Determination of Clinically Relevant Compounds using HPLC and Electrochemical Detection with Boron-Doped Diamond Electrode

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Overview

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

Methods: This method describes an approach for direct analysis of glutathione redox status and blood cholesterol using UHPLC chromatographic techniques with a robust electrochemical cell using a BDD electrode.

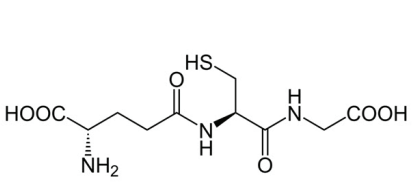
Results: The method enables the rapid separation of various aminothiols, disulfides, and cholesterol at low levels from whole blood without significant matrix interferences.

Introduction

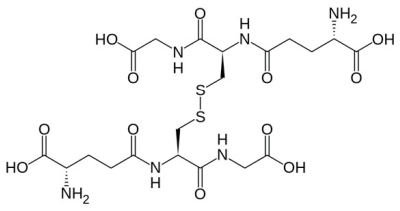
Several years ago, a novel working electrode material known as boron-doped diamond (BDD) became available, extending the range of compounds that could be measured using electrochemical detection. Although many different applications are now reported in literature, few have focused on clinical diagnostics. One possible example is the routine measurement of the cellular antioxidant glutathione (GSH) and its disulfide (GSSG). Oxidative stress is thought to be associated with many diseases. Changes in the GSH/GSSG ratio are currently used as an indicator of the level of oxidative stress. Although there are numerous approaches to measure tissue levels of GSH and GSSG, many suffer from methodological issues including specificity and may even cause an artificial change in the GSH/GSSG ratio that is being measured. Other HPLC-based approaches with electrochemical detection enable direct, selective and sensitive measurement of GSH and GSSG. Unfortunately, methods using glassy carbon working electrodes are limited by the oxidation potential used (adversely affecting the sensitivity of GSSG) and require routine maintenance due to adsorption problems. Both of these problems are overcome with the BDD working electrode. Presented here is a fast and easy way for determining the level of oxidative stress using UHPLC with BDD electrochemical detection.

Another clinically relevant compound is the sterol, cholesterol, which is a major component of cell membranes and is associated with arteriosclerosis. Although simple kits are available for cholesterol measurement in plasma or serum, these do not provide any differentiation from phytosterols that may be present in the sample. A simple UHPLC method with electrochemical detection using the BDD working electrode is presented for the measurement of cholesterol in blood with minimal sample preparation. This method is sensitive (<100 pg/mL) and provides sufficient resolution so that the presence of other phytosterols can also be determined.

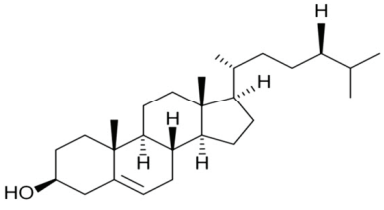
FIGURE 1. Molecular structures of A) glutathione (GSH), B) glutathione disulfide (GSSG), and C) cholesterol.



A) GSH (reduced form)



B) GSSG (oxidized form)



C) Cholesterol

Methods

1) Analysis of Glutathione Redox Status

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, 97.4% 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™ 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.

2) Analysis of Cholesterol Levels in Whole Blood

Column:	Accucore C8 column 2.6 µm, 2.1 × 100 mm
Pump Flow Rate:	1.00 mL/min
Column Temperature:	50.0 °C
Mobile Phase A:	0.1% trifluoroacetic acid, 50 mM lithium perchlorate in water
Mobile Phase B:	0.1% trifluoroacetic acid, 50 mM lithium perchlorate in acetonitrile
Injection Volume:	2 µL standards and samples
Column:	Accucore C8 column 2.6 µm, 2.1 × 150 mm
Pump Flow Rate:	1.00 mL/min
Gradient	

Time	%A	%B
-5.00	22.0	78.0
0.00	22.0	78.0
5.00	10.0	90.0
5.50	22.0	78.0
6.50	22.0	78.0

