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Short communication

Solid phase extraction procedure for urinary organic acid analysis by gas chromatography mass spectrometry

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Abstract

We have developed a solid phase extraction procedure for the detection of organic acids by GC–MS using a strong anion exchange column (Sep-Pak Vac RC, Accell Plus QMA cartridge). Extraction efficiencies of 25 organic acids were established by analyzing standards in water based solutions. High extraction efficiencies (90 to 100%) were found for many of the compounds studied. We estimated the limit of detection for 48 organic acids and glycine conjugates. They were below 5 nmole with the exception of malonic and oxalic acids and mevalonic acid lactone. This method provides the advantage of higher recoveries for a wide range of compounds 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.

Keyword: Organic acids

1. Introduction

Organic acid analysis is a powerful tool in the diagnosis of inborn errors of metabolism. It is based upon the recognition and interpretation of characteristic urinary patterns of organic acids and glycine conjugates obtained by gas chromatography/mass spectrometry. The sample preparation requires the isolation of all of the organic compounds of interest from aqueous solution [1]. Because of the large number of organic compounds detectable in urine, it is difficult to have an ideal isolation method combining high extraction recoveries and good detection sensitivities for all the compounds of interest. New isolation procedures may provide alternative methods to the study of urine organic acids, or be used in conjunction with the standard extraction methods to increase detection sensitivities for specific organic acids.

The most commonly used urine extraction procedure for organic acids involves solvent partition using ethyl acetate and/or diethyl ether, prior to sample derivatization. This extraction method yields good recoveries for a wide range of clinically significant organic acids and glycine conjugates [2,3], but compounds with higher polarities are generally extracted with lower efficiencies. Examples are *N*-acetylaspartic, homogentisic, methylcitric, and 2- and 3-hydroxy-glutaric acids. Sometimes separate methods are needed for the isolation of these compounds to facilitate quantification, especially when present in low concentrations. The application of solid phase extractions (SPE) with different stationary phases has been investigated previously. Studies using weak anion exchange [4–7], strong anion exchange [8–10], and some other [11,12] disposable columns were reported, although low recoveries for many clinically significant organic compounds limited their use in organic acid screening for metabolic disorders.

The purpose of this work was to further evaluate solid phase extraction for organic acid analysis using silica/quaternary methylamine phase strong anion exchange cartridges (Accell plus QMA strong anion exchanger). The goal was to develop a reliable sample isolation method with good sensitivities for a wide range of clinically significant metabolites and lower maintenance requirements on GC column and MS ionization source.

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2. Experimental

2.1. Chemicals

Hexanoylglycine, suberylglycine, propionylglycine, and 3-OH-glutaric acid were purchased from Dr Herman J. ten Brink (Amsterdam, The Netherlands). *N*-Isovalerylglycine, *N*-tiglylglycine, and 3-OH-propionic acid were purchased from TCI America (Portland, OR, USA). Phenylpropionylglycine was synthesized in our lab using the procedure described by Gregersen et al. [13]. The derivatizing reagent *N*-methyl-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Pierce (Rockford, IL, USA). The separation cartridges used for solid phase extraction were Sep-Pak Vac RC (500 mg) Accell^{TM} Plus QMA Cartridges (15 cc reservoirs, Part # WAT054500), manufactured by Waters Corp. (Milford, MA, USA). All other reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA). All organic solvents were of analytical reagent grade, obtained from Fisher Scientific (Houston, TX, USA).

