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A simultaneous liquid chromatography/mass spectrometric assay of glutathione, cysteine, homocysteine and their disulfides in biological samples

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Abstract

A liquid chromatography/mass spectrometric (LC/MS) method was developed for simultaneous detection and quantitation of glutathione (GSH), glutathione disulfide (GSSG), cysteine (CysSH), homocysteine (HCysSH) and homocystine in biological samples (rat brain, lung, liver, heart, kidneys, erythrocytes and plasma). Thiols were derivatized with a large excess of Ellman's reagent, a thiol-specific reagent, to ensure an instantaneous and complete derivatization. The derivatization blocked the oxidation of the thiols to disulfides, preventing errors caused by thiol oxidation. The samples were then analyzed by LC/MS. The method provides a highly selective and sensitive assay for these endogenous thiols and their corresponding disulfides. The detection limits for GSH, GSSG, CysSH, HCysSH and homocystine were 3.3, 3.3, 16.5, 29.6 and 14.9 pmol, respectively. An attempt for cystine analysis was unsuccessful due to earlier elution of the compound and strong interferences caused by other endogenous compounds. This method will be a useful tool in the investigation of the roles of these important thiol-containing compounds and their corresponding disulfides in physiological and pathological processes.

Keywords: LC/MS; Glutathione; Cysteine; Homocysteine; Disulfides

1. Introduction

Endogenous low molecular weight thiol-containing compounds, namely glutathione (GSH), cysteine (CysSH) and homocysteine (HCysSH) and their corresponding disulfides (glutathione disulfide (GSSG), cystine and homocystine) (Fig. 1) are important in a variety of physiological and pathological processes. GSH plays an essential role in protecting cells from toxic species, such as reactive oxygen intermediates and reactive electrophiles [1]. The ratio of GSH to its oxidized form,

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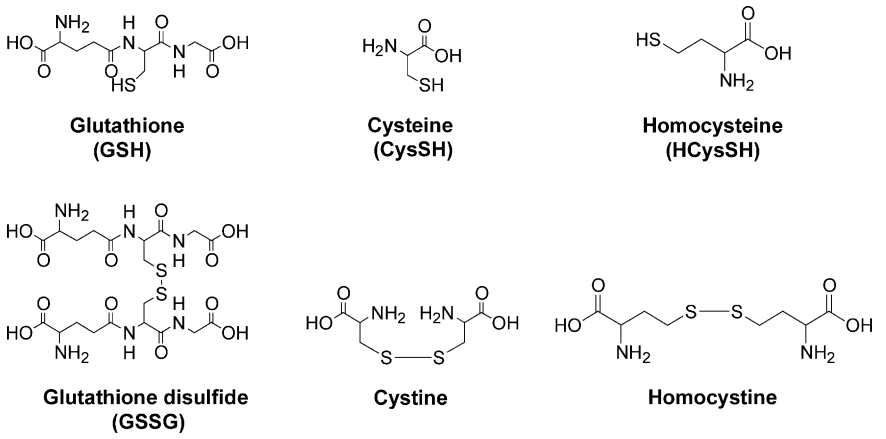


Fig. 1. Chemical structures of thiols and disulfides described in the text.

GSSG, has been shown to be an effective measure of oxidative stress in a biological system [2]. Cysteine is a synthetic precursor of GSH. Homocysteine is an intermediate in the metabolism of the indispensable amino acid methionine [3]. Increased total plasma homocyst(e)ine (tHcy), which includes all forms of homocysteine (homocysteine, homocysteine-protein disulfides and homocystine), has been recognized as an independent risk factor for cardiovascular diseases including atherosclerosis and venous thrombosis. The precise pathological mechanism by which homocysteine may cause cardiovascular diseases is currently unknown [3]. Therefore, analytical methods for the detection and quantitation of these thiol compounds and their corresponding disulfides in biological samples are desirable. The main challenges in the assay of these compounds lie in their unfavorable physicochemical properties. These compounds are highly polar and water soluble, which makes their extraction from biological matrices without derivatization impossible. The absence of chromophore for sensitive detection in LC systems presents the second problem. Third, the chemical instability, especially the liability of the thiol group to oxidation, often causes inaccurate analytical results. Most analytical methods developed for these compounds have aimed at overcoming these obstacles.

