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Metabolomic Analysis of Key Central Carbon Metabolism Carboxylic Acids as Their 3-Nitrophenylhydrazones by UPLC/ESI-MS

Jun Han¹, Susannah Gagnon¹, Tobias Eckle², and Christoph H. Borchers^{1,3,*}

¹University of Victoria - Genome BC Proteomics Centre, University of Victoria, Victoria, British Columbia, Canada

²Mucosal Inflammation Program, Department of Anesthesiology, University of Colorado Denver, CO, USA

³Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada

Abstract

Multiple hydroxy-, keto-, di-, and tri-carboxylic acids are among the cellular metabolites of central carbon metabolism (CCM). Sensitive and reliable analysis of these carboxylates is important for many biological and cell engineering studies. In this work, we examined 3-nitrophenylhydrazine as a derivatizing reagent and optimized the reaction conditions for the measurement of ten CCM related carboxylic compounds, including glycolate, lactate, malate, fumarate, succinate, citrate, isocitrate, pyruvate, oxaloacetate, and α -ketoglutarate as their 3-nitrophenylhydrazones using LC/MS with electrospray ionization. With the derivatization protocol which we have developed, and using negative-ion multiple reaction monitoring on a triple-quadrupole instrument, all of the carboxylates showed good linearity within a dynamic range of ca. 200 to more than 2000. The on-column limits of detection and quantitation were from high femtomoles to low picomoles. The analytical accuracies for eight of the ten analytes were determined to be between 89.5 to 114.8% (CV 7.4%, n=6). Using a quadrupole time-of-flight instrument, the isotopic distribution patterns of these carboxylates, extracted from a ¹³C-labeled mouse heart, were successfully determined by UPLC/MS with full-mass detection, indicating the possible utility of this analytical method for metabolic flux analysis. In summary, this work demonstrates an efficient chemical derivatization LC/MS method for metabolomic analysis of these key CCM intermediates in a biological matrix.

Keywords

Central carbon metabolism; Carboxylic acid; 3-Nitrophenylhydrazine; Chemical derivatization; LC/MS; Isotope-resolved metabolic profiling

*Correspondence should be addressed to: Prof. Christoph H. Borchers, University of Victoria - Genome British Columbia Proteomics Centre, 3101-4464 Markham St, Victoria, BC Canada V8Z 7X8, Phone (250)483-3221; Fax: (205)483-3238, christoph@proteincentre.com.

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1 Introduction

Central carbon metabolism (CCM) is a complex enzyme-mediated metabolic network which is highly conserved in biological species. The CCM pathway is composed of several metabolic sub-pathways: glycolysis, gluconeogenesis, the pentose phosphate pathway (PPP), and the tri-carboxylic acid (TCA) cycle [1]. Among the cellular metabolites of CCM, there are multiple carboxylic acids (CAs) including hydroxy-, keto-, di-, and tri-carboxylic acids. These carboxylic compounds play important roles in cellular metabolism. For example, the TCA cycle as the central hub of CCM generates reducing NADH which is fed into the oxidative PPP to produce cell usable energy in the form of adenosine triphosphate (ATP). The keto- and poly-carboxylic intermediates of the TCA cycle also act as substrates for the biosynthesis of other biomolecules, such as amino acids. Changes in the absolute concentrations of the TCA intermediates could predict defects in substrate supply into the mitochondria. Thus, measurement of the CCM-related carboxylates can provide essential information about the catabolic and anabolic processes of a cell and has been important in many metabolism studies, particularly for isotopic-labeling metabolic flux analysis (*e.g.*, with ^{13}C) [2, 3]. Powerful analytical techniques are therefore needed for precise and reliable measurement of these physiologically important carboxylates in biological matrices.

The predominant technique for analysis of CAs is gas chromatography coupled to mass spectrometry (GC/MS), in combination with chemical derivatization with boron trifluoride/butanol [4], hexamethyldisilane [5], N-methyl- N-tert-butyltrimethylsilyltrifluoroacetamide [6] or N-methyl-N-trimethylsilyltrifluoroacetamide [7, 8], etc. The derivatization reactions for GC/MS need to be conducted in non-aqueous solution, while the analytes of interest in a biological sample are usually present in aqueous cellular systems. The need for transferring the analytes from an aqueous phase to a non-aqueous phase involves evaporation to dryness and reconstitution, which can be time-consuming and may be associated with the risk of loss of the analytes [6].

LC/MS has become an attractive and commonly used technique for the analysis of organic acids, including the CCM related CAs [9, 10], for example, using anion exchange chromatography [11] or ion exclusion chromatography [12] coupled to mass spectrometry. However, the detection of the low-molecular weight CAs in negative-ion ESI, without chemical derivatization, is often problematic. Accurate and sensitive quantitation is usually difficult to achieve because of the complex matrices of biological samples. To enhance LC/UV, LC-fluorescence or LC/MS quantitation of CAs, chemical derivatization has been widely used [13–16]. For example, Kubota [17] described chemical derivatization with 4-*N,N*-dimethylaminosulfonyl-7-piperazino-2,1,3-benzoxadiazole as a pre-analytical derivatizing reagent for the LC-fluorescence determination of seven carboxylates related to the TCA cycle, and pyruvate -- an end product of the glycolysis. Kloos [18] recently introduced N-methyl-2-phenylethanamine as a reagent for the selective derivatization of several keto-, di-, and tri-carboxylic acids of the TCA cycle for LC/positive-ion ESI-MS. However, this method cannot be used for analysis of the hydroxyl CAs such as glycolic and lactic acid due to the low sensitivity for the derivatives of these two compounds in positive-ion ESI-MS.

