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Determination of GSH, GSSG, and GSNO Using HPLC with Electrochemical Detection

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Abstract

GSNO is an important intermediate in nitric oxide metabolism and mediates many NO -mediated signaling pathways through the posttranslational modification of redox-sensitive proteins. The detection of GSNO in biological samples has been hampered by a lack of sensitive and simple assays. In this work, we describe the utilization of HPLC with electrochemical detection for the detection of GSNO in biological samples. GSNO requires a high potential (>700 mV) for its electrochemical detection, similar to that of GSSG. A simple isocratic HPLC system can be used to separate and simultaneously detect GSH, GSSG, and GSNO electrochemically. This HPLC system can be utilized to measure the redox profile of biological samples and applied for the measurement of GSNO reductase activity in cells. Proper sample preparation is essential in GSNO measurements, because artifactual formation of GSNO occurs in acidic conditions due to the reaction between GSH and nitrite. Treatment of samples with ammonium sulfamate or *N*-ethylmaleimide (NEM) can prevent the artifactual formation of GSNO and accurately detect GSNO in biological samples. Overall, the HPLC with electrochemical detection is a powerful tool to measure redox status in cells and tissues.

1. Introduction

The involvement of NO as a pleiotropic signaling molecule in the regulation of numerous physiological processes, as well as its deleterious effects when generated in high amounts during pathophysiological disease, such as during neuroinflammation, has spurred a significant amount of interest in NO chemistry. Generated through the enzymatic oxidation of L-arginine to L-citrulline and NO by nitric oxide synthases (NOS), the reactivity and consequences of NO in biological systems is regulated by many complex and competing reactions; of which the reaction between NO and thiols to yield *S*-nitrosothiols is extremely important in cell signaling (Davis *et al.*, 2001; Lamas *et al.*, 2007). Of relevance to cellular redox status and signaling—through protein posttranslation modification—is the formation of *S*-nitrosoglutathione (GSNO). The intracellular formation of GSNO is complex and postulated to occur through a series of possible mechanisms (see Martinez-Ruiz and Lamas, 2007). In the presence of O_2 , NO is oxidized to dinitrogen dioxide (N_2O_3), a nitrosating species (Martinez-Ruiz and Lamas, 2007; Eqs. (6.1) and (6.2)). This reaction is accelerated in membranes due to the partition coefficient of both NO and O_2 , and may enhance the yield of thiol nitrosation in membrane-rich environments such as mitochondria (Hogg, 2002).

The formation of GSNO can also occur through a transnitrosation reaction between two thiols; in this case, the reaction between a nitrosylated protein and GSH (Eq. (6.3)).

GSNO has been identified in a variety of tissues and is considered a biologically relevant metabolite of NO due to its ability to modulate cellular signaling through the posttranslational modifications of redox-sensitive proteins, that is, *S*-nitrosylation (Eq. (6.4)) and/or *S*-glutathionylation (Eq. (6.5)). This redox modulation of numerous proteins (see Dalle-Donne *et al.*, 2008; Martinez-Ruiz and Lamas, 2007; Stamler *et al.*, 2001) has been suggested to play a substantial role in NO -regulated signaling, independent of guanylyl cyclase. Furthermore, increasing evidence points to the role of posttranslational modified proteins in disease pathology, such as in Alzheimer's disease (Dalle-Donne *et al.*, 2008).

The intracellular stability of GSNO is governed by many factors, including chemically driven degradation reactions—thiol and metal-mediated decomposition—(Hogg, 2002; Singh *et al.*, 1996; Zeng *et al.*, 2001), and enzymatically driven reactions. The main enzymatic-dependent degradation described thus far is the reduction of GSNO to GSSG by glutathione-dependent formaldehyde dehydrogenase (or alcohol dehydrogenase III); later renamed GSNO reductase (Hedberg *et al.*, 2003; Liu *et al.*, 2001; Eqs. (6.6)–(6.9)), due to its high specificity and affinity for GSNO and a reflection of the increasingly important role of GSNO in redox chemistry.

Because of the importance of GSNO in modulating NO -signaling processes, accurate and specific methods to measure GSNO in biological samples are needed. Traditionally, GSNO has been measured using HPLC with UV detection (~ 336 nm) (Steffen *et al.*, 2001; Tsikas

et al., 2001). However, this HPLC method suffers from both issues of specificity and sensitivity in measuring GSNO in biological samples. In this study, we adapted a HPLC coulometric electrochemical method for the detection of GSNO in biological samples. The coulometric HPLC method described in this work can simultaneously measure GSH, GSSG, and GSNO, thus providing a redox profile of biological samples.

