Document Number:	**SASoM/METHOD/124.v1**
Title:	**Analysis of NUC-1031, NUC-3373 and NUC-7738 by uHPLC.**
Version:	**v1**
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Effective from:	28/05/2020
Valid to:	27/05/2022

SOP History		
Number	Date	Reason for Change
v1	28/05/20	Original

1.0 Purpose –

This SOP describes the current procedures for measuring NUC-1031, NUC-3373 and NUC-7738 levels using uHPLC in Laboratory 248/249 at the St Andrews School of Medicine (SASoM).

2.0 Scope –

This SOP applies to all staff in the SASoM measuring NUC-1031, NUC-3373 and NUC-7738 levels using uHPLC in Laboratory 248 at the St Andrews School of Medicine (SASoM).

3.0 Responsibilities –

All staff measuring NUC-1031, NUC-3373 and NUC-7738 levels using uHPLC in this manner are responsible for ensuring that the methods are followed in accordance with this SOP. All staff must have read and signed the relevant risk assessment documents before performing this procedure.

4.0 Procedure –

NuCana BioMed Ltd (NuCana) currently have three lead compounds; Acelarin® (NUC-1031), NUC-3373 and NUC-7738, which are ProTide adaptations of the existing pharmaceutical nucleoside derivatives Gemcitabine, 5FU and Cordycepin respectively.

Work previously conducted by Solid Form Solutions (SFS) found that it was possible to significantly reduce the HPLC run times of NUC-1031 from 60 minutes down to 15 minutes. The method resulted in an uHPLC method that can (i) resolve the R and S isomers of NUC-1031 and (ii) can resolve degradation products when NUC-1031 drug substance was subjected to stressing with base.

Further work by SFS looked at both NUC-3373 and NUC-7738 using the uHPLC method developed for NUC-1031 and adjusted the conditions as necessary to provide a set of chromatographic conditions with which to analyse all three Pro-tides. Pre-validation was also performed on all three compounds to provide assurances that the method was fit for purpose and formally "validation ready". This Standard Operating Procedure is therefore derived from the conditions optimised by SFS.

Sample Collection and Preparation:

Standard Curves for each of the Pro-tide drugs are made up from stock solutions (10mM) using the respective Mobile Phase A as follows:

[A] 100µM = 10µL in 1000µL of Mobile Phase A
[B] 75µM = 7.5µL in 1000µL of Mobile Phase A
[C] 50µM = 5µL in 1000µL of Mobile Phase A
[D] 25µM = 2.5µL in 1000µL of Mobile Phase A
[E] 10µM = 100µL of [A] + 900µL of Mobile Phase A
[F] 5µM = 100µL of [C] + 900µL of Mobile Phase A
[G] 2.5µM = 100µL of [D] + 900µL of Mobile Phase A
[H] 1µM = 100µL of [E] + 1000µL of Mobile Phase A

Test samples are generally made up in either (i) PBS, (ii) cell culture media / 10% FCS, or (iii) Human Pooled Plasma. Samples are collected at appropriate time points before being immediately transferred to 2mL amber-coloured HPLC vials and frozen at -80°c until ready for analysis. Once thawed, all samples are kept on ice until ready for analysis.

All samples are passed through a PTFE 13mm x 0.45uM Orange syringe Filter (Conex: FIL-S-PT-045-13-100) prior to running down the column.

All samples are separated using an ACQUITY CSH C18 1.7µm 2.1 x 100mm Column (Waters; #186005297), regardless of the compound being analysed. A Guard is fitted to the column at all times.

The column is attached to a Dionex / Thermo Fisher 'Ultimate 3000' uHPLC system controlled by Chromeleon v6.80 software.

[A] HPLC Conditions for NUC-1031:

The HPLC method used for method feasibility of NUC-1031 is detailed below. This method has a back pressure of ca. 550 bar.

NUC-1031 Mobile Phase A - 0.1% Formic Acid.

- Add 1mL of Formic Acid to 1000mL of Milli Q water.

NUC-1031 Mobile Phase B - MeOH (70:30) Acetonitrile / 0.1% Formic Acid.

- Add 700mL of Methanol to 300mL of Acetonitrile.
- Add 1.0mL of Formic Acid.

Column:	Waters Acquity CSH 1.7 µm 100 mm × 2.1 mm
Mobile phase A:	0.1% Formic Acid
Mobile phase B:	Methanol / Acetonitrile (70:30 v/v) / 0.1% Formic Acid
Diluent:	Methanol : H2O (50:50 v/v)
Column temperature:	55.0°C
Sample temperature:	Ambient
Flow rate:	0.4 mL/min
Injection volume:	5µL
Detection:	267 nm
Needle wash:	Acetonitrile

Time (mins)	% B
0.00	20%
1.00	30%
5.00	45%
8.00	90%
10.00	90%
10.01	20%
15.00	20%

[B] HPLC Conditions for NUC-3373

The HPLC method used for method feasibility of NUC-3373 is detailed below. This method has a back pressure of ca. 550 bar.

