Rapid Analysis of Aminothiols by UHPLC with Boron-Doped Diamond Electrochemical Detection

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Overview

Purpose: In order to obtain satisfactory information concerning aminothiols, disulfides, and thioethers from biological samples scientists require a sensitive approach that can measure key compounds, simultaneously. A simple, accurate and rapid UHPLC method was developed for the analysis of these compounds using isocratic liquid chromatography and an amperometric electrochemical cell with a boron-doped diamond (BDD) working electrode. This allowed the accurate quantification of analytes to low picogram (pg) sensitivity.

Methods: Direct analysis of aminothiols, disulfides and thioethers using UHPLC chromatographic techniques with a robust electrochemical cell using a BDD electrode is described.

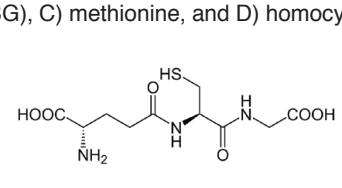
Results: The method enables the rapid separation of various aminothiols, disulfides, and thioethers at low levels from whole blood without significant matrix interferences.

Introduction

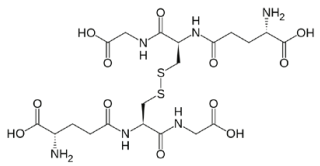
A number of biochemically important sulfur-containing compounds occur *in vivo* including: aminothiols (e.g., cysteine, glutathione [GSH], homocysteine), disulfides (e.g., cystine, glutathione disulfide [GSSG], homocystine), and thioethers (e.g., methionine) as shown in Figure 1. These aminothiols plays 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 of these exhibit technical issues. UV requires derivatization which can adversely affect the GSH/GSSG ratio, while some electrochemical approaches using glassy carbon electrodes suffer from electrode fouling and loss of response, and require routine maintenance. Although porous graphite working electrodes are more forgiving, they still need maintenance. Boron-doped diamond (BDD) however, enable the direct measurement of these analytes without electrode issues.[1,2] The rapid method presented herein is capable of accurately determining several aminothiols simultaneously using UHPLC chromatographic techniques with a BDD working electrode. Examples showing the analysis of GSH and GSSG from plasma samples using a simple "dilute and shoot" protocol are provided.

A UHPLC approach was chosen as it provides several advantages over standard HPLC conditions including: shorter cycle times between samples; improved resolution, and 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 biological samples. The use of longer UHPLC columns provides better resolution between compounds, which is important when analyzing biological samples.

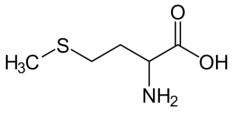
FIGURE 1. Molecular structures of A) glutathione (GSH), B) glutathione disulfide (GSSG), C) methionine, and D) homocysteine



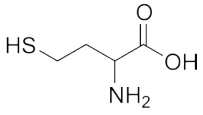
A) GSH (reduced form)



B) GSSG (oxidized form)



