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Metabolic profiling of infant urine using comprehensive two-dimensional gas chromatography: Application to the diagnosis of organic acidurias and biomarker discovery☆

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ABSTRACT

Comprehensive two-dimensional gas chromatography (GC × GC) time-of-flight mass spectrometry (ToFMS) was applied to the analysis of urinary organic acids from patients with inborn errors of metabolism. Abnormal profiles were obtained from all five patients studied. Methylmalonic academia and deficiencies of 3-methylcrotonyl-CoA carboxylase and medium chain acyl-CoA dehydrogenase gave diagnostic profiles while deficiencies of very long chain acyl-CoA dehydrogenase and mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase gave profiles with significant increases in dicarboxylic acids suggestive of these disorders. The superior resolving power of GC × GC with ToFMS detection was useful in separating isomeric organic acids that were not resolved using one-dimensional GC. A novel urinary metabolite, crotonyl glycine, was also discovered in the mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase sample which may be a useful specific diagnostic marker for this disorder. The quantitative aspects of GC × GC were investigated using stable isotope dilution analyses of glutaric, glyceric, orotic, 4-hydroxybutyric acids and 3-methylcrotonylglycine. Correlation coefficients for linear calibrations of the analytes ranged from 0.9805 to 0.9993 (R^2) and analytical recoveries from 77% to 99%. This study illustrates the potential of GC × GC–ToFMS for the diagnosis of organic acidurias and detailed analysis of the complex profiles that are often associated with these disorders.

1. Introduction

Human urine is known to contain numerous organic acids as well as many other types and classes of compounds at varying concentrations. Mass spectrometric (GC/MS, LC/MS) and NMR metabolic profiling of these organic acids can provide initial evidence for the subsequent molecular diagnosis of many inborn errors of metabolism (IEMs). IEMs result from genetic mutations that affect an enzyme involved in intermediary metabolism. Organic acids are involved in many areas of intermediary metabolism (e.g. amino and fatty acid metabolism) and there is a corresponding large number of IEMs in which organic acids accumulate *in vivo* as a result of a deficient enzyme. These IEMs can be diagnosed based on the detection in urine of abnormally elevated organic acids associated with each disorder [1]. Although IEMs on an individual level are fairly rare, collectively they (including amino acid, organic acid and urea cycle diseases) occur at a rate of 24.4 per 100,000 live births as reported from 1969 to 1996 [2]. Early detection and identification of persons affected with genetic disorders, by use of new technology, has led to unexpected discoveries related to the natural history of the disorder or options for therapy. This early detection allows for the development of therapies that include simple dietary alterations, enzyme replacement therapy, enzyme inhibitors, or bone marrow transplantation [3]. Furthermore, due to the recessive genetic nature of most IEMs there is generally a one in four chance that any subsequent children will also be affected. Therefore, early diagnosis is necessary for timely treatment and counselling. These diagnoses can be made through the use of some polar acids: for example orotate is the most effective target for the screening of six major hyperammonemias and orotic aciduria, while methylcitrate is a target for the diagnosis of propionic and methylmalonic acidaemias [4].

Certain urinary metabolites have been detected by fully automated GC/MS measurements in a clinical environment. GC/MS is crucial for both qualitative and quantitative analyses of urinary metabolites, and the specific elevated metabolites arising from many IEMs including isovaleric acidemia [5], propionic acidemia [6], pyroglutamic acidemia [7] and 3-methylcrotonylglycinemia [8] have been discovered by using this technique. By 1980, Tanaka et al. had developed a method in which 155 metabolites were identified

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putatively, which helped demonstrate the importance of GC/MS in diagnostic medicine [9]. Shoemaker et al. shortly after applied the urease enzyme in the pretreatment of urine, which drastically reduced the large amounts of urea in the sample, in order to simplify sample pretreatment and allow for the routine analysis of several compound classes (e.g. organic acids and amino acids) all in a single chromatographic run [10].

Chemical diagnoses can be made by comparing the organic acid profiles of patients who are thought to have organic acidemias, with those of control urine profiles [11]. Urinary organic acids are most commonly extracted using a liquid:liquid extraction procedure similar to the following: organic acids are extracted with diethyl ether and/or ethyl acetate under specific acidic conditions with or without the addition of sodium chloride, dehydrated with sodium sulfate and finally evaporated to dryness and derivatized to increase their volatility, so as to be compatible with GC/MS analysis. Methyl esters and trimethylsilylation are widely used derivatization methods. Trimethylsilylation is performed with or without prior oximation. The oximation reaction occurs only with keto and aldehyde functional groups and therefore it has no effect on most of the other acids in urine. In the GC/MS analysis of urine, TMS (trimethylsilyl) derivatives are generally preferred over *tert*-butyldimethylsilyl derivatives due to the latter derivatives being bulkier than the TMS moiety which may limit the complete silylation of polyols or hexoses and result in the production of multiple derivatives [3]. Most often, laboratories measure organic acids either quantitatively or qualitatively relative to a small number of internal standards. The measurement varies from laboratory to laboratory and the errors can be as high as 50%; whereas it is recommended that the error for organic acids of clinical interest should be <20% [12].

