A "Global" Method for the Determination of Tissue Thiols, Disulfides and Thioethers using an HPLC System Incorporating a Novel Boron-Doped Diamond Detector

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Abstract

Although HPLC ECD is often the preferred approach for the sensitive determination of thiols, disulfides and thioethers, it suffers from a number of issues related to chromatography, sample preparation and ECD that prevents routine use of this approach.

Chromatographic problems result from the low organic mobile phases typically used with RP separations, requiring that only columns that can withstand high-aqueous mobile phases be used. Poor sample preparation results in the build up of lipophilic materials on the column. ECD problems result from the use of high potentials with glassy carbon or graphitic electrodes that leads to instability of response; high, unstable background currents; oxidation of mobile phase; increased noise; and loss of response due to adsorption of contaminants or oxidation reaction products on the electrode's surface. The consequence of these issues is that an extraordinary amount of care and effort are required for routine analysis of these analytes.

To better enable researchers to study sulfur containing molecules a novel electrochemical detection system utilizing ESA Biosciences' Coulochem III ECD and new BDD thin-film electrode, was developed. This system mitigates many of the issues reported above. The system offers several attractive features over other traditional ECD systems including the ability to utilize high oxidation potentials for analyses in aqueous solutions without mobile phase oxidation and high background noise levels. The system provides low background currents; long-term stability of the response; low sensitivity to dissolved oxygen; inertness, and lack of fouling.

Presented here is a routine method capable of measuring a number of thiols, disulfides and thioethers (Figure 1) in <30 mins. Peak authenticity was evaluated by spiking with external standards and by treatment of the plasma sample with the reducing agent tris[2-carboxyethyl phosphine] [TCEP] to convert disulfides into thiols.

Introduction

Thiols, disulfides and thioethers (Figure 1) play numerous biochemically important roles in vivo. Glutathione (GSH) is not only a crucial antioxidant per se, but it is involved in many aspects of cellular protection: it can regenerate other antioxidants (e.g., ascorbate); it acts as a cofactor for antioxidant enzymes (e.g., GSH peroxidase); and, through its involvement in the mercapturic acid pathway, is involved in detoxification of xenobiotics. Furthermore, maintenance of a high GSH/GSSG ratio (i.e., reductive environment) is essential for normal cellular functioning (see Acworth (2003) and references therein). Homocysteine (HCYS) is a key metabolite in both sulfur amino acid biochemistry and the transfer of "activated" methyl groups (in regeneration of the "methyl-carrier" – S-adenosylmethionine (SAM)). However, it is a pro-oxidant species, forms a reactive thiolactone, and its circulating levels are regarded as an important cardiovascular risk factor.

Although electrochemical detection is often the preferred approach for the sensitive determination of thiols, disulfides and thioethers (reviewed by Acworth (2003)), it typically suffers from a number of issues related to chromatography, sample preparation and electrochemical detection:

- Chromatographic problems result from the low organic mobile phases typically used with reversed-phase separations. Only those columns that can withstand high-aqueous mobile phases can be used. Furthermore, the build up of highly lipophilic materials requires routine column cleaning in order to avoid ghost peaks and unstable baselines.
- Electrochemical detection problems result from the use of glassy carbon or graphitic electrodes and include: instability of response resulting from the high (over) potentials required for determination of the disulfide; high and unstable background currents; increased noise resulting from electrode instability and oxidation of mobile phase; and loss of response due to adsorption of matrix/mobile phase contaminants or oxidation reaction products. Routine maintenance (e.g., organic washing) and the use of fresh mobile phase are usually required in order to keep detection optimal.

Many of the electrochemical issues presented above can be overcome by the use of ESA's novel boron-doped diamond (BDD) thin-film electrode. The BDD electrode offers several attractive features over other carbon-based working electrodes including. A wide potential window in aqueous solutions enables the use of high oxidation potentials without mobile phase oxidation and high noise. The BDD provides low background currents; long-term stability of the response; low sensitivity to dissolved oxygen; inertness and lack of fouling.

Presented here is a preliminary method illustrating the ability to separate and detect a number of thiols, disulfides and thioethers in under 30mins. Peak authenticity was evaluated by spiking samples with external standards and by treatment of the plasma sample with the reducing agent tris[2-carboxyethyl)phosphine] [TCEP] to convert disulfides into thiols (Jandik *et al.*, (2001); Krijt *et al.*, (2001)).

