Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 808 (2004) 153–161
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Unified gas chromatographic–mass spectrometric method for quantitating tyrosine metabolites in urine and plasma

Albert L. Shroads [a], George N. Henderson [a,*], Jang Cheung [a], Margaret O. James [c], Peter W. Stacpoole [a,b]

[a] *Division of Endocrinology and Metabolism, Department of Medicine, University of Florida, Gainesville, FL 32610, USA*
[b] *Department of Biochemistry and Molecular Biology, Colleges of Medicine, University of Florida, Gainesville, FL 32610, USA*
[c] *Department of Medicinal Chemistry, College of Pharmacy, University of Florida, Gainesville, FL 32610, USA*

Received 28 November 2003; received in revised form 7 May 2004; accepted 10 May 2004

Available online 7 June 2004

Abstract

Tyrosine and many of its catabolites play significant roles in the in the toxicity associated with acquired and congenital forms of hypertyrosinemia. We now report a specific and sensitive GC/MS method for the simultaneous determination of tyrosine metabolites maleylacetone (MA), fumarylacetone (FA), succinylacetone (SA), fumarate and acetoacetate in urine and plasma. Tyrosine metabolites and an internal standard, 2-oxohexanoic acid (OHA), in urine or plasma samples were derivatized to their methyl esters with a 12% boron trifluoride–methanol complex (12%BF_3–MeOH). The reaction mixture was extracted with methylene chloride and analyzed by GC/MS, using a selected ion monitoring (SIM) mode. The detection limits were in the range of 0.08–0.4 ng and the quantitation limits were 0.2–2 ng. Most of the intraday and interday coefficients of variation for three concentrations (low, medium and high) of the analytes were below 10%. Sensitivity and selectivity are superior to existing HPLC or enzymatic methods and derivatization of samples is simpler than the traditional silylation of organic acids used for analysis by GC/MS or derivatization to oximes, followed by silylation in the case of the ketoacids, such as SA. Furthermore, the current procedure can be performed in aqueous solution, which results in a high percentage yield without appreciable analyte degradation or formation of side products. Thus far, the method has been successfully applied in the analysis of over 5000 urine and plasma samples from humans and rodents.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Tyrosinemia; Tyrosine; Maleylacetone; Fumarylacetone; Succinylacetone; Fumarate; Nitisione

1. Introduction

Hereditary tyrosinemia type I (HT1) is an inborn error of metabolism that may cause liver failure, renal insufficiency and peripheral neuropathy [1–4]. The underlying genetic defect is a mutation in the gene for fumarylacetoacetate hydrolase (FAH), the last enzyme in the catabolic pathway of tyrosine. Thus far, more than 30 different mutations in this gene have been identified [5] and the disease has a worldwide distribution. Mutations in FAH disrupt tyrosine catabolism (Fig. 1) and lead to the accumulation of fumarylacetoacetate (FAA), maleylacetoaetate (MAA), succinylacetoacetate (SAA), MA and FA in liver and kidney that are believed to be responsible for the hepato-renal manifestations of the disease [6]. The accumulated SAA is readily converted to SA which inhibits a proximal step in heme synthesis and leads to the build up of the putative nerotoxin δ-aminolevulinate (δ-ALA).

Pharmacologic inhibitors of tyrosine catabolism also exist and may lead to clinically important changes in the levels of tyrosine intermediates. For example, 2-(2-nitro-4-trifluoromethylbenzoyl)cyclohexane-1,3-dione; nitisione (NTBC) is a triketone that inhibits 4-hydroxyphenylpyruvate dehydrogenase and has recently been approved for the treatment of HT1 [7]. It leads to rapid and sustained reduction of SA and δ-ALA levels, but may also cause severe hypertyrosinemia, unless the dietary intake of phenylalanine and tyrosine is restricted. Dichloroacetate (DCA) is an investigational drug for the treatment of acquired and congenital disorders of mitochondrial intermediary metabolism [8] that

* Corresponding author. Tel.: +1 352 392 6193; fax: +1 352 846 0990.
E-mail address: hendegn@medicine.ufl.edu (G.N. Henderson).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.05.005