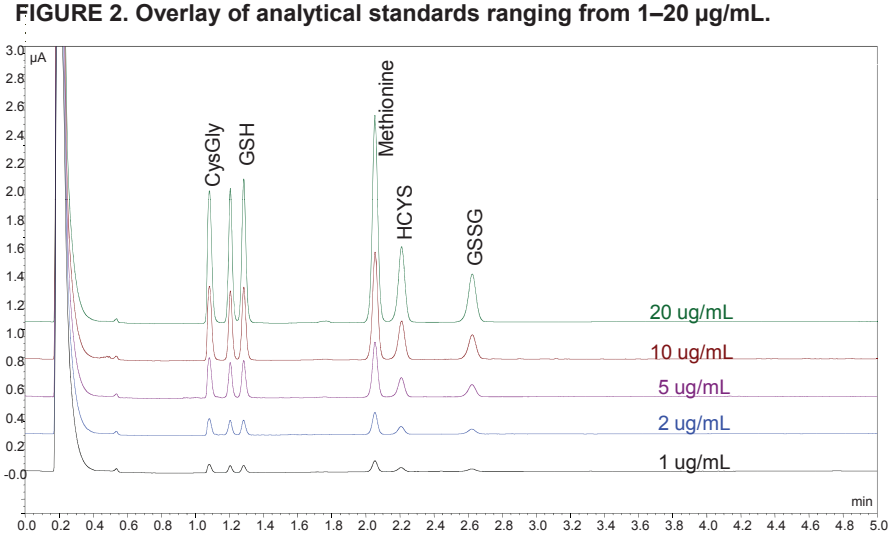
Cell Potential:	6041RS ultra Amperometric Analytical Cell with BDD electrode at +1900 mV
Clean Cell Potential	+1950 mV
Cell Clean Duration:	20.0 s
Filter Constant:	2.0 s
Sample Prep:	5–20 µL whole blood + 500 µL Mobile Phase B, mix and spin for 10 minutes at 13,000 RPM. The supernatant was transferred into an autosampler vial and placed onto the autosampler. tray

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 uses biocompatible materials in the flow path to reduce the influence of metal that contribute to elevated background currents at the electrochemical cell. Use of the 6041RS amperometric cell 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.

Analysis of Glutathione Redox Status

FIGURE 2. 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.[1,2] 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 short re-equilibration period 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 set at 10 °C. This approach enabled rapid sample processing thus minimizing issues related to the instability of the thiols. Rapid processing of these samples minimizes any major chemical transformations taking place. For biological studies, the determination of aminothiol content should include compounds such as GSH, GSSG, methionine, and homocysteine. Figure 2 illustrates the overlay of calibration standards for these compounds ranging from 1–20 µg/mL. Excellent peak resolution and retention time uniformity were observed. 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.

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 calibration curves (five concentrations in triplicate) is shown.

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	1.000	0.0509
Meth	15	3.4526	0.999	0.0736
HCYS	15	1.9282	1.000	0.0268
GSSG	15	1.2458	1.000	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 limit of detection (LOD) with S/N ratio of 5 for GSH is approximately 67 pg, while the LOD for GSSG is 175 pg on column.

FIGURE 3. Overlay of standard (black trace) vs. whole blood (blue trace).

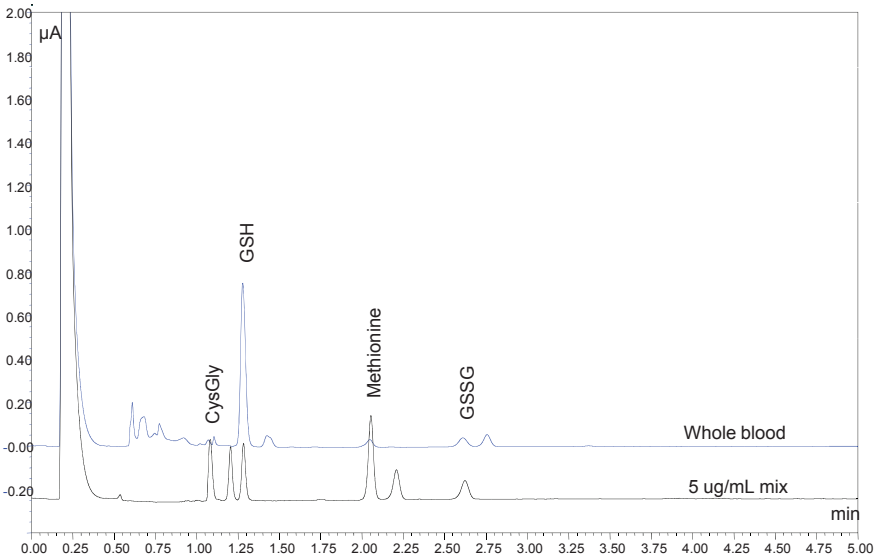


Table 2. Aminothiol levels (µM) observed in whole blood (n=3) using the BDD electrode.

