



# HILIC UPLC-QTOF Analysis of Small Molecules in Human Serum and Plasma - Proforma

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## **NPC.PRO.MS004 Version 2.1**

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Effective Date: April 2019

### **1. Purpose**

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The purpose of this proforma is to document the Ultra-Performance Liquid Chromatography (UPLC) Mass Spectrometer (MS) system Hydrophilic Interaction Liquid Chromatographic (HILIC) assay as outlined in NPC.SOP.MS004. 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**

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Prepared by Dr Verena Horneffer-van der Sluis

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Date

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Approved by Dr Maria Gomez-Romero

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Date

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Authorised by Dr Matthew Lewis

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Date

## Reagents

Assay specifics			
Analyst:			
Project:		Date:	
Chemical	Supplier	Batch/lot no.	Date opened
N-Benzoyl-d <sub>5</sub> -glycine	CDN Isotopes, D-5588		
Adenosine-2-d-1	CDN Isotopes, D-1827		
L-Phenylalanine- <sup>13</sup> C <sub>9</sub> , <sup>15</sup> N	Sigma, 608017		
Adenine-2d <sub>1</sub>	CDN isotopes, D-6291		
Taurine- <sup>15</sup> N	Sigma, 605956		
Creatine-d <sub>3</sub> .H <sub>2</sub> O	Sigma, 616249		
L-Arginine- <sup>13</sup> C <sub>6</sub>	Sigma, 643440		
L-Tryptophan-d <sub>5</sub>	Sigma, 615862		
Uracil-2- <sup>13</sup> C, <sup>15</sup> N <sub>2</sub>	Sigma, 608459		
LCMS grade water + 0.1% formic acid			
LCMS grade acetonitrile + 0.1% formic acid			
LCMS grade ammonium formate			
LCMS grade water			
LCMS grade acetonitrile			
LCMS grade isopropanol			
Comments: n/a <input type="checkbox"/>			

## PART A - Internal Standard Solution (IStd-Soln) Preparation

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

Assay specifics					
<b>Analyst:</b>					
<b>Project:</b>				<b>Date:</b>	
HILIC IStd-Soln					
Weigh both standards into a single volumetric flask (volume outlined below) using a glass weigh boat and record the mass and volume (quantities can be scaled up or down depending on requirements)					<input type="checkbox"/>
Make the volumetric flask up to volume with LCMS grade water					<input type="checkbox"/>
IStd-Stock	Mass required (mg)	Flask vol. (mL)	Target stock conc. (mM)	Actual mass (mg)	Volume made (mL)
N-Benzoyl-d <sub>5</sub> -glycine	88.4	100	4.8		
Adenosine-2-d-1	10.30		0.384		
Sonicate solution until sample dissolution observed.					<input type="checkbox"/>
Dispense 1 mL aliquots into appropriate vials and store at -80 °C until required					<input type="checkbox"/>
<b>Comments:</b> n/a <input type="checkbox"/>					

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

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

Assay specifics					
Analyst:					
Project:				Date:	
HILIC MR-Stock					
Weigh each HILIC standard into individual 2 ml Eppendorf tube and record the mass (quantities can be scaled up or down depending on requirements).					<input type="checkbox"/>
<b><i>N.B. Uracil-2-<sup>13</sup>C, <sup>15</sup>N<sub>2</sub> to be added dry to a 50 ml volumetric flask using a glass weigh boat</i></b>					
Add 1 ml water to each 2 ml tube.					<input type="checkbox"/>
MR-Stock	Mass required (mg)	Volume of water required (mL)	Target stock conc. (mg/mL)	Actual mass (mg)	Volume made (mL)
L-Phenylalanine- <sup>13</sup> C <sub>9</sub> , <sup>15</sup> N	21.0	1	2.100		
Adenine-2d <sub>1</sub>	1.29	1	0.129		
Taurine- <sup>15</sup> N	15.14	1	1.514		
Creatine-d <sub>3</sub> .H <sub>2</sub> O	1.82	1	0.182		
L-Arginine- <sup>13</sup> C <sub>6</sub>	26.00	1	2.600		
L-Tryptophan-d <sub>5</sub>	25.11	1	2.511		
Uracil-2- <sup>13</sup> C, <sup>15</sup> N <sub>2</sub>	13.81	n/a	1.381		
Sonicate stock solutions until sample dissolution is observed					<input type="checkbox"/>
Comments: n/a <input type="checkbox"/>					