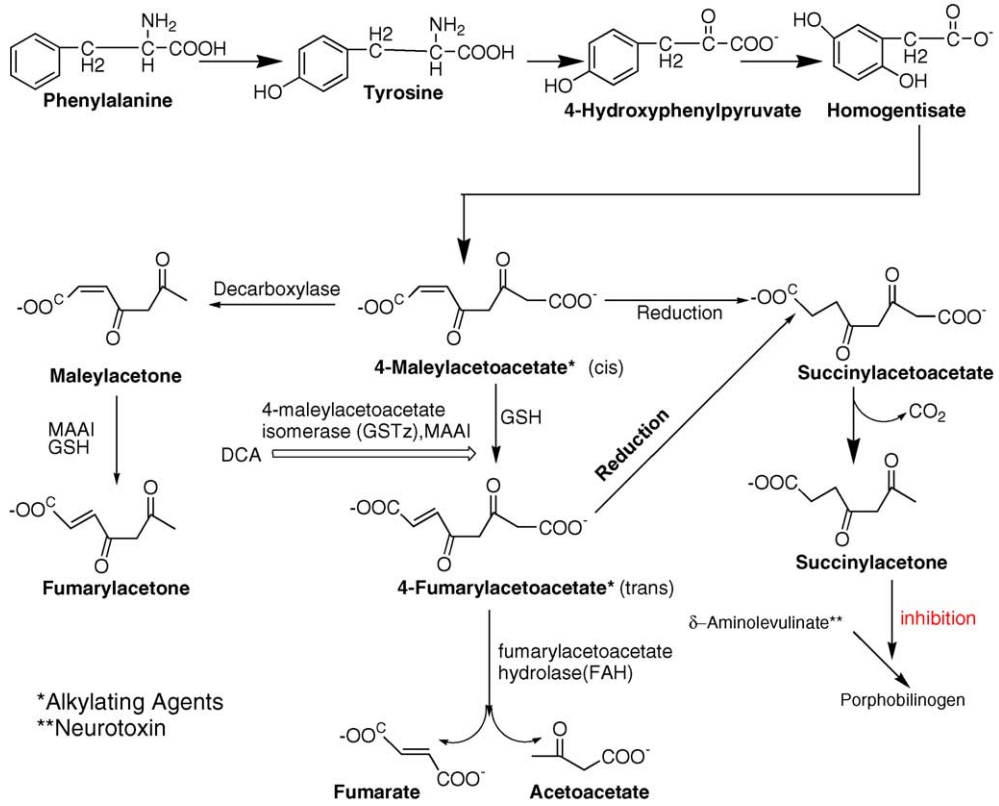

Fig. 1. Pathways of tyrosine catabolism.

inhibits MAA isomerase (MAAI), causing the build up of MA and δ-ALA [9]. MAAI also functions as the zeta isoform of glutathione *S*-transferase (GSTz) and dehalogenates a number of haloacetates [10] including DCA [11].

Insight into the pathobiology of HT1 and other causes of hypertyrosinemia would be enhanced by the ability to quantify the biochemical markers associated with these conditions. However, available analytical techniques lack sensitivity, or specificity for various tyrosine catabolites or require complex sample preparation and derivatization. Moreover, several methods are applicable to quantitating only a single catabolite. We have developed and validated a specific and sensitive GC/MS method that measures several of the clinically important metabolites of tyrosine. The method employs a simple derivatization to methyl esters prior to GC/MS analysis without prior drying or purification of the sample.

2. Experimental

2.1. Materials

SA, MA, FA, fumarate, acetoacetate and 12%BF_3–MeOH were from Aldrich Chemical Company Inc., Milwaukee, WI and methylene chloride was from Fisher Scientific, Pittsburgh, PA. All chemicals used were reagent grade, except methylene chloride, which was pesticide grade. They were used without further purification. Deionized water was prepared using a Milli-Q Water System (Millipore, Bedford, MA). Fresh frozen human plasma was purchased from Civitan Regional Blood Center (Gainesville, FL).

2.2. Samples

The blood samples were from pediatric patients with congenital lactic acidosis or from healthy volunteers who participated in clinical trials of DCA on the General Clinical Research Center at the University of Florida or from studies in rodents that had been treated with DCA. Because several metabolites of DCA are endogenous compounds, it is often administered as $^{13}C_2$-DCA for metabolic and kinetic studies. Venous blood (0.4 ml) was collected in heparinized tubes at 0, 1/4, 1/2, 1, 2, 4, 6, 8, 12 and 24 h after the DCA dose. Samples were placed on ice and then centrifuged at 1200 × *g* for 15 min at 4 °C in a Sorvall RT6000B refrigerated centrifuge (DuPont, Delaware, NJ, USA). The supernatants were stored at −80 °C before derivatization. Urine samples were either from patients with HT1 before or after treatment with NTBC or from pediatric subjects, adults subjects or rodents receiving DCA. HT1 samples were provided by Dr. G. Mitchell (University of Montreal). Urine samples were frozen at −80 °C without further processing. The human investigations were approved by the Institutional

Review Board of the Shands Hospital at the University of Florida, while those involving HT1 patients were approved by the Institutional Review Board of the Ste. Justine Hospital (Montreal). Informed consent was obtained prior study from a parent or guardian of the children and from adult volunteers. Animal studies were approved by the University of Florida's Institutional Animal Care and Use Committee.

2.3. Derivatization and extraction

Tyrosine metabolites and the internal standard OHA were derivatized to their methyl esters by reacting with 12%BF_3–MeOH complex. The derivatization procedures followed published methods [12] with minor modifications. A urine or plasma sample (200 μl) and the internal standard (OHA, 100 μg/ml in water, 100 μl) were mixed with 500 μl of 12%BF_3–MeOH in a sealed glass culture tube (16 mm × 100 mm). The mixture was heated at 115 °C for 12 min on a heating block. After cooling, 1 ml of methylene chloride and 1 ml of water were added to the reaction solution. The sample was vortexed vigorously for 2 min using a Vortex Genie 2 mixer (Fisher Scientific, Pittsburgh, PA, USA) and was centrifuged at 2500 × *g* in a Beckman J-6B centrifuge (Beckman Instruments Inc., Palo Alto, CA, USA) at 10 °C for 8 min to separate the layers. The lower methylene chloride layer was transferred to an autosampler vial for GC/MS analysis.