Biologically based samples are typically known to contain thousands of metabolites, and urine is no exception in terms of complexity. There are numerous organic acids, amino acids, amines, sugars and related chemical classes that are intermediates in cellular metabolism. Many of these metabolites are also affected by diet, age, diurnal variation and metabolic status, causing significant inter- and intra-individual differences in urinary composition. Due to the complexity of the profiles, there is the potential for peak co-elution which may result in missed or false identifications. The detection, identification and subsequent quantification of these urinary metabolites therefore requires sophisticated instrumental platforms, as classical methods suffer from underreporting of the total metabolite composition. Comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC/ToFMS) is an emerging recent and sophisticated technology which provides a two-dimensional separation in real-time, with simultaneous retention data on two columns, and a full mass spectral scan (up to 500 full scans/s) which can be correlated with the retention time co-ordinates in the two-dimensional separation space. The advantages of GC × GC over the one-dimensional GC (1D-GC) technique includes increased mass sensitivity (up to 10×), as well the fact that separations and analyses are completed in two-dimensions and therefore contain more informing power [13]. With mass spectrometry a third analysis dimension is obtained. The second dimension of chromatographic resolution is accomplished by applying two different stationary phases (either a non-polar followed by a polar phase column or *vice versa*). A modulator between the two dimensions traps the effluent from the first column and focuses it prior to thermal release into the second column. GC × GC has been demonstrated as a highly selective instrument which has been proposed as suited to complex mixture analysis [14–20]. Sinha et al. recently analyzed organic acids in infant urine by GC × GC/ToFMS through the development of an algorithm (Dotmap) for locating metabolites of interest based on their mass spectral similarity [19]. All of the 12 metabolites of interest were found by the Dotmap algorithm, and PARAFAC was implemented in order to provide pure metabolite information. In another study, Koek et al. increased the sample loading in GC × GC/MS for improved performance in metabolomics analysis, by using a setup that comprised a polar first column (BPX50) and a non-polar second column (BPX5) with a wider bore and thicker film [21]. Important drawbacks of the non-polar/polar setup (BPX5-BPX50) were overcome (including limited mass loadability and limited inertness towards the metabolites of interest) as well as improved quantification. This was achieved by the use of a thicker stationary phase film (up to 1 μm) column in the first dimension to broaden the first dimension peaks and thus inject a smaller amount in the second dimension column to reduce overloading [21].

In this report, demonstration of GC × GC with ToFMS detection as a diagnostic tool for the evaluation of urine organic acid profiles, and investigation of the qualitative and quantitative aspects of the analysis is reported. In particular, application of the technique to highly complex profiles that are sometimes encountered in routine practice and to compare the profiles obtained with one-dimensional GC/MS is of interest. The importance of using a multidimensional technique such as GC × GC/ToFMS through its improved capabilities in the field of biomarker discovery is shown through the identification of crotonyl glycine in the mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase sample, which can possibly be a useful specific diagnostic marker for this disorder.

2. Experimental

2.1. Samples and pretreatment

Anonymous urine samples were obtained from a central diagnostic metabolic laboratory servicing the states of Victoria and Tasmania, Australia. All samples had undergone comprehensive initial metabolic screening using one-dimensional GC/MS and electrospray-tandem mass spectrometry [22]. The samples were from infants and children with both normal metabolic screening results, and patients with a selection of IEMs. Organic aciduria diagnoses were confirmed by the finding of persistent metabolic abnormalities or independent testing, e.g. mutation detection or the finding of other diagnostic metabolites. The control sample was from a healthy laboratory volunteer. The GC × GC/ToFMS analyses were conducted "blind", i.e. the diagnoses and initial metabolic screening results were not known to the analyst. Samples were stored at −20 °C until needed for analysis and were then allowed to thaw out at room temperature (repeated freeze thaw cycles were minimized).

Creatinine is widely used to compensate for variations in urine volume with metabolite concentrations often expressed as their ratio relative to creatinine [23]. Urine samples were therefore diluted to a fixed creatinine concentration of 1 mmol/L prior to analysis so that the final measured concentration of metabolites in μmol/L was equivalent to μmol/mmol of creatinine to give a creatinine concentration of 1 mmol/L. This method is according to the standard method employed in the Royal Children's Hospital laboratory.

Crotonyl glycine was synthesized from crotonic acid and glycine according to a standard method [24].

2.2. Liquid–liquid extraction of urine

One hundred microlitres of internal standard solution (1 mmol/L 3,3-dimethylglutaric acid (Sigma–Aldrich, Australia) and 1 mol/L methoxyamine hydrochloride (Sigma–Aldrich, Australia)) in H_2O was added to 1 mL of diluted urine and placed in a microwave instrument (CEM MARS-5, Buckingham, UK) at 450 W for 90 s. After

cooling and saturating with solid sodium chloride, 50 μL of 6 mol/L hydrochloric acid was added and then the solution was extracted with 5 mL of ethyl acetate for 5 min on a rotary mixer. The upper organic layer was separated by centrifugation, and then transferred to clean glass tubes containing 10 μL of 25% ammonia to minimize evaporative losses of volatile organic acids and dried down under N_2 at 60 °C.

2.3. Derivatization

TMS (trimethylsilyl) derivatives were formed by adding 100 μL BSTFA (bis-(trimethylsilyl)trifluoroacetamide) containing 1% TMCS (trimethylchlorosilane) to the dried extracts from the liquid extraction. Microwave assisted derivatization was applied at 450 W for 90 s (method optimised by varying power and time settings) [25], followed by the addition of 1 mL of iso-octane. This solution was then taken for GC analysis.