Table continues

Assay specifics			
<b>Analyst:</b>			
<b>Project:</b>		<b>Date:</b>	
HILIC MR-Soln			
Transfer each MR standard stock prepared above into the 50 mL volumetric flask (containing the dry Uracil-2- <sup>13</sup> C, <sup>15</sup> N <sub>2</sub> ) and wash each tube out with additional water as described in the SOP			<input type="checkbox"/>
Make the 50 mL volumetric flask up to volume with LCMS grade water			<input type="checkbox"/>
Mix the volumetric flask until the content is visually homogenous			<input type="checkbox"/>
MR-Soln	Target concentration (mM) in 50 ml stock	Final calculated concentration (mM)	
Phenylalanine- <sup>13</sup> C <sub>9</sub> , <sup>15</sup> N	2.40		<input type="checkbox"/>
Adenine-2d <sub>1</sub>	0.19		<input type="checkbox"/>
Taurine- <sup>15</sup> N	2.40		<input type="checkbox"/>
Creatine-d <sub>3</sub> .H <sub>2</sub> O	0.24		<input type="checkbox"/>
Arginine- <sup>13</sup> C <sub>6</sub>	2.40		<input type="checkbox"/>
Tryptophan-d <sub>5</sub>	2.40		<input type="checkbox"/>
Uracil-2- <sup>13</sup> C, <sup>15</sup> N <sub>2</sub>	2.40		<input type="checkbox"/>
Dispense 1 ml aliquots (into 2 ml Eppendorf tubes) and store at -80 °C until required			<input type="checkbox"/>
<b>Comments:</b> n/a <input type="checkbox"/>			

**PART C – Analytical Study Reference (SR), analytical Long Term Reference (LTR), SR containing MR-Soln (SR+MR) 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 11.5 mL stock plasma/serum LTR from storage at -80 °C per sample batch, allow to defrost at 2-8 °C, and vortex mix before use	<input type="checkbox"/>
LTR (volume = _____) mixed with LCMS grade water (volume = _____) and MR-Soln (volume = _____) (ratio 5:5:1, analytical LTR)	<input type="checkbox"/>
Final volume dispensed into _____ 1 mL aliquots, at least one per plate.	
Aliquots stored at -80 °C in freezer	<input type="checkbox"/>
SR (volume = _____) mixed with LCMS grade water (volume = _____) and MR-Soln (volume = _____) (ratio 5:5:1, analytical SR)	<input type="checkbox"/>
Final volume dispensed into _____ 1 mL aliquots, at least one per plate.	
Start/End SR vials: prepare 2 x 165 µL aliquots of analytical SR solution	<input type="checkbox"/>
Remaining analytical SR aliquoted into _____ 165 µL aliquots	
Aliquots stored at -80 °C in freezer	<input type="checkbox"/>
SR (volume = _____) mixed with MR-Soln ( volume = _____) (ratio 10:1, SR+MR)	<input type="checkbox"/>
Instrument conditioning and DIDA vials: prepare 2x 165 µL aliquots of SR+MR solution	<input type="checkbox"/>
Aliquots stored at -80 °C in freezer	<input type="checkbox"/>
Blanks: prepare 2x blank aliquots by combining 150 µL of LCMS grade water and 15 µL of MR-Sol	<input type="checkbox"/>
Aliquots stored 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 that an action has been performed by ticking the appropriate box (☐)

Assay specifics							
<b>Analyst:</b>							
<b>Project:</b>					<b>Date:</b>		
No. of samples _____ No. of batches _____ (Sample batch is ≤1000 samples)							
Total number of dilution series sets required (sample sets x 2 + 1 backup) = _____							
The table below indicates the volumes required for a single sample batch (3 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 consists of more than a single batch.							
For ≤1000 samples							
Dilution point	Percentage of SR (%)	Vol. of SR+MR (μL)	Vol. of LCMS grade water (μL)	Vol. of IStd-Soln (μL)	Total vol. (μL)	Vol. in aliquot (μL)	
1	100	275	0	25	300	90	<input type="checkbox"/>
2	80	132	33	15	180	55	<input type="checkbox"/>
3	60	99	66	15	180	55	<input type="checkbox"/>
4	40	66	99	15	180	55	<input type="checkbox"/>
5	20	33	132	15	180	55	<input type="checkbox"/>
6	10	33	297	30	360	100	<input type="checkbox"/>
7	1	3.3	326.7	30	360	100	<input type="checkbox"/>
Blank	0	0.0	330	30	360	100	<input type="checkbox"/>
Vortex mix and spin briefly							<input type="checkbox"/>
Dispense each dilution into 3 aliquots containing the volumes detailed above (vol. in aliquot) and store at -80 °C until required							<input type="checkbox"/>
<b>Comments:</b> n/a <input type="checkbox"/>							

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

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

Assay specifics				
<b>Analyst:</b>				
<b>Project:</b>			<b>Date:</b>	
Complete all pre-project system performance checks as given NPC.SOP.MS002				<input type="checkbox"/>
Remove the appropriate aliquot of SR dilution series, conditioning, blanks and DIDA SR from storage at -80°C and allow to defrost for 2 hours at 2-8 °C				<input type="checkbox"/>
Add the corresponding volume of LCMS grade acetonitrile to the SR dilution aliquot as per the table above using pipettes				<input type="checkbox"/>
Dilution point	Percentage of SR (%)	Vol. in aliquot (µL)	Vol. ACN added (µL)	
1	100	90	270	<input type="checkbox"/>
2	80	55	165	<input type="checkbox"/>
3	60	55	165	<input type="checkbox"/>
4	40	55	165	<input type="checkbox"/>
5	20	55	165	<input type="checkbox"/>
6	10	100	300	<input type="checkbox"/>
7	1	100	300	<input type="checkbox"/>
Blank	0	100	300	<input type="checkbox"/>

