



QUANTIFICATION OF THE CELLULAR GLUTATHIONE/GLUTATHIONE DISULFIDE CONTENT BY FAST LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY ANALYSIS



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OVERVIEW

PURPOSE

To develop a fast and reliable method to measure glutathione and glutathione disulfide in cell lysate.

METHOD

N-ethylmaleimide (NEM) derivatization and LC/MRM-MS analysis coupled with stable isotope dilution.

RESULT

The LC/MS method was validated within the range of 5 to 400 μ M for GSH and 0.5 to 40 μ M for GSSG. The GSH/GSSG contents were successfully measured and quantified in THP1 cells after treated with 4-hydroxy-2-nonenal (HNE) and HNE-triacetate.

INTRODUCTION

Reduced glutathione (GSH) is a major tissue antioxidant that protects against oxidative stress in mammalian cells. When cells are exposed to increased levels of oxidative stress, GSH is converted into glutathione disulfide (GSSG). GSSG will accumulate and the ratio of GSH to GSSG (GSH/GSSG) will decrease. Therefore, the determination of the GSH/GSSG ratio is a useful indicator of oxidative stress. The accurate determination of GSH/GSSG ratios is analytical challenge due to the low cellular concentration of GSSG coupled with the need to prevent GSH oxidation during sample preparation. Here we described a simple, rapid and specific method to determine GSH/GSSG content in cell lysates by using *N*-ethylmaleimide (NEM) derivatization and LC/MRM-MS analysis coupled with stable isotope dilution.

METHOD

Preparation of calibration standards and internal standard (IS): The derivatization was done by mixing 2mM GSH (or [13 C, 15 N]GSH) solution with 20mM NEM at RT for 30min. Final concentration of GSH (represented by GSNEM) were 5 to 400 μ M and GSSG were 0.5 to 40 μ M. [13 C, 15 N]GSNEM (50 μ M) was used as internal standard.

Sample preparation: Human acute monocytic leukemia cell line (THP-1) were harvested and lysed by sonication on ice in derivatizing solution containing NEM. Internal standard was added to sample prior to injection for LC/MS analysis.

LC/MRM-MS analyses were performed on a Applied Biosystems 3200 Q-trap mass spectrometer coupled to Shimadzu LC-20AD HPLC system equipped with a Cogent Bidentate C8 column (MicroSolv, Corp.). Mobile phase A was water and B was acetonitrile, both with 0.1% formic acid. The gradient conditions were as followed: 2% B from 0 to 0.5 min, 2 to 35% B from 0.5 to 3 min, 35 to 40% B from 3 to 5 min, 40% B from 5 to 7 min, back to 2% B at 8 min, followed by a 2 min equilibration period.

RESULTS

Fig.1 GSH derivatization by using *N*-ethylmaleimide (NEM). The use of NEM prevent an underestimation of GSH and artificial GSSG production.

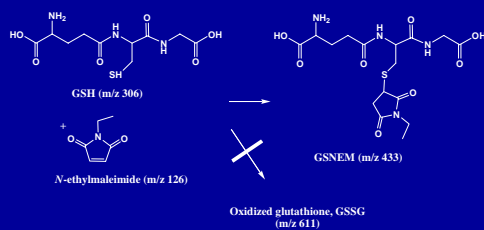


Fig.2 MRM chromatogram for GSNEM, [13 C, 15 N]GSNEM (H-GSNEM, internal standard), GSSG, GSH and H-GSH. The separation of GSSG and GSNEM were achieved by using TYPE-C Silica™ Cogent Bidentate C8 column with retention time 3.23 and 5.48 min respectively. The transition of GSNEM was used for GSH quantitation.

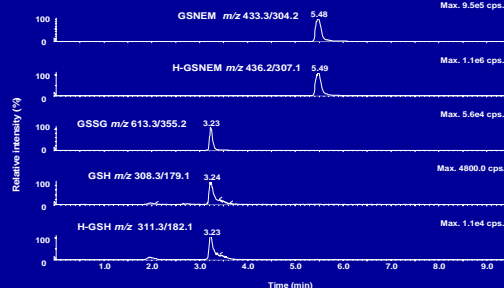


Fig. 3 Calibration curve of GSH (A) and GSSG (B). The concentration range is 5 to 400 μ M for GSNEM and 0.5 to 40 μ M for GSSG.

