Quantitation of Glutathione and Related Thiols in Acid-Preserved Samples by Hydrophilic Interaction Liquid Chromatography-Mass Spectrometry

Alan W. Taylor
Oregon State University, Corvallis, OR

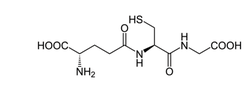
Introduction

Glutathione is the most abundant non-protein thiol in the body. Reduced glutathione (GSH) is important for protection from oxidative damage. Accurate determination of GSH, and its oxidized form (GSSG), provides an important indicator of the redox environment of cells, tissues and organisms in various states of health and disease.

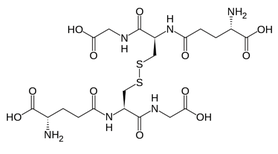
Established LC-UV and LC-fluorescence methods for quantifying GSH, GSSG, and other thiols in plasma, cells, and tissue homogenates require time-consuming sample preparation and lengthy chromatography. Detection is based on a single absorbance wavelength or fluorescence excitation/emission combination, and identification relies on retention time. Mass spectrometry provides more specific identification, enabling faster chromatography that need not resolve every analyte in time. While free thiols must be protected for accurate measurement of the redox state, no chromophore or fluorophore is needed for MS detection.

However, our investigators have a large number of samples prepared using standard PCA sample preservation, *i. e.*, the samples contain 5% w/v perchloric acid. Thus, I have the challenge of adapting these samples to a new MS-based method: neutralization of perchloric acid, derivatization with NEM to protect free thiols, separation by hydrophilic interaction liquid chromatography (HILIC), and detection by selective ion monitoring (SIM) or selective reaction monitoring (SRM) on our available mass spectrometers. As some investigators are interested in other thiols, I included the cysteine-cystine redox pair (Cys and CysSSCys, respectively) during development, in the hope of achieving a more generally applicable method.

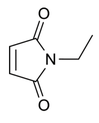
Structures



Glutathione (GSH)



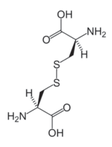
Glutathione disulfide (GSSG)



N-ethyl maleimide (NEM)

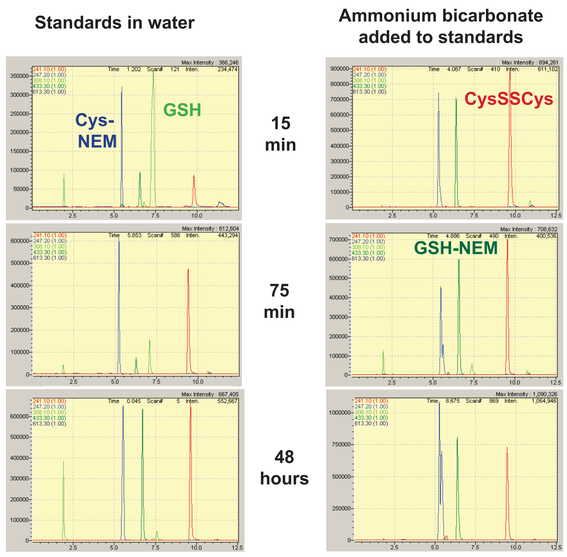
Cysteine

Cystine

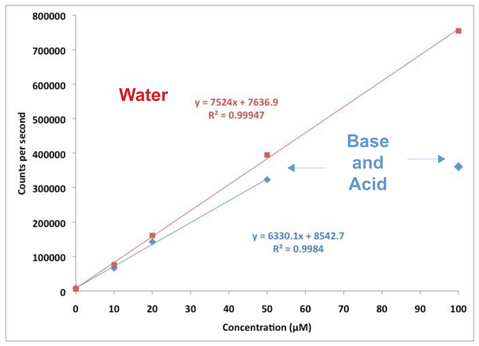


Glutathione:easiest to solve

The only problem encountered with GSH was in optimizing NEM derivatization. Ammonium bicarbonate neutralized PCA and brought the solution to pH ~7.5 for the reaction to proceed quickly. GS-NEM chromatographed well in all systems tried and was readily detected by MS and MS/MS, giving a linear response over a large range.



Disulfide response was not linear:

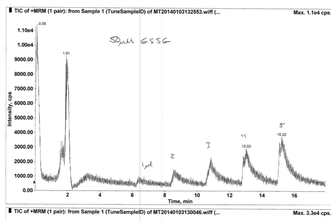


CysSScys standards gave a linear response at low concentrations. Then response leveled off, and even dropped at higher concentrations.

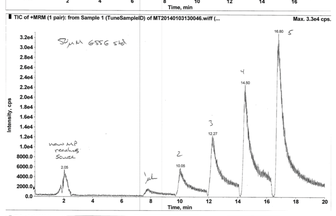
Rather than prepare standards in the normal method, ammonium bicarbonate addition followed 5 minutes later by acetic acid, a batch was prepared in pure water. The calibration curve stayed linear to the high end. Thus, the problem was simply that cysSScys is far less soluble in neutral and alkaline pHs. Since oxidized thiols are usually present in lower concentrations than the reduced forms, the concentration range of the 2 disulfides was halved in subsequent experiments.

