A GC–MS/MS method for the quantitative analysis of low levels of the tyrosine metabolites maleylacetone, succinylacetone, and the tyrosine metabolism inhibitor dichloroacetate in biological fluids and tissues

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Abstract

We developed a sensitive method to quantitate the tyrosine metabolites maleylacetone (MA) and succinylacetone (SA) and the tyrosine metabolism inhibitor dichloroacetate (DCA) in biological specimens. Accumulation of these metabolites may be responsible for the toxicity observed when exposed to DCA. Detection limits of previous methods are 200 ng/mL (1.2 pmol/µL) (MA) and 2.6 µg/mL (16.5 pmol/µL) (SA) but the metabolites are likely present in lower levels in biological specimens. To increase sensitivity, analytes were extracted from liver, urine, plasma and cultured nerve cells before and after dosing with DCA, derivatized to their pentafluorobenzyl esters, and analyzed via GC–MS/MS.
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1. Introduction

Perturbation of human tyrosine catabolism (Fig. 1) may occur through several well-known inborn errors of metabolism or by xenobiotics [1]. Loss of function mutations in the terminal catabolic enzyme, fumarylacetoacetate hydrolase, cause hereditary tyrosinemia type 1 (HT1) and the accumulation of upstream intermediates, such as fumarylacetoacetate, fumarylacetone (FA), maleylacetoacetate and maleylacetone (MA). The acetate derivatives have not been directly measured in biological tissues or fluids from affected patients and, to date, only the urinary accumulation of MA has been reported in HT1. Nevertheless, these reactive molecules are thought to be responsible for the high incidence of hepatocellular carcinoma in children with HT1 [1]. Inhibition of fumarylacetoacetate hydolase also causes diversion of tyrosine intermediates to succinylacetone (SA), the urinary accumulation of which is pathonomonic of HT1 [2,3].

In turn, SA inhibits a proximal step in heme synthesis, causing accumulation of delta-aminolevulinate. These biochemical consequences of SA are thought to account for the neuropathic complications of HT1, including peripheral neuropathy [1].

The penultimate enzyme of tyrosine catabolism, maleylacetoacetate isomerase (MAAI), catalyzes the isomerization of maleylacetoacetate to fumarylacetoacetate and of MA to FA. MAAI is identical to the zeta1 family isoform of glutathione *S*-transferase (GSTz1), which dehalogenates a number of short chain haloacids, including dichloroacetate (DCA). This xenobiotic is unusual in that it is present ubiquitously in the biosphere, in part because of its environmental contamination as a disinfection by-product of water chlorination and as a metabolite of industrial degreasing agents, such as the Superfund chemical trichloroethylene [4]. Although typical daily human exposure to DCA is probably in the low µg/kg body weight range or less, the compound is also used as an investigational drug for certain acquired and congenital metabolic diseases, in which daily oral or intravenous administration approximates 10–50 mg/kg or more [5].

Over the so-called "environmental" (µg/kg per day)—"clinical" (mg/kg/d) concentration rage, DCA inhibits its own