2.4. Instrumental analysis

The instrumentation used for the comprehensive two-dimensional gas chromatography analysis comprised of an Agilent 6890GC (Agilent Technologies, Nunawading, Australia) chromatograph with a LECO Pegasus III ToFMS system (LECO Corp., St Joseph, MI, USA) fitted with a longitudinal modulated cryogenic system (LMCS) (Chromatography Concepts, Melbourne, Australia) to effect GC × GC operation. The modulation temperature was kept constant at 0 °C and a modulation period of 5 s was applied throughout the analysis. The first GC × GC column set (non-polar/polar; NP/P) comprised a primary (^{1}D) 30 m BPX5 (5% phenyl-methylpolysilphenylene-siloxane) column, ID 250 μm, film thickness (d_f) 0.25 μm, with a second dimension (^{2}D) column of 1 m BPX50 (50% phenyl-methylpolysilphenylene-siloxane) column, internal diameter (ID) 100 μm, 0.1 μm d_f was used. The control sample was analyzed using this column set initially. Subsequently an inverse column set (P/NP) comprising a 30 m BPX50 ^{1}D column, ID 250 μm, 0.25 μm d_f and a 1 m BPX5 ^{2}D column of ID 100 μm, and 0.1 μm d_f. All columns were from SGE International, Ringwood, Australia. The second column set was found to be more effective, as it resulted in a better use of the total two-dimensional separation space (see below), and was therefore used for all further analyses. All injections were performed in splitless mode with 1 μL volume; the oven was held at an initial temperature of 70 °C for 2 min before increasing the temperature to 280 °C at 3 °C/min and held at this temperature for 5 min. The transfer line was held at 280 °C and the detector voltage at −1550 V. Mass spectra were acquired from 50 to 650 *m/z*, at an acquisition frequency of 100 spectra s^{-1}. The ToFMS detector was turned off until the TMS by-products mono-(trimethylsilyl)trifluoroacetamide and trifluoroacetamide were eluted from the column, as they are usually present in very high concentrations and can lead to saturation of the ToFMS detector. Data acquisition and processing were performed by ChromaTOF software (LECO Corp., St Joseph, MI, USA). Specific quantification ions were used to quantify certain metabolites using five different metabolite standards. Standards of glutaric acid, glyceric acid, 4-hydroxybutyric acid, 3-methylcrotonyl glycine, and orotic acid were serially diluted in order to provide calibration curves ranging from 500 to 12.5 μmol/L. Repeatability and reproducibility of second dimension retention times were also calculated (see below).

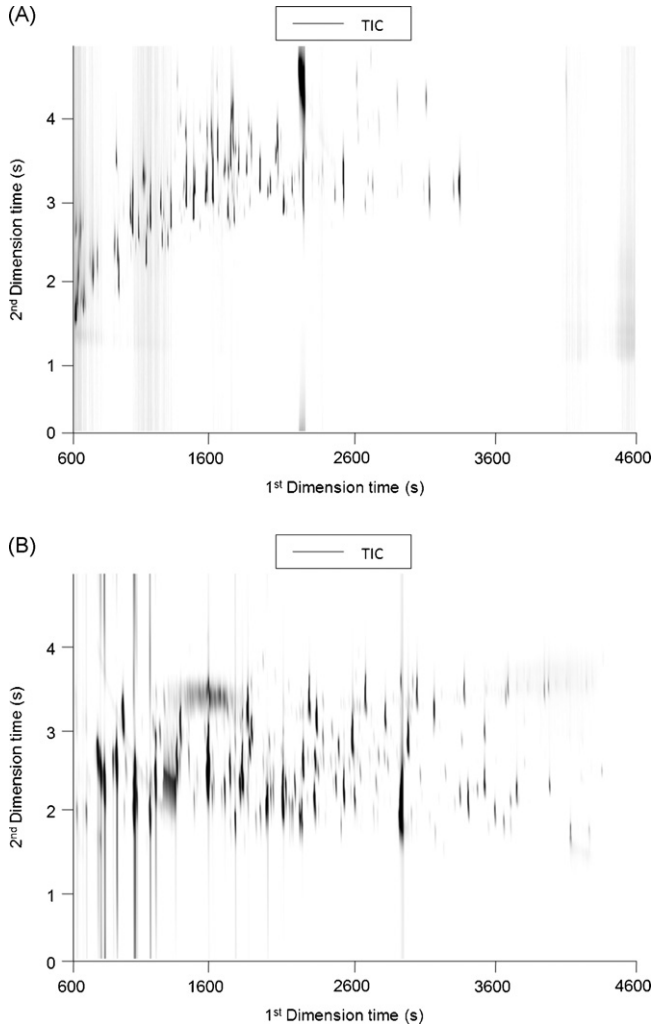

Fig. 1. Total ion chromatograms of a urine control. (A) First column set (BPX5–BPX50, 4 s modulation period); (B) second (preferred) column set (BPX50–BPX5, 5 s modulation period).

3. Results and discussion

3.1. Column comparison using a urine control

Two types of column combinations were investigated in order to perform further analyses based on the most appropriate type of column. The two column sets (NP/P and P/NP) employed 5% phenyl- and 50% phenyl-methylpolysilphenylene-siloxane phases as the respective non-polar and polar phases. Both have adequate thermal stability, and are suitable for use with derivatized samples. In general, on the NP/P column set non-polar solutes will elute at relatively low retention on the ^{2}D column, whereas on the P/NP set, polar compounds will elute at relatively lower retention on the ^{2}D column. Thus column set selectivity will be better for polar and non-polar solutes respectively, and so the most appropriate column set may be determined by which solute type requires the best selectivity of separation. Fig. 1A depicts the urine control sample using the first column set; Fig. 1B shows the same sample using the second column set. The NP/P column set shows some small measure of separation between the organic acids, although most metabolites generally elute at a similar second dimension retention time (between 2 s and 3 s). This is due to the derivatized metabolites being typically apolar, therefore possessing limited retention differences in polarity when using a polar second dimension phase. The P/NP column set shows much better use of the two-dimensional separation space, as the apolar metabolites now elute at lower temperatures (they have reduced partitioning with the polar stationary ^{1}D phase), and at these

temperatures consequently have higher retention times on the apolar ^{2}D phase with which they are more compatible. This finding coincides with the findings of Koek et al. who concluded that by using a P/NP column setup in GC × GC/MS, better quantification performance was achieved when compared to both the NP/P GC × GC/MS setup and GC/MS [21]. It was also determined that the number of peaks detected and identified when using the P/NP set was greater. Using the urine control, the NP/P column set with MS reported 621 peaks of which 167 were identified by library searching. Using the same sample and the P/NP column set, 1283 peaks were reported of which 259 were identified. In this case, peaks that were clearly not part of the sample, such as phase bleed peaks, were not included in this number. A signal-to-noise ratio of 100 and a mass threshold of 20 were used for identification, and putative identifications were performed using the NIST 2005 library (http://www.nist.gov/srd/nist1.htm), which contains 190,825 mass spectra and 163,198 compounds. NIST similarities can range from 0 (no match) to 999 (perfect match). Similarities greater than 800 were considered reasonable, acceptable matches and were therefore assigned the respective name. As extensive as these libraries may be, they do not contain an exhaustive listing of endogenous metabolites that are found in biological metabolic pathways. In this present study, only a limited number of authentic standards were available to confirm component identities through correlation of retention times and mass spectra.

