary organic acids by GC/MS are used. In this review, current chromatography/mass spectrometry method and its applications in different diseases and disorders on the basis of urinary organic acids analysis are summarized. In this article, advantages and disadvantages of extraction and derivatization methods in the investigation of organic acids are reviewed. Moreover, the choice of an internal standard and typical procedure in the analysis of organic acids are presented.

Keywords Gas chromatography/mass spectrometry, organic acids, urine, diseases

INTRODUCTION

Human urine contains numerous organic compounds at various concentrations and other chemical groups of metabolites. Organic acid analysis is a powerful tool in the diagnosis of inborn errors of metabolism (IEMs). Many inborn errors of metabolism are classified as organic acidurias. They are characterized biochemically by accumulation of metabolites that are not present under physiological conditions. They are produced as a result of activation of alternative pathways in response to the loss of function of a specific gene product (enzyme) or by accumulation of pathological amounts of normal metabolites (Luo, 2004; Lepage et al., 2006).

Urinary organic acid analysis for metabolic profiling has traditionally been used for detection of inborn errors of metabolism. Organic acids are important metabolites in major metabolic pathways, such as the Krebs cycle or the pentose phosphate pathway. The accumulation of organic acids in biological fluids, especially in urine, can also provide useful information for an early diagnosis of metabolic disorders and neurological diseases (Kumps et al., 2002; Kałużna-Czaplińska et al., 2011; Rogers, 2006). The first report in the study of organic acid metabolism was made by Klenka and Kahlke in 1963 (1963) to identify phytanic acid in a patient with Refsum's disease using only mass spectrometry. In 1966 the analytical technique GC/MS was used to identify 3-hydroxyisovaleric acid in urine (Tanaka et al., 1966). This opened the way for further studies on organic acidurias with the use of chromatographic techniques. Mainly organic acids are analyzed by gas chromatography (GC) or gas chromatography-mass spectrometry (GC/MS). The analysis by gas chromatography or gas chromatography/mass spectrometry requires suitable sample preparation, in particular derivatization of the group in order to increase volatility of the acids (Shroads et al., 2004; Jones et al., 2005; Xu et al., 2001). Applications using high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) were described in Johnson (2005) and Ramautar and Somsen (2007), with the advantage of shorter analysis time than GC. However, due to the lower resolution power of HPLC and limitations in the detection of CE sensitivity, GC methods are more suitable for the profile analysis of organic acids only. The advantages and disadvantages of these two main techniques are presented in Table 1.

The term "organic acid" refers to a broad class of compounds used in metabolic processes of the human body. Chemically, organic acids are water-soluble compounds containing one or more carboxyl groups or acidic phenolic groups. The term is generally considered to include all carboxylic acids, with or without keto, hydroxyl, or other non-amino

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TABLE 1
Advantages and disadvantages in the analysis of organic acids in urine

Characteristics			
Gas chromatography		Liquid chromatography	
Detector			
one			one (two)
Advantage	Disadvantage	Adventage	Disadvantage
Derivatization			
necessary			not necessary
Selectivity			
excellent			Low
Resolution			
excellent			Low

functional groups, but not amino acids. Some nitrogen-containing compounds are sometimes regarded as organic acids, such as pyroglutamate, or amino conjugates like hippurate (benzoylglycine). Short-chain fatty acids are also included in this group. Accumulation of organic acids in urine can indicate metabolic dysfunction, but not only that. Measurement of urine organic acid profile is used in modern nutritional medicine as a simple, sensitive test that can demonstrate functional inadequacy of specific nutrients (Gambaro et al., 2000; Burdette, 2006; Boulat et al., 2003; Kałużna-Czaplińska et al., 2010a).

The aim of this review is to present the applications of GC/MS as an important analytical tool for the diagnosis of different diseases and disorders with the use of different analytical procedures for the determination of urinary organic acids.

GAS CHROMATOGRAPHY/MASS SPECTROMETRY IN THE STUDY OF ORGANIC ACIDS

Abnormal extraction acids are not necessarily related to inborn errors of metabolism (IEMs). They are abnormally extracted metabolites, which are connected with drug therapy, dietary non-IEM diseases, or physiologic conditions. Lactic aciduria and ketonuria are good examples. Lactic aciduria is generally accompanied by other compounds: p-hydroxyphenylacetate, p-hydroxyphenyllactate, glutarate, benzoate, and hippurate, which come from bacterial metabolism and intestinal infection (Kumps et al., 2002). Ketonuria (3-hydroxybutyrate and acetoacetate) is very often accompanied by 3-hydroxyisobutyrate, 3-hydroxyisovalerate, 2-hydroxybutyrate, dicarboxylic acids, and their 3-hydroxy derivatives with chain lengths up to C14. In this case the pattern could mimic a long-chain 3-hydroxyacyl-CoA dehydrogenase or trifunctional protein deficiency profile, except for a very high excretion of ketone bodies (Paik et al., 2005).