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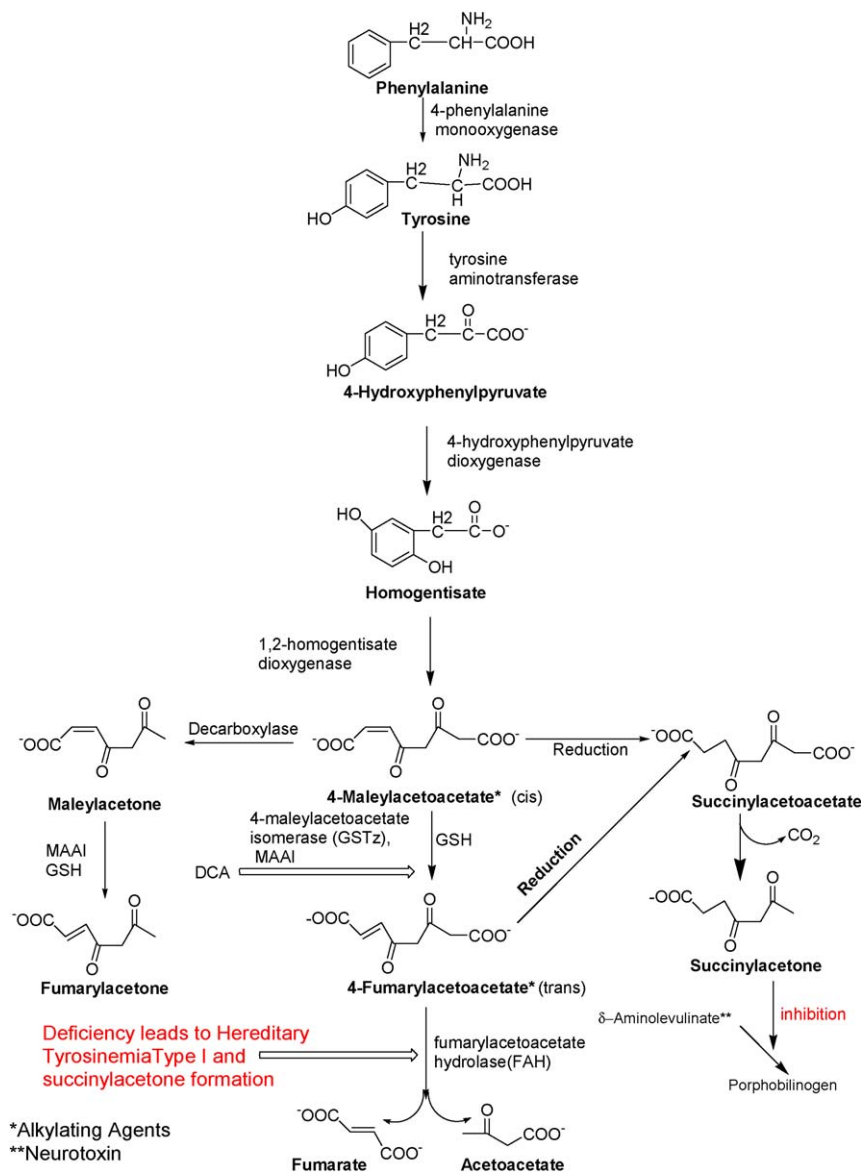*E-mail address:* mzolodz@msn.com (M.D. Zolodz).

Fig. 1. Tyrosine catabolic pathway.

metabolism, apparently by inhibiting the activity and decreasing the expression of MAAI/GSTz1 [4,6,7]. This has been demonstrated in humans [8] and rats, whereby repeated DCA exposure is associated with a decrease in its plasma clearance and an increase in its plasma half life [9]. In rats, hepatic activity and expression of MAAI/GSTz1 are reversibly decreased in a dose- and duration-dependent manner [9,10].

Current analytical techniques provide detection limits of 200 mg/mL (1.2 pmol/µL) for MA and 2.5 µg/mL (16.5 pmol/µL) for SA [11]. However, the levels of these metabolites are probably present in much lower concentrations in blood and tissues. Indeed, previous methods have not detected SA in plasma. Although DCA is readily measured in blood and urine by current techniques, [11,12] its quantitation in cells has been problematic without prior isotopic labeling of the molecule [13]. Thus, improvement in the ability to detect low concentrations of MA, SA and DCA could have broad application to the

study of normal amino acid metabolism and the effects of genetic or pharmacological perturbations thereon.

Electron capture is a highly sensitive ionization technique and can be used to improve the detection limits of electron ionization (EI) methods [14–16]. The use of a derivatizing agent with high electron capture ability and the capacity to provide stable negative ions results in increased sensitivity over positive CI or EI. A common derivatizing agent for this procedure is pentafluorobenzyl (PFB) bromide [15,16]. In the ionization source electron capture negative chemical ionization (ECNCI), gas phase PFB-analytes are bombarded with thermal electrons produced from high energy collisions of electrons with the CI gas. The five highly electronegative fluoro atoms of PFB derivatives are efficient in capturing thermal electrons. The reaction with the thermal electrons results in fragmentation of the PFB-analyte at the ester bond forming the stable anion of the carboxylic acid. These ions are then focused and analyzed in the ion trap mass

analyzer in the negative ionization mode. Furthermore, in the electron capture negative chemical ionization mode, formation of the anion is a highly efficient process resulting in a significant increase in sensitivity.

In this study we developed specific and sensitive methodology to quantitate low levels of MA, SA and DCA in rat and human liver, biological fluids and cultured nerve cells. Analytes were extracted by liquid–liquid extraction with dichloromethane (liver) or by solid phase extraction (SPE) (urine, plasma, cell lysate) and were derivatized to their pentafluorobenzyl esters, followed by GC–ECNCI-MS/MS analysis. Selected reaction monitoring (SRM) mode was used to measure DCA and metabolite concentrations in tissue, fluid and cell culture lysate. Quantitation was performed via linear regression analysis utilizing calibration curves that were prepared by spiking MA, SA, and DCA in the appropriate sample matrix, followed by extraction and derivatization.