3.2. Qualitative analysis of diseased and normal urine samples

Samples from five patients with IEMs, five children without apparent metabolic abnormalities and the urine control were all analyzed using the preferred polar/apolar column set (BPX50-BPX5). Samples from patients with the following IEMs were analyzed: 3-methylcrotonyl CoA carboxylase deficiency (3MCCD, OMIM 210200, 210210), methylmalonic acidemia (MMA, OMIM 251000), mitochondrial 3-hydroxy-3-methylglutaryl-(HMG) CoA synthase deficiency (mHSD, OMIM 605911), medium chain acyl-CoA dehydrogenase deficiency (MCADD, OMIM 201450) and very long chain acyl-CoA dehydrogenase deficiency (VLCADD, OMIM 201475). The OMIM numbers indicate the "Online Mendelian Inheritance in Man" database indices (http://www.ncbi.nlm.nih.gov/omim/). The samples from the mHSD and MCADD patients were obtained during periods of metabolic decompensation.

The enhanced sensitivity of GC × GC/ToFMS allowed the detection of trace levels of many organic acids present in the samples, which would be impossible when using a one-dimensional separation approach (Fig. 2A). Fig. 2B shows the unique separation of the trace level compounds in the ^{2}D separation space. The ability to recognize minor components is of importance, as a slight increase in a low level organic acid can be indicative of a specific deficiency, and 'masked' compounds may be simply overlooked, since their presence is neither recognized nor anticipated. Chromatograms in metabolic profiling of biological samples are typically complex, due to the large number of metabolite peaks (and multiple derivatization products). Longer analysis times are thus needed (up to 60 min) in order to attempt to obtain a better 1D-GC separation. Here, the analysis time was in the range of 70 min and, as observed by comparing Fig. 2A and B, many overlapping metabolites are now clearly separated and can therefore be better quantified.

Most organic acidurias result in the gross (>10-fold increase) excretion of marker metabolites and therefore for many laboratories a qualitative data analysis screen or 'semi-quantitative' analysis is adequate. In order to apply an objective basis to the qualitative data analysis, peak areas of the total ion chromatograms were expressed as a percentage of the total area, however this will not be suitable for precise quantification. Elevated organic acids were defined according to the following criteria: a greater than 10-fold

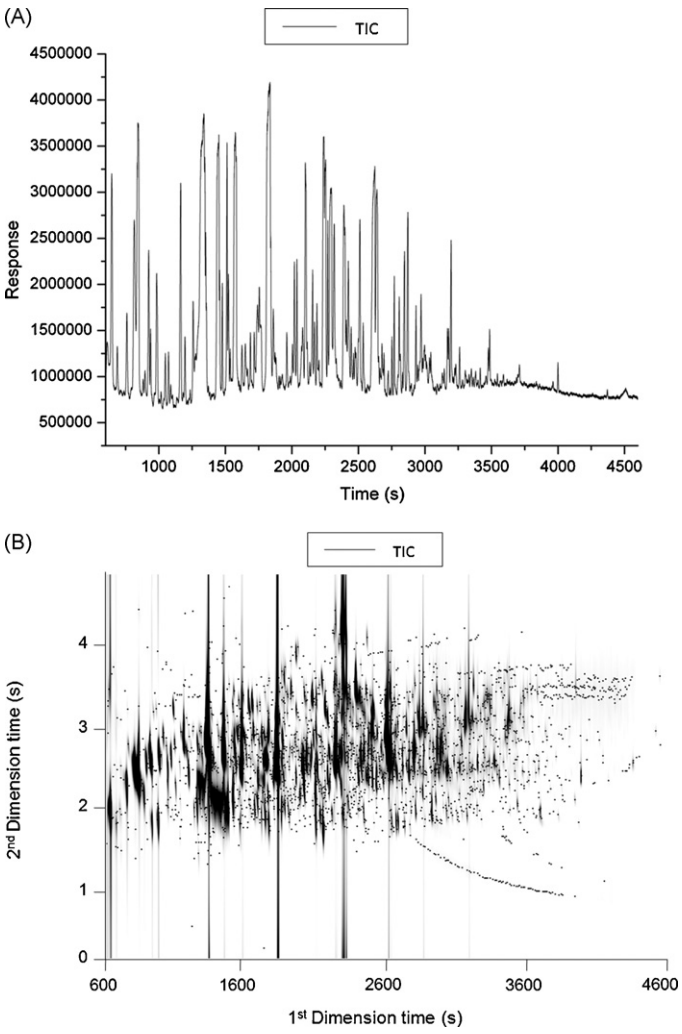

Fig. 2. Total ion chromatograms of a urine sample from a patient with mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase deficiency. (A) One-dimensional GC separation on a BPX50 column; (B) comprehensive two-dimensional GC separation on a BPX50–BPX5 column combination.

organic acid concentration increase was classified as "highly significant" increase, a 3–10-fold organic acid concentration increase was labeled as a "moderately significant" increase and up to 3-fold organic acid concentration increase was noted as a "slightly significant" increase. Relative peak area ratios (using the extracted ion chromatograms) were calculated for the increased organic acids found in each specific sample, and were compared to their corresponding relative area ratios calculated in the urine control sample. The 'extent of increase' was then calculated based on a cut-off level which was determined for the urine control. Results for individual samples are summarized in Table 1 and are discussed below; details of the expected metabolic abnormalities are summarized in [26].

