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Case - Citric Acid Cycle and Related Intermediates

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Abstract

Summary:

Analysis of the citric acid cycle (CAC) and related intermediates (such as glutamate, glutamine, GABA, and aspartate) using gas chromatography mass spectrometry (GC-MS) is an analytical approach to identifying unexpected correlations between apparently related and unrelated pathways of energy metabolism. Intermediates can be as expressed as their absolute concentrations or relative ratios by using known amounts of added reference standards to the sample. GC-MS can also distinguish between heavy labelled molecules (eg. ^{2}H- or ^{13}C-labelled) and the naturally occurring most abundant molecules. The use of metabolic tracers (eg. ^{2}H- or ^{13}C-labelled) can offer additional information that can lead to the understanding of interrelationships between the pools of intermediary metabolism, such as with the CAC, and specific metabolic pathways. Applications using tracers can also assess the turnover of specific metabolic pools under various physiological and pathological conditions.

The following SOP presents a relatively simple method that is sensitive for simultaneously measuring concentrations and heavy labelled molecules of CAC intermediates (relative and absolute) and other related intermediates of energy metabolism using GC-MS technology. Note: the following extraction procedure can also be used parallel analysis for acyl-CoA's and lipids (for details on isolating and mass spectrometry assay procedures see, refs 4-6).

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Materials

MATERIALS

N-Methyl-N-(tbutyldimethylsilyl) trifluoroacetamide (TBDMS) **Regis Technologies, Inc.**

Reagents/Materials:

Reagant/Material	Quantity Required	Vendor
*reference internal standards (IS): eg.[$^{13}C_6$]citric and [$^{13}C_4$]succinic acids (98%)	20 µL (~[1.0 mM])	Isotec (Miamisburg, OH)
*See references for additional list of reference standards		Isotec / Sigma Aldrich
Methanol:water (3:2, v/v)		stock
Glacial acetic acid:methanol mixture (5% acetic acid) in methanol/water (1:1)	3-5 ml	stock
Regisil® + 10% TMCS	70 µL	Regis Technologies, Morton Grove, IL
N-Methyl-N-(t-butyldimethylsilyl) trifluoroacetamide (TBDMS)	70 µL	Regis Technologies, Morton Grove, IL

Note:

Sigma-Aldrich,RRID:SCR_008988

Troubleshooting

Before start

Sample Preparation: Plasma or Tissue

Briefly, our approach utilizes the rapid reaction of silylating reagents with alcohols, acids and amines to form silyl derivatives (1-4). Commercially, silylating reagents are available as combinations to accelerate the reaction, as well as to react with the hindered group. For example we use either a mixture of N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) to form a TMS derivative, or a mixture of N-Methyl-N-(tbutyldimethylsilyl)-trifluoroacetamide (MTBSTFA) and t-butyldimethylchlorosilane (TBDMS) to form TBDMS derivatives.

1 **5% acetic acid in methanol/water (1:1) tissue extraction procedure**

1. Weigh about 0.1-0.5 g of powdered frozen (-80 °C) tissue; eg. use a pre-weighed/tarred 15 mL conical tube chilled over dry ice; record the tissue weight (note: when using smaller sample sizes, may use less amounts of solvents and internal standards)

2. Then spike the powdered frozen tissue with the selected internal standards (~50 nM of [$^{13}C_6$]citrate, ~30 nM and [$^{13}C_4$]succinate (may also use ~30 nM of (RS)-3-hydroxy[$^{2}H_5$]glutarate; see refs 1-4)

3. Homogenize the tissue with 3-5 mL of 5% acetic acid in methanol/water (1:1) extraction buffer (chilled on ice) for 2 min on ice bath (note: for acyl-CoA extraction, see modifications, refs 3-6)

4. Centrifuge the homogenate at 670 x g for 30 min at 4°C. Decant the supernatant into a glass test tube and save on ice and process immediately (note: may freeze the supernatant at -80°C until assaying for GC-MS (note: for acyl-CoA extraction, see modifications, refs 3-6)

5. TMSC/TBDMS derivatization procedure: pipette 100-200 µL of the supernatant collected in step 3-4 into test tube. Notes: (i) if performing parallel analysis for lipids/cholesterol or acyl-CoA assays use 80% of the supernatant collected in step 3, refs 3-6 , (ii) addition of methoxylamine-HCL is necessary if measuring analytes that contain keto groups; follow the details outlined in references (1-3)

6. Dry completely under nitrogen gas

7. Then react by adding 70 µL of either derivatizing TMSC or TBDMS reagents (in pyridine based solvent) and heat on a heating block at 80°C for 1 hour (notes: may react at room temperature overnight)

8. transfer to GC insert and cap; follow GC parameters and the monitored SIM ions (m/z) for each analyte, as outlined in refs 1-4

GC-MS Analysis: TMSC or TBDMS derivatives are analyzed using an Agilent 5973N-MSD equipped with an Agilent 6890 GC system, and a DB-17MS capillary column (30m x 0.25 mm x 0.25 um; may use 60m). The mass spectrometer is operated under electron impact mode (EI) or ammonia chemical ionization mode (CI) and selective ion monitoring

(SIM) m/z for each analyte; When stable isotopes are applied, the SIM for M0-M^+ m/z are monitored, see refs1-4