For many of the organic acids that are measured, their abnormally high levels in urine usually indicate low levels of a nutrient required to break down that compound. The presence of the urinary organic acid test helps to understand how nutrient metabolism is executed and determine where the imbalance in the metabolic cycle may be. Metabolic imbalance is a common and pervasive condition that may underlie many chronic complaints such as gastrointestinal dysfunction, muscular/joint problems, mood disorders, and headaches, which are commonly resistant to long-term treatment and sustained improvement. Organic acid testing provides a broad view of the body. One urine specimen can be used to evaluate gut, liver, and nervous system as well as energy metabolism and nutrient deficiencies.

Neurotransmitter metabolism imbalances in serotonergic or adrenergic function are frequently associated with neuroendocrine disorders such as insomnia, depression, adrenal fatigue, eating disorders, and irritable bowel syndrome (IBS). Neurotransmitter imbalances may indicate nutrient deficiencies or methylation impairments, which may have an impact on all the body systems (Burdette, 2006; Boulat et al., 2003). Cellular energy and mitochondria metabolism may be affected by metabolic imbalance too. The citric acid cycle supplies the body with its primary energy needs. Glycolysis, glycogenolysis, and beta-oxidation of fats provide precursors for this cycle. Blocks in the citric acid cycle or impairments in any of these processes may lead to chronic fatigue, muscle pain and fatigue, accelerated cell breakdown, and premature aging.

Chronic malabsorption can contribute to gastrointestinal dysfunction, nutrient insufficiencies, and dysbiosis. In turn, chronic dysbiosis may result in local inflammatory reactions, increased risk of colorectal cancers, or increased intestinal permeability, with an increased release of toxins and macromolecules into the body. All analytes and their derivatives characteristic for cellular energy, mitochondria function, and neurotransmitter metabolism imbalances are separated and identified with the use of gas chromatography/mass spectrometry method (Burdette, 2006; Boulat et al., 2003; Kałużna-Czaplińska et al., 2010a).

Homocysteine is considered to be one of many recognized risk factors for early cardiovascular (heart attack, stroke, thrombosis), neurodegenerative (Alzheimer's, macular degeneration), and other diseases. Methylation is important in converting homocysteine into methionine. Methylation is also crucial in keeping DNA from expressing cancer genes, and it is an important process in detoxication of everyday chemicals. The chemistry of folic and B12 is crucial to methylation. A more accurate determination is provided by the urinary organic acid metabolite methylmalonyl-CoA indicator, which requires B12 to break down valine into energy-yielding products, whereas B12 deficiency increases the level of methylmalonic acid in the organic acid assay. Moreover, organic acid testing is simpler than blood collection, as methylmalonic acid and other organic acids require only a single urine collection and not a blood draw (Rogers, 2006).

The presence of some organic acids in urine can be a major new factor in autism therapy. Oxalates, which may come from diet and human metabolism, are especially important in autism therapy (Shaw, 2008). In the case of autistic children, quantitative organic acid profiling can assess: mitochondrial energy production, fatty acid metabolism, carbohydrate metabolism, B-complex sufficiency, methylation of cofactors, neurotransmiter metabolism, oxidative damage, detoxification status, and bacterial and yeast overgrowth. Organic acids may also result from exogenous sources such as diet, drugs, or bacterial contamination. Biochemical anomalies connected with organic acids have been identified in many people with autism (Shaw, 2008; Neustadt, 2006; Fomina et al., 2005; Kałużna-Czaplińska et al., 2010b). A biochemical acid profile may play an important role in understanding the aetiology of autism and different developmental disorders in children. The metabolic organic acid profile analysis determines 39 organic acids, including 8 gastrointestinal metabolites, 13 cellular energy metabolites, 4 neurotransmitter metabolites, and 14 amino acid metabolites (Shaw, 2008). The analysis of organic acids in urine by GC/MS is a sensitive test that can reveal evidence of functional inadequacy of specific nutrients in autistic children. Increased citrate levels can suggest amino acid deficiency or problems with protein metabolism. Succinate cannot play its role in cellular energy production via the citric acid cycle when coenzyme Q10 (CoQ10) is inadequate. Elevated succinate excretion is a marker of deficiency of CoQ10 and riboflavin. Adipate and suberate are the products of incomplete oxidation in the omega-oxidation pathway (Shaw, 2008; Kuhara, 2007). Fig. 1 shows typical organic acid chromatograms of autistic children with biochemical anomalies connected with neurotransmitter metabolism, mitochondrial metabolism, and gastrointestinal function (Kałużna-Czaplińska, 2008).

Another developmental disorders associated with higher extraction of 3-methylglutaconic acid (3-MGC) is developmental language disorder (DLD) (Gunay-Aygun et al., 2009). The etiology of DLD is unknown. The majority of children with DLD do not have additional neurological abnormalities.