2. Experimental

2.1. Reagents

Sodium hydroxide (NaOH), potassium chloride (KCl), trifluoroacetic acid (TFA), acetonitrile (ACN), methanol (MeOH), and dichloromethane (CH_2Cl_2) were from Fischer. Succinylacetone, PFB-Br, 18-crown-6,6-oxoheptanoic acid (6-OHA), sodium sulfate (Na_2SO_4) (99%), triethylamine (TEA) were from Sigma–Aldrich (Milwaukee, WI). Methyl-*tert*-butyl ether (MTBE) was from Fluka. C_{13}-DCA was Cambridge Isotope Laboratories, Inc. (Andover, MA).

2.2. Preparation of maleylacetone

Maleylacetone was prepared according to the method of Fowler and Seltzer [17].

2.3. Nerve cell culture and lysate preparation

Rat Schwann cells were obtained from sciatic nerves of newborn pups [18]. They were cultured in Dubecco's modified Eagle Medium (Gibco, Rockville, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 10 mM HEPES (Cellgro, Mediatech, Inc., Herndon, VA), 2 µg/mL forskolin (Calbiochem, La Jolla, CA), bovine pituitary gland extract 0.1 mg/mL (Biomedical Technologies, Inc., Stoughton, MA). Cells were incubated for 48 h in a final concentration of 0, 1, 5, 10, or 40 mM DCA. Culture plates were rinsed twice then lysed with 500 µL of a solution containing 6% TFA and 1% KCl. The plates were scraped and the solution was transferred to vials and vortexed. Lysate was stored at −80 °C prior to analysis.

2.4. Plasma and urine sample preparation

Rats were anesthetized in a tank filled with carbon dioxide. Venous blood was sampled and red blood cells and plasma prepared. Equal volumes of plasma or urine were treated with a solution of 6% TFA and 1% KCl and vortexed. The solution was incubated on ice for 15 min, vortexed, and centrifuged at 14,000 rpm for 60 min at 4 °C. Two hundred microliters of sample was used for analysis.

2.5. Human liver

An adult male who was scheduled to undergo elective resection of a benign hepatic cyst volunteered to enroll in a study of DCA's effects on human tyrosine metabolism. After obtaining written, informed consent, the subject was admitted to the General Clinical Research Center at the University of Florida. He received 12.5 mg DCA by mouth every 12 h for 2 days prior to surgery, with the last dose being administered approximately 12 h before surgery. Control (untreated) liver samples were obtained from a banked repository at UF.

2.6. Liver sample preparation

An equal volume (v/w) of ice cold homogenization buffer (1N NaOH and 1% KCl) was added to pieces taken from several sections of the liver sample (total weight of approximately 500 mg) and homogenized, using a Tissue Tearer homogenizer (BioSpec). The supernatant was decanted and pH was adjusted to less than pH 2, to precipitate proteins and protonate metabolite compounds. The sample was then centrifuged in an Eppendorf centrifuge at 14,000 rpm for 60 min at 4 °C. The supernatant was transferred to a clean Eppendorf vial and kept on ice until extraction.

2.7. Extraction procedure 1: liquid–liquid

Five hundred microliters of deproteinized sample, 1 mL CH_2Cl_2, and 300 mg Na_2SO_4 (99.9%) were combined in a 5 mL glass test tube that was vortexed for 2 min, sonicated for 5 min and centrifuged at 3000 rpm, 25 °C, for 30 min. Seven hundred microliters of the bottom organic layer were transferred to a previously weighed 750 µL amber conical vial and concentrated by a gentle N_2 stream. Five hundred microliters of CH_2Cl_2 were added to the aqueous layer and extracted as above. Four hundred microliters of the bottom organic layer were transferred to a 750 µL amber conical vial. The combined extract was concentrated under N_2 to less than 10 µL. Vials were weighed to obtain a more accurate volume reading (10 µL CH_2Cl_2 = 0.0132 g). The extract was adjusted to a final volume of 100 µL of MTBE and 2–3 mg Na_2SO_4 was added prior to derivatization as a drying agent.

2.8. Extraction procedure 2: SPE

SPE Bond-Elut-PPL cartridges (100 mg, 1 mL, Varian) were first conditioned with 1 mL of methanol and equilibrated with a solution of 6% TFA and 1% KCl. The sample (200–500 µL) was added and allowed to move through the cartridge by gravity. The SPE cartridge was then rinsed with a solution of 5% ACN in 6% TFA and 1% KCl and was dried at room temperature. Analytes were eluted by allowing 500 µL (two times) MTBE to soak for one min before eluting. The eluate was concentrated to less than

5 μL. One hundred microliters of MTBE and 2–3 mg of Na_2SO_4 were added prior to addition of base and derivatization solution.

