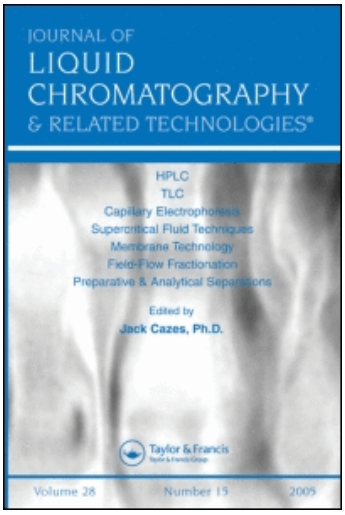


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Detection of Thiols and Disulfides in Liver Samples using Liquid Chromatography/Electrochemistry

Susan M. Lunte[a]; Peter T. Kissinger[a]
[a] Department of Chemistry, Purdue University, West Lafayette, Indiana

To cite this Article Lunte, Susan M. and Kissinger, Peter T.(1985) 'Detection of Thiols and Disulfides in Liver Samples using Liquid Chromatography/Electrochemistry', Journal of Liquid Chromatography & Related Technologies, 8: 4, 691 — 706
To link to this Article: DOI: 10.1080/01483918508067113
URL: http://dx.doi.org/10.1080/01483918508067113

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(4), 691-706 (1985)

DETECTION OF THIOLS AND DISULFIDES IN LIVER SAMPLES USING LIQUID CHROMATOGRAPHY/ELECTROCHEMISTRY

Susan M. Lunte and Peter T. Kissinger
Department of Chemistry
Purdue University
West Lafayette, Indiana 47907

ABSTRACT

The determination of thiols and disulfides independent of one another is accomplished through the use of dual electrode liquid chromatography/electrochemistry and N-ethylmaleimide. The method can be used to assess peak purity as well as to determine the oxidation state of an unknown thiol. By the addition of NEM, trace disulfides can be determined without interferences from glutathione or other thiols present in much higher concentrations in in the liver. This method is used for the detection of mixed disulfides in liver samples.

INTRODUCTION

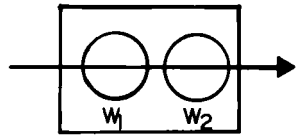
The determination of thiols and disulfides is traditionally performed by the measurement of thiols in a sample, followed by the reduction of the disulfides and then a second thiol measurement (1-2). More recently, enzymatic and chromatographic methods have been developed.

 0148-3919/85/0804-0691$3.50/0

Glutathione disulfide can be measured by the reduction of GSSG to GSH by the enzyme gluathione reductase (4). Derivatizing agents such as the Ellman reagent and OPA have been used both for simple colorimetry and for chromatographic detection of thiols (4,5). Often these assays are plaqued with interferences. Enzymatic methods are usually specific for a particular thiol or disulfide but are limited primarily to biologically important thiols. They also suffer from interferences, especially in cases where the oxidation of NADPH is used to determine the thiol or disulfide concentration.

Liquid chromatography/electrochemistry has been used previously for the determination of thiols in liver samples (6-7). Glassy carbon and gold electrodes have been employed at potentials between +800 and +1000 mV vs. Ag/AgCl (6,8,9). However, a gold mercury amalgam electrode or a mercury electrode can be used to detect the thiol at at a potential of +150 mV vs. Ag/AgCl (7,10-13). Enhanced selectivity and lower detection limits are two of the advantages gained by operating at a lower potential. Due to the nature of the reaction, only compounds which complex with mercury such as thiols, halides and chelating agents give a response in this range. The background current is also very low.

