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Methods for the Determination of Plasma or Tissue Glutathione Levels

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Abstract

We present two different methods for determining levels of glutathione in complex biological samples and plasma. The DTNB/GR enzyme recycling method is sensitive and requires no specialized equipment. The HPLC method is particularly useful for situations in which sample amounts are limited. Detailed instructions for performing each method as well as the advantages and disadvantages of each are discussed in this Chapter.

Keywords

glutathione; glutathione disulfide; glutathione reductase; HPLC; biological assay

1. Introduction

Measurement of glutathione (GSH), glutathione disulfide (GSSG), and related intermediates are important in assessing the redox and metabolic status of biological systems *in vivo* and *in vitro*. Quantitation of thiols and disulfides involved in the GSH pathway may yield clues as to mechanisms of injury of drugs and disease states, or regulation of redox sensitive pathways in an organism. Although measurements of GSH and GSSG are routinely performed, the potential errors in such measurements are often not fully recognized or appreciated. Tissue levels of both GSH and GSSG fall into a range easily measurable by most currently used methods (1–10 mM and 0.01–0.05 mM, respectively). Other biological samples such as plasma can be problematic because of the sensitivity required for quantitation (1–10 μM) and sample manipulation necessary for analysis. Simultaneous measurement of GSH and related thiols and disulfides and their mixed disulfides with GSH offer potentially important information about the redox status of a system.

Sample handling and manipulation is extremely important in assessing biologically relevant levels of any substances. Because GSH and related thiols are sensitive to oxidation and or degradation during sampling and analysis, careful attention should be paid to prevent such events from occurring. Freeze clamping tissues with liquid nitrogen-cooled tongs and storing at −80°C or acidification of plasma or tissue samples as quickly as possible minimize autooxidation and degradation.

2. Materials

2.1. DTNB and GR Recycling Method for Measurement of GSH and GSSG

1. 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)
2. *n*-Ethylmaleimide (NEM)

3. Glutathione, oxidized (GSSG) (L-Glutathione oxidized disodium salt, Sigma #G-4626)
4. β-Nicotinamide adenine dinucleotide phosphate (β –NADPH, Sigma #N-1630)
5. Sep-pak® Classic C18 cartridges (Waters #WAT051910)
6. Glutathione reductase (GR, Roche # 10105678001)
7. Methanol
8. Acetonitrile
9. 96-well clear bottom microtiter plates

2.2. GSH and GSSG measurement by HPLC (all reagents can be purchased from Sigma Aldrich using the catalog numbers indicated)

1. Methanol (34860)
2. Sodium acetate (S2889)
3. Perchloric acid (311421)
4. Boric acid (B1934)
5. Iodoacetic acid (I8136)
6. Dansyl Chloride (D2625)
7. Glutathione, reduced (G4261)
8. Glutathione, oxidized (150568)
9. L-serine (S4500)
10. sodium heparin (H4784)
11. bathophenanthroline disulfonate sodium salt (BPDS) (146617)
12. γ-glu-gly (G8390)
13. Chloroform (650498)
14. Potassuim hydroxide (KOH) (484016)
15. Sodium tetraborate (229946)
16. Potassium tetraborate (P5754)

3. Methods

The first method is a widely accepted and sensitive enzyme recycling assay based on a procedure reported by Tietze (1) and modified by Adams et al (2) that requires no specialized equipment. GSH is oxidized by 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) resulting in the formation of GSSG and 5-thio-2-nitrobenzoic acid (TNB). GSSG is then reduced to GSH by glutathione reductase (GR) using reducing equivalent provided by NADPH. The rate of TNB formation is proportional to the sum of GSH and GSSG present in the sample and is determined by measuring the formation of TNB at 412 nm. Specific changes have been described to increase assay sensitivity enabling measurements in plasma from populations with inherently low GSH or GSSG levels (3, 4).