2.9. Reaction conditions

Fifty microliters of triethylamine (TEA), 150 μL of 200 mg/mL PFB-Br, 20 mg/mL 18-c-6 solution were added to the extract. Vials were capped, vortexed, placed in a heated water bath at 50 °C and sonicated for 30 min. Vials were then centrifuged at 3100 rpm for 30 s. The reaction solution was transferred to an autosampler vial for GC–MS analysis.

2.10. Instrumental analysis

Samples were analyzed in the ECNCI mode by GC–MS using a Trace GC coupled to a PolarisQ ion trap mass spectrometer (Thermo Electron). The GC capillary column was a ZB-5 column from Phenomenex (30 m × 0.25 mm i.d., 0.25 mm thickness). The temperature program began at 35 °C for 1 min, ramped to 300 °C at 10 °C/min, and was held at 300 °C for 5 min. One microliter of sample was injected. The helium carrier gas flow rate was 1.2 mL/min, while the CI gas (methane) flow rate was 1.8 mL/min. The ionization source temperature was set to 100 °C, the inlet temperature was set to 275 °C and the transfer line from the GC to mass spectrometer was set to 275 °C. Data analysis was performed using Xcaliber software (Version 1.2). Ion trap conditions were optimized for selected reaction ion monitoring and are listed in Table 1.

2.11. Western blot

Samples of rat liver cytosol were heated in SDS-PAGE sample buffer at 95 °C for 5 min and 40 μg of total protein from each sample was resolved by SDS-PAGE on a polyacrylamide gel (12%, Bio-Rad Laboratories, Hercules, CA). The gel was then transferred to a 0.45 μm nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). After blocking for 1 h in 5% fat free milk in TBS-T buffer (Bio-Rad Laboratories, Hercules, CA), the membrane was incubated with anti-GSTz1-1 chicken polyclonal antibody (primary antibody) for 1 h, followed by anti-chicken IgG secondary antibody (Research Diagnostics, Inc., Concord, MA) for 2 h. Detection was performed using ECL Western Blotting Detection Reagents (Amersham Biosciences Corp., Piscataway, NJ) and bands were quantitated by image analysis software (ScanAnalysis, Biosoft, Ferguson, MO).

3. Results and discussion

3.1. Optimizing sample preparation

We evaluated the relative ability of basic, neutral and acidic pH solutions to homogenize liver samples. Solutions containing 100 mM phosphate buffer (pH 7.4) with 1% KCl were compared to solutions containing 6% TFA and 1% KCl solution and 1N NaOH and 1% KCl. The acidic solution was the least useful because it led to the protonation of the carboxylic acid metabolites, rendering them less hydrophilic. The basic solution provided the highest recovery of MA (Fig. 2), since at high pH, MA and SA are ionized and water soluble. The NaOH may also be involved in breaking up ionic interactions in the matrix, thereby allowing the metabolites to be more easily extracted. Urine and plasma samples were treated with 6% TFA and 1% KCl to precipitate proteins and protonate metabolites prior to extraction.

3.2. Extraction method development

We initially used a liquid–liquid extraction as the extraction method to separate analytes from aqueous solution, which decreases the complexity of the sample to be analyzed by GC–MS. MA, the most labile of the compounds, was used as the test compound to optimize extraction conditions because it can isomerize to FA. Samples were adjusted to pH < 2 with TFA to ensure that MA was protonated and extractable from the aqueous solution into the organic solvent. One milliliter of organic solvent was added to the aqueous sample and was vortexed for 2 min. Samples were then centrifuged at 4000 rpm for 20 min at 10 °C. The bottom layer was transferred to a vial and concentrated. The aqueous solution was re-extracted with 0.5 mL organic solvent and the extracts were combined and concentrated with a gentle N_2 stream. Highest extraction recovery of MA was obtained with CH_2Cl_2 as the extraction solvent, but only 29% recovery was obtained. To improve recovery, salt was added to the aqueous sample to increase the ionic strength of the solutions prior to adding dichloromethane. Both sodium chloride and sodium sulfate increased recovery of MA to 44%. Sodium sulfate was easier to remove with centrifugation and did not interfere with the mass spectral analysis. Sonicating for 5 min after vortexing yielded 100% recovery of MA.