2.4. GC/MS conditions

The GC/MS system consisted of a Hewlett-Packard (Palo Alto, CA, USA) 5890 series II plus Gas Chromatograph, a 5972A series Mass Selective detector, and a 6890 series auto-sampler. The column was a 30 m long DB-WAX (crosslinked polyethylene glycol; J&W Scientific Agilent Technologies) with 0.25 mm i.d. and 0.15 μm film thickness. The carrier gas was at a flow rate of 1.0 ml/min (30.6 cm/s) with a head pressure of 7.5 psi at 50 °C. The sample in methylene chloride solution was injected using a splitless mode and the injection volume was 1 μl. Temperatures of the injection port and mass selective detector interface were set at 250 and 280 °C, respectively. The temperature gradient of the GC oven was programmed to be initiated at 70 °C for 2 min and then raised to 240 °C at 12 °C/min and held at the final temperature for 2 min. The mass spectometer was tuned daily with perfluorotributylamine using HP Chemstation autotune software and operated in electron impact (EI) ionization mode with the ionization energy of 70 eV.

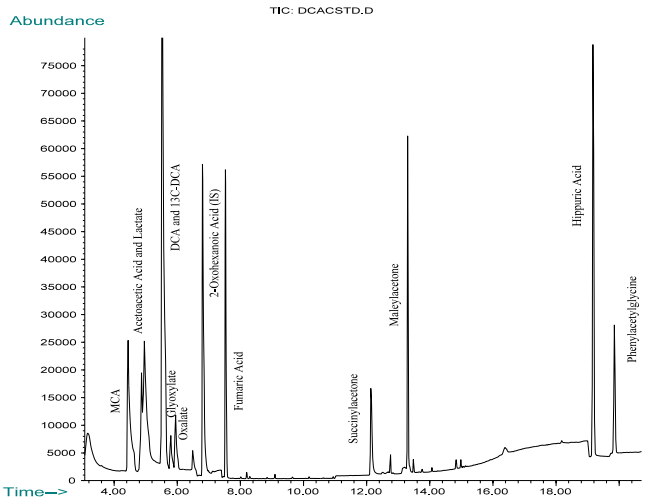

Fig. 2. TIC of the GC/MS analysis, in the selected ion monitoring (SIM) mode, of an aqueous standard of tyrosine and DCA metabolites.

Standards of each compound were prepared at higher concentrations (approximately 100 μg/ml), derivitized and analyzed utilizing full scan mass spectrometry (35–300 amu) under the same chromatographic conditions. The TIC of the authentic compounds derivatized from aqueous solution is shown in Fig. 2. The full mass spectra were needed to structurally identify the analytes and to obtain the retention times for each compound. Specific SIM groups were created in the mass spectrometer acquisition software for each analyte that included at least three representative ions from the mass spectra. The scan times of the SIM groups were changed to bracket the retention time of the compounds. Although several ions were scanned for each compound, only one ion was used for quanitation, as shown in Table 1.

2.5. Calibration curves

Calibration of tyrosine metabolites was performed by adding the compounds to human plasma (Civitan Regional

Table 1
Mass fragments for selected ion monitoring in GC/MS analysis

Compound	Acetoacetic acid	2-Oxohexanoic acid	Fumaric acid	Succinylacetone	Maleylacetone
RT (min)	4.25	6.83	7.547	12.14	13.34
Fragments (*m/z*)	57	57[a]	113[a]	43	111
	59	85	85	85	43
	116[a]	41	59	99	68
		144	114	55	170[a]
		86	144	125	97
				140	71
				172[a]	

[a] Quantitation ion.

Blood Center, Gainesville, FL). The concentration of the internal standard, OHA, was selected because its peak area is comparable to that of the highest levels of tyrosine metabolites. The plasma or urine concentrations of tyrosine metabolites were usually less than 40 μg/ml. Therefore, the concentration ranges of calibration were set between 0.5 and 40 μg/ml to bracket the expected concentrations. No standards were available for the calibration of FA and the data from its *cis* isomer MA was used for its calibration. The calibration curves were plotted as concentration versus the ratio of peak area of the compound/peak area of the internal standard.

2.6. Recovery of derivatization and extraction

The commercially available methyl ester of DCA were dissolved in methylene chloride to make a stock solution containing 1000 μg/ml of the sample. This solution was diluted to 40, 20 and 10 μg/ml with methylene chloride and three dilutions at each concentration were made. To evaluate the efficiency of extraction, the solutions were treated in a manner similar to that described in section 2.3, i.e. a 1 ml aliquot of methylene chloride solution was mixed with the derivatization reagent (0.5 ml of 12%BF_3–MeOH) and washed with 1 ml water, vortexed for 2 min and centrifuged at 2500 × *g* at 10 °C for 8 min. The methylene chloride layer was separated from the extraction mixture, and this extract and the original unextracted methylene chloride solutions were assayed by GC/MS.