Previous methods reported for the measurement of GSH and GSSG in biological samples mainly include enzymatic assay [4,5], HPLC with electrochemical detection [6–8] or ultraviolet (UV) detection without sample derivatization [9,10] and pre- or post-column thiol derivatization followed by HPLC with UV detection [11–16] or fluorometric detection [17–25]. More recently, capillary electrophoresis has been reported to be highly sensitive and selective in the measurement of GSH, GSSG [26–28] and HCysSH [26,29,30]. Quantitation of total plasma homocysteine requires the reduction of the oxidized forms (homocysteine-protein disulfides, homocystine) to homocysteine prior to its quantitation. Most reported chromatographic methods for tHcy use thiol-selective fluorogenic reagents [25,31,32] or other derivatizing agents [33] to increase sensitivity and selectivity. In addition, GC/MS has also been employed in the quantitation of homocysteine [34,35]. During the preparation of this manuscript, a highly sensitive and selective ion-exchange liquid chromatography/mass spectrometry method was reported for the measurement of GSH and GSSG in rat hepatocytes [36]. The method derivatizes GSH with iodoacetic acid at pH 10 in the dark for 1 h and then analyzes the sample by LC/MS with a specially ordered ion-exchange column.

LC/MS is a relatively new technique that employs a mass spectrometer as a detector for HPLC instead of UV, as in the conventional HPLC [37]. The use of mass spectrometry as the detection method significantly increases the selectivity of the analytical method. As a result, LC/MS requires very minimal sample treatment and no

chromophore for detection. It offers unique advantages in the assay of compounds with chemical instability, high polarity and lack of a chromophore. LC/MS has become increasingly popular and the method of choice in the pharmaceutical industry for the analysis of biological samples. We describe in this paper an LC/MS method to simultaneously quantify GSH, GSSG, CysSH, HCysSH and homocystine in biological samples (rat brain, lung, liver, heart, kidneys, erythrocytes and plasma). The method requires minimal sample treatment and offers high selectivity and sensitivity.

2. Materials and methods

2.1. Materials

Glutathione, glutathione disulfide, cysteine, homocysteine, homocystine, glutathione ethyl ester and 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) were obtained from Sigma Chemical Co. (St. Louis, MO). Trifluoroacetic acid (TFA), trichloroacetic acid (TCA) and 5-sulfosalicylic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). HPLC grade acetonitrile was obtained from Fisher Scientific (Pittsburgh, PA). Sprague–Dawley rats (200–225 g) were purchased from the National Cancer Institute (Frederick, MD).

2.2. Solutions

Stock solutions (4 mg/ml) of GSH, GSSG, CysSH, HCysSH, homocystine and glutathione ethyl ester were freshly prepared in phosphate buffer (0.1 M, pH 7) and stored immediately at −20 °C. A standard solution was prepared from the dilution of the stock solution with phosphate buffer (0.1 M, pH 7). A saturated solution of Ellman's reagent (10 mM) was prepared by dissolving 39.6 mg of the reagent in 10 ml of phosphate buffer (0.01 M, pH7). KCl (1.15%) solution was prepared in deionized water.

2.3. Equipment

LC/MS analysis was carried out on a Finnigan MAT Navigator HPLC/MS mass detector, a single quadrupole mass detector, interfaced to a SpectraSYSTEM P4000 HPLC system equipped with a SpectraSYSTEM autosampler (San Jose, CA).

