Profiling of Polar Organic Acids in Mouse Muscle Using Ion Chromatography/Mass Spectrometry

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Overview

- Ion chromatography (IC) was evaluated to demonstrate its ability to highly retain and separate polar metabolites that are poorly retained and resolved by reversed-phase chromatography.
- Ion chromatography was coupled with mass spectrometry (IC/MS) to quantitate a variety of polar organic acids in mouse muscle.
- The organic acids examined cover several metabolic pathways including glycolysis, the pentose-phosphate pathway, the TCA cycle, nucleotides, and amino acids.

Introduction

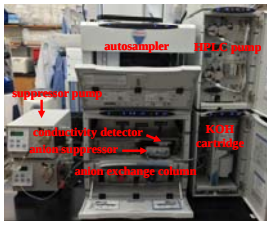
- Organic acids (OAs) are crucial metabolites that play a pivotal role in a host of different metabolic and regulatory pathways.
- We have developed a novel analytical method to quantitate 27 different polar OAs using ion chromatography/triple quadrupole mass spectrometry (IC/MS).
- The method was used to quantitate OA differences in quadriceps muscles from sedentary mice compared to mice that underwent a low intensity, long duration (LILD) or high intensity short duration (HISD) forced treadmill exercise regimen.
- This pilot study has demonstrated that IC/MS is a powerful new tool to separate and quantitate low molecular weight, polar metabolites that are difficult to analyze by other techniques.

Methods

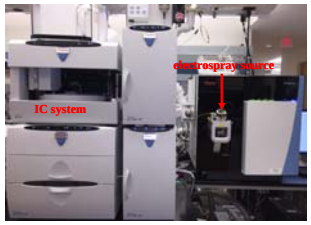
- Frozen quadriceps from mice were lyophilized with subsequent powdering and homogenization of 5 mg of powder in 500 µL of 50:50 acetonitrile/0.3% formic acid using a Precellys Evolution homogenizer.
- A 50 µL aliquot of homogenate, spiked with ^{13}C, ^{2}H, or ^{15}N internal standards, was derivatized with 50 µL of 0.2 M benzylhydroxylamine to stabilize keto-acids.
- The sample was extracted with 1 mL of ethyl acetate followed by drying down 900 µL of the organic layer with reconstitution in 100 µL of deionized water for IC/MS analysis.
- Calibration curves, spiked with ^{13}C, ^{2}H, or ^{15}N internal standards, (Cambridge Isotope Laboratories) were prepared the same as tissue samples from 0.1-250 µM or up to 5000 µM for lactic acid.
- A Thermo Scientific ICS-5000 ion chromatography system using a Dionex AS11-HC, 4 µm, 2 x 250 mm anion exchange column was used to separate the OAs using a KOH gradient from 5 mM KOH to 100 mM KOH over 11 min.
- The IC was coupled to a Thermo Scientific Quantiva triple quadrupole mass spectrometer to quantitate the OAs by single reaction monitoring via electrospray ionization in the negative ion mode.

IC/MS Instrumentation

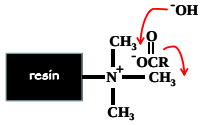
Thermo Scientific ICS-5000+ Reagent Free HPIC System



Thermo ICS-5000+ HPIC/Quantiva Triple Quadrupole MS

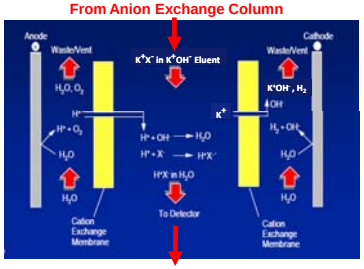


Anion Exchange Stationary Phase and Mechanism



Anion Suppressor

From Anion Exchange Column



To Electrospray Mass Spectrometer

- The anion suppressor neutralizes the KOH eluent, protonates carboxylic acids (HX), and removes potassium cations.
- Hydroxide and potassium ions can contaminate the conductivity detector. Also, potassium salts are not volatile by electrospray ionization.

Results

Precision and Accuracy of Representative Calibrators Over 3 Days

Organic Acid	Nominal Concentration (µM)	Mean Measured Conc. (µM), n = 6	S.D. (n = 6)	% CV	% Accuracy
lactic acid	50	51	2	4	101
	200	201	17	9	100
	500	502	28	6	100
	1000	951	31	3	95
	2000	2022	115	6	101
	5000	5120	369	7	102
pyruvic acid	2.5	2.5	0.2	6.2	98
	10	9.9	0.6	5.9	99
	25	25.3	0.9	3.7	101
	50	49	2	4	98
	100	104	7	6	104
	250	249	15	6	99
fumaric acid	0.25	0.23	0.03	12.05	92
	2.5	2.5	0.2	7.2	100
	10	10	1	10	101
	25	26	2	7	104
	50	49	6	11	99
	100	97	11	12	97
	250	247	15	6	99

Results cont'd

Recovery, Matrix Effect, and Freeze/Thaw Stability of Select Organic Acids in Homogenates of Mouse Quadriceps

Organic Acid	Recovery (%)	Matrix Effect (%)
lactic acid	37	11
pyruvic acid	39	37
malic acid	28	30
fumaric acid	78	21

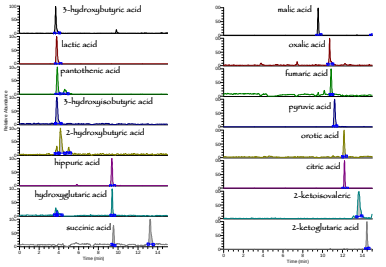
n = 3; recoveries could be improved with extractions in methanol instead of ethyl acetate

3-day Freeze/Thaw Stability of Quadriceps Homogenate

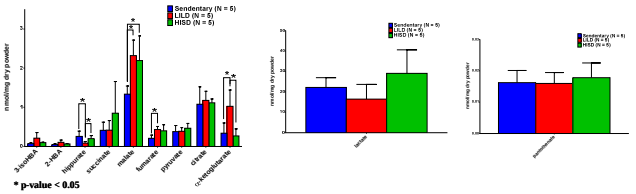
Organic Acid	Day 1 (µM)	S.D.	% CV	Day 3 (µM)	S.D.	% CV	% Stability
lactic acid	201.1	0.1	0.06	209	15	7	104
pyruvic acid	4.0	0.3	6	3.4	0.5	16	85
malic acid	15	1	9	14	2	14	93
fumaric acid	2.0	0.2	9	1.9	0.2	10	98

n = 3

IC/MS of Organic Acids in Mouse Quadriceps



Differential Profiles of Organic Acids in the Quadriceps of Exercised Mice by IC/MS



* p-value < 0.05

Conclusions and Future Directions

- Ion chromatography is a complementary separation method to HPLC (*i.e.* ion-pairing and HILIC) to retain and quantitate polar, water soluble organic acids by mass spectrometry.
- Next Steps: Develop IC/MS methods for nucleotides, glycolysis, and pentose phosphate pathway intermediates.

Acknowledgements

National Institutes of Health (U24 DK097209)