The results from the solutions, with or without extraction, were analyzed by linear regression. The efficiency of extraction was calculated as the ratio of slope of the calibration curve of each compound through extraction versus that of the calibration curve of the original solution without extraction, and multiplied by a factor of volume change during extraction (factor of extraction efficiency = ($slope_{(extract)}$/$slope_{(non\text{-}extract)}$) × factor of volume change). The calibration curves were obtained by using the same method for calibrating the compounds spiked in water, urine and plasma through derivatization. In contrast, the recovery of derivatization was calculated as the ratio of the slope of the calibration curve of each compound obtained from derivatization versus that of each reference methyl ester directly spiked in methylene chloride, and divided by the factor of extraction efficiency (recovery of derivatization reaction = ($slope_{(deriv)}$/$slope_{(non\text{-}extract)}$)/factor of extraction efficiency. Table 4 lists the recovery of derivatization and extraction efficiency in human plasma and urine.

2.7. Method validation

Precision was determined by calculating the intraday and interday coefficients of variance or relative standard deviations (R.S.D.%). Intraday and interday variances were measured using the same plasma, urine or water stock solutions prepared for calibration curves. To measure the intraday variance, five sets of derivatized and extracted samples were made at each concentration level, using the plasma, urine or water stock solutions. One set of derivatization and extraction was carried out on each of the following four successive days for measuring the interday variance. The intraday variance was calculated based on the five trial measurements accomplished in the first day, and the interday variance was calculated based on the results of five trial analyses performed on five consecutive days. Accuracy was determined by comparing concentrations measured from extracted samples with those determined from solutions to which compounds were directly added, and was expressed as bias (percentage difference between the measured and added concentrations). The intraday calibration curves were used to quantitate tyrosine metabolites in human plasma.

2.8. Sensitivity

The sensitivity limit of the instrument was evaluated by injecting decreasing amounts of each analyte and calculating the signal to noise ratio. A signal to noise ratio of 5 was defined as the sensitivity of the instrument for that particular analyte. The sensitivity of some of the endogenous compounds, such as fumarate, that were present in significant quantities in unspiked plasma and urine could not be evaluated. The values obtained from aqueous solutions are used for these compounds.

3. Results

3.1. Representative total ion chromatograms (TIC) and SIMs

The TIC of the authentic compounds spiked in water is shown in Fig. 2. The concentrations of DCA and tyrosine metabolites were approximately 10 μg/ml and OHA (methyl ester) was at a concentration of 100 μg/ml. The compounds detected were methyl esters of DCA and tyrosine metabolites. FA and SA co-elute under the GC/MS analysis conditions. However, they each have unique ions that permit quantitation without interference from each other. The GC/MS analysis of the urine of a child with HT1 before and after treatment with NTBC is shown in Figs. 3 and 4. Figs. 5 and 6 show the GC/MS analysis of urine and plasma spiked with tyrosine and DCA metabolites and demonstrate the utility of the method for analyzing the principal DCA and tyrosine metabolites in urine and plasma. Fig. 7 illustrates the application of the method the analysis of the plasma of a mouse treated with DCA.

3.2. Accuracy and precision

Quantitation was based on the peak area ratio of the target ions in the SIM mode of the compound to that of the internal standard. Since all the tyrosine metabolites are

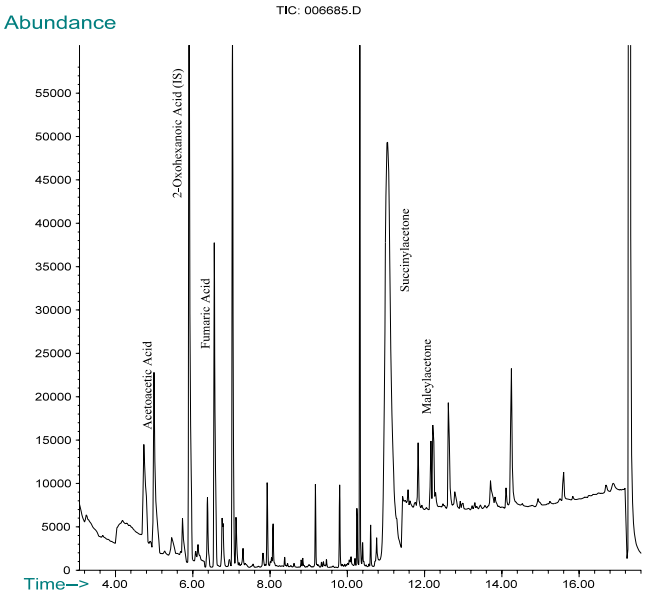

Fig. 3. TIC (SIM mode) of the GC/MS analysis of urine from a HT1 patient before the treatment with NTBC, showing elevated levels of SA, MA and other tyrosine metabolites.