The efficiency of liquid–liquid extraction is limited by the amount of time required to perform the experiment. We devel-

Table 1
Parameters for selected ion reaction monitoring

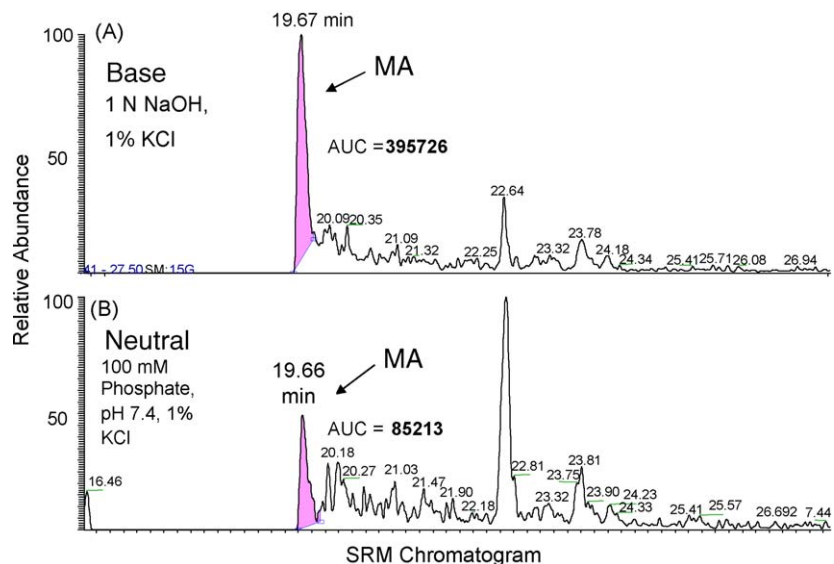| Compound	Precursor ion m/z	Isolation width	Isolation time (ms)	Excitation voltage	Excitation time (ms)	q-Value	Product ion range
MA	155	4	12	1.25	23	0.3	69, 96, 111
6-Oxoheptanoic acid (IS)	143	4	12	1.00	23	0.3	83, 99, 125
SA	157	4	12	1.00	23	0.3	71, 99, 115
DCA	129	1	8	1.20	15	0.3	34–38, 82–87

Fig. 2. Optimization of homogenization buffer for recovery of MA from rat liver after 5-day treatment with DCA at 50 mg/kg per day. Addition of base to the liver homogenization buffer provided the maximum recovery of MA (A) as compared to homogenization of liver at neutral pH (B) as shown in the SRM chromatogram for MA.

oped a solid phase extraction (SPE) method to increase the method's applicability for high throughput analysis of multiple urine and plasma samples. Retention and elution of MA, SA and DCA were tested on C_{18} (endcapped and non-endcapped), C_8, C_4, CN, phenyl and PPL SPE cartridges. C_8, C_4, CN, and phenyl showed poor recovery of the analytes, whereas the highest recovery of analytes was achieved when using PPL. The rinse solution and elution solvent were further optimized by using SPE cartridges containing the PPL stationary phase. 5% ACN, 6% TFA and 1% KCl as the rinse solution was found to be the best for retaining MA, SA, and DCA on the SPE. MTBE was most efficient in eluting MA, SA, and DCA (data not shown).

3.3. Pentafluorobenzyl esterification reaction optimization

Carboxylic acids are not easily chromatographed by GC, and often elute as broad peaks. To improve their chromatography, these compounds are often derivatized to an ester. The esterification reaction utilized in this method involves the addition of an electronegative compound such as PFB-Br to increase the ionization efficiency in NCI [15]. The carboxylic acid is first deprotonated with the addition of base followed by nucleophilic displacement of the bromine from the PFB-Br reagent and the formation of a new ester bond. 18-Crown-6 assists in driving the reaction forward by forming a complex with cations. The reaction is performed by heating the solution and excess base is imperative to neutralize the HBr produced in the reaction. This is especially important to protect MA, because it can isomerize to the more stable *trans* isomer FA with HBr and heat. The PFB derivatized analyte products provide better peak shape than the underivatized carboxylic acid in the GC separation.

Considerable effort had to be taken to obtain a high reaction yield of the ester without invoking isomerization of MA to the *trans* isomer FA. The reaction time and temperature using

three bases (KHCO$_3$, diisopropylamine, TEA) were optimized and compared. Using solid base (KHCO$_3$) and a phase transfer catalyst (18-c-6) did not neutralize the HBr produced in the reaction fast enough to prevent isomerization from occurring. In fact, several soluble bases were compared for their reaction yield and ability to prevent isomerization. The solid base KHCO$_3$ allowed the largest amount of *cis–trans* isomerization. Using diisopropylamine decreased isomerization to 50%. Triethylamine provided the highest reaction yield without producing any detectable amount of the *trans* isomer. Peak identities of MA and FA were verified by NMR (data not shown) and the elution order matched the theoretical order that the *cis* isomer had a shorter retention time as compared to the *trans* isomer.