The sample from the patient with MMA showed highly significant increases in methylmalonic, hippuric and methylcitric acids, and a moderately significant increase in adipic and 3-methyladipic acids. Methylmalonic and methylcitric acids are the main markers for MMA whereas hippuric is generally of dietary origin. Adipic and 3-methyladipic acids are not associated with MMA but may represent secondary inhibition of other metabolic pathways.

The sample from the MCADD patient contained a highly significant increase in hexanoyl glycine, succinic and trans-aconitic acids, a moderately significant increase in fumaric and isocitric acids. Hexanoyl glycine is the main marker for MCADD whereas

Table 1
Diseased samples with examples of increased organic acids (with NIST similarities) indicative of a specific IEM. Diagnostic marker metabolites are indicated in bold face while suggestive metabolites are underlined.

Diseased sample	Highly significant increase (greater than 10-fold)	Moderately significant increase (3–10-fold)	Slightly significant increase (1–3-fold)
3MCCD	**3-Methylcrotonyl glycine** (914) **3-Hydroxyisovaleric** (869)	Fumaric (825)	
MMA	**Methylmalonic** (922) Hippuric (829) **Methylcitric** (921)	Adipic (828) 3-Methyladipic (813)	
mHSD	Glutaric (828) Adipic (847) Suberic (936)	Ethylmalonic (805)	Pantothenic (813) Homovanillic (832)
MCADD	**Hexanoyl glycine** (904) Succinic (818) Trans-aconitic (843)	Fumaric (925) Isocitric (834)	
VLCADD	Adipic (897) Dehydrosebacic (828)	2-Hydroxyglutaric (879)	Hippuric (832) 3-Methylglutaconic (845)

succinic and trans-aconitic acids are not associated with IEMs. Succinic acid can result from bacterial metabolism which may indicate inadequate storage of the sample prior to receipt in the laboratory.

The sample from the mHSD patient (Fig. 2B) was the most complex sample analyzed. A large number of components were resolved and some peaks were present at high concentrations and were consequently overloaded. This sample had highly significant increases in the dicarboxylic acids glutaric, adipic and suberic acid, a moderately significant increase in ethylmalonic and slightly significant increases in pantothenic and homovanillic acids. This pattern of dicarboxylic acids has previously been described in mHSD patients although it is important to note that these metabolites are not specific for this disorder and are found in several other IEMs. Closer examination of the profile also showed the presence of a small peak identified as crotonyl glycine, which was an unexpected finding. This metabolite was not identified during initial one-dimensional GC/MS analysis of the sample because it was a relatively minor peak and incompletely separated from the much larger peak of adipic acid. The identity of the peak was confirmed by synthesis of the authentic compound and mass spectral matching.

Crotonyl glycine was undetectable in the controls or the other patients and it does not appear to have been described in either normal or pathological human urine. Crotonyl CoA is an intermediate in the final stages of fatty acid oxidation and occurs two steps upstream of 3-hydroxy-3-methylglutaryl CoA synthase. Elimination of the acyl moieties of accumulating acyl-CoA esters, such as crotonyl CoA, *via* conjugation with glycine is a common mechanism in several IEMs [26] that serves to protect cells from potentially toxic levels of these intermediates. Excretion of crotonyl glycine therefore indicates the accumulation of metabolites upstream of the metabolic block in mHSD deficiency. This is significant because the metabolic abnormalities described in this disorder to date, such as adipic and suberic, are non-specific. These metabolites are by-products of fatty acid oxidation and represent accumulation of intermediates even further upstream than crotonyl CoA. They can also occur in several disorders of fatty acid oxidation (e.g. VLCADD). Significantly, crotonyl glycine was not detected in the samples from the two patients with fatty acid oxidation disorders (VLCADD and MCADD). Glycine conjugates are important diagnostic markers for several IEMs (e.g. hexanoyl glycine and 3-methycrotonyl glycine in MCADD and 3MCCD respectively) and crotonyl glycine may therefore be a useful diagnostic marker for mHSD. Further studies are in progress to confirm this finding in other mHSD patients.

The sample from the VLCADD patient had significant increases in the dicarboxylic acids adipic and dehydrosebacic acids, a moderate increase in 2-hydroxyglutaric acid, and a slight increase in hippuric and 3-methylglutaconic acids. VLCADD is a disorder of fatty acid oxidation and the increased dicarboxylic acids are consistent with this disorder although not diagnostic.

The sample from the 3MCCD patient was found to contain highly significant increases in 3-methylcrotonyl glycine and 3-hydroxyisovaleric acid, and an increase in fumaric acid. 3-Methylcrotonylglycine and 3-hydroxyisovaleric acid are indicative of 3MCCD.

The findings explored above are in overall agreement with the results obtained from 1D-GC/MS analysis, in that the same pattern of organic acids was found, and so resulted in the same conclusions. In some cases the organic acids detected were diagnostic (MMA, MCADD, 3MCCD) and in other cases the organic acids were clearly abnormal but less specific (mHSD, VLCADD). In these latter two cases the organic acids were suggestive of the general metabolic pathways in which the defects occurred and in practice these findings should prompt additional testing for these disorders. Several additional organic acids unrelated to the primary enzymatic defect were also found to be increased. Some of these were dietary (e.g. hippuric) or related to treatment (e.g. pantothenic) while others probably represent secondary metabolic disturbances in other pathways (e.g. adipic, fumaric, 3-methylglutaconic). This phenomenon is often observed in metabolic profiles of patients with known IEMs.

