



RPC UPLC-QTOF Analysis of Small Molecules in Human Urine - Proforma

NPC.PRO.MS005 Version 2.1

Effective Date: April 2019

1. Purpose

The purpose of this proforma is to document the Ultra-Performance Liquid Chromatography (UPLC) Mass Spectrometer (MS) system reversed phase chromatographic (RPC) assay as outlined in the protocol NPC.SOP.MS005. This proforma should be used in combination with this SOP. The required sections of this proforma should be printed on the day of use, completed and then stored with all project specific documentation.

2. Proforma Approval

Prepared by Dr Verena Horneffer-van der Sluis

Date

Approved by Dr Maria Gomez-Romero

Date

Authorised by Dr Matthew Lewis

Date

Reagents

| Assay specifics | | | |
|---|---------------|---------------|-------------|
| Analyst: | | | |
| Project: | | Date: | |
| Chemical | Supplier | Batch/lot no. | Date opened |
| L-Phenylalanine - $^{13}\text{C}_9$, ^{15}N | Sigma, 608017 | | |
| N-Benzoyl-d ₅ -Glycine (Hippuric Acid-d ₅) | QMX, D-5588 | | |
| L-Glutamic Acid- $^{13}\text{C}_5$ | Sigma, 604860 | | |
| L-Isoleucine- $^{13}\text{C}_6$, ^{15}N | Sigma, 608092 | | |
| L-Leucine- $^{13}\text{C}_6$ | Sigma, 605239 | | |
| L-Tryptophan- $^{13}\text{C}_{11}$, $^{15}\text{N}_2$ | Sigma, 574597 | | |
| Octanoic Acid- $^{13}\text{C}_8$ | Sigma, 605727 | | |
| L-Glutamine- $^{13}\text{C}_5$ | Sigma, 605166 | | |
| Creatinine-Methyl-d ₃ | Sigma, 485446 | | |
| Cytidine-5,6-d ₂ | QMX, D-5424 | | |
| Citric Acid- $^{13}\text{C}_6$ | Sigma, 606081 | | |
| Benzoic Acid-Ring- $^{13}\text{C}_6$ | Sigma, 485691 | | |
| LCMS grade water + 0.1% formic acid | | | |
| LCMS grade acetonitrile + 0.1% formic acid | | | |
| LCMS grade water | | | |
| LCMS grade acetonitrile | | | |
| LCMS grade isopropanol | | | |
| Comments: n/a <input type="checkbox"/> | | | |

PART A - Internal Standard Stock (IStd-Stock) and Solution (IStd-Soln)

Preparation

Please, indicate an action has been performed by ticking the appropriate box (☐)

| Assay specifics | | | | | |
|---|-----------------------------|----------------------|---------------------------------------|------------------|--------------------------|
| Analyst: | | | | | |
| Project: | | | | Date: | |
| RPC IStd-Stock | | | | | |
| Weigh each stock standard individually into a corresponding volumetric flask as outlined below and record the weight and volume. Quantities can be scaled up or down depending on requirements. | | | | | <input type="checkbox"/> |
| Make each volumetric flask up to volume with LCMS grade water | | | | | <input type="checkbox"/> |
| IStd-Stock | Mass (mg) | Volume of water (mL) | Target stock conc. (mg/mL) | Actual mass (mg) | Actual volume (mL) |
| L-Phenylalanine- ¹³ C ₉ , ¹⁵ N | 21.01 | 200 | 0.105 | | |
| N-Benzoyl-d5-Glycine (Hippuric Acid-d5) | 18.61 | 200 | 0.093 | | |
| Sonicate stock solutions until sample dissolution observed | | | | | <input type="checkbox"/> |
| RPC IStd-Soln | | | | | |
| Combine IStd-Stock solutions prepared above into a 500 mL beaker (1:1 v/v) | | | | | <input type="checkbox"/> |
| IStd-Soln | Volume of stock in mix (mL) | | Final concentration (mg/mL) in 400 mL | | |
| L-Phenylalanine- ¹³ C ₉ , ¹⁵ N | 200 | | 0.053 | | <input type="checkbox"/> |
| N-Benzoyl-d5-Glycine (Hippuric Acid-d5) | 200 | | 0.047 | | <input type="checkbox"/> |
| Mix the beaker until the content is visually homogenous | | | | | <input type="checkbox"/> |
| Aliquot 8.5 mL internal standard solution into appropriate vials and store at -80 °C until required | | | | | <input type="checkbox"/> |
| Comments: n/a <input type="checkbox"/> | | | | | |