Recently, a dual electrode detector for the simultaneous determination of thiols and disulfides has been developed (12). This detector involves the use of two gold/mercury electrodes in the series configuration as



"GENERATOR" (E = -1.00V)

W_1: $RSSR + 2H^+ + 2e^- \longrightarrow RSH$

"DETECTOR" (E = +0.15 V)

W_2: $2RSH + Hg \longrightarrow Hg(SR)_2 + 2H^+ + 2e^-$

Figure 1: Dual electrode detector for the simultaneous determination of thiols and disulfides

is illustrated in figure 1. The first electrode is set at -1.0V vs Ag/AgCl. This electrode acts as a post column reactor to reduce disulfides to their corresponding thiols. The second, or downstream, electrode is set at +150 mV in order to detect the thiol resulting from the reduction at the first electrode, as well as thiols in the sample. Since thiols and disulfides are separated chromatographically, both the concentration of the thiol and the corresponding disulfide can be determined independently. One of the advantages of this

type of detector is that one can distinquish the thiols from the disulfides. When the generator electrode is off, only thiols are detected. If the generator electrode is turned on, both thiols and disulfides are detected. This information can be used to identify which compounds are thiols and which are disulfides. Difficulties with this sort of identification scheme occur only in cases of coeluting thiols and disulfides, since the disulfides cannot be determined independently of thiols. A method is is presented here for the determination of disulfides independently of the thiols.

In liver samples, the level of glutathione is much higher than other endogenous thiols. This can present a problem with the use of a gold/mercury electrode. In order to determine GSSG or another trace disulfide in a liver homogenate, a large quantity of glutathione must be injected along with the disulfide peak. This glutathione causes a rapid depletion of surface mercury on the electrode. The injection of several liver samples leads to a dramatic decrease in the detector response over the period of a few injections and a corresponding decrease in elecrode lifetime. Both of these factors make it difficult to quantitate GSSG or any other disulfide in liver samples

There are several ways of alleviating this problem. One method is to divert the glutathione to waste when one is determining a later eluting disulfide. This strategy has been discussed elsewhere. (14). A second method which will be discussed in this paper is to derivatize the

glutathione to a nonelectroactive conjugate. Once derivatized, glutathione is no longer detectable at +150 mV vs. Ag/AgCl.

N-ethylmaleimide has been used previously for the determination of thiols in tissue samples (15-19). The reagent absorbs at 300 nm. Thiols react with NEM to produce a conjugate which no longer absorbs at this wavelength. Total thiol content of tissue or proteins can be determined using NEM. It is most often used for the mesurement of glutathione since it is the predominant biological thiol. By reacting thiols with NEM, one can qualitatively determine which peaks are due to thiols, and which peaks are due to halides or disulfides. N-ethylmaleimide coupled with the dual electrode detector can provide valuable information regarding the identity of the peaks in a given sample, as well as an indication of peak purity. This reagent can also be used to deplete the high levels of glutathione which are present in many types of tissue samples and are responsible for the shortening of the gold/mercury electrode lifetime.

MATERIALS AND METHODS

Reagents

Chemicals were purchased from the following sources: cysteine, cystine, homocysteine, homocystine, glutathione, glutathione disulfide and N-ethylmaleimide from Sigma Chemical Company; sodium borohydride, Aldrich Chemical Company; cystinyl-bis-glycine, Vega Biochemicals;

monochloroacetic acid, Mallinkrodt; sodium hydroxide, Fisher Scientific; ethylenediamine tetracetate and sodium octyl sulfate, Kodak. Methanol was distilled in glass prior to use.

Apparatus

A Bioanalytical Systems LC-154 liquid chromatograph with tandem LC-4B aperometric controllers were used for all studies. The thin layer cell included two amalgamated gold disks in series. Preparation of the cell has been previously described (12). The system was modified to exclude oxygen. A Biophase C-18 5 μm column coupled with a C-18 guard column was employed for all studies. The mobile phase was 4% methanol and 96% monochloroacetic acid pH 3 buffer containing 1 mM sodium octyl sulfate as an ion pairing agent. The flow rate was 1 mL/min. A 20 μL injection loop was used.

