Simple, Rapid Analysis of Aminothiols with Boron-Doped Diamond Electrochemical Detection

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Key Words
Glutathione, disulfides and thioethers, electrochemical detection, BDD, neurodegenerative disease, Accucore

Introduction

A number of biochemically important sulfur-containing compounds occur in vivo including: aminothiols (e.g., cysteine, glutathione [GSH], homocysteine), disulfides (e.g., cystine, glutathione disulfide [GSSG], homocystine), and thioethers (e.g., methionine) as shown in Figure 1. These compounds play numerous physiological roles. GSH is a major cellular antioxidant and a cofactor for glutathione peroxidase, an enzyme that detoxifies hydrogen peroxide and lipid hydroperoxides. The high ratio of GSH/GSSG keeps the cell in a reducing environment, essential for its survival. Decreases in this ratio are associated with cellular toxicity and numerous diseases including neurodegeneration (e.g., Parkinson's disease). Cysteine is also a cellular antioxidant, serves as a precursor to glutathione and is often found in protein structures as a disulfide link. Methionine is an essential amino acid and serves as a methyl donor when incorporated into S-adenosylmethionine.

Although a variety of HPLC techniques have been developed for the measurement of thiols, disulfides, and thioethers, most exhibit technical issues. UV requires derivatization which can adversely affect the GSH/GSSG ratio. Electrochemical approaches allow the direct measurement of these analytes, but performance varies between electrode materials. Glassy carbon (GC) electrodes tend to foul quickly and require routine maintenance. Coulometric graphite electrodes are an improvement over GC, but the best performing electrode is the boron-doped diamond (BDD). The BDD working electrode enables the direct measurement of these analytes without electrode issues and minimal maintenance.[1,2]

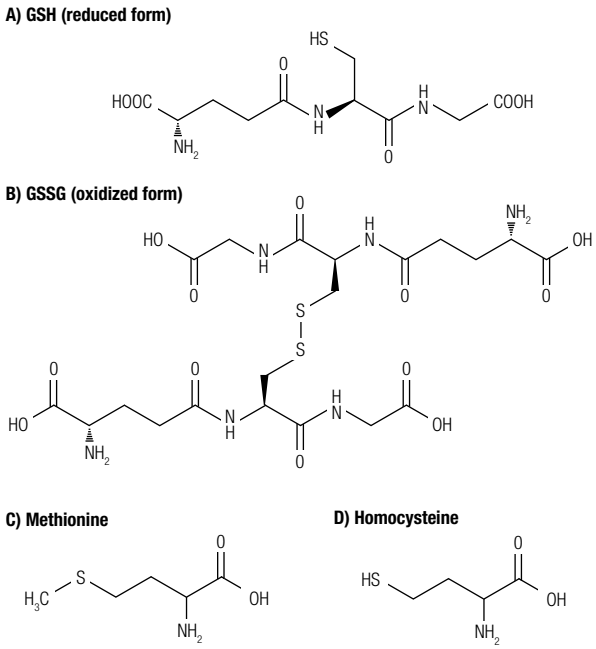

Figure 1. Molecular structures of A) glutathione (GSH), B) glutathione disulfide (GSSG) C) Methionine and D) Homocysteine.

Goal

To develop a simple, accurate, and rapid UHPLC method for aminothiols in whole blood using an isocratic LC system, an amperometric electrochemical cell with a boron-doped diamond working electrode, and an Accucore column for high resolution and rapid separation. This method must provide accurate quantification of analytes to low picogram sensitivity without significant matrix interferences.

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The rapid method presented here is capable of accurately determining several thiol compounds simultaneously using UHPLC chromatographic techniques with a BDD working electrode. Examples showing the analysis of GSH and GSSG from whole blood samples using a simple dilution protocol are provided.

A UHPLC approach provides several advantages over standard HPLC conditions including shorter cycle times between samples, improved resolution, and improved sensitivity due to sharper peaks. A sharper peak is important when measuring GSSG as it occurs at a low concentration and is one of the last peaks eluting in the chromatogram. The use of longer UHPLC columns provides better resolution between compounds, important when analyzing biological samples. The 2.6 µm solid core particles used in Accucore columns develop UHPLC efficiencies at lower backpressures than columns packed with sub-2 µm fully porous particles, making the use of longer columns practical.