The analytical procedures for determination of urinary organic acids usually consist of three steps, which will be discussed below:

- Extraction of organic acids from the urine (problems with artifacts)
- Derivatization of organic acids to more stable and volatile compounds
- GC/MS separation and identification of the derivatized compounds

EXTRACTION OF URINARY ORGANIC ACIDS AND PROBLEMS WITH ARTIFACTS

The most commonly used extraction of organic acids from biological fluids (especially urine) is an extraction with organic solvents. Solvent extraction is usually performed with ethyl acetale or diethyl ether (Grkovic et al., 2005; Peters et al., 2008). The solvent extraction involves acidification of urine, followed by saturation with NaCl and extraction with the presented organic acids. Solvent extraction has many advantages: it is an easy and rapid method that enables efficient extraction of aromatic carboxylic acid and it is associated with lower contamination by inorganic sulfates and phosphates (Niessen and Dekker, 2001). But there are disadvantages too: neutral compounds such as urea and glicerol are extracted and the extraction of polyhydroxycarboxylic acids is less efficient (Niwa, 1995). The alternative to solvent extraction is the anion exchange extraction method. After the introduction of urine to the column, neutral and basic compounds are washed with water. Organic acids are then eluted with aqueous pyridinum acetal buffer. Before the anion exchange extraction, barium hydroxide is added to urine to remove a large amount of inorganic phosphate and sulfate by precipitation. The use of weak anion exchange resin (diethylaminoethyl-Sephadex) gives higher recoveries for the more polar acids and allows higher reproducibility (Peters et al., 2008), as well as highly efficient extraction of polyhydroxycarboxylic acids. Another advantage of anion exchange extraction is lack of neutral compound extraction such as urea and glicerol. The disadvantage is time-consuming lyophilization. Moreover, inorganic phosphate is extracted even after barium hydroxide precipitation, extraction of very important citric acid and aconitic acid is less efficient if barium hydroxide precipitation is used, and complete dehydration of the extracted samples of urine is difficult. The extraction of urinary organic acids on disposable columns filled with a trimethylaminopropyl phase (strong anion-exchange material) was proposed by Kumps and coworkers. (2004). The use of solid-phase extraction on normal-phase silica columns was reported. Organic acids can be eluted by chloroform and a mixture of chloroform and t-amyl alcohol. These procedures are very rapid and reliable. Liu and coworkers (2004) developed a solid-phase extraction procedure for the detection of organic acids in urine by GC/MS using a strong anion exchange column (Sep-Pak Vac RC, Accell Plus QMA cartridge). This method provides the advantage of higher recoveries (90 to 100%) for a wide range of interest and therefore can be a potential alternative to liquid-liquid extraction for organic acid screening. It is especially sensitive for the detection of some polar compounds such as 3-OH-glutaric and *N*-acetylaspartic acids. Important advantages of the SPE method compared to the liquid-liquid extraction method are the short extraction times, lack of emulsions, use of disposable equipment, and reduced consumption of organic solvents.

Solid-phase microextraction (SPME) was first used by Liebich and coworkers in 1998 (1998). They developed a new sample preparation method for profiling organic acids in urine by GC or GC/MS. The method includes derivatization of the organic acids directly in the aqueous urine using trimethyloxonium tetrafluoroborate (TMO) as a methylating agent, extraction of the organic acid methyl esters from the urine by solid-phase microextraction using a polyacrylate fiber with a thickness of