Singly nitro-substituted phenyl hydrazines such as 2- nitrophenylhydrazine (2-NPH) have been used to derivatize carboxylic acids. For example, 2-NPH has been used as a derivatizing reagent for HPLC/UV quantitation of biotin [19], organic acids [20–22], and short-chain [23, 24] / straight-chain [24] / long-chain fatty acids [25, 26]. Another singly nitro-substituted phenyl hydrazine, 3-nitrophenylhydrazine (3-NPH), has been also utilized for quantification of ^{13}C -pyruvate and ^{13}C -lactate in dog blood by reversed-phase HPLC/ESI-MS [27]. So far, these two nitrophenylhydrazines have not been used for the simultaneous quantitation of more key carboxylates of CCM. In this work, we examined the use of 3-NPH as an efficient pre-analytical derivatizing reagent for the simultaneous determination of ten CCM carboxylates including glycolate, lactate, malate, fumarate, succinate, citrate, isocitrate, pyruvate, α -ketoglutarate, and oxaloacetate by LC/MS. With the optimized derivatization protocol, both isotope-resolved metabolic profiling and quantitation of the ten compounds in biological samples has been achieved.

2 Material and Methods

2.1 Materials and reagents

Acetonitrile, water, methanol, and formic acid were LC/MS grade. These reagents, together with three singly nitro-substituted phenylhydrazines, 2-, 3-, and 4-nitrophenylhydrazines (NPHs), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC).HCl, HPLC-grade pyridine, butylated hydroxytoluene (BHT), glycolic acid (99%), sodium DL-lactate (99.0%), DL-malic acid (99.5%), fumaric acid (99%), sodium succinate dibasic hexahydrate (99%), DL-isocitric acid trisodium (98%, 16.6% water), sodium pyruvate (99%), α -ketoglutaric acid (99%), and oxaloacetic acid (98%), were purchased from Sigma-Aldrich (St. Luis, MO, USA). The authentic reference materials for the stable isotope-labeled analogues of eight CAs, including glycolic acid-2,2-d₂ (99.7% D), sodium lactate-3,3,3-d₃ (99.8% D), malic acid-2,3,3-d₃ (98.5% D), fumaric acid-2,2,3,3-d₄ (99% D), citric acid-2,2,4,4-d₄ (98% D), succinic acid-2,2,3,3-d₄ (99% D), sodium pyruvate- $^{13}\text{C}_3$ (99% ^{13}C), and α -ketoglutaric acid-d₆ (98% D) were used as internal standard (IS) for assay development and validation. These compounds were obtained from either Sigma-Aldrich or C/D/N Isotopes (Pointe-Claire, Quebec, Canada). U- ^{13}C -Labeled glucose was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). All these chemicals and the authentic substances were used as received, without any purification.

2.2 Mouse heart specimens and analyte extraction

Mouse heart specimens were used for assay development and validation, and were obtained from Pel-Frez Biologicals (Rogers, AR, USA). According to the companying sample information, these specimens were freshly harvested and were snap-frozen in liquid nitrogen after collection. They were shipped on dry ice, and, once received, were stored at $-80\text{ }^{\circ}\text{C}$.

^{13}C -Labeled mouse heart specimens for isotope-resolved metabolic profiling were from wild-type BL/6 animals, and were obtained from the Department of Anesthesiology, School of Medicine, University of Colorado, Denver, Colorado, USA. The animals had been administered 50 mg of U- ^{13}C -glucose intraperitoneally. Ninety minutes after the U- ^{13}C -labeled glucose injection, the heart muscles were quickly freeze-clamped *in situ* with an

aluminium clamp precooled to the temperature of liquid nitrogen. The hearts were then cut out and stored at -80°C .

The whole heart specimens were diced on dry ice to fine pastes in 2-mL graduated tubes using a small handheld stainless steel homogenizer. A 125-mg aliquot of the tissue paste was precisely weighed, and 250 μL of water was added, followed by homogenization at 30 Hz for 1 min, using two 5-mm stainless steel balls on a Retsch MM400 mixer mill (Haan, near Düsseldorf, Germany). After a short spin-down at 3000 rpm ($800 \times g$) for 10 s, 750 μL of methanol was added. The samples were homogenized again for 1 min followed by centrifugation at 12,500 rpm ($14,700 \times g$) and 4°C for 10 min in a micro-centrifuge. The clear supernatant was transferred to a 5-mL volumetric flask. The tissue residue was suspended in 1 mL of 75% methanol and extracted two more times in the same way. The clear supernatants were pooled into the same volumetric flask and brought up to 5 mL with 75% methanol.

2.2.1 Ultrahigh performance LC/MS—The LC/MS system for derivatization optimization and for isotope-resolved metabolic profiling was an Acquity ultrahigh performance (UP) LC system (Waters Corp., Milford, MA), coupled to a Synapt HDMS quadrupole time-of-flight (Q-TOF) mass spectrometer (Waters Corp.). The QTOF mass spectrometer was operated in the negative-ion mode and with a detection range of m/z 100–1000. Chromatographic separation was performed on a Waters BEH C_{18} (2.1×100 mm, $1.7 \mu\text{m}$) UPLC column, using 0.01% formic acid in water (solvent A) and methanol with 0.01% formic acid (solvent B) as the mobile phase for binary gradient elution. The column flow rate was 0.3 mL/min; the column temperature was 40°C , and the autosampler was kept at 4°C . The binary elution gradient was 18% B to 90 % B in 9 minutes and then maintained at 100% B for 1 min. The column was equilibrated for 3 min with the initial solvent composition between injections. Sample injections were performed in the full-loop injection mode. Before use, the mass spectrometer was tuned with a lock-mass spray solution (50 pg/ μL leucine enkephalin in 60% aqueous methanol) and calibrated using the sodiated cluster ions from a 2-mM sodium acetate solution in 60% aqueous methanol. The ESI-MS operation parameters were spray voltage, -2.9 kV; desolvation gas (N_2) flow and temperature, 750 L/h and 350°C ; drying gas (N_2) flow and temperature, 50 L/h and 125°C ; sampling cone voltage, 35V; extraction cone voltage, 4 V; and data acquisition rate, 0.25 s. The background gas (Ar) in the collision cell was held at 0.5 mL/min. The UPLC/MS data were processed with the MassLynx software suite (Waters Corp., Milford, MA).