Reaction time and temperature had large effects on the reaction yield and were optimized using TEA as the base. The maximum reaction yields for MA and SA were obtained at 30 min. Increasing the temperature over 50 °C increased isomerization of MA to FA, whereas decreasing the reaction temperature resulted in low reaction yields (data not shown). Other parameters optimized included concentration of PFB-Br in the derivatization solution, concentration of TEA and reaction solvent (MTBE, CH$_2$Cl$_2$, hexane, and acetone were tested). Although PFB-Br must be present in excess, too high of a concentration interferes with mass spectral analysis by decreasing ionization efficiency of the analyte. A final PFB-Br concentration of 100 mg/mL provided the highest reaction yield and lowest MS interference. A final concentration of 16.6% TEA was found to optimally neutralize any TFA carried over from the extraction procedure, deprotonate the carboxylic acid of the metabolites and neutralize the HBr produced from the esterification reaction. Several reaction solvents were also tested. MTBE was determined to be the most suitable solvent for the reaction that was also compatible with GC–ECNCI-MS/MS analysis.

3.4. Internal standard

Internal standards used in GC–MS methods usually involve the isotopically labeled version of the compound of interest. However, isotopically labeled MA and SA are not commercially available. Consequently, we utilized several compounds (2-oxovaleric acid, 2-oxohexanoic acid, 6-oxoheptanoic acid 5,7-dioxooctanoic acid and 2-Bromooctanoic acid, from Sigma) that have similar structures, functional groups, and molecular weights to the analytes. These compounds were derivatized and analyzed by GC–MS. 6-Oxoheptanoic acid was chosen as the internal standard because it was readily extracted and derivatized under the reaction conditions developed for MA, had the best chromographic peak shape compared to other compounds tested and eluted at a similar retention time to MA and SA.

3.5. Method validation

Several parameters were examined to validate the method. To ensure its specificity and increase its sensitivity, we employed selected reaction monitoring mode for quantitation. The specific transitions used for each analyte are shown in Table 1. An example of an SRM chromatogram and mass spectrum of MA spiked into human liver is shown in Fig. 3. Three transitions were monitored for each compound and peak integration for quantitation was performed using two of the transition ions. To produce calibration curves, compounds were spiked into buffer, liver homogenate, plasma or urine, using at least five different concentrations. The ratio of the area under the curve (AUC) for

Table 2
Limit of quantitation (s/n = 10) in total pmol injected onto column of compounds spiked into biological matrix

Analyte	Liver (human, rat)	Plasma (rat)	Urine (rat)
MA	0.007	0.058	0.058
SA	0.588	0.588	0.588
DCA	<2.6	<2.6	<2.6