2.4. Preparation and derivatization of biological samples

Male Sprague–Dawley rats were anesthetized. Blood was collected by cardiac puncture in a heparinized tube. Lung, liver, heart, kidney and brain samples were obtained from sacrificed rats. Homogenates of lung, liver, heart, kidney and brain samples were prepared as follows. Tissues (≈ 1 g) were diced, added to ice-cold KCl solution (1.15%) (4 ml/g tissue) and homogenized with an Omni 5000 homogenizer (Omni International Waterbury, CT) over ice for 1–2 min. 50 μl of the homogenate was transferred to a 1.5 ml snap-cap conical-bottom centrifuge vial and added with glutathione ethyl ester as an internal standard (I.S.) (0.01 mg/ml, 20 μl) and Ellman's reagent (10 mM, 100 μl). The mixture was vortex-mixed for 1 min followed by the addition of 30 μl of 20% 5-sulfosalicylic acid. The precipitated proteins were removed by centrifugation (3000 $g \times 5$ min) and the supernatant was separated and stored at −80 °C before LC/MS analysis.

A control, in which the thiols were not derivatized, was conducted following the same procedure as described above except Ellman's reagent was replaced with phosphate buffer (0.01 M, pH 7).

The blood samples were immediately centrifuged at $2000 \times g$ for 5 min at 0 °C. Plasma and erythrocytes were separated. The packed erythrocytes were then washed twice with equal volumes of ice-cold isotonic saline solution. The erythrocytes were lysed by freezing and thawing three times to ensure complete cell lysis. Plasma (or erythrocytes) (1 ml) was added with ice-cold KCl solution (1.15%) (4 ml/ml tissue). 50 μl of the homogenate was transferred to a 1.5 ml snap-cap conical-bottom centrifuge vial, added with glutathione ethyl ester as an internal standard (I.S.)

(0.01 mg/ml, 20 μl) and Ellman's reagent (10 mM, 100 μl) and processed the same way as described above.

2.5. LC/MS analysis

The MS employed positive ion electrospray ionization. The HPLC eluate was introduced into the stainless-steel electrospray capillary spray held at 2.3 kV. The source and detector voltage were 20 and 650 V, respectively. The low- and high-mass resolutions were set at 12.5 during analysis. Selected ion monitoring (SIM) was set to simultaneously monitor ions with *m/z* of 269, 613, 319, 333, 505, 533, which correspond to the protonated molecular ions of homocystine, GSSG and Ellman's reagent derivatized CysSH (CysSH-Ellman), HCysSH (HCysSH-Ellman), GSH (GSH-Ellman), and internal standard (I.S.-Ellman). The HPLC conditions employed an Adsorbosil (Alltech, Deerfield, IL) C_{18} column (250 × 3.2 mm i.d., 5 μm), mobile phase A (aqueous solution with 0.1% (v/v) trifluoroacetic acid) and mobile phase B (acetonitrile). At time zero, mobile phase A was pumped isocratically for 2 min. Mobile phase B was increased from 0 to 60% from 2 to 13 min and held at 60% for an additional 2 min. All flow-rates were 0.6 ml/min. The injection volume was 20 μl.

3. Results

3.1. LC/MS analysis

Fig. 2 shows representative LC/MS chromatograms of a mixture of standards (Fig. 2a) and a rat brain tissue sample (Fig. 2b). The concentration of various compounds in the standard mixture was 5 μg/ml each (≈ 8–19 μM). As described earlier, the mass spectrometer was set to simultaneously monitor ions with *m/z* of 269, 613, 319, 333, 505 and 533, which correspond to the protonated molecular ions of homocystine, GSSG, CysSH-Ellman, HCysSH-Ellman, GSH-Ellman and I.S.-Ellman. Although CysSH-Ellman, GSH-Ellman, HCysSH-Ellman and I.S.-Ellman were eluted from HPLC very closely (with retention time of 12.05, 12.19, 13.96 and 14.30 min, respectively), there was no interference among these peaks, since they were detected in their individual channels (Fig. 2a). The LC/MS chromatogram reveals that GSSG, homocystine, GSH (as GSH-Ellman) and CysSH (as CysSH-Ellman) were detected in the brain sample (Fig. 2b). As can be seen in Fig. 2(b), peaks other than the desired analyte were also detected in each channel. These peaks were likely from other endogenous compounds with the same *m/z* as the analyte. An LC/MS analysis of a control sample in which the thiols were not derivatized was also conducted. The corresponding peaks of the Ellman's derivatives were not observed in the control sample, which provided reassurance that these peaks were from thiol derivatization with Ellman's reagent (data not shown). Quantitation of each analyte was based on the peak area ratio of the analyte to the internal standard and determined by reference to a standard curve. The standard curve was constructed by first derivatizing a thiol in 1.15% KCl solution, followed by addition of 5-sulfosalicylic acid, the same procedure as described for sample derivatization in Section 2. Due to large concentration differences of the analytes in different tissues, two standard curves covering a low concentration range (0.2–2 μg/ml) and a high concentration range (1–100 μg/ml) were constructed for each analyte. The curves demonstrated linearity ($r > 0.98$) over the concentration range. Quantitation results are presented in Table 1. For comparison, the reported values by Cotgreave et al. [19] of GSH and CysSH in rat liver, lungs and kidneys are included.

