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Biochemical Measures of Neuropathy - Glutathione Assay (GSH)

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1 Works for me dx.doi.org/10.17504/protocols.io.3qjgmun

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ABSTRACT

Summary:

Oxidative stress is highly correlated with the metabolic changes caused by hyperglycemia. Increased levels of glucose overload mitochondria and result in the production of reactive oxygen species (ROS). In addition, the flow of excess glucose through cellular pathways decreases the cell's normal ability to detoxify ROS. As a result, the neurons and axons of the peripheral nervous system contain increased levels of ROS and decreased antioxidant capacity. The following assays are used to measure these changes in rodent models of diabetic neuropathy.

Diabetic Complication:

EXTERNAL LINK

https://www.diacomp.org/shared/document.aspx?id=54&docType=Protocol

MATERIALS

NAME	CATALOG #	VENDOR
Perchloric Acid (Lot # 227044)	A469-500	Fisher Scientific
KOH Potassium hydroxide	P250-1	Fisher Scientific
Imidazole Base	I 2399	Sigma Aldrich
Potassium Chloride	P217-500	Fisher Scientific
Tris Base	604204	Fisher Scientific
Tris Hydro	812854	Fisher Scientific
EDTA (Lot 0M1037)	16-004Y	Fisher Scientific
Glutathione (Lot 070K0888)	G-6529	Sigma Aldrich
o-Phthaldialdehyde (Lot 080K2519)	P-0657	Sigma Aldrich
Methanol	A 4524	Fisher Scientific

MATERIALS TEXT

Reagent Preparation:

3 *M*HClO4 – 42 ml of 72% stock Perchloric acid into 58 ml ddH_2O

2*N*KOH, 0.4 M imidazole base, and 0.4 M KCl – 56.11 g KOH, 13.62 g Imidazole base and 14.91 g KCl. Add ddH_2O to bring up to 500 ml.

1.0 *M*Tris-HCl buffer pH 8.1 with 20mM EDTA – 50 mL 2M TRIS-HCL pH 8.1, 4mL 0.5 M EDTA and 46 mL ddH_2O.

0.1M Glutathione – 30.7 mg into 1 ml H_2O.

0-Phthaldialdehyde – 10 mg in 1 ml of methanol.

Sample Preparation:

1 **Do not let samples set at 0º for long periods of time.**

Prior to dissection, perfuse tissue with PBS pH7.4 with 0.16 mg/ml heparin

1. Label 6 sets of micro centrifuge tubes. (If only doing GSH only need 3 sets)
2. Prepare 3 M $HClO_4$ and freeze.
3. Cut tissue into segments and weigh. (~10 mg)
4. Keeping labeled tubes on dry ice, add 3 times the weight of 3 M $HClO_4$.
5. Sonicate on ~5.
6. Add 300 µl of ddH_2O **for each 100 µl** $HClO_4$ (3.3 per µl) and vortex. (May include 1 mM EDTA in ddH_2O)
7. Centrifuged at 5000 x g for 10 min.
8. Transfer supernatant to a new labeled tube. Discard protein precipitate.
9. Immediately neutralize with 290 µl/ml of a mixture of 2 *N*KOH, 0.4 *M*imidazole base, and 0.4*M*KCl and vortex. (Leaving a solution at pH 7.)
10. Centrifuge samples @ 5000 x g for 10 min @ 4º to precipitate insoluble $KClO_4$

Performing the assay:

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1. Set up Fluroskan layout and area. Read using 345nm excitation and 425nm emission.

2. Prepare standards as follows: (0-1000uM)

Concentration µM/mL	H_2O	GSH
0	500	NO GSH
15.63	500	Take 500 µL from tube 3
31.25	500	Take 500 µL from tube 4
62.5	500	Take 500 µL from tube 5
125	500	Take 500 µL from tube 6
250	500	Take 500 µL from tube 7
500	500	Take 500 µL from tube 8
1000	990	10 µL 0.1M stock

3. On a clear 96 well plate add 20 µl standards to each well in duplicate

4. Add 20 µl sample extract to each sample well in duplicate or triplicate.

5. Add 180 µl Tris-HCl buffer to each well.

6. Begin reaction by adding 2 µl methanol solution of o-phthaldialdehyde (OPD) (10 mg/ml methanol)

7. Place plate into Fluroskan holder and click **START.**

8. From the drop down menu under sheet select curve fit. Choose the appropriate data to organize (usually Measure1), then click **OK**. This calculates your standard curve.

9. Save the curve fit sheet as an Excel file into your data folder. Use the naming convention GSXXXXXX.xls, where XXXXXX is the date in yymmdd.xls.