2.2. Solid phase extraction

Water based standard solutions were prepared to establish the extraction efficiencies and evaluate detection limits. For most carboxylic acids, aliquots containing 200–250 nmole of each standard were used for extraction. For keto-acids, aliquots containing 300–500 nmoles of each acid were extracted. 3-Chloromandelic acid was used as internal standard (ISTD). Urine specimens from patients with metabolic disorders were extracted to evaluate the applicability of this procedure to organic acid screening. We used a volume of urine equivalent to 1 μmole of creatinine for analysis. The typical amount of sample used was between 50 and 2500 μL, depending on the creatinine concentration of the sample. The extraction procedure was the same for both standard solution and urine samples. Standards or urine samples were added to a fixed volume of ISTD. Keto-acids were converted to oxime derivatives before extraction by a 20-min incubation at room temperature with 100 μL of 5% hydroxylamine solution, pH 7. The samples were then diluted to 10 mL with deionized water. The SPE columns were conditioned with 5 mL of methanol followed by 5 mL of water prior to loading the samples. The solvent and sample flow rate through the column was controlled at 2–3 mL per minute under vacuum. The columns were then washed with 10 mL of methanol-water solvent (20:80, v/v) and then 1 mL of hexane. Organic acids were eluted with 4 mL of 5% (v/v) formic acid in methanol. The eluate was evaporated at 40 °C to dryness under a stream of nitrogen. Samples were derivatized by addition of 200 μLof mixture of MSTFA and acetonitrile (1:1, v/v) at room temperature.

2.3. Gas chromatography mass spectrometry

The analysis was carried out on an Agilent GC–MS system (6890N GC system and 5973N mass selective detector), equipped with the HP-5 capillary column (5% diphenyl and 95% dimethyl polysiloxane phase, 0.5 μm film thickness, 25 m × 0.2 mm i.d.). The electron ionization (El) source was operated at 230 °C. Helium was used as the carrier gas at a constant flow rate of 0.8 mL/min. Samples were introduced into GC in splitless mode. The injection volume was 0.5 μL. The injection port was maintained at 250 °C. Column temperature profile was 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. The total run time was 35 min. Data were acquired in a full scan mode with *m*/*z* range 50–550, using ChemStation software. A custom-made mass spectra library was used for identification of the organic acids.

3. Results and discussion

We studied 48 important compounds, including most of the carboxylic acids, keto-acids and glycine conjugates commonly seen in urine to evaluate the method. The extraction efficiencies of 25 organic acids were determined by comparison of the GC–MS response of an analyte after SPE to that obtained by direct injection of the same amount of the

Table 1
Solid phase extraction efficiencies of organic acids (in %)

Organic acid	Efficiency (%)	S.D. (%)	Organic Acid	Efficiency (%)	S.D. (%)
Adipic acid	102	1	4-Hydroxyphenylacetic acid	102	1
Citric acid	105	10	4-Hydroxyphenyllactic acid	97	1
Dodecanedioic acid	101	1	3-Hydroxypropionic acid	53	1
Ethylmalonic acid	109	3	Lactic acid	89	5
Fumaric acid	100	1	3-Methylglutaric acid	102	1
Glutaric acid	100	1	Methylsuccinic acid	120	1
Glyceric acid	89	1	Methylmalonic acid	52	8
Glycolic acid	80	6	Oxalic acid	4	1
Homogentisic acid	84	2	Pyroglutamic acid	98	1
3-Hydroxybutyric acid	72	3	Suberic acid	106	1
4-Hydroxybutyric acid	100	2	Succinic acid	106	1
3-Hydroxyglutaric acid	100	1	Tetradecanedioic acid	104	2
3-Hydroxyiso valeric acid	42	8			

Table 2
Organic acids studied with estimated LOD

Estimated SOD < 2 nmole		
N-Acetylaspartic acid	3-Hydroxy-3-Methylglutaric acid Orotic acid	Orotic acid
cis-Aconitic acid	4-Hydroxybutyric acid	Propionylglycine
Adipic acid	2-Hydroxyglutaric acid	Pyroglutamic acid
Citric acid	3-Hydroxyglutaric acid	Pyruvic acid
Dodecanedioic acid	4-Hydroxyphenylacetic acid	Sebacic acid
Ethylmalonic acid	4-Hydroxyphenyllactic acid	Suberic acid
		Suberylglycine
Fumaric acid	4-Hydroxyphenylpyruvic acid	Succinic acid
Glutaric acid	Isovalerylglycine	Succinylacetone
Glyceric acid	2-Ketoisocaproic acid	Tetradecanedioic acid
Glycolic acid	Lactic acid	Tiglylglycine
Glyoxylic acid	Methylcitric acid	
Hexanoylglycine	3-Methylglutaric acid	
Homogentisic acid	Methylsuccinic acid	
Estimated LOD 2–5 nmole		
Acetoacetic acid	3-Hydroxypropionic acid	Methylmalonic acid
3-Hydroxybutyric acid	2-Keto-3-Methylvaleric acid	Phenylpropionylglycine[a]
3-Hydroxyisovaleric acid	2-Ketoisovaleric acid	
Estimated LOD > 20 nmole		
Malonic acid	Mevalonic acid lactone	Oxalic acid