3.3. Improved resolving power of GC × GC

The advantage of the resolving power of GC × GC was illustrated by several metabolites which were not resolved during 1D-GC analysis. Examination of the 1D-GC profile from the patient with mHSD (Fig. 2A) indicated that a large number of components (many of them fatty acid metabolites) were present, with many incompletely resolved. Fig. 3 shows an expansion of the GC × GC profile from the same sample. Positional isomers, such as the acids, are now well resolved and 2- and 3-hydroxydicarboxylic acids are also well resolved from saturated and unsaturated carboxylic acids.

Fig. 4 shows the separation of 2-hydroxyglutaric, 3-hydroxyglutaric and 2-ketoglutaric acids chromatographically. Fig. 5 compares the 1D separation with the 2D separation, both analyses incorporating use of ToFMS. Distinguishing between these metabolites is critical because they are all markers for separate IEMs. The 1D-GC analysis of these metabolites is particularly problematic because they are inadequately resolved, as well as their having similar mass spectra, and increased amounts of 2-ketoglutaric occur in normal neonatal urine. Quantitation of these metabolites is therefore difficult using GC/MS but this could readily be achieved using GC × GC because of the complete resolution of these metabolites.

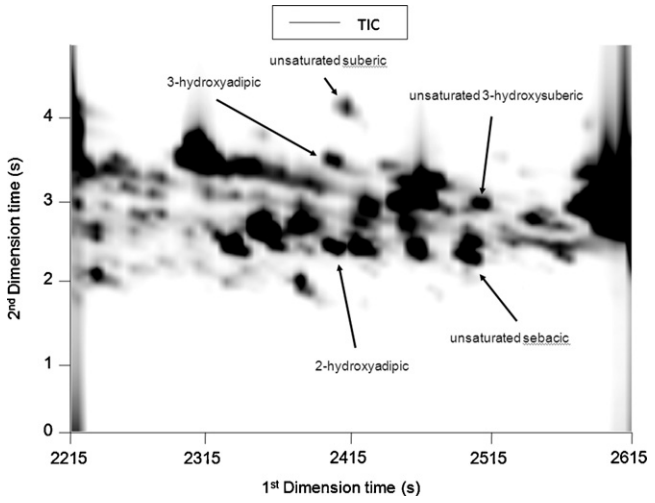

Fig. 3. Deconvolution of urinary fatty acid metabolites in a patient with mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase deficiency using GC × GC–ToFMS.

3.4. Reproducibility and quantitative performance of GC × GC–ToFMS

3.4.1. Retention time reproducibility

Seven metabolites (each having a modulation ratio (M_R) of about 3 [27]) of interest from the control sample by GC × GC/ToFMS were selected for comparing retention time shifts through ^{2}D RSD (%) for repeatability ($n = 5$) [21,22]. The performance of the instrument was investigated by observing the variability of a range of organic acids used in the identification of IEMs. The control sample was extracted and derivatized five times in order to observe any changes in peak intensity and retention time shifts. RSDs were calculated based on second dimension retention times for the three modulated peaks obtained (for each of the seven compounds) and were all within 5%; with most within 3%, which indicates that the reproducibility of the second dimension retention times are acceptable (Table 2). The repeatability and reproducibility studies performed show that minimizing the variability present is important, and the analysis has demonstrated that the differences observed between samples is probably due the varying metabolite concentrations (overloaded peaks), and not to instrumental variables.

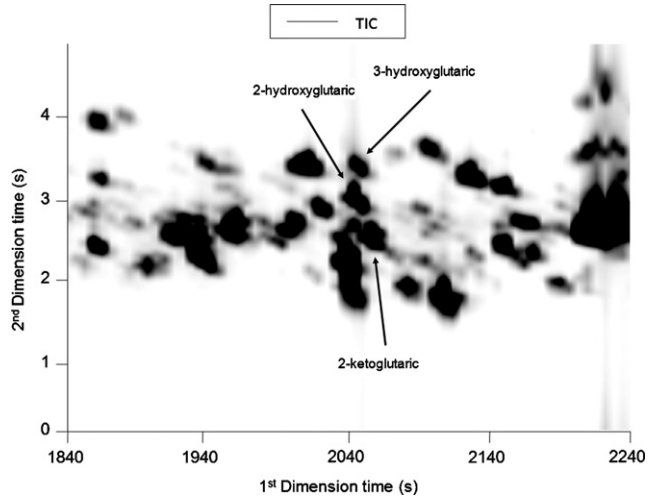

Fig. 4. Deconvolution of structurally related urinary metabolites present in the urine control using GC × GC–ToFMS.