Finally, flow injection experiments showed that mobile phase pH was the key.

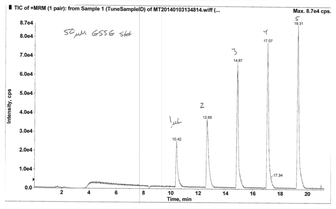
Injections of 50 µM GSSG into mobile phase of 1:1 MeCN:water, 10 mM ammonium formate, 0.1% formic acid



Mobile phase of 1:1 MeOH:water, no modifiers



Mobile phase of 1:1 MeOH:water, 0.05% ammonium hydroxide



Clearly, GSSG formed gas phase ions better from an alkaline environment.

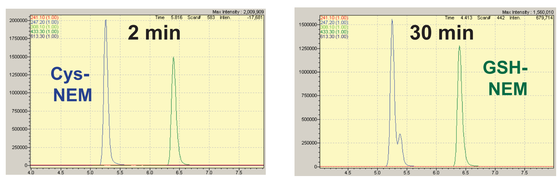
However, Cys-NEM needs an acid mobile phase for good ionization, so a pH gradient was developed using mobile phases A of 90:10 MeCN:water with 0.05% acetic acid and B of water with 0.2% ammonium hydroxide.

Cysteine was next

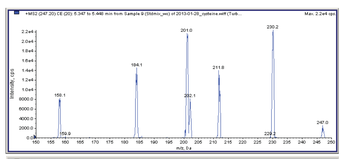
Both Cys and GSH derivatize quickly with NEM when buffered with ammonium bicarbonate, but Cys-NEM begins to show a double peak in the *m/z* 247 chromatogram within a short time. Eventually, only the later peak remains.

The change in structure yields a different product ion spectrum, as reported by Shuford *et al*. (Anal Bioanal Chem (2012) 402: 357-366). They quench the reaction with acetic acid, but too late! I prefer to use the initial reaction product, prior to the rearrangement Shuford proposed. Quenching after 5 minutes works well.

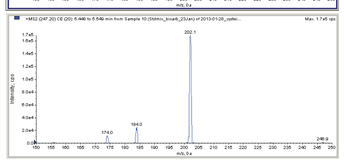
Acetic acid added after:



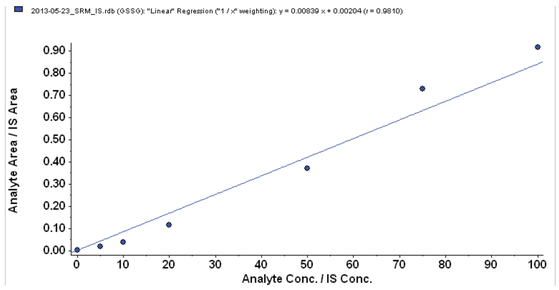
Product ions of *m/z* 247.2, early peak



Product ions of *m/z* 247.2, late peak



That was not enough to solve the GSSG problem: exponential response, often with no response at the lowest levels.

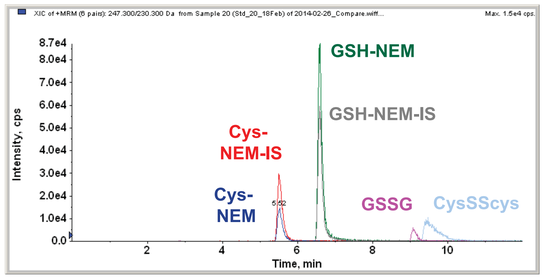


GSSG often tailed worse than other analytes, but reducing tailing did not improve response at low concentrations. This happened with multiple column and mobile combinations.

Tests for losses to vial or other surfaces showed this wasn't the cause.

Reoptimizing mass spectrometer parameters was tried. With 4 terminal carboxyl and 2 amine groups, GSSG is a very hydrophilic molecule that may stay in the center of liquid droplets in an ESI source, rather than near the surface. Thus, it may be difficult for GSSG to enter the gas phase. Increasing gas flows and temperature in the source did increase low level sensitivity, but didn't completely solve the problem.

What's left?



Final steps include:

Better protect MS from salt buildup in source

Adapt gradient from 10 to 5 cm column to halve LC-MS run time

Validation by replicate analyses of control samples, standard addition, and parallel analysis of samples with a colleague experienced in the classic LC-optical assays

Transfer sample preparation to liquid handler

And then process a backlog of hundreds of waiting samples!

Acknowledgments

Thanks to EMD Millipore Corporation and Waters Corporation for the gift of HILIC columns