The second method uses HPLC separation and fluorometric detection. The original method, developed by Reed et al (5), used iodoacetic acid (IAA) to form S-carboxymethyl derivatives with free thiols and fluorodinitrobenzene which reacts with amines to facilitate

UV absorbance detection at 365 nm. Martin and White (6) later altered this method using dansyl chloride as the derivatizing agent with fluorescence detection thereby increasing the sensitivity of the assay substantially. Finally, Jones et al (7) further refined the method to minimize artifactual oxidation and increase sensitivity. A technique using iodoactetic acid as the thiol akylating agent followed by dansyl chloride derivatization for fluorometric detection is presented. This method is advantageous because it is amenable to small sample quantities and detects thiols and disulfides of several small molecules, GSH, GSSG cystiene, cystine, and mixed disulfides in a single run using ion-paring chromatography. The alkylation and derivatization processes are rather time-consuming and iodoacetic acid (IAA) reacts rather slowly with free thiols (8, 9). While relative comparisons can be made using this method, caution should be taken in making conclusions about absolute concentrations; specifically of the disulfide species.

3.1. DTNB/GR Enzyme Recycling Method

1 Assay buffer solution (0.1M sodium phosphate with 5 mM EDTA, pH 7.4)

1. Weigh 14.196 g Na-Phosphate dibasic and dissolve in approximately 800 ml ddH2O. Add 1.86 g EDTA and dissolve completely.
2. Weigh 3.45 g Na-Phosphate monobasic and dissolve in 250 ml ddH2O. Add 0.47 g EDTA and dissolve completely
3. Add monobasic solution to dibasic solution (#2 to #1) to achieve ph of 7.4. Bring final volume to 1000 ml by adding ddH2O after ph of 7.4 has been reached.
4. Stir.

2 DTNB stock solution (10 mM)

1. Weigh 99.1 mg DTNB and dissolve in approximately 20 ml assay buffer.
2. Transfer to volumetric flask and bring volume up to 25 ml by adding assay buffer. Store in refrigerator (good for 1 month).

3 NEM stock solution (1mM)

1. Weigh 125.13 mg NEM and dissolve in 1 ml acetonitrile. Store in refrigerator.

4 GSSG standard stock solution

1. Weigh 15.3 mg glutathione (oxidized form) and dissolve in 5 ml assay buffer for final concentration of 10 mM GSH equivalence.
2. Aliquot 25 μl into 0.5 ml eppendorf tubes and store at −20 °C. Use fresh tube daily.

5 GSSG working standard

1. Add 10 μl GSSG standard to 990 μl assay buffer for concentration of 0.1 mM.
2. Add 0.5 ml of 0.1 mM GSSG standard to 4.5 ml assay buffer for concentration of 0.01 mM.

6 GSH standard curve (outlined in 4.2).

GSSG standard curve (outlined in 4.3).

7 **Reaction mixture #1:** Place 4.38 ml assay buffer in 5 ml conical tube. Add 313 μl of 10 mM DTNB stock solution. Add 50 μl glutathione reductase. Store on ice.

8 **Reaction mixture #2:** Dissolve 5 mg NADPH in 5 ml assay buffer. Store on ice.

9 GSH Assay Procedure

1. Prepare tissue samples. Homogenize 0.1 g tissue sample in 900 ml assay buffer (Store on ice). Centrifuge samples for 20 min at 12000 rpm. Remove supernatant and place in new tube. Dilute tissue supernatants as indicated in Table 1.
2. Prepare plasma samples. Add 250 μl blood to an equal volume of ice-cold 50 mM serine borate, 50 mM potassium phosphate buffer containing 17.5 mM EDTA, ph 7.4. Gently mix the sample by capping and inverting the tube 3 times. Immediately centrifuge the samples, separate the plasma, and analyze. Samples may be stored at −80°C until analysis.
3. Add 30 μl standard/tissue sample to well (in duplicate).
4. Add 120 μl of assay buffer to each well.
5. Add 50 μl of reaction mixture #1 to each well.
6. Add 50 μl of reaction mixture #2 to each well.
7. . Measure absorbance over 3 minutes at A412 nm at 30 s intervals in plate reader.

10 GSH data analysis

1. Construct a standard curve (pmol/assay) using the values in 4.2.
2. Calculate the concentration of GSH in each sample and factor in any sample dilution.