2. Methods

2.1. High-performance liquid chromatography with electrochemical detection

GSH, GSNO, and GSSG were detected using HPLC with a coulometric electrochemical detector from ESA (Chelmsford, MA). Electrochemical detection has commonly been used to measure GSH and GSSG with HPLC (Han *et al.*, 2006a; Harvey *et al.*, 1989; Rebrin *et al.*, 2007); however, its application for the measurement of GSNO has not been described. ESA offers CoulArray systems that utilize between 4 and 16 channels. We employed a 4-channel electrochemical array for the simultaneous detection of GSH, GSNO, and GSSG. The mobile phase for isocratic elution of the sulfhydryls was composed of 25 mM monobasic sodium phosphate, 0.5 mM 1-octane sulfonic acid (ion-pairing agents), and 2.5% acetonitrile, pH 2.7. All chemicals including GSH, GSSG, and GSNO were purchased from Sigma Chemicals (St. Louis, MO, USA). The pH for the mobile phase should be adjusted with 85% phosphoric acid. A flow rate of 1 mL/min was used with a C₁₈ column (5 μ M column, 4.6 $\times$ 250 mm). Acetonitrile is the key component in modulating the retention times of GSH, GSNO, and GSSG. With 2.5% of acetonitrile in the mobile phase, the retention times for GSH generally appears at ~5 min, GSNO ~16 min, and GSSG ~20 min (retention times also vary with the type of column used). Increasing acetonitrile levels in the mobile phase will decrease the elution time of the sulfhydryls, and conversely decreasing acetonitrile levels lengthen the retention time of all sulfhydryls, particularly GSNO and GSSG. It should also be noted that many other sulfhydryls (i.e., methionine, cysteine, and cystine) can be simultaneously detected with this electrochemical system.

2.2. Hydrodynamic voltammogram of GSH, GSNO, and GSSG

GSNO, like GSSG, requires a high applied potential for detection. Figure 6.1 shows that the hydrodynamic voltammogram of GSNO is similar to GSSG, requiring a potential of greater than +700 mV before a signal can be observed. The detection of GSH, on the other hand, occurs at low voltages and plateaus after +800 mV. Since both GSNO and GSSG are found at very low levels in biological samples, due to GSNO reductase and GSSG reductase activities, higher potentials (>+875 mV) are recommended for the detection of GSNO and GSSG in biological samples. In a coulometric array, there are several possible configurations for the simultaneous detection of GSH, GSSG, and GSNO. A typical setting for a four-array electrode system used was 1 = +300, 2 = +450, 3 = +600, 4 = +900 mV. Electrodes 1 and 2 serve as screening electrodes to oxidize potentially interfering compounds. GSH is detected on electrodes 3 and 4, while GSSG and GSNO are monitored in electrode 4. Figure 6.2 shows a chromatogram of GSH, GSSG, and GSNO, detected with electrodes set at +600 and +900 mV. The retention time for GSNO generally precedes GSSG by a couple of minutes (varying with the acetonitrile concentrations in the mobile phase). In a 2.5% acetonitrile concentration, the difference in the retention time between GSNO and GSSG is ~4 min. An alternative configuration for GSNO detection would be as follows: electrode 1 (+350 mV) to screen potentially interfering compounds with low potentials, electrode 2 (+500 mV) to detect GSH, electrode 3 (+700 mV) to screen potentially interfering compounds with high potentials, and electrode 4 (+900 mV) for GSNO and GSSG detection. This electrode configuration may be useful for samples where the presence of compounds that have high reduction potentials and retention times similar to GSNO that may potentially interfere with GSNO detection. However, it must be noted that

the long-term use of high potentials (>+600 mV) causes the electrode to corrode quicker and burn out at a faster rate. Consequently, the high potential required to measure GSSG and GSNO shortens the lifetime of electrodes significantly. In addition, electrode drift is a frequent problem at the high potentials required to measure GSNO. Consequently, GSNO and other standards should be injected on a regular basis to monitor electrode drift during sample analysis.