PART B - Method Reference Stock (MR-Stock) and Method Reference Solution (MR-Soln) Preparation

Please, indicate an action has been performed by ticking the appropriate box (☐)

| Assay specifics | | | | | |
|---|-----------|----------------------|----------------------------|-----------|--------------------------|
| Analyst: | | | | | |
| Project: | | | | Date: | |
| RPC MR-Stock | | | | | |
| Weigh each RPC standard individually into a corresponding volumetric flask as outlined below and record the weight and volume. Quantities can be scaled up or down depending on requirements. | | | | | <input type="checkbox"/> |
| Make each volumetric flask up to volume with LCMS grade water | | | | | <input type="checkbox"/> |
| MR-Stock | Mass (mg) | Volume of water (mL) | Target stock conc. (mg/mL) | Mass (mg) | Actual volume (mL) |
| L-Glutamic Acid- ¹³ C ₅ | 3.081 | 10 | 0.308 | | |
| L-Isoleucine- ¹³ C ₆ , ¹⁵ N | 10.380 | 10 | 1.038 | | |
| L-Leucine- ¹³ C ₆ | 10.760 | 10 | 1.076 | | |
| L-Tryptophan- ¹³ C ₁₁ , ¹⁵ N ₂ | 3.290 | 10 | 0.329 | | |
| Octanoic Acid- ¹³ C ₈ | 3.842 | 25 | 0.154 | | |
| L-Glutamine- ¹³ C ₅ | 100.000 | 50 | 2.000 | | |
| Creatinine-Methyl-D3 | 50.000 | 50 | 1.000 | | |
| Cytidine-5,6-D2 | 10.000 | 10 | 1.000 | | |
| Citric Acid- ¹³ C ₆ | 10.000 | 10 | 1.000 | | |
| Benzoic Acid-Ring- ¹³ C ₆ | 25.740 | 100 | 0.257 | | |
| Sonicate stock solutions until sample dissolution observed | | | | | <input type="checkbox"/> |

Table continues

Assay specifics

Analyst:

Project:

Date:

RPC MR-Soln

Transfer each MR-Stock solution prepared above into a 100 mL beaker according to the quantities outlined in the table below, using appropriate automatic pipettes or measuring cylinders.

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| MR-Soln | Vol. of stock in mix (mL) | Final conc. (mg/mL) in 50 mL | |
|--|---------------------------|------------------------------|--------------------------|
| L-Glutamic Acid- ¹³ C ₅ | 5 | 0.031 | <input type="checkbox"/> |
| L-Isoleucine- ¹³ C ₆ , ¹⁵ N | 1 | 0.021 | <input type="checkbox"/> |
| L-Leucine- ¹³ C ₆ | 1 | 0.022 | <input type="checkbox"/> |
| L-Tryptophan- ¹³ C ₁₁ , ¹⁵ N ₂ | 5 | 0.033 | <input type="checkbox"/> |
| Octanoic Acid- ¹³ C ₈ | 20 | 0.062 | <input type="checkbox"/> |
| L-Glutamine- ¹³ C ₅ | 1 | 0.040 | <input type="checkbox"/> |
| Creatinine-Methyl-D3 | 1 | 0.020 | <input type="checkbox"/> |
| Cytidine-5,6-D2 | 5 | 0.100 | <input type="checkbox"/> |
| Citric Acid- ¹³ C ₆ | 1 | 0.020 | <input type="checkbox"/> |
| Benzoic Acid-Ring- ¹³ C ₆ | 10 | 0.051 | <input type="checkbox"/> |

Mix the beaker until the content is visually homogenous.

☐

Aliquot into appropriate storage containers and store at -80 °C until required.

☐

Comments: n/a ☐

PART C – Analytical Study Reference (SR), analytical Long Term Reference (LTR) and Blanks Preparation - *On the Day of MS-SR preparation*