2.2.2 UPLC/MS/MS—For assay development and validation, an Ultimate 3000 RSLC system (Dionex Inc., now Thermo Scientific Dionex, Amsterdam, The Netherlands,) was used for chromatographic separation, using the same UPLC column and the same chromatographic conditions as above. A 4000 QTRAP triple quadrupole mass spectrometer (AB Sciex, Concord, ON, Canada) equipped with a Turbo Ion Spray source was operated in the negative-ion multiple-reaction monitoring (MRM) mode.

The MRM transitions for each targeted analyte were optimized *via* flow injection analysis of a solution prepared from 3-NPH derivatization of a mixed standard solution containing the ten carboxylic compounds (1 μM each). Full-mass data acquisition was performed using the

first mass analyzer (Q1) by scanning from m/z 100 to 600 in the profile mode and using a cycle time of 2 s with a step size of 0.1 Da and a pause time of 5 ms between scans. In the product ion scan experiments, MS/MS fragment ions were produced by collision-induced dissociation of selected precursor ions in the collision cell by ramping the collision energy voltages from -120 V to 0 V, and were detected using the second mass analyzer (Q3). In this way, at least two MRM transitions (Q1/Q3) per analyte were obtained and the two most sensitive transitions were used in the MRM scan mode to optimize the collision energy for each Q1/Q3 pair. The nebulizer, curtain, collision and auxiliary gas (N_2) flows, as well as the temperature of the auxiliary gas, were optimized for UPLC/MRM-MS runs after the injection of 10- μ L aliquots of the solution. The ESI-MS parameters which were optimized for UPLC/MRM-MS runs were the ESI capillary voltage, -4500 V; nebulizer gas (N_2), 30 (arbitrary units); curtain gas (N_2), 20 (arbitrary units); collision gas (N_2), 3 (arbitrary units); entrance potential, -10 V; collision cell exit potential, -23 V. The drying gas (N_2) flow and temperature were 30 (arbitrary units) and 400 °C, respectively. The Q1 and Q3 operations were set to unit resolution.

UPLC/MRM-MS data was acquired using the Analyst 1.5 software and was processed using the MultiQuant 1.2 software (AB Sciex, Concord, ON, Canada). Of the two MRM transitions per analyte, the Q1/Q3 pair that showed the higher sensitivity for each analyte was selected as the MRM transition to be used for quantitative monitoring. These quantifier MRM transitions for assay are listed in Table 1. The other transition for each analyte acted as a qualifier for the purpose of verification of the identity of the compound.

2.3 Optimization of reaction conditions

A standard solution containing 1 μ M of each of the ten carboxylates in 75% aqueous methanol was used to optimize the reaction conditions for derivatization with 3-NPH. Briefly, 100- μ L aliquots of this standard solution were sequentially mixed with 50- μ L aliquots of varying concentrations of 3-NPH, EDC, and pyridine, in 2-mL LC autosampler injection vials or in Eppendorf tubes. The 3-NPH, EDC, and pyridine solutions were freshly prepared in solvents containing different ratios of methanol/water to bring the final % methanol in the reaction mixtures to 30%, 45%, 60%, 75% and 90%, respectively. Once mixed, the solutions were individually reacted in a freezer whose temperature was adjusted to -10 °C, in an ice water bath (0 °C), in a 10 °C refrigerator, at room temperature (22 °C), or in two Eppendorf thermomixers at 30 °C and 40 °C, respectively, for different time periods ranging from 5 min to 75 min. After reaction, each solution was rapidly diluted with 700 μ L of water and 50 μ L of either 20% TEA/methanol, 2% TFA/methanol, 2 mg/mL of BHT/methanol or 100% methanol was added. Ten- μ L aliquots of each solution were injected for UPLC/QTOF MS analysis.

To maintain the consistency of the analytical results, all the reagent solutions and standard solutions were freshly prepared and were kept on ice throughout the experiment. This is particularly important for oxaloacetate, which is unstable in solution, and which could decompose even at temperatures below 0 °C [28].

2.4 Standard curves and sample assay

Stock standard solutions of the ten carboxylates were individually prepared in 50% or 75% aqueous methanol, depending on the solubility of the individual authentic substances, to give a same concentration of 2 mg/mL. Standard solutions were made in 75% aqueous methanol by mixing and stepwise dilution of each of the stock solutions by a factor of 3, to produce final concentrations covering the range of 20 µg/mL to 2 ng/mL for each compound. An IS solution was made by preparing the D or ¹³C-labeled standard analogues of eight of the ten carboxylates (with the exception of isocitrate and oxaloacetate) in 75% aqueous methanol to produce a solution with concentrations of 50 µg/mL for lactate, 0.5 µg/mL for glycolate, and 1 µg/mL for other six carboxylates. No stable isotope-labeled internal standards were available for isocitrate and oxaloacetate due to lack of a commercial availability.

Fifty µL of a mouse heart extract or a standard solution, 50 µL of the IS solution, 50 µL of 250 mM 3-NPH in 50% methanol, 50 µL of 150 mM EDC in methanol, and 50 µL of 7.5% pyridine in 75% aqueous methanol were mixed and allowed to react, either in a thermomixer at 30 °C for 30 min for quantitation of glycolate, lactate, malate, fumarate, succinate, pyruvate, and α-ketoglutarate, or in an ice water bath (0 °C) for 60 min for quantitation of isocitrate, citrate, and oxaloacetate. After this reaction, 50 µL of 2 mg/mL BHT in methanol was instantly added to these solutions, which were then diluted with 700 µL of water. A 10-µL aliquot of each solution was injected for UPLC/MS or UPLC/MS analysis of all the carboxylates except for lactate. For the analysis of lactate, the solutions were diluted by a factor of 100 with 20% aqueous methanol prior to the 10-µL injection.

2.5 Sensitivity and linearity

Analytical sensitivity was determined by the lower limits of detection (LLOD) and lower limits of quantification (LLOQ) of the UPLC/MS method. To do this, standard stock solutions of the ten carboxylates were individually diluted with 75% aqueous methanol. The resulting solutions were derivatized with 3-NPH and UPLC/MS was performed using the same protocol as described for the sample assay. To determine the LLODs and LLOQs, the signal-to-noise (S/N) ratio at the lowest concentration measured was used to estimate the on-column amounts which would yield at least three and ten times the S/N ratio, respectively, while giving CVs of the measured peak areas for individual analytes of 15% (n=3) according to the US FDA guideline for bioanalytical method validation [29]. Linear regression with IS calibration (for the eight analytes for which we had isotopically-labeled ISs) was used to determine the concentration ranges that produced a correlation coefficient (R^2) of at least 0.999. For isocitrate and oxaloacetate, external standard calibration was applied.