C) Methionine



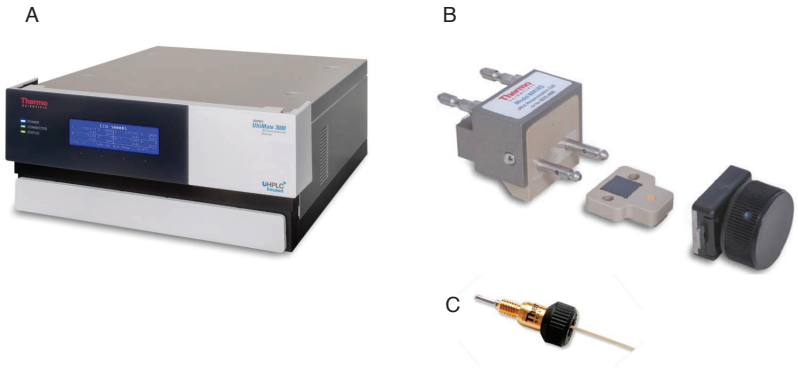
D) Homocysteine

Methods

Analytical Conditions for Aminothiol, Disulfide and Thioether Analysis

Column:	Thermo Scientific™ Accucore™ RP-MS column 2.6 µm, 2.1 × 150 mm
Pump Flow Rate:	0.500 mL/min
Mobile Phase:	0.1% pentafluoropropionic acid, 0.02% ammonium hydroxide, 2.5% acetonitrile, water
Column Temperature:	50.0 °C
Post Column Temperature	25.0 °C
Injection Volume:	2 µL standards; 4 µL samples
Cell Potential:	Thermo Scientific™ Dionex™ model 6041RS ultra Amperometric Analytical Cell with BDD electrode at +1600 mV
Filter Constant:	1.0 s
Cell Clean:	On
Cell Clean Potential:	1900 mV
Cell Clean Duration:	10.0 s
Sample Prep:	5–20 µL whole blood + 200 µL 0.4 N PCA, mix and spin for 10 minutes at 13,000 RPM. The clear supernatant was transferred into an autosampler vial and placed on the autosampler at 10 °C.

FIGURE 2A. Thermo Scientific™ Dionex™ UltiMate™ 3000 Electrochemical detector
FIGURE 2B. 6041RS ultra Amperometric Analytical Cell with BDD electrode
FIGURE 2C. Thermo Scientific™ Dionex™ nanoViper™ fingertight capillaries



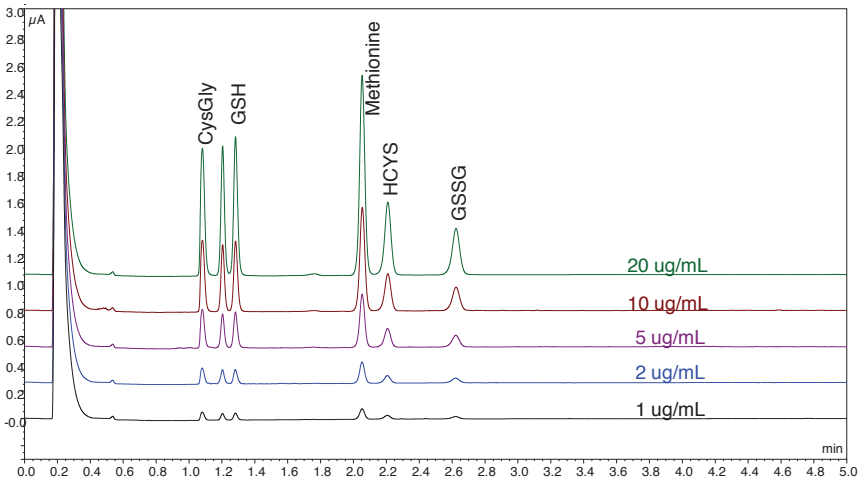
The Thermo Scientific™ Dionex™ UltiMate™ 3000 UHPLC system consists of:
- SR-3000 Solvent Rack
- DGP-3600RS Dual Gradient Pump
- WPS-3000TBPL Thermostatted Analytical Autosampler
- ECD-3000RS Electrochemical Detector
  with integrated temperature controlled column compartment
- Chromeleon 6.80 SR12 Chromatography Data System software

Results and Discussion

An instrumental prerequisite for trace analysis is that the HPLC system must be inert (free from leachable transition metals) in order to achieve optimal sensitivity using an electrochemical detector. The system shown above in Figure 2A uses biocompatible materials in the flow path to reduce the influence of metal that can contribute to elevated background currents at the electrochemical cell. The recent introduction of the ECD-3000RS detector enables multiple electrodes to be attached in series after the HPLC column.

Use of the 6041RS amperometric cell (Figure 2B) provides the unique electrochemical capabilities of the boron-doped diamond which enables the oxidation of organic compounds using higher electrode potentials than other working electrode materials. This platform provides both chromatographic and voltammetric resolution of compounds. The nanoViper (Figure 2C) fingertight fittings were employed to cope with the higher pressures due to smaller column particles. These fingertight, virtually zero-dead-volume (ZDV) capillaries can operate at pressures up to 14,500 psi and are much safer to use than PEEK™ tubing which can slip when using elevated pressures. They are made of PeekSil tubing and are available in small internal dimensions to minimize chromatographic band spreading. Capillaries used on this system were 150 micron ID for all connections made prior to the autosampler valve and 100 micron ID for those made after the injector valve.

