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An ion exchange liquid chromatography/mass spectrometry method for the determination of reduced and oxidized glutathione and glutathione conjugates in hepatocytes

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Abstract

A rugged LC-MS/MS method was developed to quantify reduced and oxidized glutathione (GSH and GSSG, respectively) in rat hepatocytes. In addition, GSH conjugates can be detected, characterized and measured in the same analysis. Samples were treated with acetonitrile and iodoacetic acid to precipitate proteins and trap free GSH, respectively. These highly polar analytes were separated by ion exchange chromatography using conditions that were developed to be amenable to electrospray ionization and provide baseline chromatographic resolution. A solvent gradient with a total run time of 13 min was used to elute the analytes, as well as any highly retained components in the samples that would otherwise accumulate on the HPLC column and degrade the chromatography. The analytes were detected using either selected ion monitoring (SIM) using an ion trap mass spectrometer or selected reaction monitoring (SRM) using a triple quadrupole mass spectrometer. The ranges for quantification of GSH and GSSG using an ion trap were 0.651–488 μM and 0.817–327 μM, respectively. Using SRM with the triple quadrupole instrument, the ranges of quantification for GSH and GSSG were 0.163–163 μM and 0.0816–81.6 μM, respectively. The accuracy and precision for both methods were within 15%. The utility of the method was demonstrated by treating rat hepatocytes with model compounds menadione and precocene I. Menadione, which contains a quinone moiety that undergoes redox cycling and induces concentration- and time-dependent oxidative stress in hepatocytes, resulted in decreased GSH concentrations with concomitant increase in concentrations of GSSG, as well as a GSH–menadione conjugate. When hepatocytes were incubated with precocene I, a time-dependent decrease in GSH concentrations was observed with concomitant increase in a GSH–precocene conjugate. GSSG concentrations did not increase in the presence of precocene I, consistent with its lack of redox activity. This analytical method has general utility for simultaneously investigating the potential of test compounds to induce both oxidative stress from redox cycling in vitro and the formation of GSH conjugates.

Keywords: Quinone moiety; GSH–menadione conjugate; Selected ion monitoring (SIM)

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1. Introduction

Glutathione (GSH) is an important intracellular cysteine-containing tripeptide that plays a key role in maintaining cellular homeostasis, as well as protecting the cell against reactive electrophiles and oxidative stress. The nucleophilic sulfhydryl group of GSH can react with potentially toxic electrophiles, such as epoxides and Michael-type acceptors, to form conjugates. Alternatively, oxidizing agents, such as hydrogen peroxide, can be reduced by GSH with concomitant formation of oxidized glutathione (GSSG). Measurement of GSH and GSSG concentrations in tissues or cells is a sensitive indicator of their redox status. GSSG is an especially sensitive means of assessing redox status because its concentration is normally maintained at very low levels relative to GSH [1,19–23].

Numerous analytical methods are available to quantify both GSH and GSSG in biological systems, and many of these involve chromatographic separation using HPLC (e.g. see references [1–9]). Since GSH and GSSG lack chromophores, the compounds are typically chemically derivatized prior to analysis to enhance detectability using UV and fluorescence detectors [1–7,9]. Derivatization is also often required to improve the retention of these highly polar molecules with reversed-phase chromatography.

Mass spectrometry offers the ability to detect and characterize polar analytes without a need for derivatization to facilitate detection, and atmospheric pressure ionization sources (electrospray and atmospheric pressure chemical ionization) are readily interfaced with reverse phase HPLC for on-line analyses. HPLC coupled with tandem mass spectrometry (LC-MS/MS) also provides outstanding sensitivity and specificity for analyses with complex (dirty) biological matrices, as well as the capability to provide both quantitative and qualitative chemical structure data ([10–12] and references therein). Numerous reports in the literature have described the use of LC-MS/MS to analyze glutathione conjugates (e.g. see references [13–17]). In most cases, the xenobiotic portion of the conjugate imparts sufficient hydrophobicity to facilitate adequate retention by reverse phase HPLC and precludes the need for derivatization.