the analyte and the AUC for the internal standard was plotted against the analyte concentration and a line was generated by linear regression. Linear calibration curves obtained in buffer, liver, plasma and urine had correlation coefficients from 0.993 to 0.999 for all three compounds. For intraday analysis (five days), each curve was prepared fresh in the appropriate matrix, extracted, derivatized and analyzed. For interday analysis, MA, SA, and DCA were spiked into appropriate control matrix (prepared five times), extracted, derivatized and analyzed. The variability was examined by calculating % R.S.D.s which ranged from 1.32 to 17.1%. Table 2 summarizes the limit of quantitation for each analyte in each matrix. The limit of quantitation for DCA was not tested below 2.6 pmol/μL because all samples contained much higher amounts of the compound. The instrument limit of detection on the full scan analysis of MA was 29 amol/μL [19]. Derivatized compounds were stable for one week when kept at −80 °C, or for 12 h at room temperature (data not shown). After repeated freeze-thaw cycles DCA treated human liver sample began to show isomerization of MA to FA. To test the stability of the compounds in liver, treated rat liver samples were exposed to several freeze-thaw cycles to test for sample loss or insta-
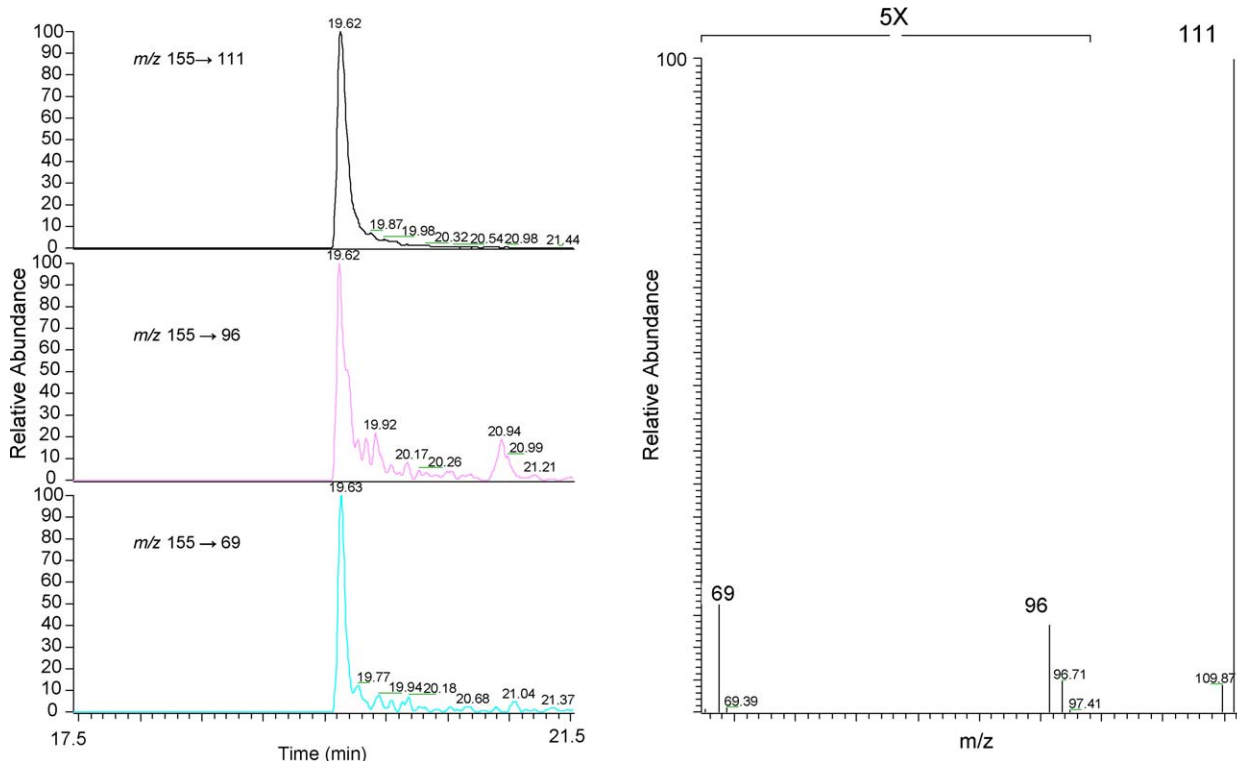

Fig. 3. Selected reaction monitoring (SRM) of MA spiked into human liver homogenate showing extracted ions. SRMs were used to increase the specificity of the methodology and the transitions at m/z 111, 96, and 69 were used to identify MA. Transitions at m/z 111 and 69 were used for quantitation.

Table 3
Primary rat Schwann cells (8.8×10^6 cells/plate) were treated with DCA for 48 h and analyzed for MA, SA, and DCA (mean of duplicate determinations)

DCA dose (mM)	DCA (pmol) in 7×10^6 cells
0	ND
1	34.0
5	88.7
10	229
40	1160

bility. After three freeze-thaw cycles, only 37% SA and 3.3% MA were recovered. DCA decreased to 59% recovery only after 5 freeze-thaw cycles (DCA content was not measured at time point zero so 1 freeze-thaw cycle was compared to five freeze-thaw cycles). Thus, samples should be frozen immediately and aliquoted while frozen to prevent isomerization from occurring, if repeated analyses are required [19].

3.6. Application to mammalian cell and tissue samples

When the liver tissue was obtained from the patient who received DCA, the SRM method had not been developed. There-fore, the hepatic MA content was analyzed in full scan mode and MA was estimated to be in the pg/g wet wt range. Repeated analysis of the human patient liver sample showed isomerization of MA to FA. The concentration of SA was 9.3 pg/g wet wt. MA and SA could not be detected in control (nondiseased, nontreated) human liver samples. Thus, we found no evidence for tissue accumulation of MA and SA in healthy liver stored under these conditions.

Accumulation of MA and/or SA may be involved in the peripheral neuropathy observed in animals and patients receiving chronic DCA. We therefore incubated primary cultures of rat Schwann cells with DCA doses of 0, 5, 10, or 40 mM for 48 h. The concentration of DCA in cell lysates increased in a dose-dependent manner, but MA and SA could not be detected (Table 3). It is possible the tyrosine catabolic pathway may not be sufficiently active in cultured rat neurons to generate measurable amounts of these intermediates, despite the presence of DCA.