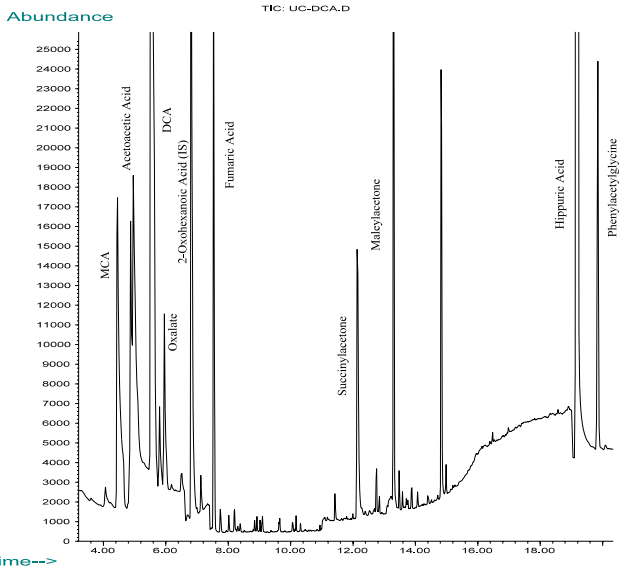

Fig. 5. TIC (SIM mode) of the GC/MS analysis of a urine sample from a healthy volunteer spiked with tyrosine and DCA metabolites, showing the separation of these compounds.

endogenous compounds, calibration samples in aqueous solutions were used for quantitation. In the case of HT1 patients, all measured tyrosine metabolites were elevated compared to control urine from healthy volunteers and therefore, standards made from control urine could be used for their quantitation. The calibration curves obtained from intraday determinations were used to quantitate the plasma and urine samples. Precision and accuracy of the method were expressed by standard deviation (S.D.), relative standard deviation (R.S.D.) and bias values listed in Tables 2 and 3. Linearity was represented by the regression coefficient value r^2. There were no significant differences between the r^2 values from different determinations for each compound.

3.3. Sensitivity and recovery of derivatization and extraction

Total recoveries from derivatization and extraction (Table 4) were calculated as the ratio of the slope of the calibration curve of each compound from derivatization

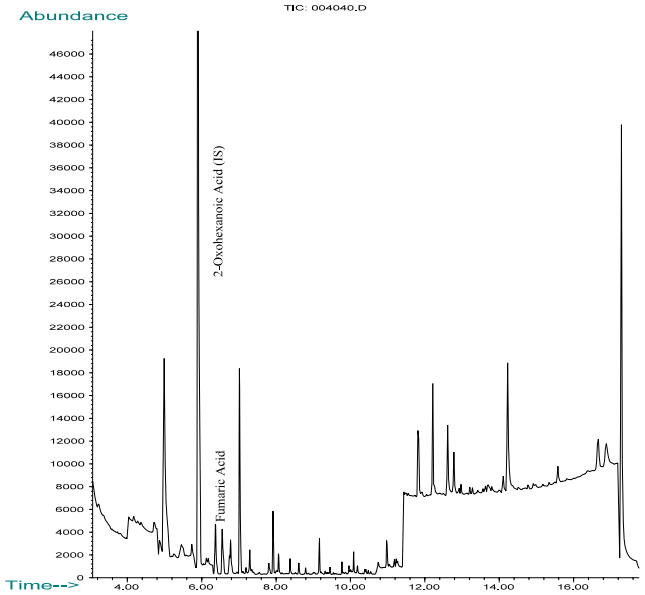

Fig. 4. TIC (SIM mode) of the GC/MS analysis of urine from a HT1 patient after treatment with NTBC (no detectable levels of tyrosine metabolites except fumaic acid was found).

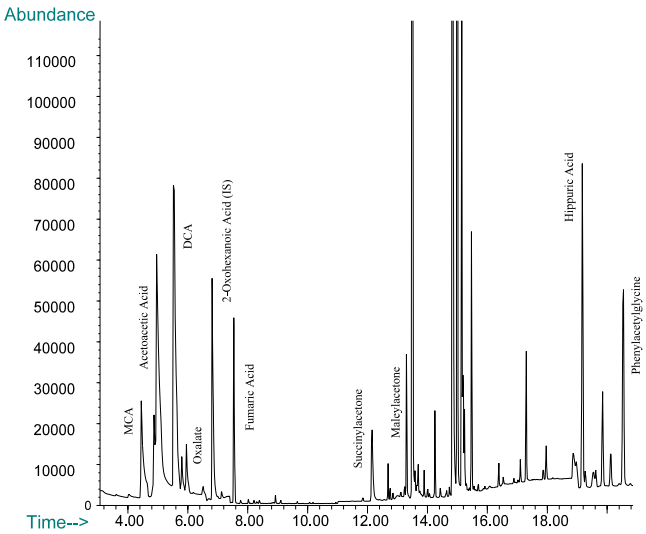

Fig. 6. GC/MS analysis (TIC-SIM) of a plasma sample from a healthy volunteer spiked with tyrosine and DCA metabolites, indicating the chromatographic resolution of these compounds in plasma.

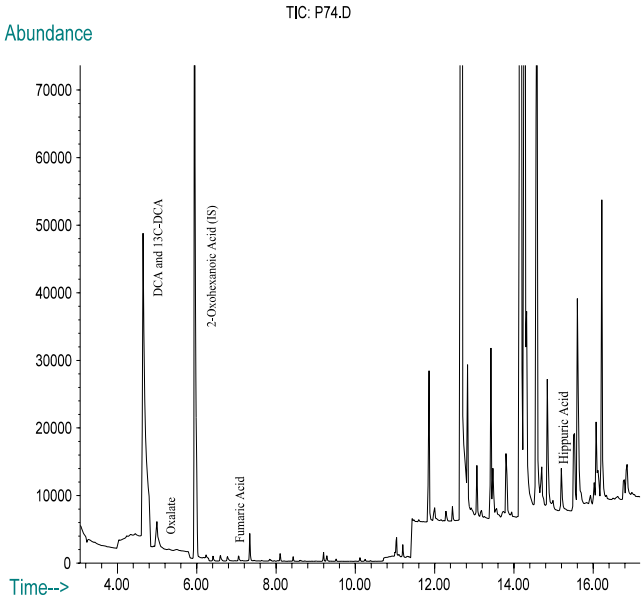

Fig. 7. GC/MS analysis (TIC-SIM) of a 30 min plasma sample from a GSTz knockout mice dosed with ^{13}C-DCA.