We have developed a rugged ion exchange LC-MS/MS method to quantify GSH and GSSG simultaneously in rat hepatocytes. In addition, conjugates formed from the reaction of glutathione with electrophiles can be detected and characterized by mass spectrometry, in the same analysis. Ion exchange chromatography was utilized to retain and separate these highly polar analytes and eliminate the need for derivatization with hydrophobic reagents. The ion exchange chromatography conditions were developed to be compatible with these relatively high-salt samples, yet were readily compatible with electrospray ionization mass spectrometry. Preparation of samples containing intact cells and culture medium utilized treatment with iodoacetic acid, to prevent oxidation or other reactions of GSH during sample preparation or storage similar to many other methods (e.g. see references [1,2,28]), along with addition of acetonitrile to precipitate proteins prior to injection into the HPLC. We have demonstrated the utility of this methodology using hepatocytes treated with two model compounds, menadione [18–24] and precocene I [25,26].

2. Materials and methods

2.1. Chemicals

GSH, GSSG, gamma-glutamylglutamic acid (γ-Glu-Glu), iodoacetic acid (IAA), menadione, and precocene I were obtained from Sigma Chemicals (St Louis, MO). An aqueous solution containing 10 mM IAA in 10 mM aqueous ammonium bicarbonate, pH 10 that was used to derivatize free thiols was prepared daily. Formic acid (90%) and acetonitrile (HPLC-grade) were purchased from Fisher Scientific (Pittsburgh, PA). Water was obtained from a Milli-Q system (Millipore, Milford, MA). L-15 medium was obtained from Gibco BRL (Grand Island, NY).

2.2. Standard solutions

The GSH in stock solutions was derivatized with IAA [1,2,28] as described below to form the

S-carboxymethyl derivative (GSH-CM) prior to dilution into calibration standards. The derivatization efficiency was shown to be quantitative and reproducible in biological samples and in stock solutions. Stock solutions of GSH-CM, GSSG, and γ-Glu-Glu (internal standard) were prepared by dissolving appropriate amounts of the compounds in water/acetonitrile (1:1). Calibration standards were prepared by adding 25 μl of an appropriate stock solution containing GSH-CM and GSSG to 100 μl of cell-free L-15 medium. The internal standard, γ-Glu-Glu, was prepared in water/acetonitrile (1:1) to a final concentration of 1 μg/ml for analyses with the triple quadrupole mass spectrometer and 100 μg/ml for analyses with the ion trap mass spectrometer.

The menadione–GSH conjugate (GS–menadione) was prepared by dissolving menadione in dimethylsulfoxide and adding GSH. GS–menadione formation was evaluated using varying ratios of GSH to menadione. In one case, the amount of GSH used was held constant (3.3 mM) and menadione was added in molar ratios of 1.8, 3.5, 8.8, and 18. The amount of GS–menadione formed reached a plateau when menadione was added in a 3.5 molar excess and did not increase with increased amounts of menadione. The reaction appeared to be complete in less than 1 h based on LC-MS analysis. At this time, the amount of GS–menadione had reached a plateau and neither unreacted GSH, nor GSSG was detected. Thus, for the purposes of this study we assumed that the reaction had reached completion. The conjugate prepared using this procedure had the same molecular weight, HPLC retention time, and product ion mass spectrum as the conjugate observed in hepatocytes following incubation with menadione.

When the amount of menadione used was held constant (5.8 mM) and GSH was present in molar ratios of 0.6, 1.1, 2.8, 5.7, and 11, the yield of GS–menadione decreased with increasing GSH concentration. The yield of GS–menadione was always lower than that observed when menadione was present in excess amounts relative to GSH. This suggested that either the excess GSH may have created a reducing environment that prevented the reaction of GSH with menadione (possibly by reducing the menadione to the hydroquinone which would not react with GSH), or excess menadione was required to oxidize the initial menadione–GSH Michael addition product to the final product that was detected in hepatocytes.