Please, indicate an action has been performed by ticking the appropriate box (☐)

| Assay specifics | |
|---|--------------------------|
| Analyst: | |
| Project: | Date: |
| SR and LTR | |
| Preparation date of MR-Soln used _____ | |
| Remove sufficient stock urine LTR from storage at -80 °C and allow to defrost at 2-8 °C (15 mL sufficient for ≤ 1000 samples) | <input type="checkbox"/> |
| LTR (volume = _____) mixed with LCMS grade water (volume = _____) and MR-Soln (volume = _____) (ratio 1:1:1, analytical LTR) | <input type="checkbox"/> |
| Final volume aliquotted into _____ 2000 µL aliquots. | |
| Store aliquots at -80°C in freezer | <input type="checkbox"/> |
| SR (volume = _____) mixed with LCMS grade water (volume = _____) and MR-Soln (volume = _____) (ratio 1:1:1, analytical SR) | <input type="checkbox"/> |
| Final volume aliquotted into _____ 2000 µL aliquots. | |
| Start/End SR vials: prepare 2x 750 µL aliquots of analytical SR solution | <input type="checkbox"/> |
| Remaining analytical SR aliquotted into _____ 750 µL aliquots. | |
| Store aliquots at -80 °C in freezer | <input type="checkbox"/> |
| SR (volume = _____) mixed with MR-Soln (volume = _____) (ratio 2:1, SR+MR) | |
| Conditioning and DIDA vials: prepare 8 aliquots of 300 µL volume, e.g. by combining 900 µL aliquots SR+MR solution (prepared above) with 300 µL of IStd-Soln twice. (Sample batch is ≤1000 samples) | <input type="checkbox"/> |
| Store 300 µL aliquots in UPLC vials at -80 °C in freezer | <input type="checkbox"/> |
| Blanks: Prepare 6 aliquots of 300 µL volume, e.g. by combining 500 µL of LCMS grade water with 250 µL of IStd-Soln and 250 µL of MR-Soln twice. (Sample batch is ≤1000 samples) | <input type="checkbox"/> |
| Store 300 µL aliquots in UPLC vials at -80 °C in freezer | <input type="checkbox"/> |
| Comments: n/a <input type="checkbox"/> | |

PART D - SR Dilution Series - *Prior Start of Analysis*

Please, indicate an action has been performed by ticking the appropriate box (☐)

| Assay specifics | | | | | | | |
|--|----------------------|--------------------|-------------------------------|-------------------|-----------------|----------------------|--------------------------|
| Analyst: | | | | | | | |
| Project: | | | | | Date: | | |
| No. of samples _____ No. of batches _____ (Sample batch is ≤1000 samples) | | | | | | | |
| Total number of dilution series sets required (sample sets x 4 + 2 backup) = _____ | | | | | | | |
| The table below indicates the volumes required for a single sample batch (6 dilution series). This will be followed for studies consisting of 1000 samples or less. Please include an appropriate table as an attachment to this document if the number of samples in the study is more than 1000 samples. | | | | | | | |
| For ≤1000 samples | | | | | | | |
| Dilution point | Percentage of SR (%) | Vol. of SR+MR (μL) | Vol. of LCMS grade water (μL) | Vol. of IStd-Soln | Total vol. (μL) | Vol. in aliquot (μL) | |
| 1 | 100 | 900 | 0 | 300 | 1200 | 190 | <input type="checkbox"/> |
| 2 | 80 | 420 | 105 | 175 | 700 | 115 | <input type="checkbox"/> |
| 3 | 60 | 234 | 156 | 130 | 520 | 85 | <input type="checkbox"/> |
| 4 | 40 | 156 | 234 | 130 | 520 | 85 | <input type="checkbox"/> |
| 5 | 20 | 105 | 420 | 175 | 700 | 115 | <input type="checkbox"/> |
| 6 | 10 | 90 | 810 | 300 | 1200 | 190 | <input type="checkbox"/> |
| 7 | 1 | 9 | 891 | 300 | 1200 | 190 | <input type="checkbox"/> |
| Blank | 0 | 0 | 990 | 330 | 1320 | 220 | <input type="checkbox"/> |
| Aliquot each dilution into 6 separate UPLC vials containing the volumes detailed above (vol. in aliquot) and store at -80 °C until required. | | | | | | | <input type="checkbox"/> |
| Comments: n/a <input type="checkbox"/> | | | | | | | |

PART E - SR Dilution Series - *On Day of Analysis*

Please indicate an action has been performed by ticking the appropriate box (☐)

| Assay specifics | |
|---|--------------------------|
| Analyst: | |
| Project: | Date: |
| Complete all pre-project checks as given NPC.SOP.MS002 | |
| Remove the appropriate aliquot of SR dilution series, and appropriate number of conditioning, blanks and/or DIDA vials from storage at -80 °C and allow to defrost for 2 hours at 2-8 °C, vortex mix and spin briefly | <input type="checkbox"/> |
| Place vials in correct positions in autosampler | <input type="checkbox"/> |
| Comments: n/a <input type="checkbox"/> | |

