Document Number: **SASoM/METHOD/126.v1**

Title: **Analysis of Glutathione 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 Glutathione by 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 Glutathione by uHPLC in Laboratory 248 at the St Andrews School of Medicine (SASoM).

3.0 Responsibilities –

All staff measuring Glutathione by 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.

NB. Be extra careful when dealing with Picric Acid and Sodium Perchlorate!

ALL USERS MUST SIGN THE RISK ASSESSMENT BEFORE CARRYING OUT THIS PROCEDURE.

4.0 Procedure –

This procedure describes the simultaneous measurement of both oxidised and reduced Glutathione by uHPLC and is based on the following paper:

Lipsa D, Cacho C, Leva P, Barrero-Moreno J & Aguar P. Development of a HPLC-UV method for the simultaneous determination of intracellular glutathione species in human cells. J Anal. Bioanal. Tech. (2015); 6(4), pp1-8.

Materials and Reagents:

Acetonitrile (3F, T)
Ammonium Sulfate (1)
Formic Acid (3A)
Glutathione (GSH) – reduced (1)
Glutathione (GSSG) – oxidised (1)
Metaphosphoric Acid (3A)
Methanol (4F, T)
Picric Acid (5T, X)
Propanol -2- (propanol iso-, propyl alcohol iso-) (2F)
Sodium Perchlorate (5X, O)
Trifluoroacetic acid (4A, T)

[A] Buffers:

0.5% Picric Acid:

Picric Acid was supplied at a concentration of 12g/L (1.2% solution) and therefore needs to be diluted prior to use (final concertation should be 0.5% solution). This will therefore mean that the stock Picric Acid needs to be diluted 1 part in 2.4 parts of DW.
(ie. Equivalent of 10mL of Picric Acid + 14mL of DW).

25mM Ammonium Sulfate:

Ammonium Sulfate (165.175mg) was weighed out and then dissolved in 0.5mL of 0.5% Picric Acid.

Mobile Phase: (H_20 : Acetonitrile (95:5) + 0.1% TFA + 12mg/mL Sodium Perchlorate).

Combine 190mL of Milli Q water and 10mL of Acetonitrile in a glass Duran bottle to give a total volume of 200mL. Trifluoroacetic acid (0.2mL) was then slowly added, followed by 2,400mg (2.4g) of Sodium Perchlorate. A plastic spatula (rather than metal) and weighing boat (rather than tin foil) were used for weighing out the Sodium Perchlorate. The cap was then loosely fitted and the mixture 'degassed' by sitting in a sonic bath for 15mins (inside the fume cabinet). The balance was then thoroughly cleaned.

[B] Cell Lysate Extraction Buffer - Ammonium Sulfate (25 mM) dissolved in 0.5% Picric Acid (0.5mL) is added to 50mL of Mobile Phase A and then stored at 4°C.

[C] Preparation and collection of cell culture lysates:

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 6-well trays, left for 24/48hrs, treated with a given drug at a range of concentrations and then left for a further 3-4 days. At the end of the time course samples are processed as follows;

1. Discard cell culture media from the cells exposed to the test agents previously solubilised into the culture media.
2. Wash the cells with ice-cold cold PBS (200 µL for 24-well plate and 300 µL for 6 well plate). Remove all residual PBS and place cells on ice.
3. Add 300 µL or 600 µL of Glutathione Extraction Buffer to the cells and then incubate the cell lysates at -18°C (in a freezer) for 10 minutes.
4. Remove lysates from the freezer, allow to thaw on ice and then mechanically scrape the cells off the plastic using a clean cell scraper.
5. Transfer the lysates to microcentrifuge tubes and sonicate over ice for 2 minutes.
6. Centrifuge at full speed in a microcentrifuge for 15 minutes at 4°C in order to remove proteins and high molecular weight compounds prior to HPLC analysis.
7. Finally, the supernatant was immediately transferred to pre-chilled dark amber vials and injected into the HPLC or stored at -80°C for further analysis of GSH and GSSG.

[D] Glutathione Standards:

Stock solutions containing 20mM GSH (mwt 307.3) or GSSG (mwt 612.6) were prepared in Glutathione Extraction Buffer as shown below:

1. Glutathione (Reduced) GSH – weigh 6.146mg into 1mL of Extraction Buffer.
2. Glutathione (Oxidised) GSSG – weigh 12.252mg into 1mL of Extraction Buffer.

Standards were then aliquoted into 10 x 100µL and stored in amber-coloured HPLC glass vials at -80°C for up to 6 months.

uHPLC Standard Curves for Glutathione (oxidised and reduced forms) are freshly made up from stock solutions (20mM) using Mobile Phase A as follows:

[A] 100µM = 2.5µL in 500µL of Mobile Phase A
[B] 75µM = 2µL in 533.33µL of Mobile Phase A
[C] 50µM = 250µL of [A] + 250µL of Mobile Phase A
[D] 25µM = 100µL of [A] + 300µL of Mobile Phase A
[E] 10µM = 100µL of [A] + in 900µL of Mobile Phase A
[F] 5µM = 250µL of [E] + 250µL of Mobile Phase A
[G] 2.5µM = 100µL of [E] + 300µL of Mobile Phase A
[H] 1µM = 50µL of [E] + 450µL of Mobile Phase A

[E] uHPLC Analysis of Glutathione:

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). 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.

uHPLC Conditions:

Column:	Waters XBridge BEH C18, 130Å, 5µm, 4.6 x150mm.
Mobile phase A:	water / acetonitrile (95/5, v/v), TFA (0.1%) and sodium perchlorate (12 mg/mL).
Mobile phase B:	n/a
Diluent:	
Column temperature:	22.0°C
Sample temperature:	6.0°C
Flow rate:	1 mL/min
Injection volume:	10µL
Detection:	215 nm
Needle wash:	Acetonitrile

Solvent Profile:

The solvent profile is shown below and consists of an isocratic elution profile of a single mobile phase running over 8 minutes.

Time (mins)	% B
0.00	0%
8.00	0%

[F] uHLC Shutdown Procedure:

At the end of the run, the system MUST BE SHUTDOWN in order to prevent precipitation within the column. 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 coat must be worn at all times. Gloves as specified in the appropriate Risk Assessment.

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 –

RA20267: Analysis of Glutathione by uHPLC

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