FIGURE 3. Overlay of analytical standards ranging from 1–20 µg/mL



The direct electrochemical detection of aminothiol compounds only using a boron-doped diamond electrochemical cell has been previously described.[1] The applied potential used for this study (+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. In the current method, the whole blood sample was added to the perchloric acid media, mixed and then centrifuged. The clear supernatant was then transferred into an autosampler vial and placed on the autosampler at 10 C. This approach enabled rapid sample processing thus minimizing issues related to the instability of the thiols. Rapid UHPLC analysis as shown in Figure 3 enables processing of these samples within three minutes before any major chemical transformations take place.

For biological studies, the determination of aminothiol content should include compounds such as GSH, GSSG, methionine, and homocysteine. Figure 3 illustrates the overlay of calibration standards for these compounds ranging from 1–20 µg/mL. Peak resolution and retention time uniformity were both excellent. The column used for this method was the Accucore RP-MS 2.6 micron solid-core material which provides 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 under 2.8 minutes as shown in Figure 3.

The 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 1) 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 standards (1–20 µg/mL, n=3)

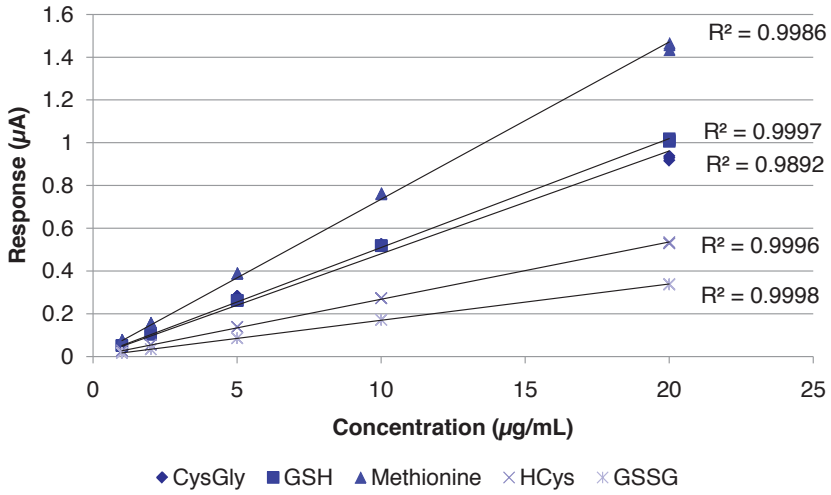


Table 1. Regression data for standard calibration curves

Peak	# Points	RSD. %	Correlation Coefficient.	Slope
CysGly	15	8.9059	0.989	0.0481
GSH	15	1.7446	1.00	0.0509
Meth	15	3.4526	0.999	0.0736
HCYS	15	1.9282	1.00	0.0268
GSSG	15	1.2458	1.00	0.0170

Enhanced peak shape (narrow peak width) in addition to improved design features of the 6041RS cell with a BDD electrode provides good sensitivity. The small cell volume of only 50 nL contributes to low background currents which helps minimize the noise of the electrochemical cell. The chromatogram shown in Figure 5 illustrates that amounts less than 400 pg on column of each compound can be determined by this method. The signal-to-noise ratios (S/N) for this standard are shown in Table 2 and range from 7.2–37. The limit of detection (LOD) with a S/N ratio of 5 are also shown in this table. The limit of detection (LOD) with S/N ratio of 5 is also shown in this table. For GSH the LOD is approximately 67 pg, while the LOD for GSSG is 175 pg on column.

