



AO-GLP-SOP-01

Valid in AcureOmics AB

Title: PLASMA SAMPLE PREPARATION FOR GC-MS ANALYSIS

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1. AIM

This SOP applies to the preparation of plasma samples prior to gas chromatography mass spectrometry (GC-MS) analysis for metabolomic studies. This SOP can also be applied to serum samples.

2. OBJECTIVE

This document describes the procedure for the extraction of metabolites from human plasma 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),
- 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 H₂O
- 4.4 1.5 ml Eppendorf tubes (Sarstedt, Germany) (Item no. 72690)
- 4.5 Gloves
- 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)

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 Vortex Genie 2 (Scientific Industries, Inc., NY, USA)
- 5.4 Speed Vac (Savant Instrument, Framingdale, NY, USA)
- 5.5 Refrigerated microcentrifuge (Hettich, Tuttlingen, Germany)
- 5.6 Oven (Memmert GmbH + Co. KG, Schwabach, Germany)
- 5.7 -80 °C Freezer

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 ₂ O	(CIL)
[² H ₄] - Succinic acid	H ₂ O	(CIL)
[¹³ C ₅ , ¹⁵ N] - Glutamic acid	H ₂ O	(CIL)
[1,2,3- ¹³ C ₃] - Myristic acid	Methanol	(CIL)
[² H ₇] - Cholesterol	Methanol	(CIL)
[¹³ C ₄] - α-Ketoglutarate	H ₂ O	(CIL)
[¹³ C ₁₂] - Sucrose	H ₂ O	(Campro)
[¹³ C ₄] - Hexadecanoic acid	Methanol	(Campro)
[² H ₄] - Putrescine	H ₂ O	(Campro)
[¹³ C ₆] - Glucose	H ₂ O	(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 (in heptane) at a concentration 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:

MeOH/H₂O (9:1 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 72.690. Tubes with an 'easy-cap' leak during the extraction procedure.

Label 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 is stored in an excel spread sheet.

9. PROCEDURE FOR METABOLITE EXTRACTION

1. Thaw plasma samples at RT (thawing times may vary depending on sample volume, but try to keep as consistent as possible and transfer to ice as soon as thawed)
2. Vortex samples briefly for 5 seconds
3. Add 900 μ l of extraction mix to 100 μ l plasma on ice
 - a. A blank sample should be prepared, containing 900 μ l Extraction mix and 100 μ l MilliQ H₂O
4. Agitate samples vigorously in a vibration mill for 2 min, at a frequency of 30 Hz
5. Cool samples in an ice bath (4 °C) for 2 h (in the fridge on ice)
6. Centrifuge for 10 min at 4 °C, 14000 rpm
7. Transfer 900 μ l of supernatant into fresh Eppendorf tubes (1.5 ml)

Note: It is possible to stop the protocol here and store the samples at -80 °C.

8. Transfer 200 μ l of the supernatant into microvials (with no cap). The remaining supernatant should be stored at -80°C.
9. Evaporate samples until dryness in a Speed Vac (no heat, should take less than 2 hours)
10. Transfer vials to a storage box (13x13x5 cm), cover with foil (no cap required) and store at -80 °C ready for GC-MS analysis.

Note, Quality control (QC) samples should also be prepared using a pooled aliquot of all plasma samples (if too many, pool a representative selection i.e. from all groups). QC samples should be prepared in exactly the same way following the protocol above and alongside all other samples.

10. DERIVATISATION FOR GCMS ANALYSIS

- 1) If taken from freezer, evaporate samples (from step 9.10) until dryness in the Speed Vac concentrator (no heat, 15 min)
- 2) Add 30 μ l methoxyamine (15 μ g/ μ l) in pyridine and cap the vial
- 3) Agitate samples for 10 min using a multi-tube vortexer
- 4) Incubate samples for 1 hour in an oven at 70 °C
- 5) Let the reaction continue for 16 h at room temperature
- 6) Add 30 μ l MSTFA, 1 % TMCS
- 7) Shake for 1 min using a multi-tube vortexer

- 8) Leave samples for 1 h at room temperature
- 9) Add 30 μ l heptane including methyl stearate (15 ng/ μ l)
- 10) Shake the samples for 1 min using a multi-tube vortexer
- 11) 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

11. DOCUMENTATION

11.1 SOP for Sample Shipment

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

12. SAFETY

12.1 Wear gloves at all times

12.2 All work with solvents (especially methanol) must be performed in the fume hood

12.3 Dispose solvents into designated waste labeled container if necessary