and extraction versus that of the calibration curve of the corresponding methyl ester directly spiked in methylene chloride. Extraction efficiency was expressed as the ratio of the slope of the calibration curve of each compound through extraction versus that of the calibration curve of original solution without extraction, and multiplied by a correction factor for volume change during extraction. In this case, 0.93 ml of methylene chloride was recovered from 1 ml used in the extraction, i.e., the correction factor for volume change was 93%. The recovery from the derivatization reaction was calculated by multiplying the value of the total recovery by the extraction efficiency.

Sensitivity of the instrument was determined as the signal of the quantitation ion to noise ratio of five for each analyte. Standards of each analyte were spiked into water and analyzed at lower concentrations until this level was reached (Table 5). An estimation of concentration of the compounds that would produce a signal to noise ratio of five in healthy urine and plasma is also included.

3.4. Application of the method for the analysis of urine and plasma samples

The present method was developed to facilitate studies on HT1 and its treatment with NTBC and to investigate the kinetics and toxicology of DCA in humans and animals. Thus far, the method has been successfully applied in the analysis of over 5000 urine and plasma samples from humans and rodents that will be reported separately. The urine data obtained from representative studies in humans are shown in Table 6 (HT1) and Table 7 (DCA). Untreated HT1 patients had high mean levels of SA and MA, whereas those in patients treated with NTBC were either lower or undetectable. In patients treated with DCA, MA levels were elevated, but SA was undetectable.

4. Discussion

Silylation and esterification are commonly used to derivatize a carboxylic acid to a non-polar group for GC/MS analysis. Other methods require multiple purification and extraction procedures followed GC/MS analysis [13–15]. Additional methods have been reported for analyzing SA, including quantitation by enzyme (δ-ALA synthase) inhibition [16]. SA and other ketoacids are first converted into

Table 2
Precision and accuracy of the assay in human urine

Urine intraday					Urine interday				
Compound spiked (μg/ml)	Mean ($n = 5$)	S.D.	R.S.D. (%)	Bias (%)	Compound spiked (μg/ml)	Mean ($n = 5$)	S.D.	R.S.D. (%)	Bias (%)
Acetoacetic acid ($r^2 = 0.9999$)					Acetoacetic acid ($r^2 = 0.9999$)				
120	124.91	2.51	2.01	4.09	120	124.98	1.57	1.26	4.15
60	63.62	1.43	2.25	6.04	60	63.85	1.22	1.92	6.41
30	32.60	0.95	2.91	8.65	30	32.14	0.31	0.98	7.12
Fumaric acid ($r^2 = 0.9999$)					Fumaric acid ($r^2 = 0.9985$)				
25	25.26	1.96	7.75	1.05	44	43.38	3.59	8.28	−1.42
15	15.75	0.78	4.95	5.03	22	19.64	3.22	16.39	−10.72
10	10.86	0.62	5.68	8.61	14	13.08	1.44	11.01	−6.59
Succinylacetone ($r^2 = 0.9975$)					Succinylacetone ($r^2 = 0.9938$)				
120	122.74	3.72	3.03	2.28	75	74.88	4.90	6.54	−0.17
50	42.20	3.50	8.29	−15.61	25	29.68	2.71	9.14	18.70
12.5	10.08	1.12	11.09	−19.33	12.5	9.92	1.77	17.86	−20.66
Maleylacetone ($r^2 = 0.9930$)					Maleylacetone ($r^2 = 0.9998$)				
50	49.24	2.86	5.80	−1.51	50	54.48	4.41	8.10	8.95
25	29.17	0.71	2.43	16.68	25	26.60	4.15	15.61	6.40
12.5	13.57	0.87	6.44	8.59	12.5	13.59	1.30	9.60	8.73