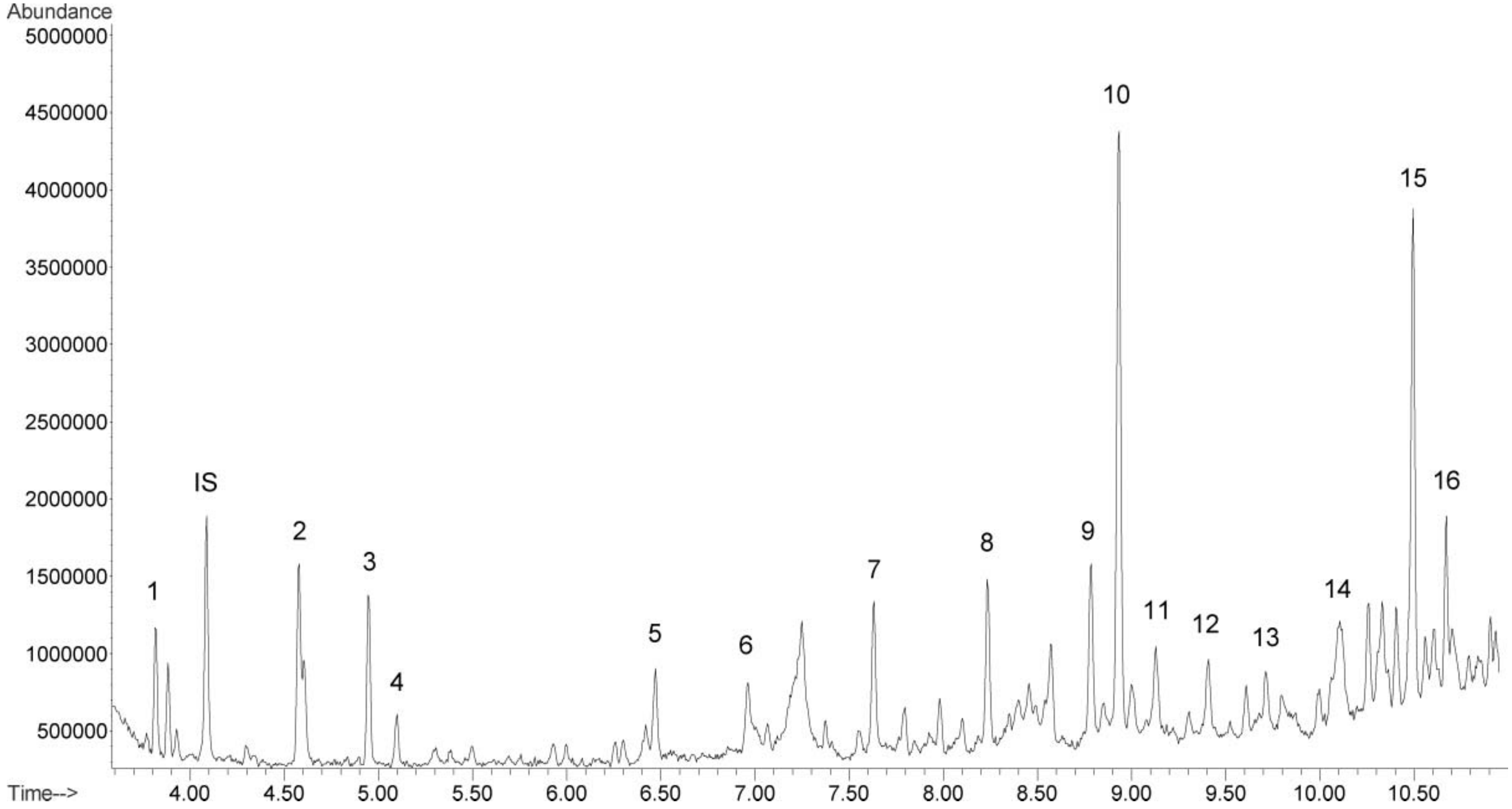


FIG. 1. Total ion chromatogram of TMS-derivatized organic acids in urine from autistic children. The numbered peaks represent the following acids: methylmalonic acid (1), ethylmalonic acid (2), succinic acid (3), methylsuccinic acid (4), methylglutaric acid (5), adipic acid (6), methyladipic acid (7), hydroxyphenylacetic (8), suberic acid (9), hydroxybenzoic acid (10), stearic acid (11), azelaic acid (12), homovanillic acid (13), citric acid (14), hippuric acid (15), sebacic acid (16), malonic acid (IS) (Kałużna-Czaplińska, 2008).

TABLE 2
GC/MS system with temperature programming and the most typical procedure used in analysis of organic acids