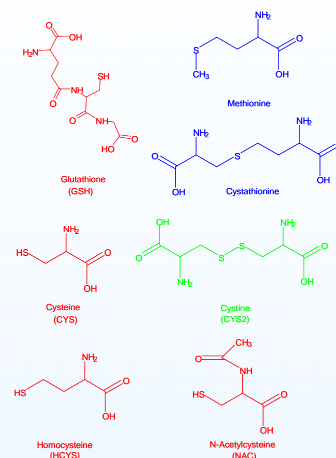

Figure 1. Structures of Thiols (red), Thioethers (blue) and Disulfides (green). Glutathione Disulfide (GSSG), Cysteinylglycine (CYSGLY) and Homocystine (HCYS2) are not shown.

Materials and Methods

The isocratic HPLC system consisted of a Model 584 pump, a Model 542 refrigerated autosampler, a thermostatic organizer, and a Model 5300 Coulochem III detector equipped with: an amperometric cell (Model 5040) with a BDD working electrode and a 25µm gasket.

LC Conditions:

Column, Guard:	Shiseido C18 MG, 4 x 20mm; 3µm
Column, Analytical:	Inertsil C18, 3 x 150mm; 3µm
Injection Volume:	10µL (tray at 5°C)
Mobile Phase:	25mM Sodium dihydrogen phosphate; 1.4mM 1-octanesulfonic acid; 6% (v/v) acetonitrile; final pH 2.65 (with phosphoric acid)
Temperature:	35 °C
Flow Rate:	0.75mL/min

Detector Conditions:

Detector:	Coulochem III (Model 5300)
Applied Potential:	+1400mV (vs. Pd reference)
Clean Cell:	+1900mV, 30sec. 5 min re-equilibration
Guard Cell:	Model 5020, +900mV

Reagents and Standards:

In order to minimize background currents, the highest quality EC-compatible reagents must be used. Sodium phosphate (EM Science); OSA (JT Baker); ACN (EM Science); Phosphoric acid (Fisher Scientific, HPLC grade). All standards were from Sigma. Individual stock standards (1mg/mL) were prepared in water and stored at 4 °C. Further dilutions were in water.

Sample Preparation:

A 100 µL volume of fresh plasma was mixed with 100 µL water and vortexed for 30 seconds. The sample was then placed on ice for 10 minutes. A 200 µL volume of 0.1N PCA was then added and the sample vortexed. The sample was then transferred to a centrifuge microfilter tube (VectraSpin Micro, polysulfone 30K MWCO) and was centrifuged 13k rpm, for 30 minutes at 5 °C. A 100 µL volume of filtrate was transferred to a conical autosampler vial and a 10 µL volume was injected into the HPLC system.*

**For over-spiking plasma samples, 50 µL of the 1µg/mL standard mix was added to the 100µL plasma filtrate. A 10µL volume was injected onto the HPLC system.*

Reduction of Plasma by TCEP:

A 100 µL volume of fresh plasma was mixed with 75 µL water and 25 µL TCEP solution (50 mg TCEP/mL water; Pierce) and vortexed for 30 seconds. The sample was then left for 10 minutes to allow reduction reaction to be completed. A 200 µL volume of 0.1N PCA was then added and the sample vortexed. The sample was then transferred to a centrifuge micro-filter tube (VectraSpin Micro, polysulfone 30K MWCO) and was centrifuged 13k rpm, for 30 minutes at 5 °C. A 100 µL volume of filtrate was transferred to a conical autosampler vial and a 10 µL volume was injected into the HPLC system.

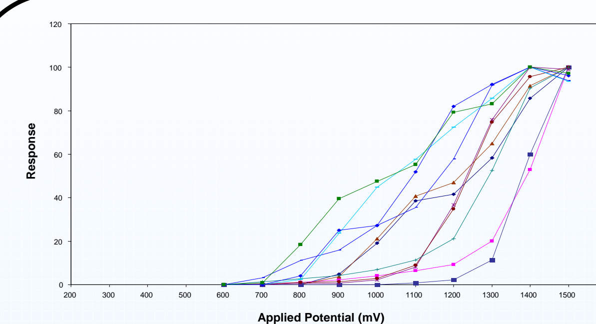

Figure 2. Current-voltage curves for thiols, disulfides and thioethers (10 µg/mL mixture on column). The potential applied to the BDD electrode started at +1500mV, and was decreased by 100mV with each subsequent injection. The signal (current) produced for each analyte was plotted as a function of applied potential. The oxidation potential of 1400mV was chosen for all further experimentation.