Experimental

Equipment

- Thermo Scientific™ Dionex™ UltiMate™ 3000 LC System, including:
  - SRD-3600 Integrated Solvent and Degasser Rack
  - DGP-3600RS Pump Dual Gradient Separation Pump
  - WPS-3000TBPL Rapid Separation Wellplate Autosampler
  - TCC-3000RS Rapid Separation Thermostatted Column Compartment
- Thermo Scientific™ Dionex™ UltiMate 3000 Electrochemical Detector
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software version 6.8

Reagents and Standards

Deionized (DI) water

Perchloric Acid, GFS Chemicals® P/N 67

Hydrochloric Acid, J.T. Baker® P/N 9535-03

Pentafluoropropionic Acid, Fisher Scientific P/N AC41692

Ammonium Hydroxide, Fisher Scientific P/N A470-250

Acetonitrile, Fisher Scientific P/N A996-4

CysteinylGlycine (CysGly) (354.40 MW), Sigma-Aldrich® C0166

Glutathione, reduced (GSH,307.32 MW), Sigma-Aldrich G4251

Glutathione, oxidized (GSSG, 656.59 MW), Sigma-Aldrich G4376

L-Homocysteine (135.18 MW), Sigma-Aldrich 69453

L-Methionine (149.21 MW), Sigma-Aldrich M6039

Separation Conditions

Column:	Thermo Scientific™ Accucore™ RP-MS, 2.6 µm, 2.1 × 150 mm (Part Code: 17626-152130)
Pump Flow Rate:	0.500 mL/min
Mobile Phase:	0.1 % Pentafluoropropionic Acid, 0.02 % Ammonium hydroxide, 2.5 % Acetonitrile, 97.4 % Water
Column Temperature:	50.0 °C
Injection Volume:	2 µL standards; 4 µL samples
Cell Potential:	Thermo Scientific™ Dionex™ 6041RS ultra Amperometric Analytical cell with boron-doped diamond working electrode at +1600 mV
Filter Constant:	1.0 sec
Cell Clean:	On
Cell Clean Potential:	1900 mV
Cell Clean Duration:	10.0 s with a 1.5 minute recovery before next injection

Preparation of Solutions and Reagents

- ***0.4 N Perchloric Acid Solution***- A solution of perchloric acid facilitates the deproteinization of blood samples. Prepare 0.4 N perchloric acid (PCA) solution by dissolving 3.42 mL PCA (70%) in a 100 mL volumetric flask containing 95 mL water. Bring to volume with distilled (DI) water.
- ***0.1 N Hydrochloric Acid Solution***- This acidic solution provides a suitable reducing environment to protect labile compounds. Make a 1 N hydrochloric acid (HCl) solution by taking 8.25 mL concentrated hydrochloric acid (37%) and slowly adding this to a 100 mL volumetric flask containing 95 mL water. Bring to volume with DI water. Dilute 10 mL of this 1 N HCl solution in a 100 mL volumetric flask and bring to volume with DI water to prepare a 0.1 N HCl solution.
- ***CysteinylGlycine (CysGly) 1 mg/mL CysGly (MW 354.40) Stock Solution***- Dissolve 10.0 mg into a 10.0 ml volumetric flask using 0.1 N HCl. Store at 4 °C and prepare fresh every 3–4 weeks.
- ***Reduced Glutathione (GSH) 1 mg/mL GSH (MW 307.32) Stock Solution***- Dissolve 10.0 mg into a 10.0 ml volumetric flask using 0.1 N HCl. Store at 4 °C and prepare fresh every 3–4 weeks.
- ***Oxidized Glutathione (GSSG) 1 mg/mL GSSG (MW 656.59) Stock Solution***- Dissolve 10.0 mg into a 10.0 ml volumetric flask using 0.1 N HCl. Store at 4 °C and prepare fresh every 3–4 weeks.
- ***Homocysteine 1 mg/mL (MW 135.2) Stock Solution***- Dissolve 10.0 mg homocystine into a 10.0 ml volumetric flask using 0.1 N HCl. Store at 4 °C and prepare fresh every 3–4 weeks.
- ***Methionine 1 mg/mL (MW 149.21) Stock Solution***- Dissolve 10.0 mg into a 10.0 ml volumetric flask using 0.1 N HCl. Store at 4 °C and prepare fresh every 3–4 weeks.