2.3. GSNO detection in biological samples: Effect of sample preparation

The degradation of GSNO by GSNO reductase occurs at diffusion-controlled rates; hence, half-life of GSNO is short and rarely accumulates to high levels in cells. However, using the HPLC coulochemical array system, we were able to observe GSNO formation in neurons and astrocytes following NO treatment (Yap *et al.*, 2010). For the measurement of GSNO, proper sample preparation is critical for accurate measurement. Although *S*-nitrosothiols are relatively stable, due to the slightly polar covalent bond between sulfur and nitrogen, the bond is susceptible to homolysis by strong, direct light (Hogg, 2002). Hence, during the measurement of GSNO, GSH, and GSSG, it is particularly important to use dark amber vials for all experiments, under minimal light exposure. In addition, GSNO can be artifactually generated during sample processing, particularly under acidic conditions.

For the measurement of GSH and GSSG in biological samples (i.e., cells, tissues, and plasma), acid treatment (i.e., 5% *o*-metaphosphoric acid and trichloroacetic acid), to prevent GSH autoxidation and to precipitate proteins, has been frequently used (Han *et al.*, 2006a; Rebrin *et al.*, 2007). Following acid treatment, samples are centrifuged ($12,000 \times g$ for 5 min) and the supernatant injected into the HPLC for GSH and GSSG measurements. GSH is stable in acids, and the low pH prevents GSH from deprotonating and acting as a strong nucleophile to form glutathionylated proteins (Han *et al.*, 2006b). However, acid treatment for GSNO measurements creates a problem since nitrite and GSH react in acidic conditions to form GSNO (Eqs. (6.9)–(6.10)) (Tsikas, 2003). Because nitrite is a major oxidation product of NO, it will be present in biological samples when NO is produced. Consequently, the acidification of biological samples containing GSH and nitrite result in the artifactual formation of GSNO.

For GSNO measurements, nonacidic buffer must be utilized or additional steps must be taken to neutralize GSH or nitrite in samples. We investigated the utilization of ammonium sulfamate or *N*-ethylmaleimide (NEM) for the measurement of GSNO in neurons. Ammonium sulfamate neutralizes nitrite in biological samples under acidic conditions (Tsikas *et al.*, 2001). Conversely, NEM binds to the free thiol groups of GSH, preventing any possible reaction with nitrites under acidified conditions (Asensi *et al.*, 1994) to prevent any possible reaction with nitrites. The effect of sample preparation on GSNO measurements is illustrated in Table 6.1. The treatment of primary cultured neurons with the NO donor, DETA-NO (20 μM), for 1 h causes GSNO formation in neurons, but the levels vary depending on sample preparation. The addition of only 5% *o*-metaphosphoric acid to neurons results in very high levels of GSNO. Clearly, the high GSNO levels in NO-exposed neurons treated with only *o*-metaphosphoric acid are partially due to artifactual formation since the treatment of neurons with ammonium sulfate (25 mM) dissolved in *o*-

metaphosphoric acid resulted in significantly lower GSNO levels. Similarly, NEM treatment to neurons (20 mM), which chelates GSH, followed by *o*-metaphosphoric acid treatment, resulted in equally low levels of GSNO. Neither NEM or ammonium sulfamate resulted in the degradation of GSNO in standards or spiked biological samples. Sample preparation using NEM or ammonium sulfamate produced slightly varying GSNO values in neurons treated with DETA-NO, although the difference was not significant. An advantage of using ammonium sulfamate over NEM in sample preparation is the ability to simultaneously measure GSH, GSNO, and GSSG in the same sample, while NEM treatment only allows for GSNO and GSSG measurements. Consequently, we recommend ammonium sulfamate treatment for the measurement of GSNO in biological samples, although measurements with NEM pretreatment should also be done to ensure that the values obtained for GSNO and GSSG are correct. Overall, HPLC with electrochemical detection plus treatment with ammonium sulfamate allows for the simultaneous measurement of GSH, GSSG, and GSNO, thus providing an accurate measurement of the redox status in cells. In addition to increasing GSNO levels, NO treatment to neurons caused an oxidation in the neuronal redox potential (increasing ~42 mV) due to loss of GSH and increase in GSSG formation (Yap *et al.*, 2010).