3.2. Detection limit of LC/MS

A detection limit was determined for each analyte (Table 2). Compared with other reported methods, this method is much more sensitive than HPLC with UV detection and comparable to or more sensitive than HPLC with fluorescence detection or electrochemical detection [8], or capillary electrophoresis [26,28].

3.3. Reproducibility of LC/MS

A reproducibility study was also carried out with a sample containing 5 μg/ml of each analyte

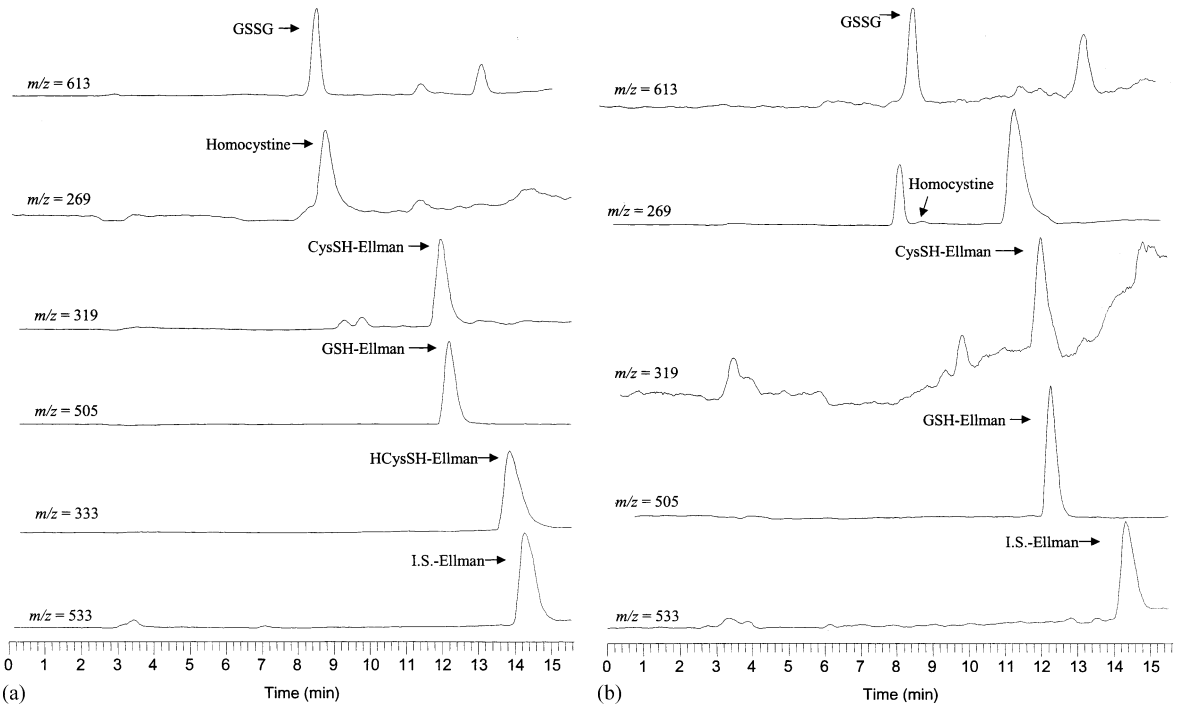


Fig. 2. Representative LC/MS chromatograms of (a) a mixture of standards (GSSG, homocystine, CysSH (as CysSH-Ellman), GSH (as GSH-Ellman), HCysSH (as HCysSH-Ellman)) and the internal standard (as I.S.-Ellman) at a concentration of 5 μg/ml each; and (b) a rat brain sample. The *m/z* of the ions being monitored in each channel are indicated in the figure.