PART F - Sample Preparation

Please, indicate an action has been performed by ticking the appropriate box (☐)

| Assay specifics | | |
|--|--|--------------------------|
| Analyst: | | Date: |
| Project: | | Plate Identity: |
| 1 | Thaw the sample plate, analytical LTR and analytical SR at 2-8 °C for a minimum of 2 hours | <input type="checkbox"/> |
| 2 | Centrifuge sample plate at 3486 g for 1 minute at 4 °C | <input type="checkbox"/> |
| 3 | Add 150 µL LCMS grade water to each sample well (excluding columns 11 and 12) | <input type="checkbox"/> |
| 4 | Dispense 225 µL of analytical LTR to column 11 | <input type="checkbox"/> |
| 5 | Dispense 225 µL of analytical SR to column 12 | <input type="checkbox"/> |
| 6 | Add 75 µL of IStd to each well on the plate | <input type="checkbox"/> |
| 7 | Seal sample plate with cap mat | <input type="checkbox"/> |
| 9 | Mix for 2 minutes on a plate mixer at 1200 rpm at 4 °C | <input type="checkbox"/> |
| 10 | Centrifuge sample plate at 3486 g for 10 minutes at 4 °C | <input type="checkbox"/> |
| 11 | Label two analytical plates with a unique plate barcode label | <input type="checkbox"/> |
| 12 | Carefully remove cap mat from the sample plate (<i>without disturbing the pelleted material</i>) | <input type="checkbox"/> |
| 13 | Transfer 135 µL of each well to both analytical plates; RPOS and RNEG | <input type="checkbox"/> |
| 14 | Seal both analytical plates with heat seal foil | <input type="checkbox"/> |
| 15 | Place RPOS and RNEG plate in relevant autosampler | <input type="checkbox"/> |
| Comments: n/a <input type="checkbox"/> | | |

PART G - Preparation of Mobile Phases and Wash Solutions

Please indicate an action has been performed by ticking the appropriate box (☐)

| Assay specifics | |
|---|--------------------------|
| Analyst: | |
| Project: | Date: |
| Preparation of RPC Mobile phase A and B | |
| N.B. Volumes can be scaled up or down depending on requirement | |
| Mobile phase A: Preparation of 0.1% formic acid in water | |
| 0.1% formic acid in water as supplied | <input type="checkbox"/> |
| Label appropriately | <input type="checkbox"/> |
| Mobile phase B: Preparation of 0.1% formic acid in acetonitrile | |
| 0.1% formic acid in acetonitrile as supplied | <input type="checkbox"/> |
| Label appropriately | <input type="checkbox"/> |
| Comments: n/a <input type="checkbox"/> | |

Table continues

Assay specifics

Analyst:

Project:

Date:

Preparation of RPC Wash Solutions

N.B. Volumes can be scaled up or down depending on requirement

Seal Wash (isopropanol:water 1:9 v/v)

Transfer 100 mL of isopropanol into a Duran bottle

☐

Add 900 mL of LCMS grade water into the Duran bottle with mixing

☐

Mix until the content is homogenous

☐

Sonicate for 5 minutes, seal the bottle and assign an expiry of 1 month

☐

Weak needle wash (isopropanol:water 1:9 v/v)

Transfer 100 mL of isopropanol into a Duran bottle

☐

Add 900 mL of LCMS grade water into the Duran bottle with mixing

☐

Mix until the content is homogenous

☐

Sonicate for 5 minutes, seal the bottle and assign an expiry of 3 months

☐

Strong needle wash (isopropanol)

Use isopropanol as supplied and assign an expiry of 3 months

☐

Comments: n/a ☐

PART H - Acquisition

Please indicate an action has been performed by ticking the appropriate box (☐)

| Assay specifics | |
|--|--------------------------|
| Analyst: _____ | |
| Project: _____ | Date: _____ |
| Instrument number: _____ | |
| Column: Waters Acquity UPLC HSS T3 1.8µm, 2.1 x 150mm, P/N: 186003540 LOT: _____ Serial Number: _____ | |
| Ionisation mode required: _____ | |
| Instrument check performed (See separate Proforma sheet NPC.PRO.MS002) | <input type="checkbox"/> |
| All solvent lines match the assay specific buffers and solutions | <input type="checkbox"/> |
| Check the following against NPC.SOP.MS006: | |
| Tune file used: _____ | |
| Acquisition method used: _____ | |
| Inlet method used: _____ | |
| Sequence loaded into Masslynx | <input type="checkbox"/> |
| Sample plate loaded into tray position | <input type="checkbox"/> |
| Comments: n/a <input type="checkbox"/> | |