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Organic Acid Quantitation in Mouse Muscle by Ion Chromatography-Mass Spectrometry with Isotopically Labeled Standards

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Highlights

- New IC-MS method developed for organic acid detection and quantification
- Stable isotope-labeled organic acids employed for improved precision and accuracy
- 28 polar, low molecular weight organic acids quantified in mouse muscle
- Statistically significant differences in levels of organic acids found in fatigued vs. sedentary mice

Introduction

Organic acids (OAs) are important metabolites that play an essential role in an array of energy metabolism pathways (e.g., glycolysis and tricarboxylic acid cycle).[1,2] In addition, short chained OAs are emerging as important regulators of host immune responses and transcriptional regulation.[3,4] Their significance to cellular metabolism is heightened by their association with diseases, such as cancer and diabetes.[5-7] As a result, research has been focused on quantifying OAs in various biological samples (e.g., urine,[8] plasma,[9] serum[10]). In these studies, measurements of OAs were accomplished by liquid chromatography (LC) or capillary electrophoresis (CE) coupled to mass spectrometry (MS).[11,12] The commonly utilized modes of chromatography include reversed-phase (with C_{18} bonded silica), ion pair, and hydrophilic interactions. Despite that, the efficiency of separating polar OAs with these techniques can be challenging. An attractive complementary technique for untargeted metabolomics of polar metabolites is ion chromatography (IC)-MS.[13]

In this note, a targeted IC-MS method using stable isotope-labeled standards (SIS) was used to quantify a panel of polar OAs in mouse muscle.[14] The SIS OAs served as internal standards for enhanced precision and accuracy of OA measurements. Statistically significant quantitative differences were observed for four OAs in the quadricep muscle of sedentary and fatigued mice. Overall, this study demonstrated the ability of IC-MS with stable isotope-labeled OAs to separate and quantify a collection of low molecular weight polar metabolites that are difficult to analyze by other techniques.

Methods

Mouse Exercise Protocol

Mice (C57BL/6; 13 weeks of age) were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and divided into three groups. One group of mice served as controls and remained sedentary. The other two groups were subjected to low intensity, long duration (LILD) or high intensity, short duration (HISD) treadmill protocols.

Sample Preparation

Mice were intraperitoneally injected with sodium phenobarbital (200 mg/kg; Premier Pharmacy Laboratories; Weekeewachee, FL, USA). After flash freezing the harvested quadricep muscles with liquid nitrogen, the frozen muscles were lyophilized with subsequent powdering and homogenization in aqueous/organic solvent.[14] OAs were extracted from the homogenates and spiked with a SIS OA mixture (comprised of 16 standards with ^{13}C, D, and/or ^{15}N labels; ≥98% isotopic enrichment; see **Table 1**).

To stabilize the α-keto acids, the homogenates were derivatized with *O*-benzylhydroxylamine (0.2 M). Following derivatization, the samples were extracted with ethyl acetate and dried down before reconstitution in 100 µL of deionized water for IC-MS/MS.

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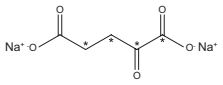
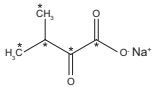
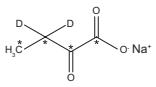
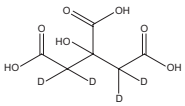
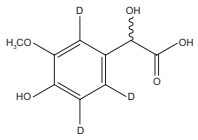
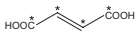
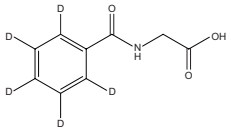
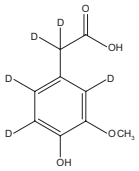
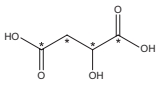
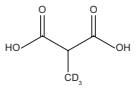
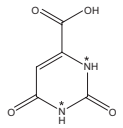
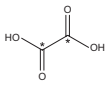
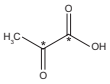
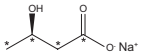
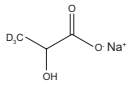
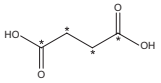
*For further information on original content, please refer to reference 14 (PMID: 27782384). Corresponding author C. Petucci (cpetucci@sbpdiscovery.org).
†These authors contributed equally to this application note.