Extraction/derivatization	GC conditions (temperature programming)	MS conditions and analyzer	Ref.[a]
SPME (solid-phase microextraction) fibers with different coating of PA, CW–DVB, PDMS–DVB, PDMS, CAR–PDMS were used to obtain the best extraction efficiency. Derivatization reagent: DES as well as the ion pairing agent, tetrabutylammonium hydrogensulfate (TBA–HSO_4).	GC split/splitless injector, 250°C. Temperature program: 40°C (held for 1 min) to 200°C at a ramp of 10°C/min followed by a ramp of 20°C/min to 280°C and held for 5 min. Columns: HP-5MS (5% diphenyl and 95% dimethyl polysiloxane phase, 30 m × 0.25 mm ID × 0.25 μm film). Gas flow: 1.0 mL/min	Electron impact ionization (EI) with a nominal electron energy of 70 eV. MS ion source temperature: 230°C. Quadrupole temperature and transfer line temperature: 150 and 280°C, respectively. Scan range: m/z 40–350.	(1)
SPME (solid-phase microextraction) fibers: 65 μm poly(dimethylsiloxane)/divinylbenzene (PDMS/DVB), 75 μm carboxen/polydimethylsiloxane (CAR/PDMS), 100 μm poly(dimethylsiloxane) (PDMS), 65 μm carbowax/ divinylbenzene (CW/DVB), and 85 μm polyacrylate (PA) were used. Derivatization reagent: TFAA (trifluoroacetic anhydride) to form o-trifluoroacetylcreatinine was performed.	GC split 1:10 injector, 230°C. Temperature program: 200°C for 1 min, 200°C to 240°C at 8°C/min, and hold for 7 min. Columns: DBWAX (30 m × 0.25 mm I.D. 0.25 μm film) Gas (helium) flow: 1.0 mL/min	Electron impact ionization (EI) with a nominal electron energy of 70 eV. MS ion source temperature: 200°C. Quadrupole temperature and transfer line temperature: 150 and 280°C, respectively. Scan range: m/z 40–450.	(2)
SPE (solid phase extraction) using CHEMELUT extraction column. Derivatization reagent: *N*, *O*-Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane for 10 min at 60°C.	GC split 50:1 injector, 300°C. Temperature program: 60°C to 300°C at a ramp of 24°C/min. Columns: DB-1 fused-silica capillary column (30 m × 0.25 mm × 0.25 μm)	Ion trap. The transfer line is held at 260°C.	(3)
SPE (solid phase extraction) using a strong anion exchange column (Sep-Pak Vac RC, Accell Plus QMA cartridge). Derivatization reagent: *N*-methyl-trimethylsilyltrifluoroacetamide (MSTFA) and acetonitrile (1:1, v/v) at room temperature.	GC split/splitless injector, 250°C. Temperature program: 80°C for 2.0 min, ramp 6.5°C/min to 200°C, and then 12°C/min to 300°C, held for 6 min. Columns: HP-5 capillary column (5% diphenyl and 95% dimethyl polysiloxane phase, 0.5 μm film thickness, 25 m × 0.2 mm i.d.) Gas (helium) flow: 0.8 mL/min	Electron impact ionization (EI) with a nominal electron energy of 70 eV. MS ion source temperature: 230°C. Quadrupole temperature and transfer line temperature: 150 and 280°C, respectively. Scan range: m/z 50–550.	(4)
SPE (solid phase extraction) using strong anion-exchange (SAX) cartridges. Derivatization reagent: Trimethyloxonium tetrafluoroborate (TMO).	GC split 1:30 injector, 280°C. Temperature program: 40°C to 260°C at 2°C/min, then held at 260°C for 30 min. Column: OV1701 (25 m × 25 mm fused-silica column). Gas (helium) flow: 1 mL/min	Triple stage quadrupole (TSQ) Electron impact (EI); temperature of ion source, 150°C; pressure, 1.33 Pa; emission current, 200 mA. Mass scan from 40 to 400 m/z.	(5)

(Continued on next page)

TABLE 2
GC/MS system with temperature programming and the most typical procedure used in analysis of organic acids *(Continued)*

Extraction/derivatization	GC conditions (temperature programming)	MS conditions and analyzer	Ref.[a]
LLE (liquid-liquid extraction), with tetrahydrofuran (THF) and ethyl acetate (EtOAc) as extraction solvents. Derivatization reagent: N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) pyridine at 60°C for 15 min.	GC split 1:10 injector, 250°C. Temperature program:Columns: HP-5 capillary column (5% diphenyl and 95% dimethyl polysiloxane phase, 0.5 μm film thickness, 25 m × 0.2 mm i.d.)	Quadrupole Mass spectra (70 eV) in the electron-impact ionization mode. Source and quadrupole temperatures were maintained at 230°C and 150°C, respectively, and the capillary direct interface was heated to 280°C. Scan range: m/z 50–550 amu.	(6)
LLE (liquid-liquid extraction), with ethylacetate. Derivatization reagent: BSTFA + 1% TMCS (trimethylchlorosilane) at 60°C for 60 min.	GC split/splitless injector, 250°C. Temperature program: 90°C to 280°C with an increment of 3°C per min. Columns: CP-Sil 8 CB capillary column (length 30 m, internal diameter 0.25 mm, film 0.25 μ).	Quadrupole transfer line 280°C, ion source 150°C, and mass analyzer 35°C. Mass spectrometer was programmed from m/z 10 to 650 amu.	(7)
LLE (liquid-liquid extraction), with diethyl ether and ethyl acetate. Derivatization reagent: N−methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) at 60°C for 1 h.	GC split 1:10 injector, 260°C. Temperature program: 100°C for 2 min, programmed to 260°C at a rate of 3°C/min and finally to 300°C at a rate of 20°C/min with holding time for 10 min. Columns: DB-5 and DB-17 (OV-17 bonded) fused-silica capillary columns (30 m × 0.25 mm i.d., 0.25 m film thickness) Gas (helium) flow: 1.0 mL/min	The temperature interface and ion source were 300 and 230°C, respectively. The mass range scanned was 50–650 amu at a rate of 0.99 scan/s.	(8)

a (1) Li et al., 2007; (2) Tsai and Huang, 2008; (3) Hagena et al., 1999; (4) Liu et al., 2004; (5) Liebich and Gesele, 1999; (6) Wittmann et al., 2008; (7) Wajner et al., 2009; (8) Paik and Kim, 2004.

85 μm, and transfer of methyl esters into the GC or GC/MS instrument. Desorption of the analytes takes place in the heated injection port. The suggested sample preparation is very simple. The organic acids are directly methylated in the aqueous urine. There is no need for any evaporation step and for the use of an organic solvent. Table 2 (first column) presents the most typical extraction procedures used in the analysis of organic acids in urine (Liu et al., 2004; Li et al., 2007; Tsai and Huang, 2008; Hagena et al., 1999; Liebich and Gesele, 1999; Wittmann et al., 2008; Wajner et al., 2009; Paik and Kim, 2004).