2.3. Hepatocyte experiments

Rat hepatocytes were prepared by collagenase (Sigma Chemical Co, St Louis, MO) perfusion [27], suspended in L-15 medium to a density of 1×10^6 cells/ml, and maintained at 37°C by gently swirling in flasks in a water bath under an atmosphere of O_2/CO_2 (95% 5%). The cell suspensions were preincubated for 60 min and then treated with two concentrations of either menadione (30 or 300 μM) or precocene I (250 or 1000 μM) and 100 μl samples were removed at various times for analysis of GSH, GSSG, and potential GSH-conjugates. Menadione and precocene I were dissolved in DMSO prior to addition to hepatocytes and final concentration of DMSO in the hepatocyte incubation was $< 1\%$.

2.4. Sample preparation

Hepatocyte incubation samples (100 μl containing cells and medium) were treated with 100 μl of 10 mM IAA in 10 mM aqueous ammonium bicarbonate (pH 10, pH adjusted with ammonium hydroxide), and allowed to react in the dark for 1 h. An aliquot of internal standard solution (25 μl) and 25 μl of acetonitrile/water (1:1) (or 25 μl of GSH/GSSG stock solution for calibration standards) were added, followed by 400 μl of acetonitrile to precipitate proteins. Water (200 μl) was added so that the sample solvent proportions were similar to the initial chromatographic conditions, and the samples were centrifuged at 14 000 rpm for 10 min. An aliquot (5–25 μl) of the supernatant was injected onto the HPLC.

2.5. High–performance liquid chromatography

The HPLC system consisted of a Perkin Elmer (Norwalk, CT) Series 200 autosampler and pumps (high pressure gradient mixing) that were inter-

faced to the mass spectrometer. Chromatographic separation was performed on a Hamilton PRP-X110S anion exchange 100×2.1 mm, 7 μm column at 30°C using either isocratic or gradient methods. At the time of these studies, columns of this size were special ordered from the supplier (Phenomenex, Torrance, CA). Solvent A was 0.1% aqueous formic acid/acetonitrile (1:1) and Solvent B was 2.0% aqueous formic acid/acetonitrile (1:1). Analytes were eluted from the HPLC column using the following linear gradient conditions: 0 min, 0% B, flow 0.25 ml/min; 1.0 min, 0% B, flow 0.25 ml/min; 4.0 min, 100% B, flow 0.25 ml/min; 6.5 min, 100% B, flow 0.25 ml/min; 7.0 min, 0% B, flow 0.5 ml/min; 12.0 min, 0% B, flow 0.5 ml/min; 13.0 min, 0% B, flow 0.25 ml/min. It was critical that the HPLC pumps had a very low dead volume to provide efficient delivery of the gradient solvents to the HPLC column and rapid equilibration of the HPLC column between injections. Isocratic conditions were also evaluated using the same HPLC column with a mobile phase composed of 0.5% aqueous formic acid /acetonitrile (1:1) and a flow rate of 0.25 ml/min.

2.6. Mass spectrometry

Electrospray-ionization mass spectrometry was performed in positive ion mode using either a Finnigan LCQ Ion Trap Mass Spectrometer (ThermoQuest, San Jose, CA) or a Sciex API 3000 Triple Quadrupole Mass Spectrometer (Thornton, Ontario, Canada).

The Finnigan LCQ was operated with the capillary temperature at 215°C, sheath gas at 90 (arbitrary units) and auxiliary gas at 40 (arbitrary units). The electrospray voltage was set to 4 kV with a tube lens offset of 10 V. The HPLC flow was diverted from 0 to 2.8 min and after 4.3 min to prevent contamination of the heated capillary by the large amounts of salts in the samples. Analyses were done in MS^1 mode scanning from m/z 190 to 650. Analytes were quantified based on peak areas from their respective reconstructed ion chromatograms for the protonated molecular ions (GSH-CM, m/z 366; GSSG m/z 307 + m/z 613 (sum of the singly and doubly charged ions); Internal Standard, m/z 277). A significant signal at m/z 307 was observed for doubly charged GSSG using the Finnigan electrospray source.