Table 3
Precision and accuracy of the assay in human plasma

Plasma intraday					Plasma interday				
Compound spiked (μg/ml)	Mean ($n = 5$)	S.D.	R.S.D. (%)	Bias (%)	Compound spiked (μg/ml)	Mean ($n = 5$)	S.D.	R.S.D. (%)	Bias (%)
Acetoacetic acid ($r^2 = 1.0000$)					Acetoacetic acid ($r^2 = 0.9999$)				
36.54	36.93	1.06	2.87	1.07	36.54	36.60	0.51	1.39	0.15
18.42	18.74	0.69	3.67	1.72	18.42	18.63	0.34	1.84	1.15
4.6	4.83	0.13	2.72	4.89	4.6	4.82	0.16	3.40	4.87
Fumaric acid ($r^2 = 0.9716$)					Fumaric acid ($r^2 = 0.9710$)				
20.12	24.67	1.04	4.22	22.61	20.12	24.09	1.03	4.28	19.72
10.06	6.62	0.53	8.03	−34.19	10.22	6.82	0.29	4.18	−33.22
2.52	1.77	0.15	8.27	−29.63	2.52	1.97	0.20	10.30	−21.96
Succinylacetone ($r^2 = 0.9999$)					Succinylacetone ($r^2 = 0.9995$)				
20.44	22.23	2.02	9.07	8.76	20.44	22.23	1.95	8.77	8.76
10.22	11.37	0.46	4.05	11.29	10.22	11.62	0.47	4.00	13.69
2.52	2.97	0.27	8.95	17.66	2.52	2.78	0.29	10.54	10.48
Maleylacetone ($r^2 = 0.9995$)					Maleylacetone ($r^2 = 0.9998$)				
8	10.22	0.48	4.72	27.73	8	10.22	1.07	10.44	27.77
2	1.87	0.20	10.48	−6.50	2	1.96	0.26	13.09	−1.87
1	0.83	0.03	3.58	−17.17	1	0.81	0.09	11.67	−19.33

Table 4
Recovery of derivatization, extraction efficiency in urine and plasma

Sample	Recovery of derivitization, mean ± S.D. (%)	Extraction efficiency, mean ± S.D. (%)	Total recovery, mean ± S.D. (%)
Urine	91.89 ± 2.5	79.56 ± 1.7	73.1 ± 2.5
Plasma	100.7 ± 3.5	85.1 ± 4.7	85.7 ± 4.7

Table 5
Method sensitivity in water, urine and plasma

Compound	Metabolite	Retention time (min)	Sensitivity (μg/ml at S/N = 5)		
			Standard	Urine	Plasma
MCA	DCA	4.5	2.5	2.5	2.5
Acetoacetic acid	Tyr	6.5	1.0	1.0	2.0
DCA	–	5.6	0.7	1.0	1.0
^{13}C-DCA	–	5.6	0.7	1.0	1.0
Glyoxylate	DCA	5.8	1.0	Present at higher levels	Present at higher levels
Oxalate	DCA	5.9	1.0	Present at higher levels	Present at higher levels
Fumaric acid	Tyr	7.6	0.5	Present at higher levels	Present at higher levels
Succinylacetone	Tyr	12.1	2.6	2.6	7.5
Acetylglycine	DCA	12.8	2.5	5.0	5.0
Maleylacetone	Tyr	13.3	0.2	0.2	0.2
Hippuric acid	DCA	17.5	0.8	0.8	0.8
Phenylacetyl-glycine	DCA	19.9	1.4	1.4	2.8

Tyr: tyrosine.

oxime derivatives that are extracted, dried and converted into trimethyl silyl derivatives under dry conditions [17,18]. Prior to derivatization to oxime, prepurification by solid phase extraction [19] or other means are employed [17]. The silylated oxime derivatives are then analyzed by GC/MS using direct quantitation or isotope dilution method [18]. The formation of oxime derivatives is often performed in the presence of strong acids that may isomerize or degrade sensitive compounds such as MA.

The precision (expressed in R.S.D.%) and accuracy (expressed in %bias) are below 10% for most of the compounds, but, there are exceptions which require further comment. Fumaric acid has relatively poor accuracy in plasma (>10%) but has acceptable accuracy in urine. The calibration curve of fumaric acid is not as linear in plasma ($r^2 = 0.9710$) as in urine ($r^2 = 0.9985$) and the variation of the curve could also be responsible for this discrepancy. Fig. 3 is a TIC of the sample and there appears to be another compound in this sample that is nearly coeluting with acetoacetic acid. The sum of the two responses of the two compounds makes the trace produce an appearance that the acetoacetic acid is tailing. In Fig. 5, a compound elutes closely to acetoacetic acid

Table 6
Mean urine concentration of tyrosine metabolites in HT1 children before and after treatment with NTBC

Tyrosine metabolite	Pre NTBC treatment		Post NTBC treatment	
	Concentration (μg/ml)	STD	Concentration (μg/ml)	STD
Acetoacetic acid	Not detected	–	Not detected	–
Fumaric acid	5.2	3.6	0.93	0.48
Succinylacetone	57	29	Not detected	–
Fumarylacetone	Not detected	–	Not detected	–
Maleylacetone	0.77	0.63	Not detected	–

Table 7
Mean values for tyrosine and DCA metabolites in children treated with DCA for congenital lactic acidosis