Preparation of the mixed disulfides

Mixed disulfides were prepared by mixing equimolar amounts of the two corresponding thiols together in 3% hydrogen peroxide (20). The reaction was allowed to proceed overnight. The cysteinylglycine was produced by the reduction of the disulfide with sodium borohydride.

Preparation of liver cytosol

Male swiss mice were decapitated and their livers removed. The liver was then homogenized in Tris/KCl buffer of pH 7.4. The mixture was centrifuged for 20 minutes at

9,000 g. The supernatant was then centrifuged for 60 minutes at 100,000 g. The cytosolic supernatant was used to show the utility of NEM for the qualitative identification of thiols and disulfides in these experiments.

Use of NEM

A 0.24 M solution of NEM was prepared in methanol and water. 200 μL of this reagent was added to 500 μL of cytosol supernatant. The reaction was allowed to proceed for approximately 15 minutes. After this time the reaction was quenched with 1 M perchloric acid and the sample was centrifuged to remove protein. Following centrifugation the supernatant was injected directly into the liquid chromatograph. A similar procedure was used in the determination of the mixed disulfides, except that perchloric acid was not added to the reaction mixture.

RESULTS AND DISCUSSION

The utility of NEM as a qualitative tool for the determination of disulfides is shown in Figure 2a. The reaction mixture of cysteine and glutathione appeared to yield only the homogenous disulfides. With both electrodes on, peaks for glutathione, cysteine, cystine and GSSG are readily apparent. The small peak eluting at about 10 minutes was determined to be a thiol and later identified as cysteinylglycine, a hydrolysis product of glutathione. Figure 2b shows the same reaction mixture after adding

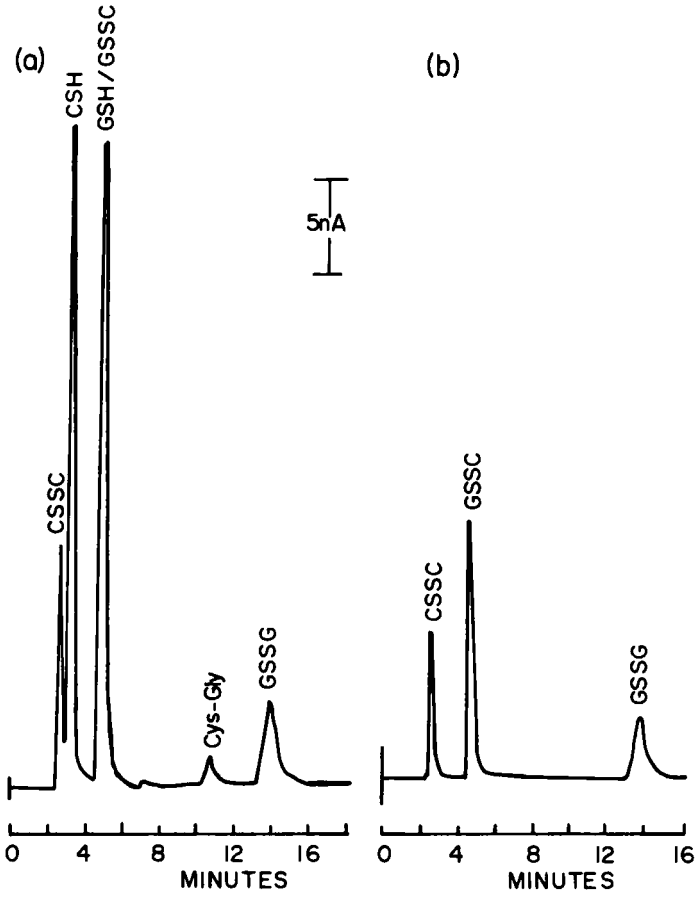


Figure 2a: Reaction mixture of glutathione and cysteine with hydrogen peroxide. Mobile phase 4% methanol and 96% Monochloroacetic acid pH 3 with 1 mM SOS. Both W(1) and W(2) are on. GSH-glutathione, CSH-cysteine, Cys-gly-cysteinylglycine 2b: Same sample as figure 2 with NEM added. GSSG-glutathione disulfide, GSSC-glutathione-cysteine mixed disulfide, CSSC-cystine.