11 GSSG Assay Procedure

1. Preparation of tissue samples: Homogenize 0.1 g tissue sample in 890 μl assay buffer and 10 μl NEM (Store on ice). Centrifuge for 20 min at 12000 rpm. Remove supernatant and place in new tube.
2. Preparation of plasma samples: Add 250 μl blood to an equal volume of ice-cold 50 mM serine borate, 50 mM potassium phosphate buffer containing 17.5 mM EDTA and 10 mM NEM, ph 7.4. Gently mix the sample by capping and inverting the tube 3 times. Immediately centrifuge the samples, separate the plasma, and analyze. Samples may be stored at −80°C until analysis.
3. Sep-pak tissue samples and standards.
    a. Wash new column with 3 ml ddH_2O (1 drop per second).
    b. Wash column with 3 ml methanol (1 drop per second).
    c. Wash column with 3 ml assay buffer (1 drop per second).
    d. Flush column with air.

e. Place column into microcentrifuge tube and insert 1 ml syringe.

f. Add 200 μl of standard/tissue supernatant to syringe and push through.

g. Add 800 μl assay buffer to syringe and push through.

h. Flush column with air.

i. Vortex tube.

j. Clean column by washing with 3 ml methanol followed by 3 ml assay buffer.

k. Flush column with air.

l. Repeat steps for additional samples. Columns should not be reused more than 3 times for samples and more than 2 times for tissue supernatants.

4. Add 200 μl of Sep-pak eluent to well (in duplicate).

5. Add 50 μl of reaction mixture #1 to each well.

6. Add 50 μl of reaction mixture #2 to each well.

7. . Measure absorbance over 3 minutes at A412 nm at 30 s intervals in plate reader.

12 GSSG data analysis

1. Construct a standard curve (pmol/assay) using the values in 4.3.

2. Calculate the concentration of GSSG in each sample and factor in any sample dilution.

3.2. GSH and GSSG measurement by HPLC

1. Plasma buffer solution

   8 mL 100 mM boric acid (0.62 g/100 mL)

   2 mL 100 mM sodium tetraborate (3.81 g/100 mL)

   105 mg L-serine

   5 mg sodium heparin

   10 mg BPDS

   20 mg iodoacetic acid

2. **Sample buffer**

	Final concentration
71 mL of 70% perchloric acid	10%
6.2 g boric acid	0.2 M
1.38 mg γ-glu-gly	10 μM

Dissolve in a total volume of 500 ml of ddH_2O.

3. **Iodoacetic acid**

   14.8 mg in 2 mL of distilled H_2O, made fresh daily

4. **KOH/tetraborate.** Add 5.6 g KOH to a plastic bottle containing 5 g $K_2B_4O_7$•4H2O and 100 mL H_2O, mix thoroughly. Let stand overnight and remove the supernatant and discard the precipitant.

5. Dansyl chloride 20 mg/ml in acetone.

6. Chloroform

7. Mobile phase

   80% MeOH, 20% H2O

   Acetate-buffered MeOH, pH 4.6 [640 mL MeOH, 200 mL acetate stock*, 125 mL glacial acetic acid, 50 mL H2O]

   *acetate stock, 272 g Na acetate trihydrate, 122 mL H2O, 378 mL glacial acetic acid

8. **Preparation of standards:** Standards are made in concentrations from 10 to 40 nmol per assay for GSH and 1 to 4 nmol per assay for GSSG dissolved in sample buffer. The thiols are unstable to freeze-thaw and should be made fresh each time. The standards are derivatized using the same methods as the experimental samples.

9. **Preparation of samples: Plasma** samples are drawn into plasma buffer, mixed thoroughly, and centrifuged to separate plasma from cells. Once separated, 200 ml of the plasma/buffer mixture is added to 200 ml of the sample buffer and mixed. After 5 min the mixture is centrifuged to precipitate the proteins and the plasma supernatant may be frozen at −80 °C for future analysis.

   Cells require that the media is removed and the cells washed at least 3 times in PBS. Sample buffer should be placed directly on the cells before harvesting. The acidity of the buffer will precipitate the proteins and cause the cells to be thick and granular. The mixture should be scraped off of the plate, placed in a tube and centrifuged to precipitate the proteins.

   Tissues are harvested and snap-frozen prior to analysis. Homogenize approximately 10 mg of tissue in 0.5–1.0 mL of sample buffer. The sample must be kept cold during homogenization. The homogenate is centrifuged to precipitate proteins. If samples sizes exceed 50 mg, the tissue should be ground under liquid nitrogen in cooled mortar and pestle and transferred directly into the sample buffer solution.