In organic acid analysis there are problems with artifacts due to either medication or dietary manipulation. For example, in the case of people receiving valproic acid, it can be well masked by other important metabolites including organic acids. Children who receive milk formulas supplemented with medium-chain triglycerides exhibit a characteristic urinary acid profile, which is very similar to patients with a mitochondria fatty acid β-oxidation defect (Korman et al., 2000). There are great difficulties in identifying some organic acids because they occur in very small amounts or because they are not completely extracted prior to derivatization. The trimethylsilyl (TMS) derivative of 4-hydroxybutyric acid elutes very close to the urea peak, and in the case of some patients who excrete very small amounts this acid may be missed (Hori et al., 2005). Another example is succinyl acetone, which is a major marker of tyrosinemia type 1 (Zhang et al., 2000; Yoshida et al., 2005). This compound may disappear in alkaline urine samples and can be missed when it is present in small amounts in urine. Organic acids are very difficult for analysis in the case of children with different disorders. These children receive many medications, vitamins, and supplements that can give a false organic acid profile.

DERIVATIZATION OF ORGANIC ACID EXTRACTS

Organic acids are polar and thermally unstable and they have low volatility, so it is necessary to convert them into polar, volatile, and thermally stable derivatives prior to GC/MS analysis. A very popular method used in derivatization is trimethylsilylation. The following trimethylsilylating reagents are commercially available:

- N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)
- N,O-bis(trimethylsilyl) acetamide (BSA)
- N-trimethylsilylimidazole (TMSI)
- trimethylchlorosilane (TMCS)
- hexamethyldisilazane (HMDS)
- N-trimethylsilylacetamide (TMSA)
- N-methyl-N-trimethylsilylacetamide (MTMSA)
- N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA)
- N-trimethylsilyldimethylamine (TMSDMA)
- N-trimethylsilyldiethylamine (TMSDEA)

The most common derivatives are methyl esters. One of their advantages is that their mass spectra recorded by GC/MS are easy to interpret and they are preferable for the examination of organic acids in clinical chemistry and biochemistry (Jones and Chalmers, 2000; Purevsuren et al., 2008).

TMS($-Si(CH_3)_3$) derivatives display good separation on the chromatographic column and characteristic fragment ions $[M-15(CH_3)]^+$ in electron impact (EI) mass spectra are observed. The EI mass spectra of the TMS derivatives often show relatively intense fragment ions at m/z 73, $[Si(CH_3)_3]^+$, and m/z 147, $[(CH_3)_2\text{-}Si{=}O\text{-}Si(CH_3)_3]^+$. The appearance of an intense ion at m/z 147 indicates that there are more than two TMS groups in a molecule. TMS derivatives are unstable under humid conditions and susceptible to temperature and fragmentation of the TMS derivatives. Thus, it is difficult to obtain enough information for the analysis of molecular structures. Another method th isat very often used is trimethylsilylation with N, O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS). Derivatization by tert-butyldimethylsilyl (t-BDMS) was first reported in 1982 (Fernandes et al., 2006). T-BDMS reacts with active hydrogen in -OH, -COOH, $-NH_2$, and -SH groups. The advantages of t-BDMS compared to TMS are: stability against moisture, good separation by GC/MS, and an intense fragment peak at $(M-57)^+$, which is received by the dissociation of the tert-butyl group from a molecular ion. The disadvantages are: less data in mass spectra of organic acids and longer retention time on GC, especially in the case of polyhydroxy carboxylic acids (Bengtsson and Lehotay, 1996). Methylation using diazomethane (CH_2N_2) is easy to perform because diazomethane reacts easily with carboxyl groups and phenolic hydroxyl groups (Niessen and Dekker, 2001). Diazomethan is highly toxic, explosive, and unstable after storage for a long period of time. It is synthesized when necessary. Ketoacids are analyzed as their oxime (hydroxime, HO-TMS), methooxime (methyloxime, MO-TMS) (Purevsuren et al., 2008), ethoxime (ethyloxime, EO-TMS), or methooxime-methyl ester derivatives (Cyr et al., 2000). Keto acids are unstable and should be stabilized by the formation of oxime before esterification. Hydroxylamine hydrochloride is used for oximation, O-methylhydroxylamine hydrochloride for methoximation, and O-ethylhydroxylamine hydrochloride for ethoximation. For example, O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride was used to convert oxoacids, aldehydes, and ketones present in urine into oximes (Niessen and Dekker, 2001). Table 2 (first column) presents the most typical derivatization procedure used in the analysis of organic acids in urine (Liu et al., 2004; Li et al., 2007; Tsai and Huang, 2008; Hagena et al., 1999; Liebich and Gesele, 1999; Wittmann et al., 2008; Wajner et al., 2009; Paik and Kim, 2004).