2.6 Assay precision and accuracy

The precision of the quantitation was measured as the intra-day coefficient of variation (CV) by preparing and analyzing eight analytical replicates of a pooled mouse heart extract every 3 hours within a same day. The accuracy of quantitation was evaluated by determining the recoveries of known amounts of standard compounds for each of the quantified carboxylates to different aliquots of a pooled mouse heart tissue extract. The spiked amounts

corresponded to 100%, 200%, and 300% of the measured concentrations and represented low, medium, and high concentration levels of the analytes, respectively. The IS solution was added to each sample, and subjected to 3-NPH derivatization and LC/MS assay. The percent recoveries were calculated as $[(\text{mean observed concentration}) / (\text{spiked concentration})] \times 100\%$.

3 Results and Discussion

3.1 Optimization of derivatization

Water-soluble carbonyl compounds, including aldehydes, ketones, and carboxylic acids, can be coupled to hydrazines in aqueous solution to form hydrazones using carbodiimides such as EDC as the condensing reagent. This type of condensation reaction, as shown in Figure 1, follows a nucleophilic addition-elimination mechanism. The purpose of this study was to develop a useful chemical derivatization approach with a singly nitro-substituted phenylhydrazine for multiplexed quantitation of seven carboxylic intermediates of the TCA cycle (malate, fumarate, succinate, isocitrate, citrate, oxaloacetate, and α -ketoglutarate), plus three other CCM-related carboxylates (glycolate, lactate, and pyruvate) using UPLC/MS. First, we compared three singly nitro-substituted phenylhydrazines (2, 3, and 4-NPHs) for derivatizing the ten analytes. The 4-NPH reagent showed much lower reactivity with all the analytes than did the other two NPHs. The 2-NPH showed better reactivity than 4-NPH, but comparisons done in parallel indicated that the LC/MS detection sensitivities of the 2-NPH derivatives of the ten carboxylates was generally 2–30 times lower than that of the 3-NPH derivatives, though 2-NPH has been widely used for HPLC/UV or HPLC/MS analysis of carboxylic acids previously [19–26]. Therefore, 3-NPH was chosen for derivatization of the CAs to form their 3-nitrophenylhydrazones in the final UPLC/MS assay.

Various derivatization conditions were optimized with 3-NPH as the derivatizing reagent. Reaction temperature and time are important factors that affect a chemical reaction. Although all of the ten carboxylates showed good reactivity with 3-NPH, our experiment demonstrated that there was not a single set of reaction temperature and reaction time that could be applied to achieve good quantitation for all the ten analytes. Figure 2 shows how the reaction temperature and reaction time influence the peak areas of the derivatives of the ten analytes, under the optimum of the other reaction conditions. It was interesting that the two hydroxyl carboxylic compounds (glycolate and lactate), three di-carboxylic compounds (malate, fumarate and succinate), two ketone-containing mono- and di-carboxylic compounds (pyruvate and α -ketoglutarate, respectively) showed the highest peak areas at 30 °C and when the reaction time was between 20 min to 60 min. However, for another ketone-containing di-carboxylic compound (oxaloacetate) and two tri-carboxylic compounds (isocitrate and citrate), a lower reaction temperature at 0 °C was the optimum for their complete derivatization and a minimum of 60 min reaction time was needed for complete conversion of these three carboxylates to their 3-nitrophenylhydrazones. Therefore, 30 °C for 30 min and 0 °C for 60 min were chosen as the two combinations of reaction temperature and time for optimal derivatization of the first group of seven carboxylates and for the second group of three carboxylates, respectively. Under these two different reaction

conditions, no partially-derivatized products were detected for any of the carboxylates, as determined by examining the extracted ion chromatograms for their predicted partially derivatized products. Interestingly, in our experiments, all of the carboxylates had optimal reaction temperatures and reaction times under milder conditions than those described by Uran, et al. [27], where 60 °C and 20 min were used to derivatize lactate and pyruvate with 3-NPH for analysis by LC/ESI-MS.

The organic solvent of choice and the percent water in the reaction mixture proved to have a significant influence on the reaction. Comparison between acetonitrile and methanol as an organic solvent in the reaction mixture showed that derivatization efficiency was higher in methanol than in acetonitrile (data not shown). Therefore, methanol was used for the remainder of the study. Figure 3A shows the influence of the percent methanol in water in the reaction medium on the derivatization of the ten CAs, under the optimal reaction temperatures and the corresponding reaction times and other reaction conditions. The plots indicate that a reaction medium containing methanol:water (75:25, v/v) gave the best derivatization efficiency for all the CAs. Using the methanol:water (75:25, v/v) solvent as the reaction medium, Figures 3B to 3D show how the concentrations of 3-NPH, EDC and pyridine in the reaction mixture influence the chromatographic peak areas of the ten derivatives by UPLC/QTOF MS. Based on these plots, 50 mM of 3-NPH, 30 mM of EDC, and 1.5% pyridine in the reaction mixture were the optimum conditions.