Table 1. Stable isotope-labeled standards used to quantify OAs in mouse quadricep muscle. Chemical purities and isotopic enrichments were ≥98%. The asterisks represent the positions of ^{13}C or ^{15}N labeling.

Catalog No. / Description	Structure
CLM-4442 α-Ketoglutaric acid, disodium salt (1,2,3,4-$^{13}C_4$)	
CLM-4418 α-Ketoisovaleric acid, sodium salt ($^{13}C_5$)	
CDLM-4611 α-Ketobutyric acid, sodium salt ($^{13}C_4$; 3,3-D_2)	
DLM-3487 Citric acid (2,2,4,4-D_4)	
DLM-4794 DL-Vanilmandelic acid (ring-D_3)	
CLM-1529 Fumaric acid ($^{13}C_4$)	
DLM-7703 Hippuric acid (D_5)	
DLM-2738 Homovanillic acid (phenyl-D_3, 2,2-D_2)	

Catalog No. / Description	Structure
CLM-8065 L-Malic acid ($^{13}C_4$)	
DLM-387 Methylmalonic acid (methyl-D_3)	
NLM-1048 Orotic acid (1,3-$^{15}N_2$)	
CLM-2002 Oxalic acid (1,2-$^{13}C_2$)	
CLM-9505 Pyruvic acid (1,2-$^{13}C_2$)	
CLM-3853 Sodium D-3-hydroxybutyrate ($^{13}C_4$)	
DLM-3317 Sodium L-lactate (3,3,3-D_3)	
CLM-1571 Succinic acid ($^{13}C_4$)	

IC-MS/MS

The IC-MS platform consisted of an ICS-5000+ HPIC ion chromatography system and a TSQ Quantiva triple quadrupole (QqQ) mass spectrometer (both from Thermo Scientific, San Jose, CA, USA). The IC system utilized guard (2 × 50 mm, 4 µm; IonPac AG11-HC; Dionex, Sunnyvale, CA, USA) and anion exchange (2 × 250 mm, 4 µm; IonPac AS-11-HC; Thermo Scientific) columns. The analytical column was maintained in a temperature-controlled compartment at 35°C. OA mixtures (5 µL replicate injections; n = 2 for calibration curves and n = 5 for mouse muscle) were separated at 0.35 mL/min over 17.1 min (**Table 2**).

Table 2. KOH gradient for IC-MS/MS.

Gradient Conditions	
Time (min)	OH^- Conc. (mM)
0	5
3	10
5	20
8	50
10	80
11	100
17	100
17.1	5

Following the gradient, the column was re-equilibrated for 2 min at the starting eluent conditions of 5 mM KOH. The column effluent passed through an anion suppressor (AERS 500, 2 mm; Thermo Scientific) before reaching the ESI source of the mass spectrometer. The source was operated in the negative ion mode at 2.5 kV.[14] All MS measurements were conducted in MRM mode with unit resolution (0.7 Da) and 0.8 s cycles.

Data Analysis

Quantitative analysis of the target OAs was performed with TraceFinder 3.2 software (Thermo Scientific). The standard curves were generated using linear or quadratic functions with 1/x or $1/x^2$ weighting. OA concentrations in the mouse muscle tissue are expressed as nmol/mg dry weight. Surrogate SISs were used in select cases where no authentic SIS was available. Statistical significance between fatigued and sedentary mice was defined as $p < 0.05$.

Results and Discussion

Assay Development and Validation

Preliminary optimization of the sample preparation/processing steps was first conducted to maximize detectability, verify surrogate utility, and confirm system suitability. From a starting panel of 37 OAs, 28 were found to qualify. These provided interference-free signals in the mouse quadricep tissue measurements, a finding that was confirmed by the use of stable isotope-labeled standards. Their retention times spanned a 3.5 to 14.1 min range and eluted according to the following order: monocarboxylic acids (e.g., lactic), dicarboxylic acids (e.g., succinic), and tricarboxylic acids (e.g., citrate). Metrics evaluated during assay validation included recovery, stability, precision, accuracy, and limits of quantitation (LOQ). Regarding the stability, for example, the OAs in stored muscle homogenates were found to be stable over three freeze-thaw cycles with average stabilities of 96% obtained for an OA subset. The precision and accuracy were evaluated through standard curves that were run in duplicate each day over the course of three days. These were generated using SIS (10-125 µM) and unlabeled organic acid (typically 0.05-250 µM) calibrators.[14] The average accuracies were 100% (range: 82 to 115%), while the average precision was 7% CV (range: 1-19%). The linear curves had R^2 values of 0.99 or greater and LOQs ranging from 0.25 to 50 µM. **Figure 1** shows three representative curves for three different concentration ranges of OAs.

