AO-GLP-SOP-02

Valid in AcureOmics AB

Title: **PREPARATION OF TISSUE FOR METABOLIC PROFILING BY GC-MS**

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1. AIM

This Standard Operating Procedure (SOP) applies to the preparation of tissue for metabolic profiling by gas chromatography mass spectrometry (GC-MS).

2. OBJECTIVE

This document describes the procedure for the extraction of metabolites from tissue prior to GC-MS analysis. These samples have been shipped and stored following the procedures detailed in the SOP for Sample Shipment.

3. RESPONSIBILITY

3.1 Each employee is responsible for the work performed in this SOP
3.2 This SOP should be reviewed and revised within 24 months from the validity date.

4. MATERIALS

4.1 **Solvents:** Methanol (HPLC grade) (Fisher Scientific, Loughborough, UK), Chloroform (Merck, Darmstadt, Germany)
4.2. **Reagents:** N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), 1 % trimethylchlorosilane (TMCS) and pyridine (Thermo Fisher Scientific, Rockford, IL, USA). Methoxyamine (Sigma-Aldrich, St Louis, MO, USA).
4.3 MilliQ H_2O
4.4. 1.5 ml Eppendorf tubes (Sarstedt, Germany) (Item no. 72690)
4.5. Tungsten Carbide Beads, 3mm (Qiagen, Hilden, Germany) (Cat.no. 69997)
4.6. 300 µl microvials (Chromacol, Herts, UK) (Part no. 03-FISV)
4.7 GC vial caps (Chromacol, Langerwehe, Germany) (Part no. 9-SC(B)-8RT1)
4.8. Protective gloves and goggles

5. EQUIPMENT

5.1 MM400 Vibration Mill (Retsch GmbH & Co. KG, Haan, Germany)
5.2 VWR VX-2500 Multi-Tube Vortexer (VWR International, Stockholm, Sweden)
5.3 Speed Vac concentrator (Savant Instrument, Framingdale, NY, USA)
5.4 Refrigerated microcentrifuge (Hettich, Tuttlingen, Germany)
5.5 -80 ^{o}C Freezer
5.6 Pestle and mortar

5.7 Ice box with secure lid
5.8 Aluminum foil
5.9 Metal spatula/spoon
5.10 Magnet
5.11 Liquid nitrogen

6. STANDARDS

Eleven internal standards (IS) are used for GC-MS analysis and are obtained from Cambridge Isotope Laboratories (CIL) (Andover, MA, USA), Campro Scientific (Veenendaal, The Netherlands) and Icon (Summit, NJ, USA) (stated below). The standards are stored in the following solutions:

[$^{13}C_5$] - Proline	H_2O	(CIL)
[$^{2}H_4$] - Succinic acid	H_2O	(CIL)
[$^{13}C_5$,^{15}N] - Glutamic acid	H_2O	(CIL)
[1,2,3-$^{13}C_3$] - Myristic acid	Methanol	(CIL)
[$^{2}H_7$] - Cholesterol	Methanol	(CIL)
[$^{13}C_4$] - α-Ketoglutarate	H_2O	(CIL)
[$^{13}C_{12}$]- Sucrose	H_2O	(Campro)
[$^{13}C_4$] - Hexadecanoic acid	Methanol	(Campro)
[$^{2}H_4$] - Putrescine	H_2O	(Campro)
[$^{13}C_6$] - Glucose	H_2O	(Campro)
[$^{2}H_6$] - Salicylic acid	Methanol	(Icon)

Stock solutions of each standard are stored in the fridge at a concentration of 500 ng/µl. Methyl stearate is used as a standard for the sensitivity test and is stored in the fridge at concentrations of 5 ng/µl and 15 ng/µl. Methyl stearate is obtained from Sigma-Aldrich (St Louis, MO, USA).

7. PREPARATION OF SOLUTIONS

The following extraction mix is prepared:
Methanol/Chloroform/H_2O (3:1:1 v/v) + each internal standard (2.5 ng/µl).

A solution of methoxyamine (15 µg/µl) in pyridine is stored in the fridge.

8. TUBES AND LABELLING

Use Eppendorf tubes with a 'Safety Cap', for example Sarstedt type 72690. Tubes with an 'easy-cap' leak during the extraction procedure.