and 1 μg/ml of I.S. in 1.15% KCl with 3% 5-sulfosalicylic acid. Reproducibility of the assay expressed as the R.S.D. for each analyte was 5.2, 4.3, 3.7, 2.8 and 5.8% ($n = 6$) for GSH (as GSH-Ellman), GSSG, cysteine (as CysSH-Ellman), homocysteine (as HCysSH-Ellman) and homocystine, respectively.

3.4. Recovery study

A recovery study was conducted. The recovery study for each analyte involved preparation of two samples—sample 1 derived from plasma and sample 2 obtained by spiking sample 1 with a known amount of an analyte (100 μM for a thiol

Table 1
Concentrations (nmol/g or mL tissue) of thiols and disulfides in various rat tissues determined by LC/MS

Sample	GSSG	GSH	GSH[a]	CysSH	CysSH[a]	Homocystine
Brain	11±1	950±59		639±163		308±14
Plasma	8±2	20±22		246±42		115±146
Heart	34±19	1346±385		55±39		n.d.[b]
Lung	31±8	1388±317	1010±149	281±28	14±7	n.d.[b]
Liver	348±325	7940±950	5198±278	220±140	62±11	n.d.[b]
Erythrocytes	18±2	2338±121		59±31		148±22
Kidney	12±7	110±90	2115±70	3710±350	77±82	n.d.[b]

Values are given as mean±S.E.M. ($n = 3$ separate rats).
[a] Literature values [19].
[b] Not detected.

Table 2
Detection limits of various analytes by the LC/MS analysis

Sample	Sample concentration (μM)	Injected analyte (pmol)
GSH-Ellman	0.16	3.3
GSSG	0.16	3.3
CysSH-Ellman	0.8	16.5
HCysSH-Ellman	1.5	29.6
Homocystine	0.75	14.9

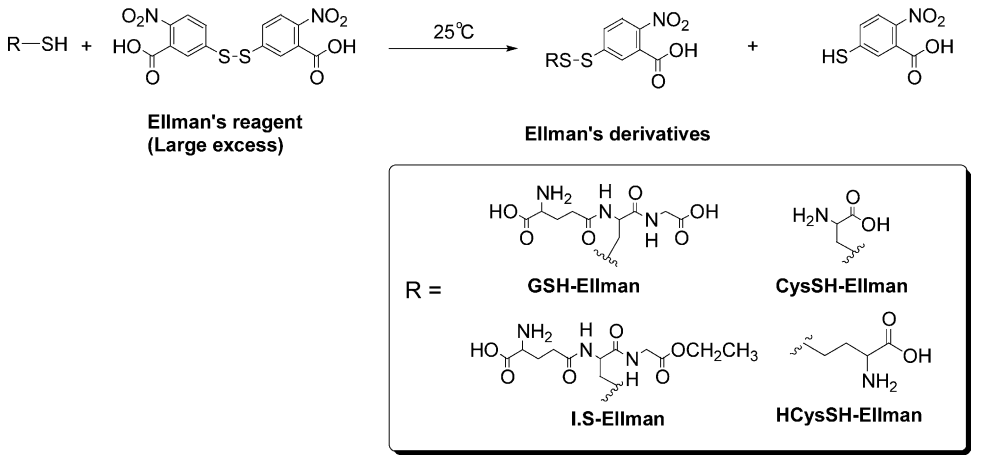
and 10 μM for a disulfide) [19]. The analyte in both samples was determined by LC/MS. The data obtained from subtraction of the analyte in sample 1 from that in sample 2 were used for recovery calculation. The recovery yields for GSH, GSSG, cysteine, homocysteine and homocystine were 112 ± 6, 110 ± 4, 86 ± 7, 99 ± 5 and $98 \pm 6\%$ ($n = 3$), respectively.