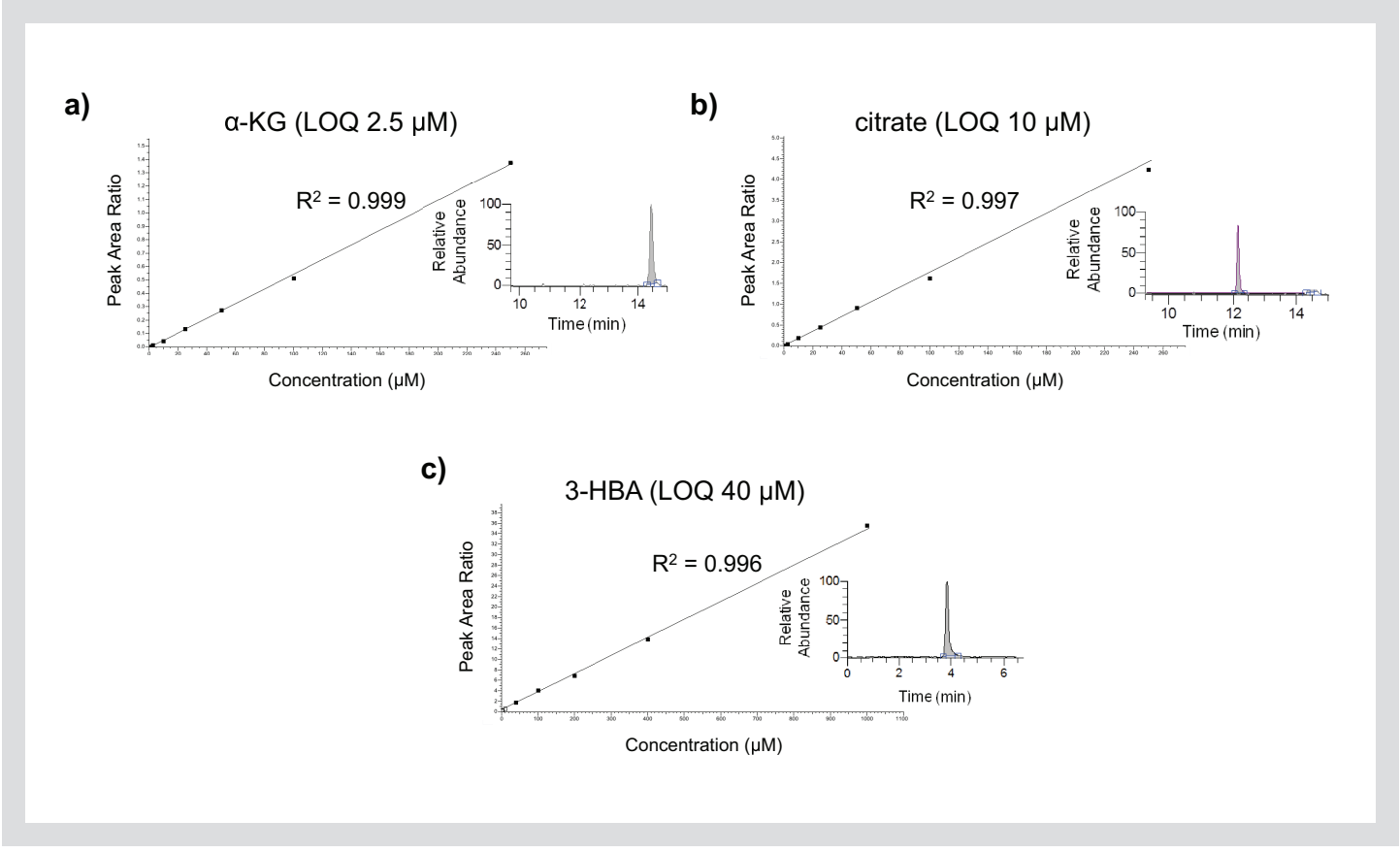


Figure 1. Representative standard curves from the IC-MS/MS analysis. Shown is α-ketoglutarate (α-KG) in **a**), citrate in **b**), and 3-hydroxybutyrate (3-HBA) in **c**). The insets represent extracted negative ion chromatograms for the deprotonated organic acids.