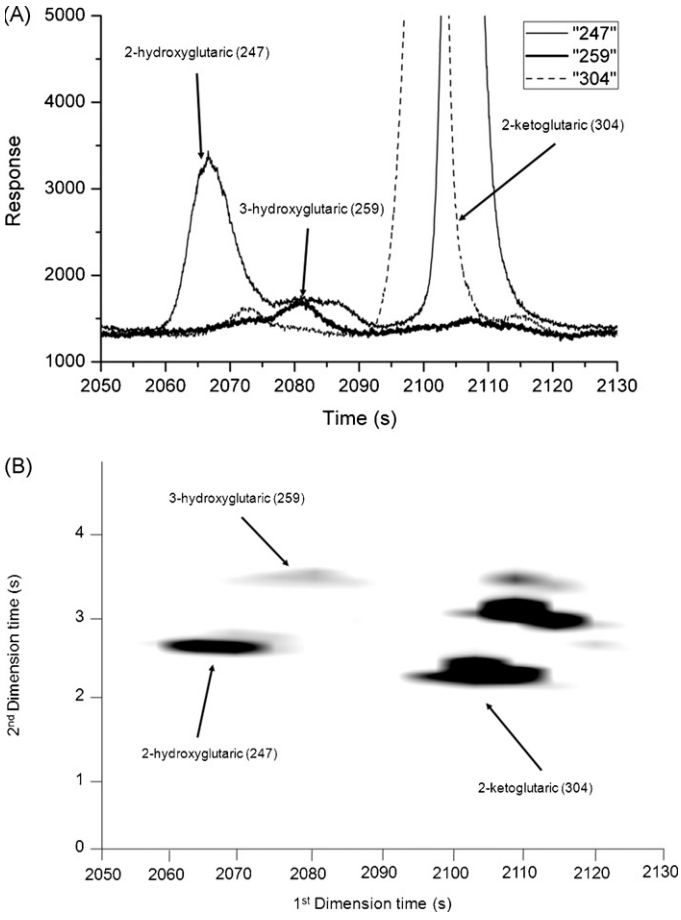

Fig. 5. Extracted ion chromatograms (m/z 247, 259, 304) comparing (A) one-dimensional and, (B) two-dimensional separations.

3.4.2. Quantification of diseased urine samples

Quantification of the acid compounds in urine samples is important in the study of IEMs, as an increase in specific organic acids is indicative (i.e. a marker) of a particular type of IEM. In GC × GC, integration is achieved using the raw chromatogram, which may be performed by summation of each modulated peak, or a representative subset of the modulated peaks [27] derived for that metabolite.

Five metabolite standards (glutaric, glyceric, 4-hydroxybutyric, 3-methylcrotonyl glycine and orotic acids) were used to generate calibration curves in order to quantify the metabolites of interest in the analyzed samples. Quantification was performed by calculating relative peak area ratios of the five isotopically labeled metabolite standards ($^2H_4$-glutaric, 2,3,3-$^2H_3$-glyceric, $^2H_6$-4-hydroxybutyric, $^{13}C_2$-3-methylcrotonyl glycine, and $^{15}N_2$-orotic) against the internal standard (3,3-dimethylglutaric acid); quantification ions were selected for each isotopically labelled metabolite, and upper concentration (in relation to the linear concentration levels) were used for the determination of the calibration. Table 3 shows the quantification ions, the upper and lower calibrators (including dilutions), calibration equation (and R^2), and %RSD ($n = 5$). The original metabolite standard solution was diluted by a factor of 2 and 4 respectively, to obtain upper and lower calibrators ranging from 500 down to 12.5 μmol/L. RSDs and R^2 values were all within acceptable limits; RSDs all being below 7% with R^2 values greater than 0.98.

To study the recovery of metabolites from the urine extracts, standard solutions of the five metabolite standards (glutaric, glyceric, 4-hydroxybutyric, 3-methylcrotonyl glycine and orotic acids)

Table 2
Repeatability ($n = 5$) of 10 metabolites of interest in respect of peak retention times (three modulated peaks per compound; RSD%).

Metabolite	^{1}D, ^{2}D mean retention time (s)	Mean retention time RSD (%)	Modulated peak 1 RSD (%)	Modulated peak 2 RSD (%)	Modulated peak 3 RSD (%)
(R*,R*)-2,3-Dihydroxybutanoic acid, tris(trimethylsilyl)-	1170, 3.420	1.23	3.33	1.61	4.16
Benzoic acid trimethylsilyl ester	1250, 2.310	2.30	3.55	1.45	4.41
Pentanedioic acid, 3,3-dimethyl-, bis(trimethylsilyl) ester (I.S.)	1560, 3.270	1.01	4.12	4.56	3.85
Malic acid, tris(trimethylsilyl) ester	1605, 3.830	1.34	3.50	4.02	2.64
2-Ketoisocaproic acid, trimethylsilyl ester	1675, 2.130	4.65	2.15	5.05	4.82
Malonic acid, bis(2-trimethylsilylethyl) ester	2085, 2.380	2.00	1.39	4.67	5.41
Sebacic acid, bis(trimethylsilyl) ester	2570, 2.870	1.83	1.99	5.03	3.38

Table 3
Quantification and recoveries (using relative area response ratios) for the five metabolite standards and isotopic labeled metabolites.

Metabolite standards	Q ion	Original upper concentration (μmol/L)	Equation and R^2	%RSD	Isotopically labeled metabolites	Q ion	Conc (μmol/L)	Recovery (%)
Glutaric	261	500	$y = 0.1798x - 0.0675$ $R^2 = 0.9971$	3.94	$^2H_4$-glutaric	265	2000	95
Glyceric	307	200	$y = 0.1486x - 0.0743$ $R^2 = 0.9805$	4.25	2,3,3-$^2H_3$-glyceric	310	1000	99
4-Hydroxy butyric	233	100	$y = 0.0852x - 0.0352$ $R^2 = 0.9993$	5.09	$^2H_6$-4-hydroxybutyric	239	500	94
3-Methyl crotonyl glycine	214	50	$y = 0.0295x - 0.0111$ $R^2 = 0.996$	6.49	$^{13}C_2$-3-methylcrotonyl glycine	216	250	78
Orotic	254	50	$y = 0.0244x - 0.0085$ $R^2 = 0.9844$	5.76	$^{15}N_2$-orotic	256	250	77

were spiked to the urine control at concentration levels ranging from 2000 to 250 μmol/L and recoveries for all the metabolites were found to be satisfactory (i.e. 77–99%) (Table 3).