NEM. NEM reacts with the thiols and not the disulfides. Upon the addition of NEM, it was found that the mixed disulfide was coeluting with the glutathione. By turning the generator electrode off, we can further verify that the peak is due to a disulfide. With W(1) off, the glutathione peak is smaller. If NEM is added to the reaction mixture and only W(2) is on, then we see no endogenous peaks or unreacted thiols (Figure 3). Table 1 summarizes the use of NEM for the determination of peak purity. With both electrodes on we will see both thiols and disulfides. By turning W(1) off we will detect only thiols. The use of NEM further aids in this identification process. If NEM is added to the reaction mixture and both electrodes are on, then only disulfides and components which do not react with NEM will be detected. Halides and chelating agents can be distinquished by the addition of NEM and only W(2) on. In this case, only halides and chelating agents will be detected.

Figure 4 shows the utility of this method for a liver sample. With both electrodes on, several thiols and disulfide peaks are discernible, with GSH being the most

TABLE 1

Determination of Thiols and Disulfides using NEM and Dual Electrode Liquid Chromatography/Electrochemistry

W(1)	W(2)	REAGENT	DETECTS
+1000	+150	none	RSH, RSSR
+1000	+150	NEM	RSSR
off	+150	none	RSH
off	+150	NEM	nothing

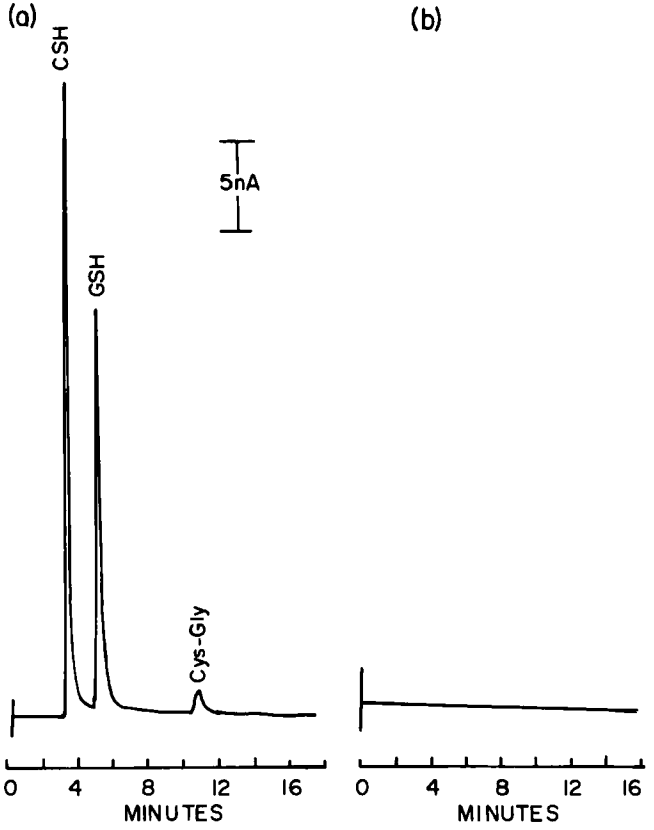


Figure 3: (a) Same sample as figure 2 with only the "detector" electrode on. (b) After the addition of NEM

prominent. This large GSH peak can cause some problems in the determination of trace disulfides such as GSSG or the mixed disulfide GSSC. Not only does it obscure some peaks, but it also fouls the mercury electrode by quickly depleting the surface mercury after only a few injections. The addition of NEM solves both of these problems. The mixed disulfide of glutathione and cysteine can be