3.2 Post-reaction treatment

2- and 3-NPHs have previously been used for quantitation of some carboxylic group-containing compounds by HPLC/UV and HPLC/ESI-MS. In the literature, a high-pH quenching protocol (*i.e.*, either addition of 1% TEA to the reaction products or the use of potassium hydroxide to quench the reaction at 80 °C for 10 min) was used. Our experiments indicated that this high-pH quenching approach led to accelerated degradation of the 2 or 3-nitrophenylhydrazones of the ten carboxylates. In order to find an appropriate protocol for improving the chemical stability of the 3-nitrophenylhydrazones produced from the targeted carboxylates, we compared four post-reaction treatment protocols. These four protocols were (1) addition of 1% TEA to the reacted mixture; (2) addition of 0.1% TFA (pH ~2) to the reacted mixture; (3) addition of 0.1 mg/mL of BHT -- a common antioxidant -- to the reacted mixture; and (4) no post-reaction treatment. Figure 4 shows the time-course changes of the measured chromatographic peak areas for four representative structurally-different carboxylates (glycolate, pyruvate, fumarate, and oxaloacetate) from a mixed standard solution containing 1 μ M of each analyte. During the experiment, all of the samples were kept at 4 °C in the UPLC autosampler. As shown, all of the derivatives showed improved chemical stability when a trace amount of BHT was included in the solution, or when no post-reaction treatment was used. In addition, although the derivative of glycolate showed good chemical stability when either "no treatment" or BHT was used, the chemical stability of the derivatives of other three carboxylates were better when BHT was added. Therefore, the BHT protocol was chosen as the optimum post-reaction treatment in this work. Since BHT is an anti-oxidant, this experiment implies that oxidation may be responsible, at least in part, for the time-related degradation of the 3-nitrophenylhydrazones. In our experiments, we also compared the chemical stabilities of the derivatives by spiking BHT before or after

the derivatization. This made no difference to the measured peak areas for all the ten carboxylates.

3.3 *Cis(E)*- and *trans(Z)*-isomerization of nitrophenylhydrazones

Figure 5 is a UPLC/MS profile of the ten derivatives prepared from a mixed standard solution. As observed, glycolate, lactate, malate, fumarate, succinate, isocitrate, and citrate each show a single chromatographic peak in the profile. However, due to the existence of the C=N double bonds in the 3-nitrophenylhydrazones formed after derivatization, and other asymmetric functional groups attached to the C=N double bonds, *cis(E)*- and *trans(Z)*-stereoisomers are observed for the derivatives of pyruvate and oxaloacetate. This phenomenon is similar to the situation when 2,4-dinitrophenylhydrazine was used for derivatizing some carbonyl compounds [30]. In our experiment, two doublet peaks would be also observed for α -ketoglutaric derivative if acetonitrile was used as the organic solvent for chromatographic separation (data not shown), but when methanol was used, the *cis(E)*- and *trans(Z)*-stereoisomers for α -ketoglutaric were not separated and only a singlet peak was observed, as shown in Figure 5. The *cis(E)*- and *trans(Z)*-stereoisomers for pyruvate and oxaloacetate were always observed in our experiment, either with 2-NPH or 3-NPH as the derivatizing reagent. However, observation of the *cis(E)*- and *trans(Z)*-stereoisomers of pyruvate has not previously been mentioned in the literature [27].

The two doublet peaks from a single analyte could possibly cause analytical errors due to the existence of the *cis(E)*- and *trans(Z)*-stereoisomers —similar to the situation that occurs when 2,4-dinitrophenylhydrazine is used for derivatizing ketones and aldehydes for HPLC/UV detection [31–33]. To compensate for the possible analytical errors resulting from the existing *cis(E)*- and *trans(Z)*-stereoisomers by UPLC/MS, stable isotope (D or ^{13}C)-labeled standard compounds were used as IS for the accurate assay of eight of the ten carboxylates in this study (with the exception of isocitrate and oxaloacetate, as mentioned previously). Internal standard calibration was not used for isocitrate and oxaloacetate because there were no isotopically-labeled authentic compounds available for these two analytes.

3.4 Sensitivity and linearity

The analytical sensitivity was defined as the on-column LLOD and LLOQ using the UPLC/MS method described above. Table 2 lists the resulting on-column LLODs and LLOQs of the ten analytes, which is in the range of high femtomoles to low picomoles. Table 2 also includes the coefficients of linear regression from the calibrators of the ten analytes, with IS calibration for eight of these and with external calibration for the remaining two, *i.e.*, isocitrate and oxaloacetate. As shown, the linear regression coefficients (R^2) were >0.9992 in all cases. Table 2 also lists the linear ranges of quantitation as the on-column amount (pmol) for each of the analytes, with dynamic ranges from >200 for lactate to >2000 for α -ketoglutarate.

3.5 Assay precision and accuracy

In order to achieve accurate quantitation by UPLC/MS and to correct for any other influences that can affect the signal obtained, isotopically-labeled authentic compounds were

used as IS for eight of the ten carboxylates. For pyruvate which showed doublet peaks following the derivatization, the total areas of the *E* and *Z* isomers from the respective natural and isotopically-labeled derivatives were used for the internal calibration-based quantitation, and a good calibration curve was created. Table 3 shows the assay results for a mouse heart tissue sample.

Quantitation precision was evaluated by UPLC/MRM-MS as the intra-day reproducibility of eight analytical replicates of a pooled mouse heart extract prepared and analyzed every 3 hours for 24 hours. As shown in Table 2, eight of the ten analytes showed intra-day CVs that were below 7.1% (n=8). Larger CVs were observed for the two low-concentration carboxylates (isocitrate and oxaloacetate), whose concentrations in all the tested samples was determined to be lower than 0.002 nM.

To determine the analytical accuracy, the recoveries of the spiked standards were measured by UPLC/MRM-MS for the eight precisely-quantifiable carboxylates. Table 3 lists the measured recoveries of these eight carboxylates at three spiking levels, corresponding to 100%, 200%, and 300% of their measured tissue concentrations. The recoveries of the spiked-in standards were determined to be from 89.5 % (CV%=5.7, n=6) for citrate at the 100% spiking level to 114.8% (CV = 4.1%, n=6) for fumarate at the 300% spiking level. These recoveries indicate the high accuracy of this analytical method for quantitation of these key carboxylic compounds of the CCM, although the accuracies for isocitrate and oxaloacetate could not be measured due to their extremely low concentrations in the mouse heart tissue.