CHOICE OF AN INTERNAL STANDARD IN ORGANIC ACID ANALYSIS

The choice of at least one suitable internal standard is essential in organic acid analysis. Whether quantitative analysis of certain acids is performed or whether semiquantitative analysis is used to assess the metabolite profile, the internal standards (IS) should be added at the very beginning of the sample preparation (Niessen and Dekker, 2001; Hübschmann, 2009). The most popular internal standards are: tricarballylic acid, tropic, undecanoic acid, hexadecandioic acid, pentadecanoic acid, heptadecanoic acid, isopropylmalonic acid (Niessen and Dekker, 2001), malonic acid (Kałużna-Czaplińska et al., 2010b), 2-hydroxyvaleric acid, 2-phenylbutric acid (Niessen and Dekker, 2001), and 2-ketocaproic acid (Liu et al., 2004). These internal standards are tested in urine organic acid because their chemical structure and absence in normal and pathological urine are known (Hübschmann, 2009). Sometimes interferences such as similar retention time and common specific ion with acids are likely to be found in samples, leading to the rejection of the candidate as an internal standard (Niessen and Dekker, 2001). Malonic acid can be unsuitable for internal standard in some urine organic analysis because it interferes with detection of malonic aciduria in malonyl-CoA decarboxylase deficiency (Fernandes et al., 2006). Analysis using stable isotope dilution is available, but a high price and wide spectrum of compounds determined in organic acid analysis make it less often used in laboratory analysis (Yoon et al., 2005; Kuhara, 2001).

GAS CHROMATOGRAPHY COLUMNS

Different capillary columns are available for organic acid separation and analysis. For the analysis of TMS derivatives of extracted organics acids, it is suitable to use columns coated with the dimethylsiloxane-type chase, DB-1, DB-5, DB-5MS (Wittmann et al., 2008; Yoon et al., 2005), and in the case of oxime-TMS capillary columns with stationary phases such as OV-1, OV-5, OV-17 (Niessen and Dekker, 2001; Fernandes et al., 2006). In considering the length, diameter, and film thickness of the column, it is necessary to account for sample capacity, duration of analysis, required separating power, and requirements of the mass spectrometers. In the urine analysis of organic acids, especially inborn errors of metabolism (IEMs), enantiomeric form of acids and their metabolites are observed. The use of a conventional nonchiral column cannot determine which enantiomer is present in the sample. However, the development of new chiral columns using modified cyclodextrin made it possible to analyze different classes of enantiomers (Niessen and Dekker, 2001). Usually the 30 m columns for organic acid analysis in urine are preferred. The column diameter depends upon the MS requirements and mostly the widebore columns (0.32 mm ID) are used. Narrowbore columns (0.22 mm ID) are preferable to split/splitless injection when the concentration of organic acids is low. Film thickness affects resolution (with thick films increasing resolution, retention, and sample capacity). For many organic acid analyses a standard film thickness of 0.25–0.5 μm is good enough, and helium as a carrier gas is used at a flow rate of 1–2 mL/min (Liu et al., 2004; Li et al., 2007; Tsai and Huang, 2008; Hagena et al., 1999; Liebich and Gesele, 1999; Wittmann et al., 2008; Wajner et al., 2009; Paik and Kim, 2004).

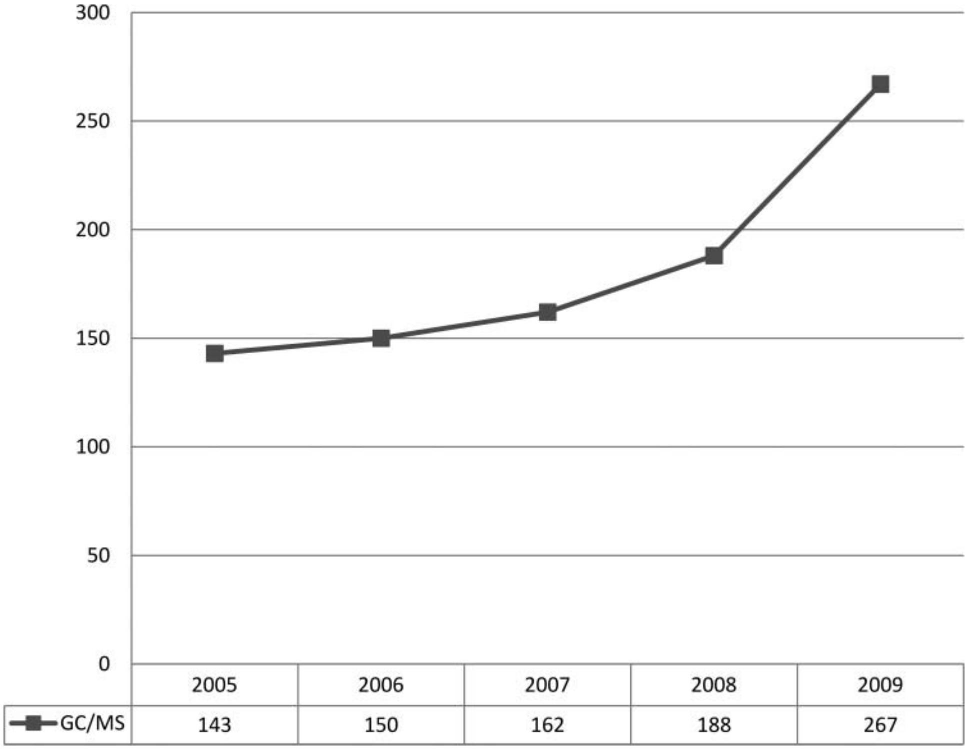
SAMPLE INJECTION, TEMPERATURE PROGRAMMING, AND MOST TYPICAL PROCEDURES USED IN ANALYSIS OF ORGANIC ACIDS