4. Discussion

Our initial aim was to develop an LC/MS method to measure thiol-containing compounds directly without derivatization. This was achieved without difficulty for GSH and GSSG (data not shown). However, we noticed that there was minor thiol oxidation during sample treatment, storage and transport. Oxidation of GSH to GSSG did not significantly affect the quantity of GSH due to its high concentration (millimolar range) in biological samples [1]. However, due to low levels of GSSG, which is $\approx 1\%$ of GSH in the body, the oxidation did significantly increase GSSG concentration. Therefore, we decided to block the oxidation by derivatizing the thiols with Ellman's reagent. Ellman's reagent has been extensively used to specifically derivatize thiols [38] and chromatographic methods have been reported for the derivatized GSH and CysSH [12,16,26]. Scheme 1 shows the chemical reaction and the structures of the derivatized GSH, CysSH, HCysSH and I.S. GSSG and homocystine have no thiol group and will not react with Ellman's reagent. An excess amount of Ellman's reagent is needed to ensure a complete derivatization of all thiols in the sample [38]. Without the excess Ellman's reagent, the formed derivatives, which are mixed disulfides, can also react with residual thiols (e.g. protein thiols) to form a different disulfide (Scheme 2) resulting in an inaccuracy of the assay. We have used twice as much Ellman's reagent per sample as in the procedures reported earlier [16,26]. Under this condition, the derivatization with both standards and biological samples was shown to be instantaneous and the concentrations of the formed Ellman's derivatives remained constant for 1 day at room temperature and 1 month when stored at -80 °C (data not shown). The increased chemical stability of the analytes provided convenience and increased the accuracy of the assay. It is worth noting that although the derivatization was added in the sample treatment process, as described in Section 2, this additional step required very little extra effort and time.

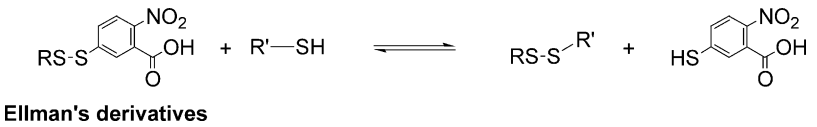
Our results revealed that GSH (as GSH-Ellman), cysteine (as CysSH-Ellman) and GSSG were detected in all tissues. Homocystine was found in the brain, plasma and erythrocytes, but not in the other tested tissue samples. No homocysteine (as HCysSH-Ellman) was detected in any of the tissue samples. The failure to detect homocysteine in normal rat tissues is not unexpected. Although we could not find data on the level of homocysteine in normal rat tissues, a total homocyst(e)ine (tHcy), which includes all forms of homocysteine (homocysteine, homocysteine-protein disulfides and homocystine), was reported to be only in the range of 0.5–6 nmol/g wet weight (liver: 3.79 ± 0.55; kidney: 1.21 ± 0.12; brain: 0.76 ± 0.07; heart: 0.97 ± 0.08; lung: 1.13 ± 0.08; spleen: 1.05 ± 0.07) [39]. In addition, homocysteine in human plasma was reported to be near absence [40]. Therefore, homocysteine, if present, was below the detection limit of this method. An attempt to detect cystine in tissues was unsuccessful due to earlier elution of the compound (retention time: 3.2 min with 100% water containing 0.1% TFA as mobile phase) where strong interferences caused by other endogenous compounds occurred.

Quantitation of the samples revealed that there was a good agreement of the quantity of GSH determined by our method and Cotgreave's in the liver and lungs (Table 1). However, the quantity of



Scheme 1. Derivatization of GSH, CysSH, HCysSH and the internal standard with Ellman's reagent.