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

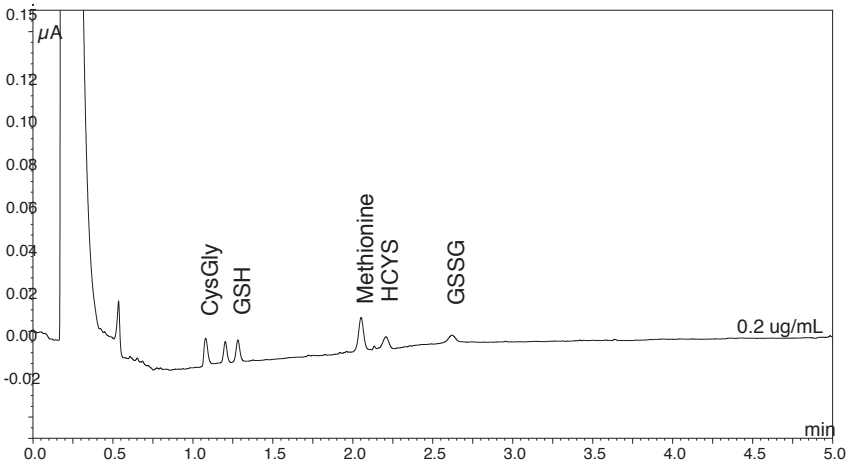


Table 2. Signal-to-noise ratio values calculated for 400 pg on column

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

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

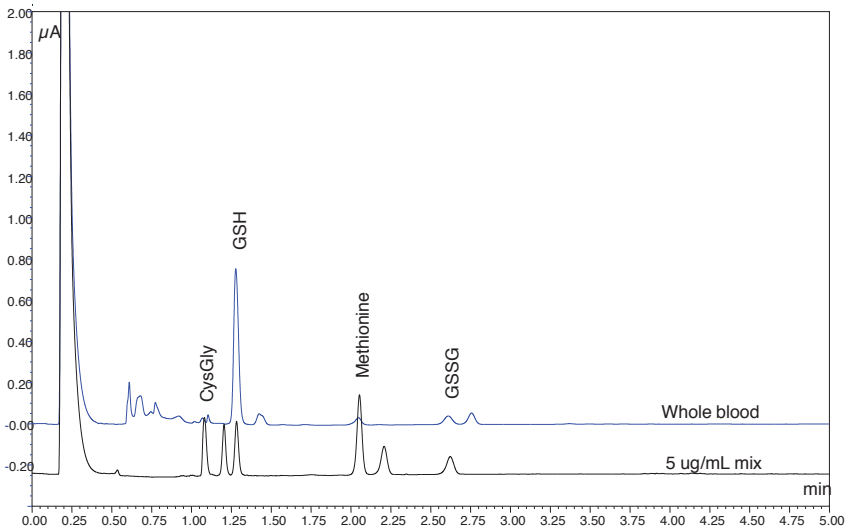


Table 3. Aminothiol levels (μM) observed in whole blood (n=3) using the BDD electrode

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

Figure 6 shows an overlay of chromatograms for a standard mixture of aminothiols and a sample of deproteinized whole blood. The levels of GSH and GSSG was easily measured in small samples of whole blood (<20 μL) using the UHPLC method described. The levels detected as shown in Table 3 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 collected and processed.

Conclusions

- The method for the analysis for the measurement of thiols, disulfides and thioethers proved to be simple and reliable with sufficient sensitivity for their measurement in deproteinized whole blood. Technical issues related to GSH autoxidation were minimized by rapid sample preparation techniques.
- Although the analysis of aminothiols in this work was related to whole blood, the method could be easily adapted to determine the levels of these compounds in plasma and tissue samples as well.
- The levels of GSH and GSSG detected are within the range of reported levels using other techniques.

References

1. Bailey, B.A. et al. Direct determination of tissue aminothiol, disulfide, and thioether levels using HPLC-ECD with a novel stable boron-doped diamond working electrode. *Advanced Protocols in Oxidative Stress II, Methods in Molecular Biology*, **2011**, 594, 327-339.
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3. Raffa, M. et al. Decreased glutathione levels and impaired antioxidant enzyme activities in drug-naïve first-episode schizophrenic patients *BMC Psychiatry*, **2011**, 11**,** 124-131.

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