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Biochemical Measures of Neuropathy - GSSG (Non-Enzymatic)

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We use this protocol and it's working

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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:

Neuropathy

Materials

MATERIALS

- Perchloric Acid (Lot # 227044) **Fisher Scientific Catalog #**A469-500
- N-ethylmaleimide **Merck Millipore (EMD Millipore) Catalog #**34115-5GM
- KOH Potassium hydroxide **Fisher Scientific**
- Imidazole Base **Fisher Scientific**
- Potassium Chloride
- Tris
- HCL
- EDTA **BioWhittaker Catalog #**16-004Y
- O-phthaldialdehyde (Lot 80K25190) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P-0657
- GSSG (Glutathione (Oxidized)) (Lot 93148120) **Roche Catalog #**105 635

Reagent Preparation:

3 *M* $HClO_4$ – 42 ml of 72% stock Perchloric acid into 58 ml ddH_2O

0.04M *N*- ethylmaleimide – 5mg in 1 mL ethanol (N- ethylmaleimide complexes with GSH (Hissin and Hilf, 1976) thus preventing interference from GSH oxidation Per Dr. Obrosova)

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.

***0.1* 0.1 *M* imidazole-HCl buffer pH 7.6 with 20 mM EDTA –** 68 mg in 50 ml ddH_2O. Add 400 µL 0.5 M EDTA and pH to 7.6 with HCl then bring volume up to 10 mL.

GSSG Standard 100mM – 61.26 mg in 1 mL ddH_2O.

***O*-Phthaldialdehyde** – 10 mg in 1 ml of methanol.

Note:

Roche (RRID:SCR_001326)

Thermo Fisher Scientific (RRID:SCR_008452)

Sigma-Aldrich (RRID:SCR_008988)

Troubleshooting

Sample Preparation:

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

1. If using extract from GSH add 100 µl 0.04 M N-ethylmaleimide to 200 µl homogenate then skip to Performing the Assay or

2. Label 3 sets 1.5 ml and 1 set of 0.6 ml micro centrifuge tubes.

3. Prepare 3 *M* $HClO_4$ and freeze.

4. Keeping labeled 1.5ml tubes on dry ice, add 3 volumes of 3 *M* $HClO_4$.

5. Cut tissue into segments and weigh. (~10 mg)

6. Place weighed segment in tube on top of $HClO_4$.

7. Place tubes in an alcohol bath maintained at –8º to –10ºC. and agitate 15 minutes or until the acid completely penetrates the tissue fragments. (Make sure the temperature does not go below –10º to completely extract the ice. (2 *M* $HClO_4$ will freeze at -12ºC.)

8. Add 333 µl of ddH_2O for each 100 µl $HClO_4$ and shake at 4ºC for 10 min. (May include 1 mM EDTA in ddH_2O)

9. Pull off 25 µl and place in a 0.6 ml micro-centrifuge tube for protein analysis.

10. Centrifuge samples at 5000 x g for 10 min at 4º.

11. Transfer supernatant to a new labeled tube. Discard protein precipitate.

12. Immediately neutralize with 290 µl/ml of a mixture of 2 N KOH, 0.4 *M* imidazole base, and 0.4*M* KCl . (Leaving a solution at pH 7.)

13. Centrifuge at 4000 x g for 5 min to precipitate insoluble $KClO_4$.

Performing the assay:

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

Concentration µM/mL	H_2O	GSSG
0	500	NO GSSG
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

1. On a clear 96 well plate add 40 µl standards to each well in duplicate.

2. Add 40 µl sample extract to each sample well in duplicate or triplicate.

3. Add 160 µl imidazole-HCl buffer to each well. Add 0.2-10 µM NADPH

4. Begin reaction by adding 0.3 U of glutathione reductase.

5. Set up Fluroskan layout and area..

6. Read using 340 nm excitation and 460 nm emission.

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.