A list showing the dilutions of stock standards for preparation of the calibration curves is in Table 1.

Table 1. Preparation of working standard solutions (prepare fresh daily).

Concentration	1st Addition	2nd Addition	Instruction
10 µg/mL	1 volume of 20 µg/mL solution	Add 1 volume of 0.1 N HCl	Mix well
5 µg/mL	1 volume of 20 µg/mL solution	Add 3 volumes of 0.1 N HCl	Mix well
2 µg/mL	1 volume of 20 µg/mL solution	Add 9 volumes of 0.1 N HCl	Mix well
1 µg/mL	1 volume of 2 µg/mL solution	Add 1 volume of 0.1 N HCl	Mix well
0.1 µg/mL	1 volume of 1 µg/mL solution	Add 9 volumes of 0.1 N HCl	Mix well

Prepare a 20 µg/mL working solution of the five different stock solutions shown above by placing 1.0 mL each of CysGly, GSH, Methionine, Homocysteine, and GSSG into a 10 mL container, cap and mix.

Sample Preparation

Mix 5–20 µL fresh whole blood and 200 µL 0.4N PCA. Vortex and spin for 10 minutes at 13000 RPM. Transfer the clear supernatant into an autosampler vial and place on the autosampler at 12 °C. See procedure below for the detailed procedure, Figure 2.

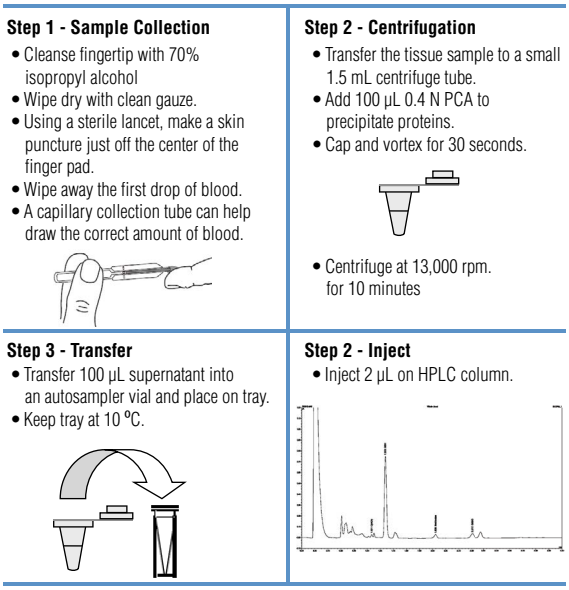

Figure 2. Whole blood extraction procedure.

Results and Discussion:

Maximum performance comes not just from the detector but also from its integration with the UltiMate 3000 UHPLC+ system optimized for EC detection. This system provided inert fluidic flow-paths to minimize noise and unwanted auto-oxidation. This is extremely important when measuring labile analytes such as GSH in order to prevent artifactual changes in the GSH/GSSG ratio. The system uses biocompatible materials in the flow path to reduce the influence of metal that can contribute to elevated background currents at the BDD electrochemical cell. The unique electrochemical capabilities of the boron-doped diamond enable the oxidation of organic compounds using higher electrode potentials than other working electrode materials.

Biocompatible Thermo Scientific™ Dionex™ nanoViper™ fingertight fittings were used to handle the higher pressures resulting from columns containing smaller stationary phase particles. These fingertight capillaries can operate at elevated pressures (up to 1000 bar, 14,500 psi) and are much safer to use than PEEK™ tubing which may slip at elevated pressures. They make connections that have virtually zero-dead-volume and are available in small internal dimensions to help minimize chromatographic band spreading. This enables the operator to obtain sharp chromatographic peaks with the highest resolution possible. Capillaries used on this system were 150 µm i.d. for all connections made prior to the autosampler valve and 100 µm i.d. for those made after the injector valve.