GSH in the kidneys determined by our method was much lower than that determined by Cotgreave (110±90 vs. 2115±70 nmol/g tissue). In contrast, the quantity of CysSH in the kidney determined by our method was much higher than that determined by Cotgreave (3710±350 vs. 77±82 nmol/g tissue). In a separate report, the quantities of GSH and CysSH in the rat kidneys were reported to be 1049±940 and 239±19 nmol/g tissue, respectively [7], which is in a closer agreement with the data obtained by Cotgreave. GSH quantities obtained by enzyme assays were also in line with Cotgreave's report [41,42]. This big discrepancy between our results and the literature values prompted us to conduct experiments: (a) to verify the identity of the peak assigned to be CysSH-Ellman; and (b) to find out whether the low kidney level of GSH determined by our method was a real reflection of kidney GSH or an error related to our method. To verify the identity of the peak assigned as CysSH-Ellman, an LC/MS/MS analysis of the kidney sample was conducted. LC/MS/MS can provide the mass spectrum of the analyte and conclusively verify the identity of the peak. LC/MS/MS analysis of the kidney samples revealed that the peak assigned as CysSH-Ellman from the sample exhibited an identical mass spectrum as that of a synthetic standard of the CysSH-Ellman (Fig. 3). Thus, we concluded that the detected peak in the sample was, indeed, from CysSH-Ellman and our values of CysSH in the rat kidney were correct. Several approaches were taken to verify the low concentration of GSH in the kidney samples. First, we performed an LC/MS analysis of the kidney control samples, where no derivatization was conducted, by monitoring GSH (*m*/*z* 308) directly instead of monitoring GSH-Ellman. The GSH level was found to be 102±73 nmol/g tissue, consistent with the quantitation obtained through derivatization (110±90 nmol/g tissue, Table 1). We also checked the same sample with a different LC/MS instrument and similar data was obtained (data not shown). Then a question was raised whether our sample treatment process could cause a loss of GSH in the rat kidney samples. There was a minor difference in the order of protein precipitation between our method and most other methods for GSH determination. With most other methods, tissues were homogenized under an



Scheme 2. Decomposition of Ellman's derivatives without excess Ellman's reagent.