NUC-3373 Mobile Phase A - 0.1% Formic Acid.

- Add 1mL of Formic Acid to 1000mL of Milli Q water.

NUC-3373 Mobile Phase B - Methanol / 0.1% Formic Acid.

- Add 1.0mL of Formic Acid to 1000mL of Methanol.

Column:	Waters Acquity CSH 1.7 µm 100 mm × 2.1 mm
Mobile phase A:	0.1% Formic Acid
Mobile phase B:	Methanol / 0.1% Formic Acid
Diluent:	Methanol : H2O (50:50 v/v)
Column temperature:	55.0°C
Sample temperature:	Ambient
Flow rate:	0.4 mL/min
Injection volume:	10µL
Detection:	270 nm
Needle wash:	Acetonitrile

Time (mins)	% B
0.00	20%
1.00	30%
3.00	45%
20.00	55%
22.00	90%
25.00	90%
25.01	20%
30.00	20%

[C] HPLC Conditions for NUC-7738:

The HPLC method used for method feasibility of NUC-7738 is detailed below. This method has a back pressure of ca. 550 bar.

NUC-7738 Mobile Phase A - 0.1% Formic Acid.

- Add 1mL of Formic Acid to 1000mL of MilliQ water.

NUC-7738 Mobile Phase B - MeOH (70:30) Acetonitrile / 0.1% Formic Acid.

- Add 700mL of Methanol to 300mL of Acetonitrile.
- Add 1.0mL of Formic Acid.

Column:	Waters Acquity CSH 1.7 µm 100 mm × 2.1 mm
Mobile phase A:	0.1% Formic Acid
Mobile phase B:	Methanol : Acetonitrile (70:30 v/v) / 0.1% Formic Acid
Diluent:	Methanol : H2O (50:50 v/v)
Column temperature:	55.0°C
Sample temperature:	Ambient
Flow rate:	0.4 mL/min
Injection volume:	2µL
Detection:	260 nm
Needle wash:	Acetonitrile

Time (mins)	% B
0.00	20%
1.00	30%
5.00	45%
8.00	90%
10.00	90%
10.01	20%
15.00	20%

uHLC Shutdown Procedure:

At the end of the run, the system MUST BE SHUTDOWN in order to prevent precipitation of the Ammonium Dihydrogen Phosphate buffer (Mobile Phase A). Failure to go through this shutdown procedure correctly will result in blockage of the tubes or the piston seals within the pump modules.

Shutdown should be carried out as follows:

1. At the end of the experimental run, ensure that the column heater has been switched off and the column returned to ambient temperature.
2. Stop the flow to both pumps, remove the bottles containing 'Mobile Phase A' and 'B' and then replace each of them with fresh Acetonitrile.
3. Separately purge Pumps A and B with Acetonitrile.
4. Flush the column with Acetonitrile for 15mins, checking the backpressure in case it should increase. This should be done with a 50:50 mix from pumps A/B. This will equilibrate the column with acetonitrile (the default storage medium for the columns)
5. Stop the flow to both pumps, remove the bottles containing 'Mobile Phase A' and 'B' and then replace each of them with fresh Iso Propyl Alcohol / Propan-2-ol.
6. Separately purge Pumps A and B with Propan-2-ol.
7. Close the Purge Valve and turn off the instrument.

5.0 Personal protection –

A Howie coat must be worn at all times. Gloves as specified in the appropriate COSHH RA.

6.0 Spillages –

Always clean up any spills immediately after use, only you know what you have spilt and are aware of its hazard. Spillages should be mopped up with paper towel, disinfected with 70% ethanol and finally washed with detergent.

7.0 Training –

All staff should undergo training in this technique before performing the procedure.

8.0 Related documents –

8.1 Risk assessments –
RA20110 – uHPLC Analysis of Protides

9.0 Approval and sign off –

Author:

Name: Peter Mullen

Position: Research Fellow

Signature: Date:

Management Approval:

Name: Peter Mullen

Position: SOP Administrator

Signature: Date:

QA release by:

Name: Alex MacLellan

Position: QA Manager

Signature: Date:

STANDARD OPERATING PROCEDURE

Please sign below to indicate you have read this S.O.P and understand the procedures involved.

NAME	POSITION HELD	SIGNATURE	DATE