The Sciex API 3000 was operated with Turbo Ionspray® at a temperature of 400°C and a gas flow of 7 L/min. The ionspray voltage was 4.5 kV with the nebulizer gas set to 8 (arbitrary units) and curtain gas set to 10 (arbitrary units). The collision energy was 35 eV with the collision gas set to 8 (arbitrary units) and an orifice potential of 50 V. Analytes were detected in selected reaction monitoring (SRM) mode at unit resolution using the following transitions: GSH-CM, m/z $366 \rightarrow 237$; GSSG, m/z $613 \rightarrow 355$; γ-Glu-Glu (Internal Standard), m/z $277 \rightarrow 148$. There was no significant signal at m/z 307, representing doubly charged GSSG, observed with Turbo Ionspray. The transitions of m/z $366 \rightarrow 237$ for GSH-CM and m/z $277 \rightarrow 148$ for γ-Glu-Glu both corresponded to the elimination of pyroglutamic acid (129 amu). Similarly, the transition of m/z $613 \rightarrow$ 355 for GSSG represented loss of two pyroglutamic acid moieties (258 amu).

3. Results and discussion

3.1. Chromatography

Ion exchange chromatography conditions were developed to accommodate the highly polar nature of glutathione and its related products. The isocratic conditions described in Section 2 provided good chromatographic peak shape and a relatively short analysis time of under 4 min (data not shown). Analysis of hepatocyte or control medium samples, however, resulted in progressive and severe degradation of chromatographic performance characterized by decreasing retention times with complete loss of analyte peaks after 6–8 injections. This problem was attributed to a cumulative saturation of the column by strongly retained components in these samples. Chloride (Cl^-) was present in concentrations in excess of 100 mM, and bicarbonate (used to buffer the hepatocyte medium) concentrations were high as well. Both of these components bind to anion exchange columns more tightly than carboxylates [29] and require more strongly eluting conditions

to prevent them from accumulating on the column. Gradient conditions were, therefore, developed to clean the column more thoroughly between injections. An example chromatogram is shown in Fig. 1(A). The analytes described in this paper eluted in less than 5 min, although the total analysis lasted 13 min to provide thorough washing and re-equilibration of the column between injections. The gradient resulted in significantly narrower peaks than were obtained using isocratic conditions and, therefore, improved sensitivity. Retention times and analyte responses were stable