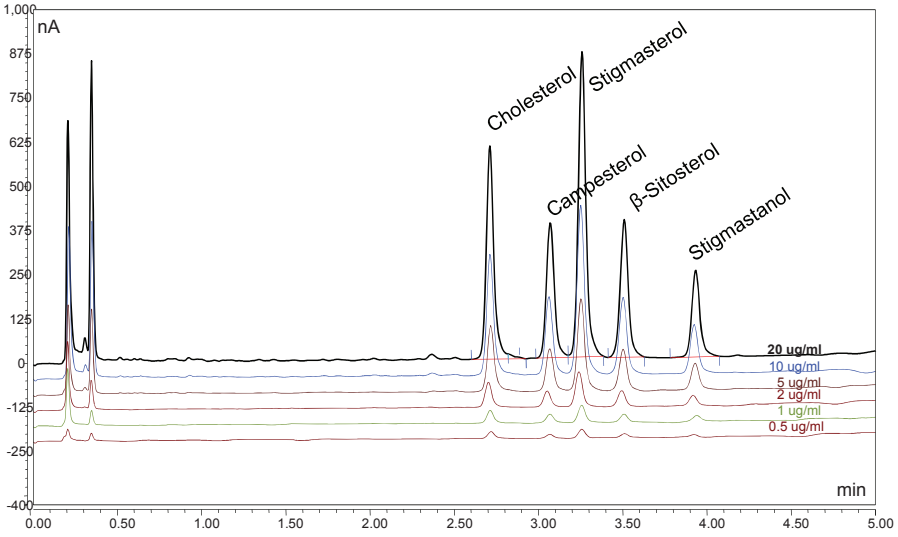
	CysGly	GSH	Methionine	GSSG
Level µM	55.9±13.0	1017.2±26.2	107.4±52.7	71.1±25.9

Figure 3 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 2 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.

Analysis of Cholesterol Levels in Whole Blood

Rapid UHPLC analysis as shown in Figure 4 enables processing of samples within five minutes. The overlay of calibration standards of five phytosterols from 0.5 – 20 µg/mL is shown in Figure 4. Good linearity of response to different concentrations was obtained with correlation coefficients ranging from R^2 = 0.992–0.997 for the five compounds evaluated (Table 3) over the range of 0.5 – 20 µg/mL. The RSD values

FIGURE 4. Overlay of analytical standards (1 – 40 ng on column).

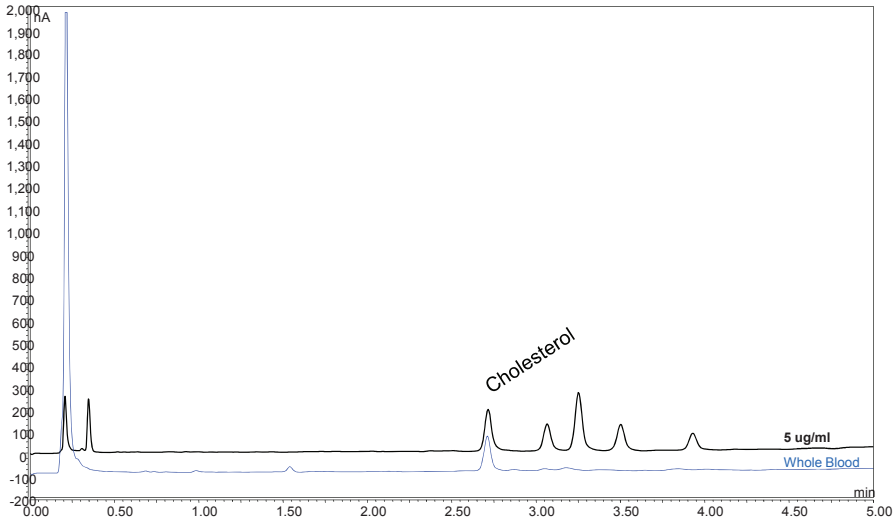


ranged from 7.2% to 9.5%, indicating that the BDD electrode provided suitable stability during this study. Samples can also be prepared for HPLC analysis by saponification with potassium hydroxide to reduce acylglycerols to fatty acids prior to analysis in order to remove potential buildup of lipophilic material being retained on the analytical column (data not shown). The analysis of cholesterol in whole blood was performed and is shown in Figure 5. The mean level of cholesterol in one subject was calculated at 182.3 mg/dL with a %RSD of 1.91% (n=6).

Table 3. Regression data for standard calibration curves.

Peak Name	Ret.Time (min)	Number of Points	Rel.Std.Dev. %	Coeff.of Determination	Slope
Cholesterol	2.72	12	8.73	0.9943	31.74
Campesterol	3.068	12	9.4045	0.9942	20.43
Stigmasterol	3.258	12	7.1536	0.9965	44.72
Sitosterol	3.51	12	8.4131	0.9943	20.38
Stigmastanol	3.923	12	9.5449	0.9923	12.82

FIGURE 5. Overlay of standard (red trace) vs. whole blood (blue trace).



Conclusions

- UHPLC with electrochemical detection using a BDD electrode provides a suitable means of determining clinically relevant compounds.
- 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. The levels of GSH and GSSG detected are within the range of reported levels using other techniques
- The levels of cholesterol detected in whole blood 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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