The use of the BDD electrode for the direct electrochemical detection of thiols, disulfides, and thioether compounds was described previously.[1] The applied potential (+1600 mV) was sufficient to oxidize both thiol and disulfide analytes. Advantages of this approach include a stable electrode surface and method simplicity since no sample derivatization is required. After each analysis the electrode surface was regenerated by a 10 second clean cell pulse at +1900 mV. After a 1.5 minute re-equilibration at +1600 mV the electrode was once again stable and could be used for the analysis of the next sample. Rapid UHPLC analysis as shown in Figure 3 enabled processing of these samples within three minutes, minimizing any major chemical transformations taking place.

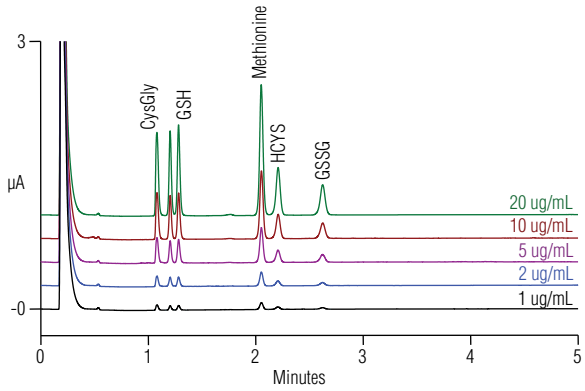

Figure 3. Molecular structures of A) glutathione (GSH), B) glutathione disulfide (GSSG) C) Methionine and D) Homocysteine.

For the analysis of aminothiols in biological studies, compounds such as GSH, GSSG, methionine, and homocysteine, need to be included. Figure 4 illustrates the overlay of calibration standards for these compounds ranging from 1–20 µg/mL. Peak resolution (6.36 for GSH, 2.44 for Meth, and 5.38 for HCys) and retention time uniformity (GSH Peak Average RT of 1.29 minutes with %RSD of 0.463 %) were both excellent. The Accucore RP-MS column provided fast, high resolution separations but with lower system pressures. When operated at 0.5 mL/min at 50.0 °C the backpressure was less than 300 bar with the last compound (GSSG) eluting in less than 2.8 minutes.

Calibration curves for aminothiol standards are shown in Figure 4. Good linearity of response to different concentrations was obtained with correlation coefficients ranging from R^2 = 0.989–1.00 for the five compounds evaluated (Table 2) over the range of 1–20 µg/mL. The percent relative standard deviation (%RSD) for the calibration curves (five concentrations in triplicate) is also shown in Table 1. The RSD values ranged from 1.2% to 8.9%, indicating that the BDD electrode provided good stability during this study.

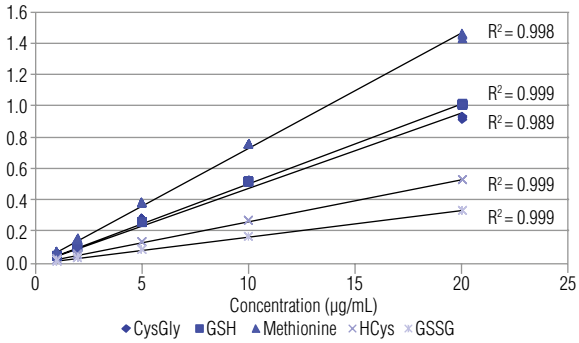

Figure 4. Calibration curves for aminothiol standards (1 – 20 µg/mL; N=3).

Table 2. Regression data for aminothiol standard calibration curves

Peak	# Points	RSD %	Correlation Coefficient	Slope
CysGly	15	8.9059	0.989	0.0481
GSH	15	1.7446	0.999	0.0509
Meth	15	3.4526	0.999	0.0736
HCYS	15	1.9282	0.999	0.0268
GSSG	15	1.2458	0.999	0.0170

The enhanced peak shape (narrow peak width) obtained with the BDD electrode increased sensitivity. The small cell volume of only 50 nL contributed to low background currents which helped minimize noise. Lower noise is an important aspect of the assay since it enables improved sensitivity. The chromatogram shown in Figure 5 illustrates that amounts less than 400 picograms on column of each compound can be determined by this method. The signal-to-noise ratios (S/N) for these compounds are shown in Table 3 and range from 7.2–37. The limit of detection (LOD) using an S/N ratio of 5 is also shown in this table. For example, the LOD is approximately 67 picograms on column for GSH and 175 picograms for GSSG.