Number the Eppendorf tubes from 1-number of samples. Label on the cap and the side of the tube. All information including sample number, sample name, age, disease state, genetic information etc is stored in an excel spread sheet.

9. PROCEDURE FOR SAMPLE PREPARATION PRIOR TO GCMS ANALYSIS

9.1 Grinding tissue

1. Transfer tubes containing tissue samples from the -80 ^{o}C freezer into an ice box containing liquid nitrogen
2. Place the mortar and pestle on aluminum foil and cool with liquid nitrogen
3. Immediately add the tissue sample to the mortar and grind to a fine powder using the pestle.
4. Transfer the powder immediately into a fresh Eppendorf tube (cooled in liquid nitrogen) using a spoon (also cooled) and keep in liquid nitrogen prior to storage in the -80 ^{o}C freezer.
   Note: Each sample must be processed using a clean pestle, mortar and spoon. Equipment can be washed with RBS and Milli Q water and rinsed in ethanol for rapid drying.

9.2 Weighing tissue

1. Transfer tubes containing tissue samples (powder form) from the -80 ^{o}C freezer to an ice box containing liquid nitrogen
2. Tare the weight of a fresh Eppendorf tube (do not cool) and transfer 10-12 mg tissue into the tube using a cooled spatula. Record exact weight.
3. Transfer the tube into liquid nitrogen. This process should be done as fast as possible.
   Note: the spatula must be cleaned with Milli Q water between each weighing.
4. Also prepare a blank sample with an empty tube.
5. All samples should be stored in the -80 ^{o}C freezer until metabolite extraction.

9.3 Metabolite extraction

Note: Keep all samples on ice whenever possible.

1. Add 1000 µl of extraction mix to ~10 mg tissue using a 100µl–1000µl volume pipette (also add to the blank sample) **NB, minimise the distance when transferring the extraction mix from the storage bottle to the Eppendorf as chloroform can easily be lost from the pipette tip.**
2. Add a 3 mm tungsten carbide bead to the Eppendorf tube
   Note: ensure that the Eppendorf cap is securely closed
3. Agitate samples vigorously in a vibration mill for 3 min, at a frequency of 30 Hz
4. Remove the metal bead with a magnet. IMPORTANT!
5. Centrifuge at 14000 rpm for 15 min, at 4 ^{o}C
6. Transfer 950 µl supernatant into fresh Eppendorf tubes (1.5 ml). **Note: be gentle not to disturb the pellet.**

   Note: It is possible to stop the protocol here and store the samples at -80 ^{o}C.

7. Transfer 200 µl of the supernatant into glass microvials (with no cap).
8. Evaporate samples until dryness in a Speed Vac concentrator (no heat, should take less than 2 hours)
9. Transfer vials to a storage box (13x13x5 cm), cover with foil (no need for vial caps) and store at -80 ^{o}C.
10. The remaining supernatant from step 7 should be stored at -80^{o}C for future use.

9.4 Derivatisation for GCMS analysis

1) If taken from freezer, evaporate samples (from step 9.3.9) until dryness in the Speed Vac concentrator (no heat, 15 min)
2) Add 30 µl methoxyamine (15 µg/µl) in pyridine and cap the vial
3) Agitate samples for 10 min using a multi-tube vortexer
4) Let the reaction continue for 16 h at room temperature
5) Add 30 µl MSTFA, 1 % TMCS
6) Shake for 1 min using a multi-tube vortexer
7) Leave samples for 1 h at room temperature
8) Add 30 µl heptane including methyl stearate (15 ng/µl)
9) Shake samples for 1 min using a multi-tube vortexer
10) Samples are now ready for GC-TOF analysis (must be analysed on the same day and cannot be stored)

A SOP for the use of the GC-MS instrument for metabolic profiling will take over here

10. DOCUMENTATION

10.1 SOP for Sample Shipment

10.2 SOP describing the Guidelines for the use of the GC-MS instrument

11. SAFETY

11.1 Wear gloves at all times
11.2 Wear protective gloves and goggles when handling liquid nitrogen
11.3 All work with solvents must be performed in the fume hood
11.4 Dispose solvents into designated waste labelled container if necessary