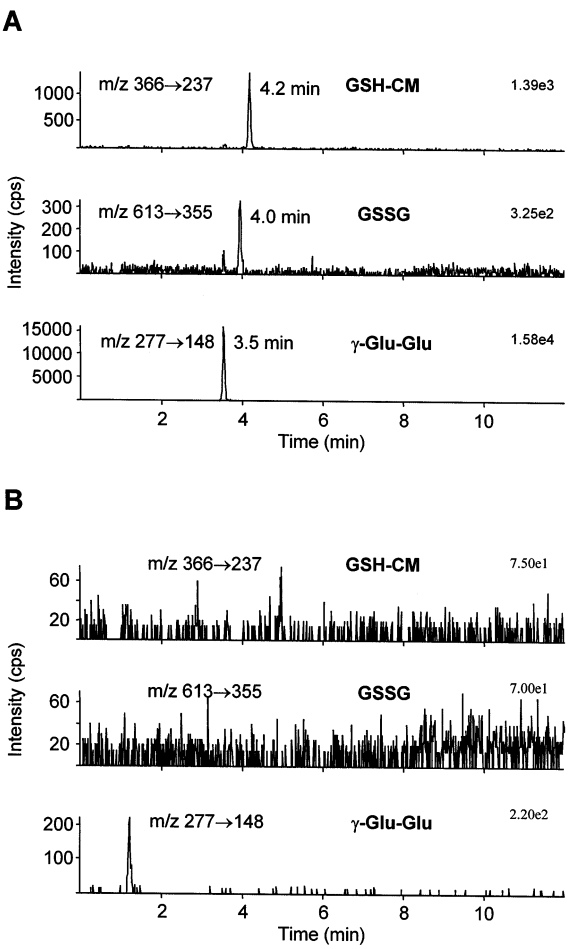

Fig. 1. (A) Mass chromatogram from SRM analysis of control medium spiked with 100 ng/ml GSH-CM (m/z 366 → 239), 100 ng/ml GSSG (m/z 613 → 355), and 500 ng/ml γ-Glu-Glu (internal standard, m/z 277 → 148). (B) Chromatogram from control medium.

even after 36 h of continuous analyses. For example, analyses were performed on the LCQ system continuously for approx. 36 h and the internal standard response had a 6% RSD during that time. Columns from different production lots were used and provided essentially identical chromatographic results. The chromatogram in Fig. 1(B) shows that no interfering signals were observed with control medium.

3.2. Derivatization and recovery

Samples were treated with IAA to derivatize the free thiol of GSH and prevent oxidation of GSH to GSSG during the sample preparation and analysis [1,2,28]. In order to evaluate the derivatization efficiency of GSH and the recovery of GSH-CM and GSSG from hepatocyte samples, a known amount of a freshly prepared solution containing GSH and GSSG was spiked into a hepatocyte mixture and then derivatized with IAA to verify that derivatization was complete and recovery was quantitative. The endogenous GSH and GSSG concentrations in the hepatocytes were also determined and were subtracted from the amounts measured in the spiked hepatocyte samples. The results were compared to a calibration line prepared using GSH-CM and GSSG as described in Materials and Methods. Recovery of GSH and GSSG from hepatocytes was $> 80\%$.

3.3. Calibration: sensitivity and linearity

The method was validated using both ion trap and triple quadrupole mass spectrometers. As expected, the triple quadrupole operated in SRM mode provided better sensitivity than the ion trap operated in scanning MS^1 mode. The ion trap was operated in scanning MS^1 mode because selected ion monitoring methods did not provide additional sensitivity; however, the use of scanning MS^1 on the ion trap provided the opportunity to re-evaluate data post-acquisition for the presence of metabolites formed via glutathione conjugation. Analytes were quantified using reconstructed ion chromatograms. Results from a series of calibrations performed on consecutive days are summarized in Table 1. Using the ion trap, the

Table 1
Precision and accuracy of calibration standards for GSH (measured as the S-carboxymethyl derivative, GSH-CM) and GSSG analyzed on consecutive days by MS^1 using a Finnigan LCQ ion trap mass spectrometer[a]

Nominal concentration	0.200	0.500	1.00	2.00	5.00	10.0	25.0	50.0	100	150	200
GSH concentration ($\mu g/ml$)											
Curve 1	0.214	0.422	0.930	2.03	5.24	10.7	26.9	51.0	87.1	147	
Curve 2	0.197	0.518	0.990	1.98	5.03	10.8	25.6	44.7	89.3	134	
Curve 3	0.198	0.510	0.986	2.05	4.97	10.6	26.8	43.4	85.2	142	
Curve 4	0.228	0.342	0.832	2.24	5.51	11.6	27.6	45.4	88.6	140	
Curve 5	0.208	0.482	0.854	1.92	5.38	10.9	28.0	46.9	92.2	139	
Mean	0.209	0.455	0.918	2.04	5.23	10.9	27.0	46.3	88.5	140	
C.V. (%)	6.02	16.2	7.97	5.79	4.40	3.59	3.39	6.36	2.96	3.41	
Accuracy	105	91.0	91.8	102	105	109	108	92.6	88.5	93.5	
GSSG concentration ($\mu g/ml$)											
Curve 1	ND[b]	0.498	1.01	2.01	4.83	10.1	25.1	51.6	96.2	151	201
Curve 2	0.186	0.600	0.966	1.97	4.87	9.61	25.9	47.2	96.4	150	213
Curve 3	ND	0.494	1.01	2.08	4.89	10.0	26.2	47.2	95.1	143	231
Curve 4	ND	0.484	1.07	1.98	4.98	9.83	26.0	48.2	95.7	150	210
Curve 5	ND	0.500	1.03	1.85	5.23	9.74	26.5	48.5	100	146	205
Mean	0.186	0.515	1.02	1.98	4.96	9.86	25.9	48.6	96.7	148	212
C.V. (%)	NA	9.28	3.58	4.18	3.24	1.97	1.92	3.72	2.04	2.30	5.33
Accuracy	93.0	103	102	98.9	99.2	98.6	104	97.1	96.7	98.7	106