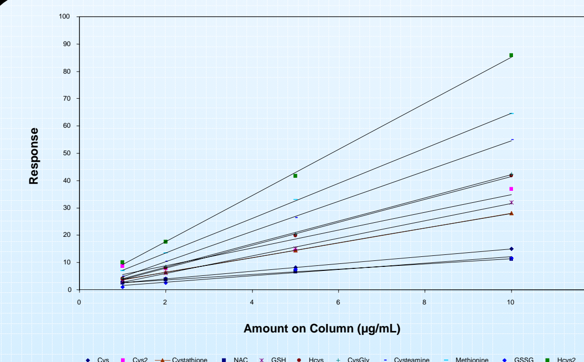

Figure 3. Linear Curves for the 11 Thiols, Disulfides and Thioethers.

Results and Discussion

The optimum applied potential for analytes was +1400mV vs Pd reference (Figure 2). The assay showed good linearity (Figure 3). The LOD was ~500pg on column.

Unlike other working electrode materials, BDD electrodes do not easily foul and demonstrated excellent stability over time and multiple runs. The response was stable for at least 65 hours for standards with 1.5% RSD for GSH and 6.5% RSD for GSSG. Routine analysis of biological extracts (over months) did not adversely affect performance.

The analysis of plasma is shown in Figure 4. Spiking with standard helped verify analyte authenticity. However, unless the patient is consuming NAC, it is unlikely that the peak corresponding to NAC in plasma is genuine. Analyte authenticity was further explored by disulfide reduction with TCEP (a reducing agent that liberates thiols from disulfides, mixed disulfides and protein thiol adducts). As shown in Figure 5, thiol levels increased and disulfide levels decreased following TCEP treatment. Unfortunately, in this chromatographic system excess TCEP reagent eluted at the same retention time as HCYS.

Plasma GSH levels were not determined in this method. due to the handling of the samples that did not take precautions to preserve the GSH/GSSG ratio (see Acworth (2003) and references therein).

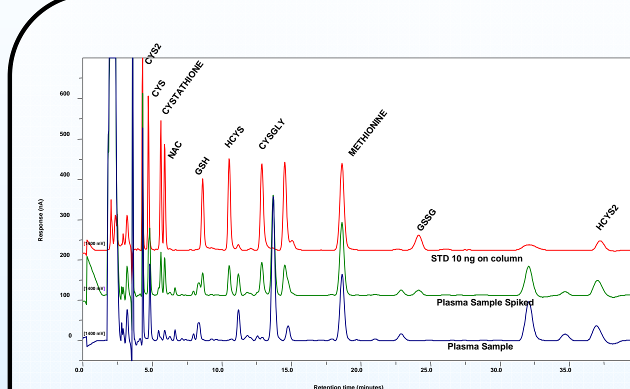

Figure 4. Chromatograms showing the resolution of numerous thiols, disulfides and thioethers in under 40mins, for standards (10ng, each on column), plasma, and standard-spiked plasma samples.

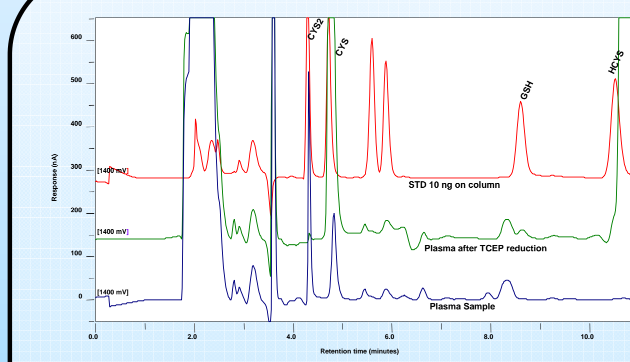

Figure 5. Data showing the effect of treating plasma with TCEP. As expected thiol levels increased (due to their liberation from disulfides, mixed-disulfides and protein thiol adducts).

Conclusions

The BDD working electrode offers many advantages over other carbon-based materials. It exhibits chemical and physical stability even at potentials as high as +1400mV, conditions impossible to use with traditional carbon electrodes. The BDD electrode is compatible with aqueous mobile phases typically used for the analysis of thiols, even at high potentials. The BDD electrode has shown resistance to fouling and remained responsive after months of use.

This ability to operate at extreme potentials and resistance to contamination now makes the electrochemical detection of thiols, disulfides and thioethers routine.

References

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