[a] This compound was synthesized in our lab. The estimated purity was 75%.

analyte. Both samples were derivatized under the same conditions before injection. To compensate for the variability in extraction and injection volumes, the responses were all normalized against the ISTD (A), 3-chloromandelic acid, which had a recovery close to 100% with this procedure. All the samples were studied in triplicates. Table 1 reports the average of the extraction efficiency and the standard deviation (S.D.) of each compound presented as percentage. Among the 25 compounds listed in the table, 16 of them had extraction efficiencies close to 100%. The recoveries for compounds with multi-peak formation, such as keto-isovaleric acid, were not calculated.

We estimated the limit-of-detection (LOD) for the compounds studied. The LOD was estimated as the amount of the analyte, in nmoles extracted from an aliquot of sample, which would result in a peak with total ion abundance equivalent to 0.5% of the abundance of the ISTD (A), 3-chloromandelic acid. The SPE conditions minimized the background noise and allowed good quality of mass spectra for compound identification. Table 2 summarizes the estimated LOD for the 48 compounds. With the three exceptions of malonic and oxalic acids and mevalonic acid lactone, all the other acids studied had estimated LOD below 5 nmole, most of them around 1–2 nmole, which should be sufficient for organic acid screening. Higher LOD (∼2 nmole) were observed for *N*-acetylaspartic, homogentisic, methylcitric, 4-OH-butyric, 2- and 3-OH-glutaric acids, succinylacetone and some of the glycine conjugates including tiglylglycine and propionylglycine. This represents the strength of this method, since these compounds are generally not extracted well with organic solvents.

The recoveries of several organic acids from urine were also evaluated. Known amounts of acids were added to urine samples prior to extraction. The difference between the measured (after subtracting endogeneous contribution from urine matrix) and the added amounts was less than 20% for each of the acids studied, confirming the data obtained in aqueous solutions of standards (data not shown). In order to evaluate the applicability of the SPE to clinical screening, we analyzed a number of urine samples from patients with known metabolic disorders including organic acidemias and fatty acid oxidation defects. In all cases, the abnormal metabolites, characteristic of each disease, were recovered and allowed a correct diagnosis. Three examples are presented here in comparison with solvent extraction (SE) method.

Fig. 1 shows two GC–MS chromatograms of the same urine specimen from a patient with 3-OH-3-methylglutaryl-CoA lyase deficiency (HMG-CoA). Chromatogram (A) was obtained after SPE and (B) after SE. (In this case, the sample prepared with SPE procedure was injected in a split mode with a ratio of 1:10. Therefore the absolute abundances in the two chromatograms are not comparable). The abnormal metabolites, characteristics of this disease, include 3-OH-3-methylglutaric acid, 3-methylglutaconic acid, 3-methylglutaric acid, 3-OH-isovaleric acid, and glutaric acid. In both solid phase and solvent extractions, all the characteristic organic acids were detected. The relative abundances of 3-methylglutaric, 3-methylglutaconic and 3-OH-3-methylglutaric acids compared to ISTD(A) after SPE were greater than those after SE.

Fig. 2 shows two chromatograms obtained from a urine specimen of a patient with Glutaric Acidemia Type I (GA I). Chromatogram (A) was obtained after SPE and (B) after SE. Elevated glutaric and 3-OH-glutaric acids were detected by both methods. The relative abundance of 3-OH-glutaric acid compared to ISTD (A) after SPE was several times