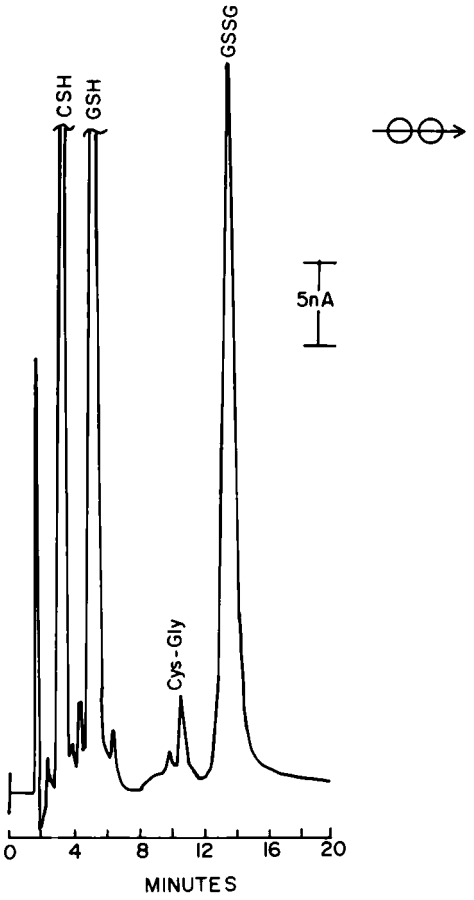


Figure 4: Liver cytosol sample, both electrodes on

detected once NEM is added to remove the glutathione peak (figure 5). Although chromatographic conditions would be changed if one was looking particularly for this compound, NEM can assist in assesing peak purity of any thiol peak in any complex matrix. Secondly, NEM eliminates the large GSH peak so that trace disulfides can be determined more

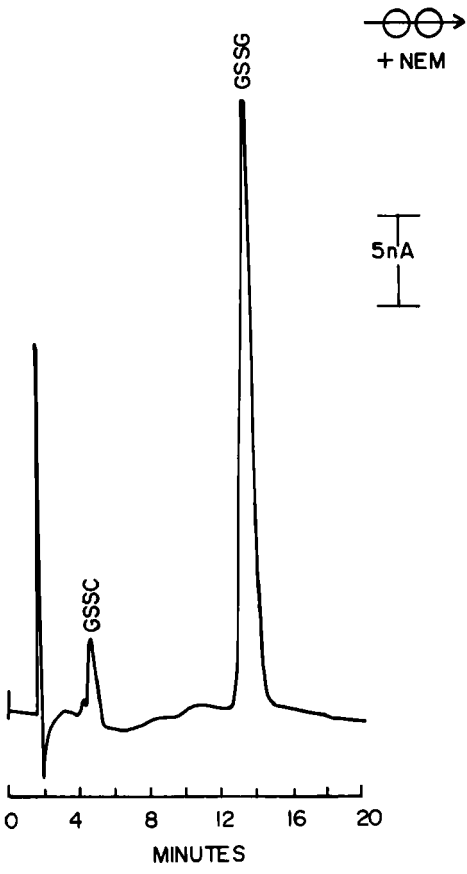


Figure 5: Same conditions as figure 5 with NEM added

reproducibly. If only thiols are to be quantitated, the generator electrode can be turned off producing a chromatogram for the liver sample as shown in figure 6.

The use of the traditional thiol reagent, NEM, coupled with dual electrode liquid chromatography/ electrochemistry can provide an indispensable tool for the independent determination of thiols and disulfides in