*Table continues*



**Continuation - PART E - SR and Dilution Series - On Day of Analysis.**

Conditioning, blanks and DIDA aliquots: thaw appropriate number of conditioning, blanks and DIDA aliquots required for study, add 15 µL of IStd-Soln and 360 µL of cold acetonitrile	<input type="checkbox"/>
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Blank: Vortex mix	<input type="checkbox"/>
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Conditioning and DIDA: Mix for 2 minutes on a plate/tube mixer (1400 rpm at 2-8 °C) and incubate for 2 hours at 2-8 °C	<input type="checkbox"/>
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Centrifuge at 3486 g for 10 minutes at 4 °C	<input type="checkbox"/>
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Aliquot supernatant into appropriate labelled UPLC vials	<input type="checkbox"/>
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Place vials in correct positions in autosampler	<input type="checkbox"/>
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**Comments:** n/a ☐

## PART F- Sample Preparation

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

Assay specifics		
Analyst:		Date:
Project:		Plate Identity:
1	Thaw sample plate, analytical LTR, analytical SR and IStd-Soln at 2-8 °C for a minimum of 2 hours, vortex mix and spin briefly	<input type="checkbox"/>
2	Centrifuge sample plate (3486 g for 1 minute at 4 °C)	<input type="checkbox"/>
3	Add 60 µL LCMS grade water to each well (excluding columns 11 and 12)	<input type="checkbox"/>
4	Dispense 110 µL of analytical LTR (containing MR) to column 11	<input type="checkbox"/>
5	Dispense 110 µL of project specific analytical SR (containing MR) to column 12	<input type="checkbox"/>
6	Add 10 µL of IStd-Soln to each well	<input type="checkbox"/>
7	Add 360 µL of ice cold acetonitrile to each well	<input type="checkbox"/>
8	Seal sample plate with heat seal foil	<input type="checkbox"/>
9	Mix for 2 minutes on a plate mixer (1400 rpm at 2-8 °C)	<input type="checkbox"/>
10	Centrifuge sample plate (3486 g for 10 minutes at 4 °C)	<input type="checkbox"/>
11	Label two analytical plates with a unique barcode label	<input type="checkbox"/>
12	Carefully remove heat seal foil from the sample plate ( <i>without disturbing the pelleted material</i> )	<input type="checkbox"/>
13	Transfer 125 µL of each sample to both analytical plates; HPOS and backup	<input type="checkbox"/>
14	Seal both analytical plates with heat seal foil	<input type="checkbox"/>
16	Place HPOS plate in relevant autosampler position	<input type="checkbox"/>
15	Store backup plate at -80 °C	<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	
<b>Analyst:</b>	
<b>Project:</b>	<b>Date:</b>
<b>Preparation of HILIC Mobile phase A and B</b>	
N.B. Volumes can be scaled up or down depending on requirement	
<b>Mobile phase A: preparation of 2 L (20 mM ammonium formate in water + 0.1% formic acid)</b>	
Weigh 2.52 g of ammonium formate into a glass weigh boat. Mass = _____	<input type="checkbox"/>
Transfer the ammonium formate into a 2 L volumetric flask	<input type="checkbox"/>
Make up to volume with LCMS grade water + 0.1% formic acid	<input type="checkbox"/>
Mix until the solution is completely homogenous	<input type="checkbox"/>
Slowly transfer to a 2 L Duran bottle, sonicate, and label appropriately	<input type="checkbox"/>
Measure the pH of the mobile phase ensuring the pH is $3.5 \pm 0.1$ pH = _____	<input type="checkbox"/>
<b>Mobile phase B: preparation of 2 L (acetonitrile with 0.1% formic acid)</b>	
Transfer LCMS grade acetonitrile in 0.1% formic acid to a 5 L Duran bottle	<input type="checkbox"/>
Label appropriately	<input type="checkbox"/>
<b>Comments:</b> n/a <input type="checkbox"/>	

**Table continues**

### Assay specifics

**Analyst:**

**Project:**

**Date:**

### Preparation of HILIC 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.

☐

Mix until the content is homogenous.

☐

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

☐

#### Weak needle wash (water:acetonitrile 1:3 v/v)

Transfer 100 mL of LCMS grade water into a Duran bottle.

☐

Add 300 mL of LCMS grade acetonitrile.

☐

Mix until the contents are homogenous.

☐

Transfer to autosampler weak wash bottle and assign an expiry date of 1 month.

☐

#### Strong needle wash (isopropanol)

Transfer isopropanol as supplied to autosampler strong wash bottle and assign an expiry date of 3 months.

☐

**Comments:** n/a ☐

## PART H – Acquisition

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

Assay specifics	
<b>Analyst:</b>	
<b>Project:</b>	<b>Date:</b>
Instrument number: _____	
Column: <b>Acquity UPLC BEH HILIC, 1.7µm, 2.1 x 150 mm, P/N: 186003462</b> LOT: _____ Serial Number: _____	
Ionisation mode required: _____	
Instrument check performed (Please, 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.MS004:	
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"/>
<b>Comments:</b> n/a <input type="checkbox"/>	