Document Number:	**SASoM/METHOD137.v1**
Title:	**Analysis of Adenine, Adenosine, Inosine and Cordycepin levels by uHPLC**
Version:	**v1**
Author:	**Peter Mullen**

Effective from:	19/08/2021
Valid to:	19/08/2023

SOP History		
Number	Date	Reason for Change
V1	19/08/2021	Original Document

1.0 Purpose –
This SOP describes the current procedure for measuring Adenine, Adenosine, Inosine and Cordycepin levels by uHPLC in Laboratory 248 at the St Andrews School of Medicine (SASoM).

2.0 Scope –
This SOP applies to all staff in the SASoM involved in measuring Adenine, Adenosine, Inosine and Cordycepin levels by uHPLC in Laboratory 248 at the St Andrews School of Medicine (SASoM).

3.0 Responsibilities –
All staff involved in measuring Adenine, Adenosine, Inosine and Cordycepin levels by uHPLC 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 –

This methodology is based on a paper by Lee et al, 2014 (*Cordycepin-enriched WIB801C from Cordycepin militaris inhibits collagen-induced Ca2+ mobilisation via cAMP-dependent phosphorylation of Inositol 1, 4, 5-Triphosphate receptor in human platelets*). The paper describes a protocol for simultaneously measuring Adenine, Adenosine and cordycepin by uHPLC.

Method –

Materials and Reagents:

Adenine (Sigma; A8626-1G) – make up at 10mM in 0.1M HCl.
Adenosine (Sigma; A9251-5G) – make up at 10mM in 1M NaOH at 37°c.
Adenosine (Stratech; B1877-APE; 10mM ready-made solution in 1mL DMSO).
Cordycepin (Sigma; C3394-25MG)
Inosine (Sigma; I4125-1G)
XBridge BEH C18 Column, 130Å, 5 µm, 4.6 mm X 150 mm (Waters; 186003116;).
VanGuard Cartridge Holder (Waters; 186007949).
Column Guards

Buffers:

0.01M Potassium dihydrogen orthophosphate
Weigh out 680.45mg of Potassium dihydrogen orthophosphate and then make up to a final volume of 500mL with MilliQ water.

[A] Preparation and collection of Cell culture media samples:

Cells can be cultured and treated in various petri-dishes as per standard procedures already documented. In general, cells are plated into petri dishes or 96/48/24/12-well trays, left for 24/48hrs, treated with a given drug at a range of concentrations and then left for a further number of days. At the end of the time course the media can be removed, transferred to amber-coloured glass vials (to prevent non-specific binding to plastic) and stored at -80°C until ready for analysis. Samples may require concentrating using a rotary evaporator prior to analysis.

[B] uHPLC Analysis of ATP, ADP, AMP and other nucleotides:

- All samples are analysed using a (Dionex) Thermo Fisher 'Ultimate 3000' uHPLC system controlled by Chromeleon v6.80 software.
- All samples are separated using an XBridge BEH C18 column, 130Å, 5 µm, (4.6 mm X 150 mm), regardless of the nucleotide being analysed. A column guard is fitted to the column at all times to prevent sample blockage.
- All samples are passed through a PTFE 13mm x 0.45uM Orange syringe filter device (Conex: FIL-S-PT-045-13-100) prior to running down the column.

[C] Standard Curve Preparation:

uHPLC Standard Curves for each of the nucleotides (eg. Adenine, Adenosine, Inosine and Cordycepin) are made up from stock solutions (10mM) using Mobile Phase A as follows:

[A] 50µM = 5µL in 1000µL of Mobile Phase A
[B] 25µM = 2.5µL in 1000µL of Mobile Phase A
[C] 10µM = 1.5µL in 1500µL of Mobile Phase A
[D] 5µM = 100µL of [A] + 900µL of Mobile Phase A
[E] 2.5µM = 100µL of [B] + 900µL of Mobile Phase A
[F] 1µM = 100µL of [C] + 900µL of Mobile Phase A
[G] 0.5µM = 100µL of [D] + 900µL of Mobile Phase A
[H] 0.1µM = 100µL of [F] + 1000µL of Mobile Phase A

[D] HPLC Conditions:

Mobile Phase A (0.01M Potassium dihydrogen orthophosphate).

- Weigh out 680.45mg of $KH_4H_2PO_4$ into a 1L glass beaker.
- Make up the volume to 1L with fresh Milli Q water.
- Filter into a clean 1L Duran Bottle prior to use.