2.4. Measurement of GSNO reductase activity using HPLC

Another application of the HPLC electrochemical system is the measurement of GSNO reductase activity in cells and tissues. For the measurement of GSNO reductase activity in primary cultured neurons, the following protocol was used. Neurons were washed with ice-cold PBS three times before lysis in reductase buffer (20 mM Tris-HCl, 0.5 mM EDTA, 0.1% NP-40, and 1 mM PMSF, pH 8). Lysate was sonicated three times (20 s, setting at 3.0, 100% pulse rate) at intervals with 1 min rest time on ice to disrupt cellular membranes and solubilize all proteins. To detect GSNO metabolizing activity, 1 mg/mL lysate was incubated with 100 μ M of GSNO in the absence or presence of 200 μ M NADH at room temperature (25 °C). One hundred microliters of the lysate was removed at 5 min intervals and added into equal volumes of ice-cold 10% *o*-metaphosphoric acid. Samples were spun down at 10,000 $\times$ g for 10 min at 4 °C to precipitate proteins and the supernatant was then collected and analyzed by HPLC for GSNO and GSSG formation. Figure 6.3 demonstrates that GSNO is only degraded by neuronal lysate when NADH is present. This suggests that GSNO degradation in neurons is mediated by the NADH-dependent GSNO reductase (Eqs. (6.6)–(6.8)). The decrease in GSNO in neuronal extracts was associated with the increase in the formation of GSSG that was also observed by HPLC (data not shown). GSNO reductase activity has traditionally been measured by monitoring NADH levels, which is a substrate for many other enzymatic systems. HPLC with electrochemical detection is advantageous as a complementary approach for the measurement of GSNO reductase activity since GSNO levels can be directly measured.

3. Summary

HPLC with electrochemical detection is a simple (no derivatization required) and sensitive method for the simultaneous measurement of GSH, GSSG, and GSNO. This HPLC system can be utilized to measure the redox profile of biological samples and applied to the measurement of GSNO reductase activity in cells. The drawback of HPLC with electrochemical detection is that a high potential is required to measure GSNO and GSSG, which will shorten the lifetime of electrode and cause electrode drift. Proper sample preparation is essential in GSNO measurements, since artifactual formation of GSNO will occur in acidic conditions due to a reaction between GSH and nitrite. Treatment of samples with ammonium sulfamate or NEM can prevent the artifactual generation of GSNO and accurately assesses GSNO levels in biological samples. Overall, the HPLC with electrochemical detection is a powerful tool to measure the redox status of cells and tissue.