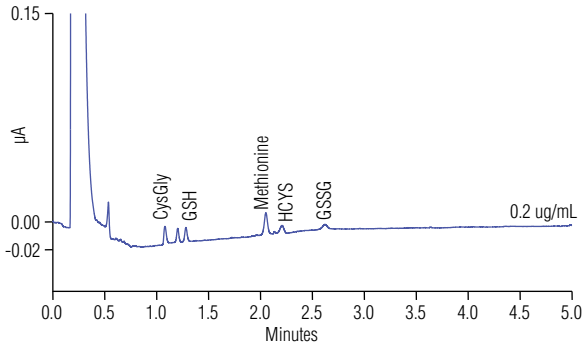

Figure 5. Sensitivity of low level analytical standard (400 pg on column).

Figure 6 shows an overlay of chromatograms for a standard mixture of aminothiols and a sample of deproteinized whole blood. This method enables rapid sample processing thus minimizing issues related to the instability of the thiols. The levels detected as shown in Table 4 are within the range of reported levels using other techniques.[3] Although the level of homocysteine was below the assay LOD using these small sample volumes, it would be possible to detect this compound by simply increasing the volume of sample analyzed.

Chromeleon chromatography data system software provided results quickly, efficiently, and accurately. The Chromeleon eWorkflow concept captured the unique aspects of the chromatography workflow, and guided the operator through a predetermined and streamlined process from injection to final report.

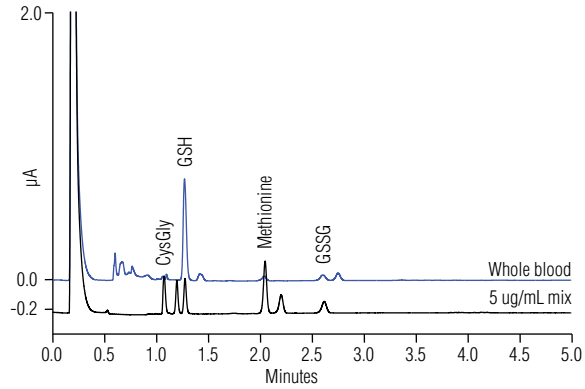

Figure 6. Overlay of chromatograms of standard (black trace) vs. whole blood (blue trace).

Table 3. Signal to noise ratio values calculated for 400 pg on column.

Compound	CysGly	GSH	Methionine	Hcys	GSSG
S/N Ratio	37.0	29.4	20.4	7.2	11.4
LOD (pg, S/N of 5)	54	67	98	278	175

Table 4. Aminothiol levels (µM) observed in whole blood (n=3) using the BDD electrode.

	CysGly	GSH	Methionine	GSSG	GSSG
Level µM	55.9±13.0	1017.2±26.2	107.4±52.7	71.1±25.9	11.4

Conclusion

This method proved to be simple and reliable with sufficient sensitivity for the analysis of thiols, disulfides and thioethers in small samples of deproteinized whole blood. Technical issues related to GSH autoxidation instability were minimized by rapid sample preparation techniques. Although the analysis of aminothiols in this work was related to whole blood, the method can be easily adapted to determine the levels of these compounds in plasma and tissue samples. The levels of GSH and GSSG detected are within the range of reported levels using other analytical techniques. With UHPLC compatibility, the UltiMate 3000 UHPLC+ system provided high data acquisition rates for conventional or fast, high resolution chromatography with electrochemical cells that provide ultralow peak dispersion. This enabled the analysis of complex samples such as whole blood using shorter run cycles. All thiol-related analytes were analyzed within 3 minutes with on-column limits of detection less than 70 picograms for GSH.

References

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