3.6 Stable isotope-resolved metabolic profiling

Our 3-NPH derivatization method was next evaluated for LC/MS-based isotope-resolved metabolic profiling of the ten carboxylates which are involved in the CCM pathway [1]. A ^{13}C -labeled mouse heart from an animal which had been administered 50 mg $\text{U-}^{13}\text{C}$ -glucose was analyzed to determine the isotopic distribution patterns of the ten analytes. The analyzed heart tissue extract used for this experiment was similar to those used for the study of myocardial metabolism within the heart tissues of wild-type BL/6 mice and *Per2*^{-/-} animals [34]. After the infusion of $\text{U-}^{13}\text{C}$ -labeled glucose, the extent of incorporation of the ^{13}C isotope into the CCM pathway of the animal biological system were successfully detected by UPLC-high resolution MS on a QTOF instrument, even for the two carboxylates present at extremely low concentrations - isocitrate and oxaloacetate. Figure 6 shows the comparison of the isotope patterns from four representative carboxylates detected in a heart tissue sample with those from the natural authentic compounds. These four carboxylates are lactate, pyruvate, fumarate, and citrate. Lactate is an end product of anaerobic cellular respiration with pyruvate as a precursor in the cytosol of a cell. Pyruvate is the end product of the glycolysis through CCM. In addition to being the precursor for anaerobic respiration, yielding lactate and ethanol, and being a substrate for biosynthesis of several amino acids, pyruvate is transported into the matrix of the mitochondria, where it is oxidized by pyruvate dehydrogenase to produce acetyl-coenzyme A which is used in the TCA cycle. Fumarate and citrate, together with malate, succinate, isocitrate, α -ketoglutarate, and oxaloacetate, are the major intermediates of the TCA cycle. With successful detection of the isotope patterns

of these analytes, the incorporation of ^{13}C atoms derived from the $\text{U-}^{13}\text{C}$ -glucose into these carboxylic intermediates of CCM can then be followed. This demonstrates the applicability of our developed method and its potential for use in isotope-labeling metabolic flux analysis.

4 Conclusions

Optimization of the protocol for chemical derivatization of the ten hydroxy-, keto-, and poly-carboxylates with 3-NPH, and the choice of appropriate post-reaction treatment, enabled successful quantitative analysis of these key CCM carboxylic metabolites by UPLC/MRM-MS. This pre-analytical chemical derivatization, in combination with UPLC/MRM-MS, has sufficient analytical sensitivity for accurate determination of eight of the ten compounds in mouse heart tissue, with the on-column LLODs and LLOQs in the high femtomole to low picomole range. The addition of a trace amount of BHT to the reacted mixtures improved the chemical stability of the 3-nitrophenylhydrazones formed from the carboxylates, which made this LC/MS-based method suitable for high-throughput analysis where the analysis of a large number of samples is required. Additionally, this technique has the potential to be valuable for metabolic flux analysis when isotope-labeling is used.

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Abbreviations

UPLC	ultrahigh performance liquid chromatography
HPLC	high performance liquid chromatography
MS	mass spectrometry
LC/UV	liquid chromatography with ultraviolet detection
GC/MS	gas chromatography-mass spectrometry
LC/MS	liquid chromatography coupled to mass spectrometry
MS/MS	tandem mass spectrometry
MRM	multiple reaction monitoring
QTOF	quadrupole time-of-flight
IS	internal standard
ESI	electrospray ionization
CV	coefficient of variation
Ai	peak area of internal standard
As	peak area of analyte standard

LLOD	lower limit of detection
LLOQ	lower limit of quantitation
CCM	central carbon metabolism
PPP	pentose phosphate pathway
TCA	tri-carboxylic acid
2-NPH	2-nitrophenylhydrazine
3-NPH	3-nitrophenylhydrazine
4-NPH	4-nitrophenylhydrazine
ATP	adenosine triphosphate
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
BHT	butylated hydroxytoluene
CA	carboxylic acid
S/N	signal-to-noise ratio.

References

- Valdes J, Pedroso I, Quatrini R, Dodson RJ, Tettelin H, Blake R 2nd, Eisen JA, Holmes DS. Acidithiobacillus ferrooxidans metabolism: from genome sequence to industrial applications. BMC Genomics. 2008; 9:597. [PubMed: 19077236]
- Metallo CM, Walther JL, Stephanopoulos G. Evaluation of ¹³C isotopic tracers for metabolic flux analysis in mammalian cells. J Biotechnol. 2009; 144:167–174. [PubMed: 19622376]
- Usui Y, Hirasawa T, Furusawa C, Shirai T, Yamamoto N, Mori H, Shimizu H. Investigating the effects of perturbations to pgi and eno gene expression on central carbon metabolism in Escherichia coli using ¹³C metabolic flux analysis. Microb Cell Fact. 2012; 11:87. [PubMed: 22721472]
- Li YC, Yu JZ. Simultaneous determination of mono- and dicarboxylic acids, omega-Oxo-carboxylic acids, midchain ketocarboxylic acids, and aldehydes in atmospheric aerosol samples. Environ Sci Technol. 2005; 39:7616–7624. [PubMed: 16245835]
- Ghassempour A, Nojavan S, Talebpour Z, Amiri AA, Najafi NM. Monitoring of the fermentation media of citric acid by the trimethylsilyl derivatives of the organic acids formed. J Agric Food Chem. 2004; 52:6384–6388. [PubMed: 15478996]
- Guo K, Li L. High-Performance Isotope Labeling for Profiling Carboxylic Acid-Containing Metabolites in Biofluids by Mass Spectrometry. Anal Chem. 2010
- Alonso AP, Raymond P, Hernould M, Rondeau-Mouro C, de Graaf A, Chourey P, Lahaye M, Shachar-Hill Y, Rolin D, Dieuaide-Noubhani M. A metabolic flux analysis to study the role of sucrose synthase in the regulation of the carbon partitioning in central metabolism in maize root tips. Metab Eng. 2007; 9:419–432. [PubMed: 17869563]
- Phillips MM, Case RJ, Rimmer CA, Sander LC, Sharpless KE, Wise SA, Yen JH. Determination of organic acids in Vaccinium berry standard reference materials. Anal Bioanal Chem. 2010; 398:425–434. [PubMed: 20582402]
- Ehling S, Cole S. Analysis of organic acids in fruit juices by liquid chromatography-mass spectrometry: an enhanced tool for authenticity testing. J Agric Food Chem. 2011; 59:2229–2234. [PubMed: 21361392]
- Luo B, Groenke K, Takors R, Wandrey C, Oldiges M. Simultaneous determination of multiple intracellular metabolites in glycolysis, pentose phosphate pathway and tricarboxylic acid cycle by