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References

- Asensi M, Sastre J, Pallardo FV, Estrela JM, Vina J. Determination of oxidized glutathione in blood: High-performance liquid chromatography. *Methods Enzymol* 1994;234:367–371. [PubMed: 7808308]
- Dalle-Donne I, Milzani A, Gagliano N, Colombo R, Giustarini D, Rossi R. Molecular mechanisms and potential clinical significance of S-glutathionylation. *Antioxid Redox Signal* 2008;10:445–473. [PubMed: 18092936]
- Davis KL, Martin E, Turko IV, Murad F. Novel effects of nitric oxide. *Annu Rev Pharmacol Toxicol* 2001;41:203–236. [PubMed: 11264456]
- Han D, Hanawa N, Saberi B, Kaplowitz N. Hydrogen peroxide and redox modulation sensitize primary mouse hepatocytes to TNF-induced apoptosis. *Free Radic Biol Med* 2006a;41:627–639. [PubMed: 16863996]
- Han D, Hanawa N, Saberi B, Kaplowitz N. Mechanisms of liver injury. III. Role of glutathione redox status in liver injury. *Am J Physiol Gastrointest Liver Physiol* 2006b;291:G1–G7. [PubMed: 16500922]
- Harvey PR, Ilson RG, Strasberg SM. The simultaneous determination of oxidized and reduced glutathiones in liver tissue by ion pairing reverse phase high performance liquid chromatography with a coulometric electrochemical detector. *Clin Chim Acta* 1989;180:203–212. [PubMed: 2743574]
- Hedberg JJ, Griffiths WJ, Nilsson SJ, Hoog JO. Reduction of S-nitrosoglutathione by human alcohol dehydrogenase 3 is an irreversible reaction as analysed by electrospray mass spectrometry. *Eur J Biochem* 2003;270:1249–1256. [PubMed: 12631283]
- Hogg N. The biochemistry and physiology of S-nitrosothiols. *Annu Rev Pharmacol Toxicol* 2002;42:585–600. [PubMed: 11807184]
- Lamas S, Lowenstein CJ, Michel T. Nitric oxide signaling comes of age: 20 years and thriving. *Cardiovasc Res* 2007;75:207–209. [PubMed: 17574221]
- Liu L, Hausladen A, Zeng M, Que L, Heitman J, Stamler JS. A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. *Nature* 2001;410:490–494. [PubMed: 11260719]
- Martinez-Ruiz A, Lamas S. Signalling by NO-induced protein S-nitrosylation and S-glutathionylation: Convergences and divergences. *Cardiovasc Res* 2007;75:220–228. [PubMed: 17451659]
- Rebrin I, Forster MJ, Sohal RS. Effects of age and caloric intake on glutathione redox state in different brain regions of C57BL/6 and DBA/2 mice. *Brain Res* 2007;1127:10–18. [PubMed: 17113050]
- Singh SP, Wishnok JS, Keshive M, Deen WM, Tannenbaum SR. The chemistry of the S-nitrosoglutathione/glutathione system. *Proc Natl Acad Sci USA* 1996;93:14428–14433. [PubMed: 8962068]
- Stamler JS, Lamas S, Fang FC. Nitrosylation. the prototypic redox-based signaling mechanism. *Cell* 2001;106:675–683. [PubMed: 11572774]
- Steffen M, Sarkela TM, Gybina AA, Steele TW, Trasseth NJ, Kuehl D, Giulivi C. Metabolism of S-nitrosoglutathione in intact mitochondria. *Biochem J* 2001;356:395–402. [PubMed: 11368766]
- Tsikas D. Measurement of physiological S-nitrosothiols: A problem child and a challenge. *Nitric Oxide* 2003;9:53–55. [PubMed: 14559432]
- Tsikas D, Denker K, Frolich JC. Artifactual-free analysis of S-nitrosoglutathione and S-nitroglutathione by neutral-pH, anion-pairing, high-performance liquid chromatography. Study on peroxynitrite-mediated S-nitration of glutathione to S-nitroglutathione under physiological conditions. *J Chromatogr A* 2001;915:107–116. [PubMed: 11358239]
- Yap L-P, Garcia JV, Han D, Cadenas E. Role of nitric oxide-mediated glutathionylation in neuronal function: Potential regulation of energy utilization. 2010 (submitted).
- Zeng H, Spencer NY, Hogg N. Metabolism of S-nitrosoglutathione by endothelial cells. *Am J Physiol Heart Circ Physiol* 2001;281:H432–H439. [PubMed: 11406512]

Figure 6.1. Hydrodynamic voltammogram of GSH, GSSG, and GSNO. The signal generated by GSH, GSSG, and GSNO standards at different voltages are shown.

Figure 6.2.

HPLC chromatogram of GSH, GSSG, and GSNO. The analysis shows GSH, GSSG, and GSNO signals generate at +600 and +900 mV. GSNO and GSSG cannot be generally detected until the potential reaches greater than +700 mV. Insert shows the electrode settings for all channels for optimal detection of GSH, GSNO, and GSSG.

Figure 6.3.

Measurement of GSNO reductase in neurons activity using HPLC with electrochemical detection. GSNO ($100\ \mu\text{M}$) is degraded by primary cultured neuronal lysates only in the presence of NADH ($200\ \mu\text{M}$), suggesting that GSNO was being mediated by the NADH-dependent GSNO reductase. Values are expressed as percent of GSNO concentration at the start of the experiment.

Table 6.1

Effect of sample preparation on GSNO levels in primary cultured neurons

	GSH	GSNO	GSSG
<i>MPA treatment only</i>			
Control	13.8 ± 5.1	0	0.041 ± 0.035
NO treatment	7.15 ± 5.8	2.81 ± 1.21	0.24 ± 0.19
<i>AS plus MPA treatment</i>			
Control	13.2 ± 6.1	0	0.038 ± 0.029
NO treatment	7.8 ± 6.0	0.21 ± 0.14	0.18 ± 0.15
<i>NEM plus MPA treatment</i>			
Control	–	0	0.049 ± 0.039
NO treatment	–	0.30 ± 0.23	0.23 ± 0.19

All values expressed as nmol per million cells. Primary cultured neurons were treated with a NO donor, DETA-NO (20 μ M) for 1 h. AS, ammonium sulfamate (25 mM); NEM, *N*-ethylmaleimide (20 mM); MPA, *o*-metaphosphoric acid (5%).