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OA Quantitation in Mouse Muscle

The validated IC-MS/MS method was used to quantify OA differences in quadricep muscles of sedentary and fatigued (LILD and HISD) mice. The average values for a representative subset of OAs for each group of mice are shown in **Figure 2**.

The concentrations of OAs determined in sedentary mouse muscle, approximately varied between 0.02 (for 3-HBA) and 1.34 (for malate) nmol/mg. Statistically significant differences were observed for hippurate, malate, fumarate, and α-ketoglutarate between different groups of mice (**Figure 2**). This application demonstrated that a targeted IC-MS method with SIS OAs can be used to successfully quantify polar OAs in mouse quadriceps muscle of key biological pathways. The capacity to quantify specific OAs can, however, be extended beyond the study of how different forms of exercise influence the concentration of OAs arising from various metabolic pathways. For example, this developed IC-MS method with stable isotope-labeled OAs can be used to identify specific check points that associate with metabolic disorders in similar or dissimilar biological samples. Regardless of the application, the use of stable isotope-labeled standards is essential to confirm the identity of metabolites and for precise and accurate quantification.

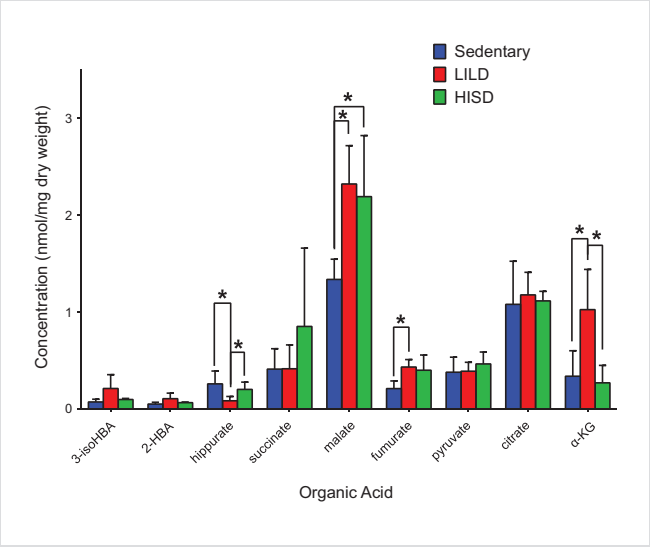

Figure 2. Histograms of OAs quantified in the quadricep muscles of sedentary and fatigued (LLID and HISD) mice by IC-MS/MS. The standard deviations are the result of five technical measurements, while the asterisks denote $p < 0.05$. Abbreviations: 3-isoHBA, 3-hydroxyisobutyrate; 2-HBA, 2-hydroxybutyrate, α-KG, α-ketoglutarate.

Acknowledgements

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Related Products

Catalog No.	Description
MSK-OA-1	Labeled Organic Acid Mix
MSK-OA1-1	Labeled Keto Acid Mix
MSK-OA2-1	Labeled Diacid Mix
MSK-OA3-1	Labeled Monoacid Mix
MSK-OA4-1	Labeled Hydroxy Acid Mix
MSK-OA5-1	Labeled Aromatic Acid Mix
MSK-OA6-1	Labeled Other Acid Mix
MSK-OA7-1	Labeled Other Organic Acid Mix

Companion unlabeled standard (US) mixes are also available (e.g., MSK-OA-US-1). Please refer to our Metabolomics Organic Acid Mixtures flyer for the compositions and see isotope.com for pricing.

For a complete listing of organic acids, please visit isotope.com → Products → Organic Acids and Conjugate Salts.

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