10. Derivatization

    1. Mix 300 μL of plasma, cell or homogenate supernatant with 60 μL of IAA.

    2. Adjust the pH to 9.0±0.2 with KOH/tetraborate (~220 μL) and incubate the sample at room temperature for 20 min.

    3. Add 300 μL of dansyl chloride, mix the solution, and place it in the dark for 16 to 24 h.

    4. Add 500 μL of $CHCl_3$ to the solution, mix, centrifuge briefly and remove the upper layer containing the derivatized sample. This process removes excess dansyl chloride from the sample.

5. The sample can be stored at −80°C with the $CHCl_3$ layer for up to 12 months.

11. HPLC

    1. Centrifuge samples for 2 min prior to loading into either autosampler vials or manually injecting into the HPLC. The typical injection volume is 25–35 μL of prepared sample.

    2. Gradient:

        Initial conditions: 80% A, 20% B at 1 ml/min

        Hold at initial conditions for 10 min

        Linear gradient to 20% A, 80% B form 10 to 30 min

        Hold at final conditions for 15 min

        Return to initial conditions for column re-equilibration, at least 15 min

    3. Detection:

        Peaks are detected by fluorescence using an excitation wavelength of 328 nm and an emission wavelength of 541 nm.

    4. Quantification

        Sample concentrations are determined by experimentally derived standard curves.

4. Notes

4.1. Recommended dilutions for tissue supernatants for measurement of GSH and GSSG by DTNB and GR Recycling Method

Lung	1:100
Kidney	1:100
Intestine	1:100
Liver	1:200

4.2. GSH Standard Curve

nM/mL	pmol/assay	GSSG working std. (μL)	assay buffer (μL)
0	0	0	1000
1	30	100	900
2	60	200	800
3	90	300	700
4	120	400	600
5	150	500	500
7.5	225	750	250
10	300	1000	0

4.3. GSSG Standard Curve (if GSSG concentrations are anticipated to be low, use 10 pmol/assay standard. Otherwise, use 400 pmol/assay standard)

pmol/assay	GSSG working std. (μL)	assay buffer (μL)	NEM (μL)
0	0	990	10
10	25	965	10
20	50	940	10
40	100	890	10
80	200	790	10
120	300	690	10
160	400	590	10
200	500	490	10
400	1000	0	10

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References

1. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem. 1969; 27:502–522. [PubMed: 4388022]
2. Adams JD Jr, Lauterburg BH, Mitchell JR. Plasma glutathione and glutathione disulfide in the rat: regulation and response to oxidative stress. J Pharmacol Exp Ther. 1983; 227:749–754. [PubMed: 6655568]
3. Smith CV, Hansen TN, Martin NE, McMicken HW, Elliott SJ. Oxidant stress responses in premature infants during exposure to hyperoxia. Pediatric research. 1993; 34:360–365. [PubMed: 8134179]
4. Adams JD Jr, Lauterburg BH, Mitchell JR. Plasma glutathione and glutathione disulfide in the rat: regulation and response to oxidative stress. J Pharmacol Exp Ther. 1983; 227:749–754. [PubMed: 6655568]
5. Reed DJ, Babson JR, Beatty PW, Brodie AE, Ellis WW, Potter DW. High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. Anal Biochem. 1980; 106:55–62. [PubMed: 7416469]
6. Martin J, White IN. Fluorimetric determination of oxidised and reduced glutathione in cells and tissues by high-performance liquid chromatography following derivatization with dansyl chloride. J Chromatogr. 1991; 568:219–225. [PubMed: 1770099]
7. Jones DP, Carlson JL, Samiec PS, Sternberg P Jr, Mody VC Jr, Reed RL, Brown LA. Glutathione measurement in human plasma. Evaluation of sample collection, storage and derivatization conditions for analysis of dansyl derivatives by HPLC. Clin Chim Acta. 1998; 275:175–184. [PubMed: 9721075]
8. Rogers LK, Leinweber BL, Smith CV. Detection of reversible protein thiol modifications in tissues. Anal Biochem. 2006; 358:171–184. [PubMed: 17007807]
9. Santori G, Domenicotti C, Bellocchio A, Pronzato MA, Marinari UM, Cottalasso D. Different efficacy of iodoacetic acid and N-ethylmaleimide in high-performance liquid chromatographic measurement of liver glutathione. J Chromatogr B Biomed Sci Appl. 1997; 695:427–433. [PubMed: 9300880]