Mobile Phase B (Methanol).

- All 250mL of Methanol to a clean Duran Bottle.

uHPLC Conditions:

Column:	Waters XBridge BEH C18, 130Å, 5µm, 4.6 x150mm.
Mobile phase A:	0.1M $KH_4H_2PO_4$
Mobile phase B:	Methanol
Diluent:	Methanol
Column temperature:	25.0°C
Sample temperature:	25.0°C
Flow rate:	1.00 mL/min
Injection volume:	10µL
Detection:	254 nm
Needle wash:	Acetonitrile

Solvent Profile:

The solvent profile was 15mins (up to 30 mins) and is kept constant at 15% Mobile Phase B for the duration of the run.

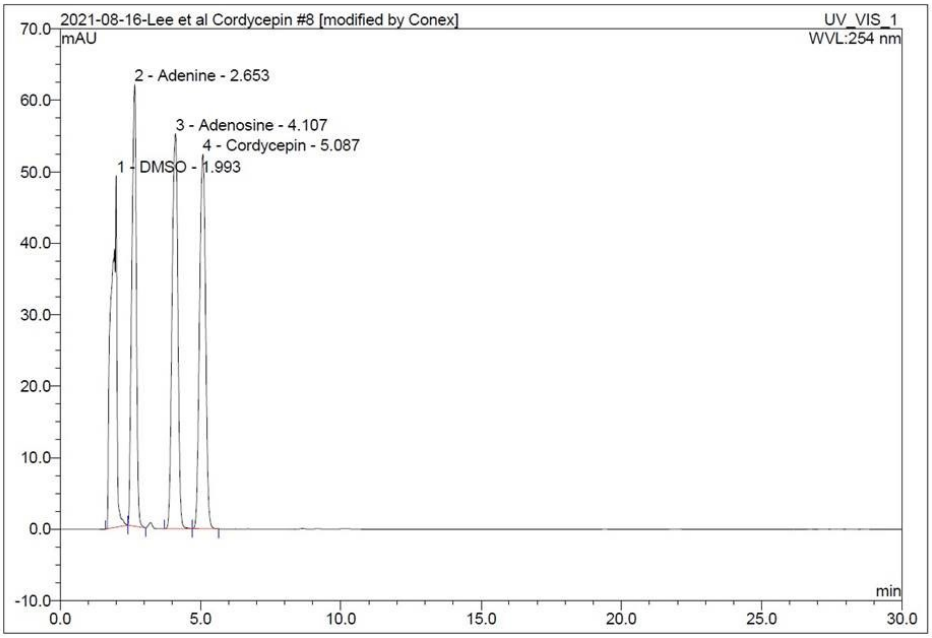
[E] uHLC Shutdown Procedure:

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 Milli Q water.
3. Separately purge Pumps 'A' and 'B' with Milli Q water.
4. Flush the column with Milli Q water for 15mins, checking the backpressure in case it should increase. This should be done with a 50:50 mix from pumps A/B.
5. Stop the flow to both pumps, remove the bottles containing 'Mobile Phase A' and 'B' and then replace each of them with fresh Acetonitrile.
6. Separately purge Pumps A and B with Acetonitrile.
7. 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)
8. 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.
9. Separately purge Pumps A and B with Propan-2-ol.
10. Close the Purge Valve and turn off the instrument.



5.0 Personal protection -

A Howie laboratory coat and lab gloves must be worn at all times.

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 a paper towel, disinfected with Virkon or Haztabs, and finally wiped with 70% ethanol.

7.0 Training -
All personnel using this method must have received cell-culture and sterile lab-practice training.

8.1 Risk assessments –
RA23755_Analysis of Adenine, Adenosine, Inosine and Cordycepin levels by uHPLC.

8.2 Standard Operating Procedures –
SASoM-METHOD-124-Analysis of NUC-1031, NUC-3373 and NUC-7738 by uHPLC.
SASoM-METHOD-125-Analysis of ATP, ADP, AMP and other nucleotides by uHPLC.
SASoM-METHOD-112-Preparation of Cell Lines for HPLC analysis of GSSG and GSH.

9.0 Approval and sign off –

Author:

Name: Peter Mullen

Position: Research Fellow

Signature:  Date: 09/09/2021

Management Approval:

Name: Peter Mullen

Position: Research Fellow

Signature:  Date: 09/09/2021

QA release by:

Name: John O'Connor

Position: Research Technician

Signature:  Date: 08/09/2021

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