Compound	Placebo group ($N = 204$)		DCA group ($N = 202$)	
	Concentration (μg/ml)	STD	Concentration (μg/ml)	STD
MCA	0.00	0.00	0.00	0.00
Acetoacetic acid	0.65	0.97	0.47	0.91
DCA	1.30	9.62	13.44	36.07
^{13}C-DCA	0.21	1.76	8.06	9.74
Glyoxylate	8.64	10.91	6.41	5.91
Oxalate	103.7	108.7	95.5	105.2
Fumaric acid	14.19	33.94	6.88	15.03
Succinylacetone	0.00	0.00	0.00	0.00
Fumarylacetone	0.21	0.44	0.24	0.59
Acetylglycine	16.12	157.06	5.33	6.67
Maleylacetone	0.14	0.34	2.87	3.68
Hippuric acid	1342	1660	1366	1893
Phenylacetylglycine	1.21	2.27	1.62	5.46
^{13}C-MCA	0.00	0.01	0.00	0.01
^{13}C-Glyoxylate	0.22	0.57	0.59	2.72
^{13}C-Oxalate	4.93	6.22	10.08	8.07
^{13}C-Phenylacetylglycine	0.02	0.20	0.12	0.53
^{13}C-Hippuric acid	142.3	178.6	162.8	244.9
^{13}C-Acetylglycine	0.02	0.29	0.08	0.40

and because the column resolution is better one can clearly see that there are two compounds present. Acetoacetic acid is measured using a selected ion chromatogram of mass 116 and the nearby peak does not interfere with accurate quantitation.

SA has a lower than expected accuracy in both urine and plasma, especially in lower concentrations. SA is a difficult compound to quantitate because the peak generated tended to tail, which makes accurate integration and measurement difficult at low levels. All samples are bracketed with standards before and after the samples and are quantitated with the average of the two calibration curves. SA values were considerably higher in patients than normals and they were within the linear range of the concentration curve. Thus, the method can be used for measuring patient samples, which is its intended use.

The present method uses Lewis acid as a catalyst and methylation occurs under relatively mild conditions. Only one step is involved in preparing the sample for GC/MS analysis and no sample preprocessing or anhydrous conditions are required for derivatization or analysis. The technique is ideally suited for high throughput analysis, compared to methods that require multiple steps. Since the reaction mixtures can be purged with water and extracted with organic solvents, the final extract will be cleaner than those obtained from any method that uses direct silylation of the organic acids, and this minimizes background noise and contamination of the column and detector. We chose BF_3–MeOH for the methylation reaction, because other methylating (alkylating) agents, such dimethyl sulfate, diazomethane and diazoethane, have low solubilities in aqueous samples. BF_3–MeOH can precipitate plasma proteins and dissolve the analytes and it can also be washed out with water after the derivatization reaction.

Acknowledgements

This study was supported by NIH grants R01ES07355, P42ES07375 and MO1RR00082.

References

[1] H. Schierbeek, G.J. Beukeveld, H. van-Faassen, F.J. van-Spronsen, K. Bijsterveld, E.E. Venekamp-Hoolsema, B.G. Wolthers, G.P. Smit, J. Inherit. Metab. Dis. 16 (1993) 1034.

[2] E.A. Kvittingen, J. Inherit. Metab Dis. 14 (1991) 554.
[3] P.A. Russo, G.A. Mitchell, R.M. Tanguay, Pediatr. Dev. Pathol. 4 (3) (2001) 212.
[4] G. Mitchell, M. Lambert, R. Tanguay, in: C.R. Schriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, Health Professions Division, McGraw-Hill Inc., New York, NY, 1995, pp. 1077–1106.
[5] S.T. Pitkanen, M.K. Salo, M. Heikinheimo, Ann. Med. 32 (2000) 530.
[6] J.L. Aponte, G.A. Sega, L.J. Hauser, M.S. Dhar, C.M. Withrow, D.A. Carpenter, E.M. Rinchik, C.T. Culiat, D.K. Johnson, PNAS 98 (2001) 641.
[7] M. Grompe, Semin Liver Dis. 21 (2001) 563.
[8] P.W. Stacpoole, G.N. Henderson, Z. Yan, R. Cornett, M.O. James, Drug. Metab Rev. 30 (1998) 499.
[9] R. Cornett, M.O. James, G.N. Henderson, J. Cheung, A.L. Shroads, P.W. Stacpoole, Biochem. Biophys. Res. Commun. 262 (1999) 752.
[10] Z. Tong, P.G. Board, M.W. Anders, Chem. Res. Toxicol. 11 (1998) 1332.
[11] M.O. James, R. Cornett, Z. Yan, G.N. Henderson, P.W. Stacpoole, Drug. Metab. Dispos. 25 (1997) 1223.
[12] Z. Yan, G.N. Henderson, M.O. James, P.W. Stacpoole, J. Chromatogr. B 703 (1997) 75.
[13] P. Divry, C. Vianey-Liaud, J. Cotte, Biomed. Environ. Mass Spectrom. 14 (1987) 663.
[14] H.M. Liebich, E. Gesele, J. Chromatogr. A. 843 (1999) 237.
[15] K.R. Kim, H.G. Park, M.J. Paik, H.S. Ryu, K.S. Oh, S.W. Myung, H.M. Liebich, J. Chromatogr. B Biomed. Sci. Appl. 712 (1998) 11.
[16] A. Grenier, A. Lescault, C. Laberge, R. Gagne, O. Mamer, Clin. Chim. Acta 123 (1982) 93.
[17] X. Fu, M. Kimura, M. Iga, S. Yamaguchi, J. Chromatogr. B Biomed. Sci. Appl. 758 (2001) 87.
[18] H. Schierbeek, R. Berger, Clin. Chim. Acta 184 (1989) 243.
[19] T. Hagen, M.S. Korson, M. Sakamoto, J.E. Evans, Clin. Chim. Acta 283 (1999) 77.