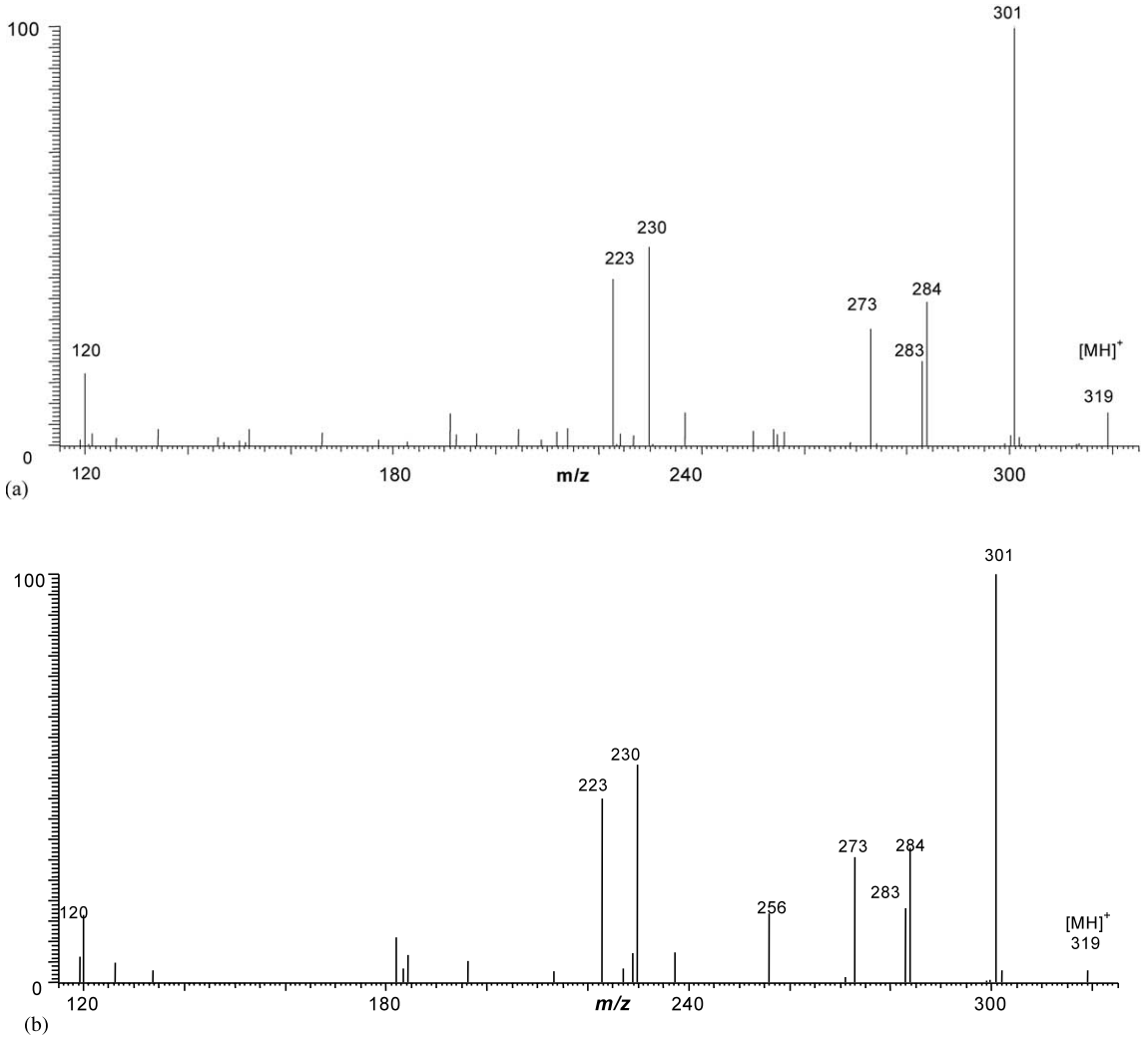


Fig. 3. Product ion spectra of the ion at *m/z* 319 [$(MH)^+$ of CysSH-Ellman)] obtained using an ion trap mass spectrometer from (a) a synthetic standard of CysSH-Ellman; and (b) a rat kidney sample.

acidic condition in which proteins precipitate during homogenization. Our samples were homogenized under a neutral condition for 1–2 min before derivatization of thiols. There was evidence that kidney GSH could be degraded by γ-glutamyl transpeptidase of renal tubules [43]. The question was whether during the homogenization the enzyme could significantly reduce GSH levels. To check this possibility, we homogenized samples in 6.5% (w/v) trichloroacetic acid (TCA) as reported by Cotgreave et al. [19]. After removal of proteins, the sample was neutralized with 8% NaOH before the addition of the internal standard and Ellman's reagent. An LC/MS analysis of the rat kidney samples revealed that the quantity of GSH was 188 ± 105 nmol/g tissue consistent with our earlier GSH values (Table 1). We also determined GSH levels in plasma (46 ± 14 nmol/g tissue) and brain (172 ± 188 nmol/g tissue) with this sample treatment condition. These data confirmed that no significant degradation of GSH occurred during the 2-min homogenization of tissue samples under a neutral condition compared with the homogenization conducted under an acidic condition.

Therefore, we conclude that the GSH and cysteine levels in the kidney samples determined by our method were accurate.

Although no homocysteine (as HCysSH-Ellman) was detected in any of the tissue samples, the homocystine quantity determined by our method in rat plasma was higher than that reported for total homocyst(e)ine (115 ± 146 vs. 6–10 nmol/ml plasma) [44]. It is worth noting that with an additional step in sample treatment, that is to reduce homocystine and homocystine-protein disulfides to homocysteine with a reducing agent [25], our method will also be able to measure total homocyst(e)ine in biological samples. Being able to determine homocysteine, homocystine and total homocyst(e)ine will be an added advantage of this method over others in delineating the role of this amino acid in cardiovascular diseases.

5. Conclusion

In summary, we have developed an LC/MS method to simultaneously detect and quantify GSH, GSSG, CysSH, HCysSH and homocystine in biological samples. The assay requires minimal sample treatment and offers high sensitivity and selectivity. One disadvantage of this method is the cost and availability of the LC/MS equipment. However, with the appearance of less expensive benchtop LC/MS instruments (the one used in this investigation), LC/MS has become increasingly available in most research laboratories and hospitals. Our method will be a useful tool in the investigation of the roles of these endogenous thiol-containing compounds and their corresponding disulfides in physiological and pathological processes.

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