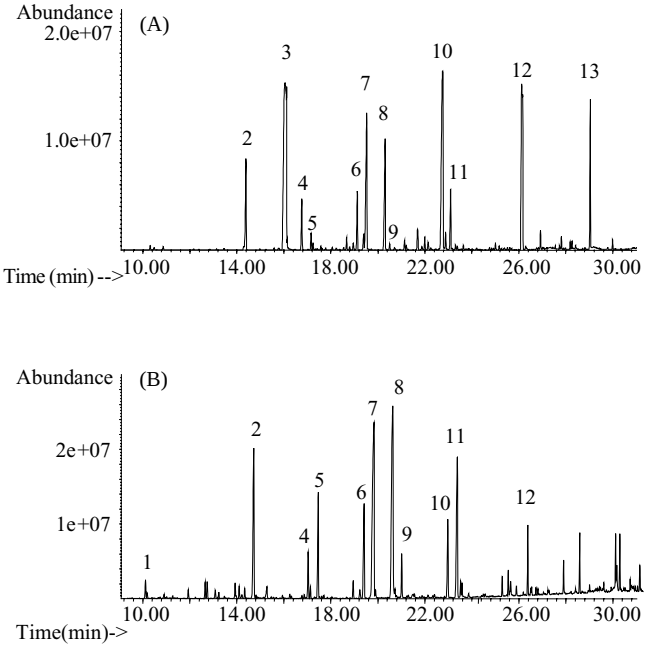

Fig. 1. Full scan chromatograms of a urine sample from a patient with 3-OH-3-methylglutaryl-CoA lyase deficiency obtained from Agilent GC–MS after (A) the solid phase extraction procedure and (B) the solvent extraction procedure. (1) 3-OH-isovaleric acid, peak 1; (2) 3-OH-isovaleric acid, peak 2; (3) phosphoric acid; (4) succinic acid; (5) 2-ketocaproic acid, ISTD (B); (6) 3-methylglutaric acid; (7) 3-methylglutaconic acid, peak 1; (8) 3-methylglutaconic acid peak 2; (9) adipic acid; (10) 3-OH-3-methylglutaric acid; (11) 3-chloromandelic acid, ISTD (A); (12) citric acid; (13) uric acid.

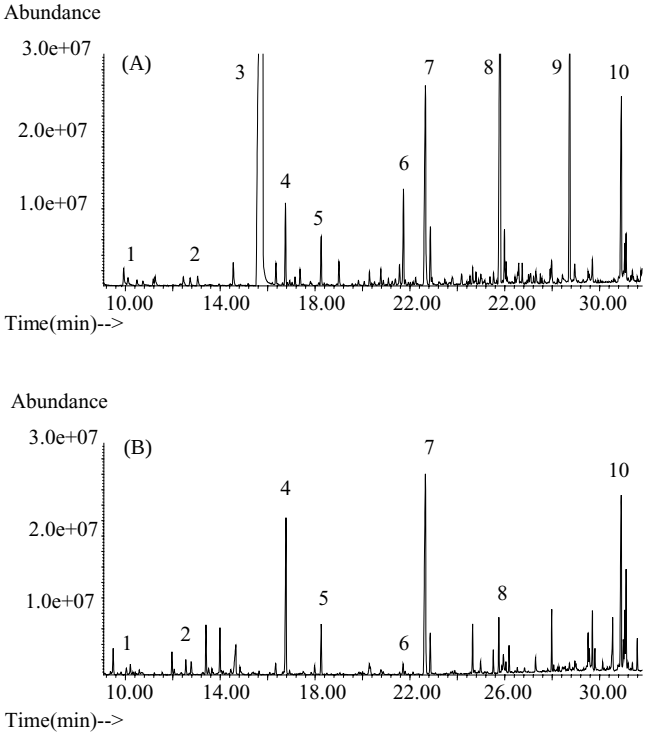

Fig. 2. Full scan chromatograms of a urine specimen from a patient with Glutaric Acidemia Type I. Chromatogram (A) was obtained after the solid phase extraction procedure and (B) after the solvent extraction procedure. (1) Lactic acid; (2) Pyruvic acid; (3) phosphoric acid; (4) 2-ketocaproic acid [ISTD (B)]; (5) glutaric acid; (6) 3-OH-glutaric acid; (7) 3-chloromandelic acid [ISTD (A)]; (8) citric acid; (9) uric acid; (10) tetracosane [ISTD (C)].

greater than that after SE. The extraction efficiency for this acid was greatly improved with SPE.