- liquid chromatography-mass spectrometry. *J Chromatogr A*. 2007; 1147:153–164. [PubMed: 17376459]
11. Rellan-Alvarez R, Lopez-Gomollon S, Abadia J, Alvarez-Fernandez A. Development of a new high-performance liquid chromatography-electrospray ionization time-of-flight mass spectrometry method for the determination of low molecular mass organic acids in plant tissue extracts. *J Agric Food Chem*. 2011; 59:6864–6870. [PubMed: 21619056]
 12. Bylund D, Norstrom SH, Essen SA, Lundstrom US. Analysis of low molecular mass organic acids in natural waters by ion exclusion chromatography tandem mass spectrometry. *J Chromatogr A*. 2007; 1176:89–93. [PubMed: 18001753]
 13. Halket JM, Zaikin VV. Derivatization in mass spectrometry-3. Alkylation (arylation). *Eur J Mass Spectrom (Chichester, Eng)*. 2004; 10:1–19.
 14. Santa T, Al-Dirbashi OY, Fukushima T. Derivatization reagents in liquid chromatography/electrospray ionization tandem mass spectrometry for biomedical analysis. *Drug Discov Ther*. 2007; 1:108–118. [PubMed: 22504396]
 15. Santa T. Derivatization reagents in liquid chromatography/electrospray ionization tandem mass spectrometry. *Biomed Chromatogr*. 2011; 25:1–10. [PubMed: 21058414]
 16. Deng P, Zhan Y, Chen X, Zhong D. Derivatization methods for quantitative bioanalysis by LC-MS/MS. *Bioanalysis*. 2012; 4:49–69. [PubMed: 22191594]
 17. Banos CE, Silva M. In situ continuous derivatization/pre-concentration of carbonyl compounds with 2,4-dinitrophenylhydrazine in aqueous samples by solid-phase extraction Application to liquid chromatography determination of aldehydes. *Talanta*. 2009; 77:1597–1602. [PubMed: 19159770]
 18. Kloos D, Derks RJ, Wijtmans M, Lingeman H, Mayboroda OA, Deelder AM, Niessen WM, Giera M. Derivatization of the tricarboxylic acid cycle intermediates and analysis by online solid-phase extraction-liquid chromatography-mass spectrometry with positive-ion electrospray ionization. *J Chromatogr A*. 2012; 1232:19–26. [PubMed: 21862021]
 19. Yomota C, Ohnishi Y. Determination of biotin following derivatization with 2-nitrophenylhydrazine by high-performance liquid chromatography with on-line UV detection and electrospray-ionization mass spectrometry. *J Chromatogr A*. 2007; 1142:231–235. [PubMed: 17234197]
 20. Henderson GC, Tuazon MA. Separation of positional and geometrical fatty acid isomers as 2-nitrophenylhydrazide derivatives by high-performance liquid chromatography. *Anal Biochem*. 2011; 413:66–68. [PubMed: 21324304]
 21. Peters R, Hellenbrand J, Mengerink Y, Van der Wal S. On-line determination of carboxylic acids, aldehydes and ketones by high-performance liquid chromatography-diode array detection-atmospheric pressure chemical ionisation mass spectrometry after derivatization with 2-nitrophenylhydrazine. *J Chromatogr A*. 2004; 1031:35–50. [PubMed: 15058566]
 22. Miwa H. High-performance liquid chromatographic determination of mono-, poly- and hydroxycarboxylic acids in foods and beverages as their 2-nitrophenylhydrazides. *J Chromatogr A*. 2000; 881:365–385. [PubMed: 10905720]
 23. Miwa H, Yamamoto M. High-performance liquid chromatographic analysis of serum short-chain fatty acids by direct derivatization. *J Chromatogr*. 1987; 421:33–41. [PubMed: 3429573]
 24. Miwa H, Yamamoto M. Reversed-phase ion-pair chromatography of straight- and branched-chain dicarboxylic acids in urine as their 2-nitrophenylhydrazides. *Anal Biochem*. 1988; 170:301–307. [PubMed: 3394930]
 25. Miwa H, Yamamoto M. Liquid chromatographic determination of free and total fatty acids in milk and milk products as their 2-nitrophenylhydrazides. *J Chromatogr*. 1990; 523:235–246. [PubMed: 2090661]
 26. Miwa H, Yamamoto M. Rapid liquid chromatographic determination of fatty acids as 2-nitrophenylhydrazine derivatives. *J AOAC Int*. 1996; 79:493–497. [PubMed: 8920138]
 27. Uran S, Landmark KE, Hjellum G, Skotland T. Quantification of ¹³C pyruvate and ¹³C lactate in dog blood by reversed-phase liquid chromatography-electrospray ionization mass spectrometry after derivatization with 3-nitrophenylhydrazine. *J Pharm Biomed Anal*. 2007; 44:947–954. [PubMed: 17482415]