We also investigated the effect of DCA on the expression of GSTz1 in liver and on the concentrations of tyrosine metabolites in rat plasma, urine and liver before and after oral treatment of 50 mg/kg per day for 5 days (Table 4). DCA decreased the expression of GSTz1 in treated animals 85% below that found

Table 4
Effect of DCA on hepatic GSTz1 expression, determined by Western immunoblotting, and on levels of MA, SA, and DCA in rat liver, plasma, and urine, using GC–ECNCI-MS with SRMs

Analyte	Liver mean ± S.D.	Plasma mean ± S.D.	Urine mean ± S.D.
GSTz1 (control)	18.3 ± 1.19 μg/g wet weight liver	NA	NA
GSTz1 (treated)	2.73 ± 1.62 μg/g wet weight liver	NA	NA
MA	17.1 ± 11.99 pg/g wet wt liver	11.8 ± 2.85 pg/mL	224 ± 87.0 pg/mL
SA	6.42 ± 3.39 pg/g wet wt liver	300 ± 160.6 pg/mL	2.19 ± 1.23 ng/mL
DCA	7.20 ± 2.74 ng/g wet wt liver	54.4 ± 26.2 ng/mL	1.83 ± 0.43 ng/mL

MA, SA, and DCA concentrations were undetectable in liver, plasma, and urine of untreated rats ($n = 5$ per group).
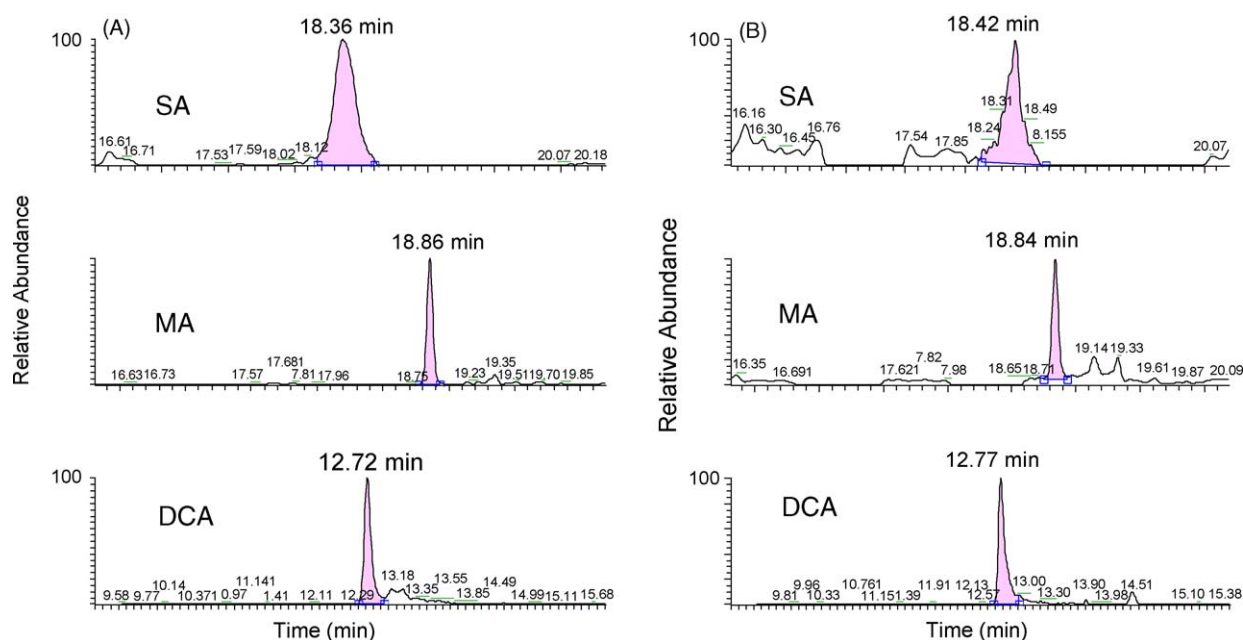

Fig. 4. Rats ($n = 5$ control, $n = 5$ treated) were treated for 5 days with 50 mg/kg per day of DCA. Example SRM chromatograms of A urine and B liver analysis after DCA.

in control livers. Five control rats and five treated rats were analyzed for MA, SA, and DCA. No untreated (control) samples contained detectable amounts of MA, SA or DCA. However, these molecules were readily quantitated in the plasma, urine and liver of dosed animals. Fig. 4A and B shows representative chromatograms of urine and liver analysis of SA, MA and DCA.

4. Conclusions

We developed a sensitive GC–MS/MS method to measure MA, SA, and DCA in biofluids and tissue. Previously recorded limits of quantitation were improved almost 200 times for MA and 30 times for SA and enabled for the first time the detection of these compounds in plasma and liver. The method is readily applicable to investigating the tyrosine catabolic pathway, particularly when influenced by congenital or acquired deficiencies of its cognate enzymes.

Acknowledgment

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