N-Acetylaspartic acid is the diagnostic marker for Canavan disease, a neurodegenerative leukodystrophy, often lethal in the first decade of life. This compound has poor extraction efficiency with traditional SE procedures. In order to evaluate the extraction efficiency of *N*-acetylaspartic acid with SPE, we spiked a normal urine sample with *N*-acetylaspartic acid. The *N*-acetylaspartic acid concentration in this sample was 300 mmol/mol of creatinine, corresponding to the lower end of the concentration range usually seen in patients with Canavan disease. Fig. 3 shows the chromatograms obtained from this urine sample after (A) SPE and (B) SE. There is a significant improvement on the recovery for this compound with the SPE when compared with SE method.

The performance of this SPE procedure is comparable to that of the more widely used SE procedure. It provides good extraction recoveries for most of the pathological compounds. This is in contrast to the observations made by Mardens et al. [9], who observed no recovery on some of the polar compounds like suberic, aconitic, citric, methylcitric and 3-hydroxysebacic acids and low recoveries on the oxime derivatives of pyruvic, glyoxylic, and 2-ketoglutaric acids. With our strong anion exchange column, the more polar compounds were actually extracted with better efficiencies.

There were drawbacks observed with this procedure. Very low extraction recoveries were found for oxalic acid, malonic acid, and mevalonic acid lactone. Complementary procedures may be needed for a better detection of these acids when necessary. The largely extracted strong polar compounds phosphoric, uric and citric acids were often the dominant peaks in urine chromatograms obtained after SPE, see Figs. 1–3. Uric and citric acids should interfere minimally with the detection and quantification of clinically significant compounds with our chromatographic separation program. However the co-elution of phosphoric acid with ethylmalonic acid is significant, although these two compounds can be resolved by ion extraction technique when processing the MS data.

In summary, SPE using the strong anion exchange column Sep-Pak (Accell Plus QMA) provided high extraction efficiencies for a wide range of pathological compounds. It can be a potential alternative to solvent extraction for organic acid screening. Although some limitations exist, the high extraction recoveries for many compounds not extracted well with solvent extraction make this procedure especially useful as a complement to solvent extraction, to confirm and quantitate some organic acids, such as 3-OH-glutaric and *N*-acetylaspartic acids.

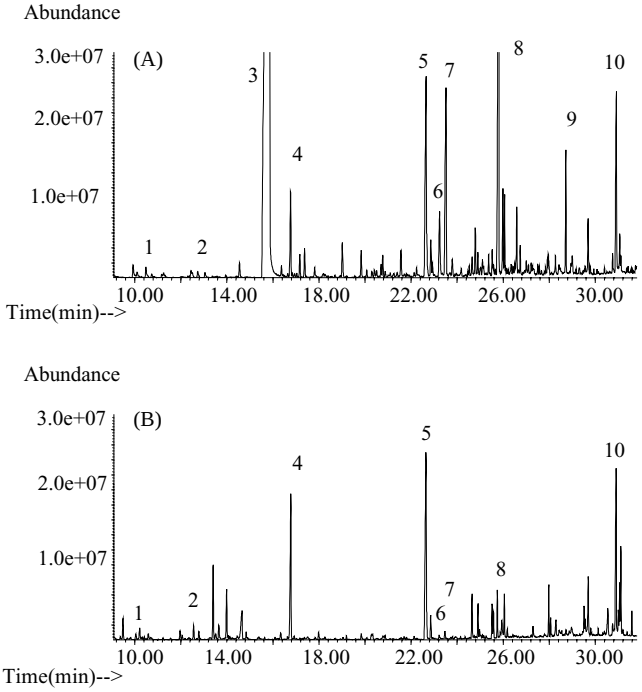

Fig. 3. Full scan chromatograms of a normal urine sample spiked with *N*-acetylaspartic acid at 300 mmol/mol of creantinine, obtained from Agilent GC–MS after (A) the solid phase extraction procedure and (B) the solvent extraction procedure. (1) Lactic acid; (2) pyruvic acid; (3) phosphoric acid; (4) 2-ketocaproic acid [ISTD (B)]; (5) 3-chloromandelic acid [ISTD (A)]; (6) *N*-acetylaspartic acid, peak 1; (7) *N*-acetylaspartic acid, peak 2; (8) citric acid; (9) uric acid; (10) tetracosane [ISTD (C)].

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