28. Chassagnole C, Noisommit-Rizzi N, Schmid JW, Mauch K, Reuss M. Dynamic modeling of the central carbon metabolism of *Escherichia coli*. *Biotechnol Bioeng*. 2002; 79:53–73. [PubMed: 17590932]
29. USFDA. Draft Guidance for Industry: Bioanalytical Method Validation. Rockville, MD, USA.: US Food and Drug Administration; 1999.
30. Uchiyama S, Inaba Y, Kunugita N. Derivatization of carbonyl compounds with 2,4-dinitrophenylhydrazine and their subsequent determination by high-performance liquid chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2011; 879:1282–1289.
31. Chavarria M, Kleijn RJ, Sauer U, Pfluger-Grau K, de Lorenzo V. Regulatory tasks of the phosphoenolpyruvate-phosphotransferase system of *Pseudomonas putida* in central carbon metabolism. *MBio*. 2012; 3(2):1–9.
32. Costenoble R, Picotti P, Reiter L, Stallmach R, Heinemann M, Sauer U, Aebersold R. Comprehensive quantitative analysis of central carbon and amino-acid metabolism in *Saccharomyces cerevisiae* under multiple conditions by targeted proteomics. *Mol Syst Biol*. 2011; 7:464. [PubMed: 21283140]
33. David H, Akesson M, Nielsen J. Reconstruction of the central carbon metabolism of *Aspergillus niger*. *Eur J Biochem*. 2003; 270:4243–4253. [PubMed: 14622289]
34. Eckle T, Hartmann K, Bonney S, Reithel S, Mittelbronn M, Walker LA, Lowes BD, Han J, Borchers CH, Buttrick PM, et al. Adora2b-elicited Per2 stabilization promotes a HIF-dependent metabolic switch crucial for myocardial adaptation to ischemia. *Nat Med*. 2012; 18:774–782. [PubMed: 22504483]

Figure 1. Reaction mechanism for the derivatization of carbonyl compounds, including carboxylic acids, to form 3-nitrophenylhydrazones, using EDC as a coupling reagent and pyridine as a catalyst.

Figure 2.
Influence of reaction temperature and reaction time on 3-NPH derivatization of ten carboxylates.

Figure 3.

Influence of the concentrations of 3-NPH (A), EDC (B), pyridine (C) and methanol (D) in the reaction mixture on chromatographic peak areas of the derivatives of ten carboxylates, as determined by UPLC-QTOF MS.

Figure 4. Comparison of four post-reaction treatments on the chemical stabilities of the 3-nitrophenylhydrazones of glycolate, fumarate, citrate, and pyruvate, as the representative of hydroxyl, di-, tri- and ketone carboxylates, respectively, after 3-NPH derivatization.

Figure 5.

UPLC/MRM-MS profile of the 3-phenylhydrazones of a mixture of ten carboxylates (1 μ M each): (1) glycolate, (2) lactate, (3) malate, (4) fumarate, (5) succinate, (6) isocitrate, (7) citrate, (8) (E) and (Z)-pyruvate (after derivatization), (9) oxaloacetate, and (10) α -ketoglutarate.

Figure 6.

(A) Isotopic distribution patterns of lactate, pyruvate, fumarate and citrate analyzed by UPLC/QTOF MS from the heart tissue of a wild-type BL/6 mouse administered 50 mg of U- ^{13}C -glucose, compared to (B) the patterns of the natural standard compounds of four carboxylates.

Table 1

MRM transitions of ten 3-nitrophenylhydrazine-derived carboxylates

Compound	Q1 m/z	Q3 m/z	Declustering potential (V)	Collision energy (V)
Glycolate	210.1	152.1	−65	−22
Lactate	224.2	137.0	−70	−26
Malate	403.3	137.0	−85	−51
Fumarate	385.3	152.1	−90	−25
Succinate	387.3	152.1	−90	−32
Isocitrate	596.4	234.1	−105	−43
Citrate	596.4	222.1	−100	−44
Pyruvate	357.3	150.0	−88	−28
α-Ketoglutarate	550.4	233.2	−105	−40
Oxaloacetate	536.1	247.2	−110	−34

Table 2
On-column LLOD, LLOQ, linearity range and precision of the assay by UPLC/MRM-MS

Compound	LLOD [pmol]	LLOQ [pmol]	Linear range (pmol)	R ²	Intraday CV % (n=8)
Glycolate	0.2	0.5	0.5~340	0.9995 [*]	5.3
Lactate	0.2	0.6	4~870	0.9995	3.3
Malate	0.1	0.3	0.3~135	1.0000	2.2
Fumarate	0.06	0.2	0.3~210	1.0000	3.8
Succinate	0.02	0.04	2~1570	0.9999	5.5
Citrate	0.07	0.2	8~10550	0.9998	4.9
Isocitrate	0.2	0.6	20~16800	0.9992	20.2
Pyruvate	0.06	0.2	2~1700	0.9999	2.9
α-Ketoglutarate	0.02	0.06	1~2400	0.9999	6.4
Oxaloacetate	0.02	0.06	0.8~550	0.9997	26.1

(^{*}) Coefficient of linear regression with internal standard calibration, except for isocitrate and oxaloacetate for which external standard calibration was used

Table 3

Tissue concentrations of eight carboxylates in a mouse heart and accuracy of quantitation measured by UPLC/MRM-MS

Compound	Concentration (n=6)*		Recovery % $\pm$ CV % (n=6) at 3 spike-in levels		
	$\mu\text{mol/g}$	CV%	100%	200%	300%
Glycolate	0.003	5.6	96.7 $\pm$ 4.2	96.6 $\pm$ 2.0	97.5 $\pm$ 3.0
Lactate	45.7	3.9	111.3 $\pm$ 4.1	111.0 $\pm$ 6.4	108.6 $\pm$ 2.3
Malate	1.86	1.8	110.1 $\pm$ 4.4	104.9 $\pm$ 4.3	107.4 $\pm$ 2.9
Fumarate	0.59	2.7	109.0 $\pm$ 5.0	113.2 $\pm$ 3.1	114.8 $\pm$ 4.1
Succinate	0.83	5.3	111.1 $\pm$ 3.0	106.9 $\pm$ 5.1	108.6 $\pm$ 6.2
Isocitrate	<0.002	20.9	N/D*	N/D	N/D
Citrate	0.018	5.7	91.5 $\pm$ 7.4	89.5 $\pm$ 5.7	92.9 $\pm$ 2.5
Pyruvate	0.048	3.6	89.8 $\pm$ 1.2	96.0 $\pm$ 4.7	101.6 $\pm$ 2.5
α -Ketoglutarate	0.005	7.1	97.7 $\pm$ 4.5	102.6 $\pm$ 4.0	104.0 $\pm$ 2.8
Oxaloacetate	<0.002	27.1	N/D	N/D	N/D

* Not determined due to lack of isotopically-labeled internal standards and their very low tissue concentrations.