In the case of organic acid analyses in urine, the use of capillary columns and sample injection with splits from 1:20 to 1:60 is recommended. The injection temperature is between 200° and 250°C. Injection volume of 1–2 μL is often used. Analyses of organic acids in urine include a wide range of compounds. In order to separate and identify these compounds, temperature programming is advisable. Table 2 presents the most common sample injection, temperature programs, and procedures used in identification and quantification of organic acids in urine (Liu et al., 2004; Li et al., 2007; Tsai and Huang, 2008; Hagena et al., 1999; Liebich and Gesele, 1999; Wittmann et al., 2008; Wajner et al., 2009; Paik and Kim, 2004).

GC/MS SEPARATION AND IDENTIFICATION OF THE DERIVATIZED COMPOUNDS

Several instrument configurations are commercially available. They allow positive identification of organic acids in urine by their chromatographic retention time and the mass spectra usually obtained from either quadrupole filter or ion trap mass spectrometers in the electron impact ionization mode. A typical acquisition setup for organic acid analysis in scan mode with an MSD (Mass Selective Detector) is as follows: impact voltage 70 eV, electron multiplier voltage 2200, scan range m/z 50–550 at 2 scans/1.3 second. Software for data analysis is available and recommended. The use of a computer library of mass spectra for comparison and visualization of the spectra is essential for identification and interpretation of each urine sample.

Sometimes in the laboratory there are attempts to automate sample preparation for analysis of organic acids by gas chromatography-mass spectrometry using a computer-controled, automated robotic workstation, which is integrated and connected to the gas chromatography/mass spectrometry



	2005	2006	2007	2008	2009
■ GC/MS	143	150	162	188	267

FIG. 2. The diagram shows the frequency of total publications from 2005 to 2009 about gas chromatography/mass spectrometry in the study of organic acids. Bibliographic research in Chemical Abstracts Plus containing the keywords "organic acids and GC/MS or LC/MS or CE/MS" using SciFinder Scholar. A total of 910 journal articles were found.

system. Bengtsson and Lehotay (1996) first developed two methods: one employed solvent extraction, while the other utilized a silica, solid-phase extraction cartridge. Both automated methods were compared to a manual solvent extraction procedure, which is used routinely by researchers in the laboratory. Normal, spiked urine, and urine from patients with a variety of metabolic abnormalities were analyzed. The robotic workstation did not meet all requirements for a rapid, reliable laboratory device. Recoveries with the automated procedure were less than with the manual method, and some organic acids important in the diagnosis of inborn errors of metabolism were not detected. Additionally, the robotic device had mechanical and design problems that made it slower and less reliable than the manual procedure.

CONCLUSIONS

An increasing interest in the application of gas chromatography and mass spectrometry methods in organic acids analyses has not changed, despite the passage of time (Fig. 2). Gas chromatography/mass spectrometry (GC/MS) has become an important analytical tool in the determination of organic acids in biological fluids, especially in urine. Many organic acidurias were first discovered by GC/MS, which is even today an essential technique for the final diagnosis of most organic acidurias. Capillary GC allows the most efficient separation of organic acids and gives chromatographic information indicating very precise retention times for these compounds of endogenous and exogenous origin. Mass spectrometry coupled with gas chromatography, with full scan mass spectra and automated identification, quantification, or semiquantification can be used to evaluate 200 metabolites. GC/MS analysis is effective for detecting abnormal metabolics of organic acids and assessment of: mitochondrial energy production, fatty acid metabolism, carbohydrate metabolism, B-complex sufficiency, methylation of cofactors, neurotransmitter metabolism, oxidative damage, detoxification status, and bacterial and yeast overgrowth.

In the near future, the number of diagnosable diseases connected with the organic acid profile will increase. GC/MS provides an absolute identification of the metabolites in the complex profiles of urinary organic acids and is used for screening or routine analysis of the acids in laboratories. In the future organic acid analysis by GC/MS may enable the determination of individual nutrient insufficiencies affecting our health.

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