3.4.3. Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) and limit of quantification (LOQ) were determined manually using the standard with the lowest concentration, i.e. 12.5 μmol/L (which was still detectable at this lower level), and reconstructing the respective EIC for each metabolite of interest (glutaric, glyceric, 4-hydroxybutyric, 3-methylcrotonyl glycine and orotic acids). Detection limits were calculated by determining the S/N ratio and extrapolating to the S/N = 3 level (for the LOD) and S/N = 10 level (for the LOQ). The LOD achieved using this method was 0.04–0.20 μmol/L and the LOQ attained was 0.81–1.03 μmol/L across the five standard metabolites. The LOQ was below the lowest standard, which is in agreement with the concentrations chosen to construct the calibration curve. It must be noted though, that in GC × GC the analyst must be very careful when determining LOD and LOQ as S/N depends on the modulation period and the phase shift (therefore figures can be taken only as an approximate measurement), as well on the concentration as suggested by Ong and Marriott [28].

4. Conclusions

This paper shows the value of GC × GC/ToFMS urinary organic acid profiling for the diagnosis and investigation of suspected IEMs. Diagnostic or suggestive profiles were obtained from five patients with known inborn errors of metabolism which recapitulated the findings of 1D-GC/MS. The results also demonstrated that GC × GC is a useful tool for biomarker discovery. This was exemplified by the unexpected finding of increased crotonyl glycine in the sample from the child with mHSD deficiency. Importantly, the dicarboxylic acids identified in mHSD are not specific for this disorder whereas crotonyl glycine may prove to be a useful diagnostic marker for this disorder. The enhanced resolving power of GC × GC was also beneficial in the detailed analysis of complex profiles obtained when patients are in metabolic decompensation as well as resolving key isomeric organic acids with similar spectra that co-elute using 1D-GC.

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References

[1] R.A. Chalmers, A.M. Lawson, Organic Acids in Man: Analytical chemistry, Biochemistry and Diagnosis of the Organic Acidurias, Chapman & Hall, London, 1982.
[2] D.A. Applegarth, J.R. Toone, R.T. Lowry, R. Brian, Pediatrics 105 (2000) e10.
[3] T. Kuhara, Mass Spectrom. Rev. 24 (2005) 814.
[4] T. Kuhara, in: T. Masaru, N. Takaaki (Eds.), Metabolomics, Springer, Tokyo, 2005, p. 167.
[5] K. Tanaka, M.A. Budd, M.L. Efron, K.J. Isselbacher, Proc. Natl. Acad. Sci. U.S.A. 56 (1966) 236.
[6] F.A. Hommes, J.R. Kuipers, J.D. Elema, J.F. Jansen, J.H. Jonxis, J. Pediatr. Res. 2 (1968) 519.
[7] E. Jellum, T. Kluge, H.C. Borresen, O. Stokke, L. Eldjarn, Scand. J. Clin. Lab. Invest. 26 (1970) 327.
[8] L. Eldjarn, E. Jellum, O. Stokke, H. Pande, P.E. Waaler, Lancet 2 (1970) 521.
[9] K. Tanaka, D.G. Hine, A. West-Dull, T.B. Lynn, Clin. Chem. 26 (1980) 1839.
[10] J. Shoemaker, W. Elliott, J. Chromatogr. 562 (1991) 125.
[11] D. Issachar, J.F. Holland, C.C. Sweeley, Anal. Chem. 54 (1982) 29.
[12] L. Sweetman, in: F.A. Hommes (Ed.), Techniques in Diagnostic Human Biochemical Genetics: A Laboratory Manual, Wiley-Liss, 1991, p. 143.
[13] P. Marriott, R. Shellie, Trends Anal. Chem. 21 (2002) 573.
[14] C. Vendeuvre, F. Bertoncini, L. Duval, J.-L. Duplan, D. Thiebaut, M.-C. Hennion, J. Chromatogr. A 1056 (2004) 155.
[15] R. Shellie, P. Marriott, P. Morrison, Anal. Chem. 73 (2001) 1336.
[16] J. Dalluge, L.L.P. van Stee, X. Xu, J. Williams, J. Beens, R.J.J. Vreuls, U.A.Th. Brinkman, J. Chromatogr. A 974 (2002) 169.
[17] P. Korytar, L.L.P. van Stee, P.E.G. Leonards, J. de Boer, U.A.Th. Brinkman, J. Chromatogr. A 994 (2003) 179.
[18] A.E. Sinha, C.G. Fraga, B.J. Prazen, R.E. Synovec, J. Chromatogr. A 1027 (2004) 269.
[19] A.E. Sinha, J.L. Hope, B.J. Prazen, E.J. Nilsson, R.M. Jack, R.E. Synovec, J. Chromatogr. A 1058 (2004) 209.

[20] K.M. Pierce, J.C. Hoggard, J.L. Hope, P.M. Rainey, A.N. Hoofnagle, R.M. Jack, B.W. Wright, R.E. Synovec, Anal. Chem. 78 (2006) 5068.
[21] M.M. Koek, B. Muilwijk, L.L.P. van Stee, T. Hankemeier, J. Chromatogr. A 1186 (2008) 420.
[22] J.J. Pitt, M. Eggington, S.G. Kahler, Clin. Chem. 48 (2002) 1970.
[23] C. Zuppi, I. Messana, F. Forni, C. Rossi, L. Pennacchietti, F. Ferrari, B. Giardina, Clin. Chim. Acta 265 (1997) 85.
[24] N. Gregerson, S. Kolvraa, K. Rasmussen, Biomed. Mass Spectrom. 6 (1979) 439.
[25] K. Kouremenos, J. Harynuk, P. Morrison, P.J. Marriott, J. Chromatogr. B, submitted for publication.
[26] Physician's Guide to the Laboratory Diagnosis of Metabolic Diseases, Springer-Verlag, Berlin, Heidelberg, 2003.
[27] W. Khummueng, J. Harynuk, P.J. Marriott, Anal. Chem. 78 (2006) 4578.
[28] R.C.Y. Ong, P. Marriott, J. Chromatogr. Sci. 40 (2002) 276.