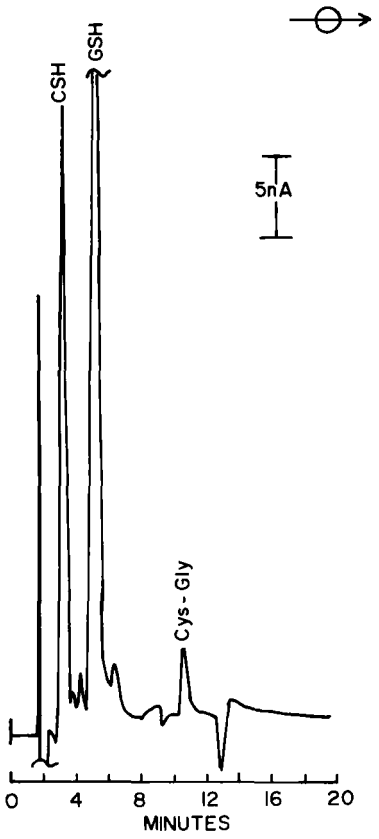


Figure 6: Detection of thiols in liver cytosol. Only W(2) is on.

complex samples. Table 2 shows the capacity factors of a number of important liver thiols and disulfides. All of these thiols react with NEM. Both the oxidized and reduced forms of these compounds can be detected using the dual electrode detector. By utilizing both the dual electrode detector and NEM, thiols and disulfides can be determined in any biological sample including any tissue samples

TABLE 2

Capacity Factors for Selected Hepatic Thiols and Disulfides

Compound	k'	Reaction with NEM
cysteine	1.06	yes
cystine	0.75	no
homocysteine	3.38	yes
homocystine	9.60	no
glutathione	2.38	yes
glutathione disulfide	9.25	no
cysteine-glycine	4.50	yes
cystinyl-bis-glycine	23.0	no
homocysteine-cysteine disulfide	3.13	no
glutathione-cysteine disulfide	1.50	no
glutathione-homocysteine disulfide	8.70	no

which contains large amounts of glutathione or another endogenous thiol. The detection of the mixed disulfides of homocysteine and cysteine, as well as those of glutathione and cysteine can also be accomplished using this technique.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the Purdue Cancer Center for their generous financial support of this

project. We would also like to thank George Barone for his assistance in sample preparation and Dr. Robert Van Etten for the use of the ultracentrifuge.

REFERENCES

1. Woodward, G.E. and Fry, E.A., J. Biol. Chem., 97, 465 (1932)

2. Ellman, G.L., Arch. Biochim. Biophys., 82, 70 (1959)

3. Tietze, F., Anal. Biochem., 27, 502 (1969)

4. Reeve, J., Kuhlenkamp J., Kaplowitz N., J. Chromatogr., 194, 424 (1980)

5. Hissen, P.J. and Hilf, R., Anal. Biochem., 74, (1976) 214

6. Mefford, I. and Adams, R.N., Life Sci., 23, 1167 (1978)

7. Demaster, E.G., Shirata, F.N., Redfern, B., Goon, D.J.W., and Nagasawa, H.T., J. Chromatogr., 308, 83 (1984)

8. Kreuzig F. and Frank, J., J.Chromatogr., 218, 615 (1981)

9. Cox, J.A. and Przyjany, A., Anal. Lett., 10, 869 (1977)

10. Rabenstein, D.L. and Saetre, R., Anal. Chem., 49, 1036 (1977)

11. Saetre, R. and Rabenstein, D.L., Anal. Biochem., 40, 684 (1978)

12. Allison, L.A. and Shoup, R.E., Anal. Chem., 55, 8 (1983)

13. Eggli, R.and Asper, R., Anal. Chim. Acta, 101, 253 (1978)

14. Lunte, S.M. and Kissinger, P.T., J. Chromatogr., in press

15. Gregory, J.D., J. Am. Chem. Soc., 77, 3922 (1955)

16. Benesch, R., Benesch, R.E., Gutcho, M. and Laufer, L., Science, 123 (1956)

17. Leslie, J., Williams, D.L., Corin, G., Anal. Biochem., 3, 257 (1962)

18. Roberts, E. and Rouser, G., Anal. Chem., 30, 1291 (1958)

19. Alexander, N., Anal. Chem., 30, 1293 (1958)

20. Jocelyn, P.C., Biochemistry of the SH Group, Academic Press, London, 1972, 94-100