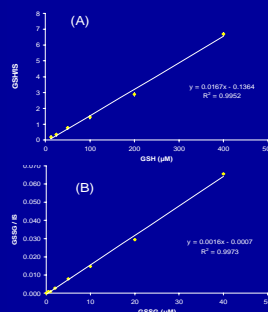
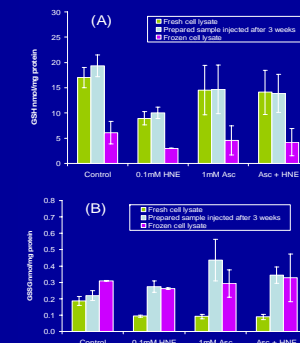


Table. 1 The method were found accurate and precise in the range of 5 to 400 μ M for GSNEM and 0.5 to 40 μ M for GSSG. At all QC sample concentrations examined, the accuracy was within 100±16% and precision values were less than 9%.

	GSH (μ M)			
	5.00 (n=3)	10.00 (n=3)	100.00 (n=3)	400.00 (n=3)
Mean	4.45	10.22	101.5	400.7
SD	0.38	0.54	4.07	5.29
Precision (%)	8.5	5.3	4.0	1.3
Accuracy (%)	88.9	102.3	101.5	100.2
	GSSG (μ M)			
	0.50 (n=3)	1.00 (n=3)	10.00 (n=3)	40.00 (n=3)
Mean	0.68	1.02	9.80	39.98
SD	0.04	0.07	0.20	0.19
Precision (%)	6.8	7.0	2.0	0.5
Accuracy (%)	115.8	101.8	98.0	99.9

Fig.4 GSH (A) and GSSG (B) concentration in THP-1 cells after treatment with HNE and Ascorbic acid (n=4).

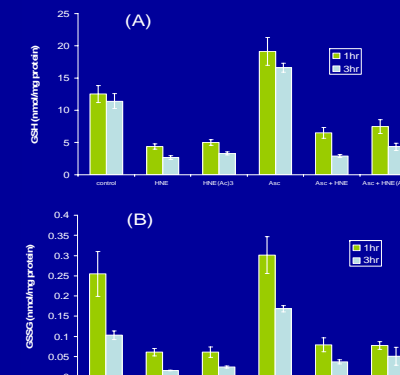


Adding HNE caused a significant drop of GSH concentration when comparing to control. If cells were preincubated with ascorbic acid, the level of GSH will be close to normal concentration.

The storage stability of prepared samples have also been tested. When GSH was converted to GSNEM, it was stable at least up to 3 weeks in the refrigerator. However, GSSG concentration were slightly increased during storage.

Sample preparation was critical when processing the cells. If cells have been frozen, significant lower amount of GSH and higher amount of GSSG were found.

Fig.5 GSH (A) and GSSG (B) concentrations in THP-1 cells treated with HNE, HNE triacetate and ascorbic acid for 1 and 3 hr (n=4).



HNE triacetate (HNE(Ac)₃) is a chemically protected form of the HNE. It mimics intracellular formation of HNE to reach deeper cellular targets such as nuclear DNA. The results showed that both HNE and HNE(Ac)₃ can cause depletion of intracellular GSH and GSSG. The drops of GSH level caused by HNE and HNE(Ac)₃ were similar. The concentration of GSH and GSSG decreased more when incubation time increased.

CONCLUSION

- A fast LC/MRM-MS method has been established to specifically and robustly determine cellular GSH and GSSG content.
- The method was used to monitor the change of the GSH / GSSG in cultured cells model after HNE or HNE(Ac)₃ treatment. However, sample preparation is critical for accurate measurement.
- HNE or HNE(Ac)₃ treatment caused significant decreased GSH level while pretreated with antioxidant can protect cells from HNE or HNE(Ac)₃ treatment.
- This method can be adapted for quantitation of GSH and GSSG in tissues and possibly fresh mitochondria.

ACKNOWLEDGEMENT

The authors wish to acknowledge the Mass Spectrometry core facility of the Environmental Health Sciences Center (P20 ES00210) at Oregon State University. We also like to thank Dr. Brandi Langsdorf for the synthesis of HNE(Ac)₃ and all the help from the Ken F. Stevens' lab.

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