[a] Standard solutions prepared by spiking carboxymethylated GSH (GSH-CM), GSSG, and γ-Glu-Glu (internal standard) into L-15 medium were analyzed by LC-MS using an ion trap operated in scanning (m/z 190-650) MS^1 mode. Ion chromatograms were reconstructed for GSH-CM (m/z 366), GSSG (m/z 613+307), and Glu-Glu (m/z 277) for determination of peak areas for quantitation. Calibration lines were weighted $1/X^2$ with quadratic curve fitting.

[b] ND — Not detected

Table 2
Precision and accuracy of calibration standards for GSH (measured as the S-carboxymethyl derivative, GSH-CM) and GSSG Analyzed by SRM using a Sciex API 3000 triple quadrupole mass spectrometer[a]

Nominal concentration	0.0500	0.100	0.200	0.500	1.00	2.00	5.00	10.0	25.0	50.0
GSH concentration (μg/ml)										
Curve 1	0.0536	0.937	0.192	0.471	1.01	2.00	5.24	10.3	24.2	50.7
Curve 2	0.0561	0.984	0.198	0.475	1.01	1.98	5.20	10.6	24.6	50.7
Curve 3	0.0515	0.895	0.192	0.491	1.04	2.06	5.19	10.5	24.0	49.7
Curve4	0.0483	0.892	0.187	0.495	1.02	2.04	5.19	10.7	24.3	48.9
Mean	0.0524	0.927	0.192	0.483	1.02	2.02	5.21	10.5	24.3	50.0
C.V. (%)	6.32	4.65	2.36	2.42	1.25	1.86	0.4	1.86	0.96	1.69
Accuracy	105	92.7	96.2	96.7	102	101	104	105	97.2	100
GSSG concentration (μg/ml)										
Curve 1	0.0587	0.0864	0.185	0.533	0.975	2.09	5.23	10.6	23.2	50.7
Curve 2	0.0546	0.0910	0.187	0.536	1.02	2.07	5.31	10.7	23.7	50.1
Curve 3	0.0500	0.0933	0.186	0.538	0.963	2.02	4.72	9.66	25.4	50.4
Curve4	0.0501	0.0797	0.196	0.491	1.04	2.07	5.00	10.2	25.7	49.2
Mean	0.0535	0.0876	0.188	0.524	1.00	2.07	5.07	10.3	24.5	50.1
C.V. (%)	7.42	6.85	2.68	4.29	3.66	1.71	5.22	4.61	5.06	1.34
Accuracy	107	87.6	94.1	105	100	103	101	103	98.1	100

[a] Standard solutions prepared by spiking carboxymethylated GSH (GSH-CM), GSSG, and γ-Glu-Glu (internal standard) into L-15 medium were analyzed by LC-MS using a triple quadrupole mass spectrometer operated in SRM mode. Ion chromatograms were reconstructed for GSH-CM (m/z 366→237), GSSG (m/z 613→355), and Glu-Glu (m/z 277→148) for determination of peak areas for quantitation. Calibration lines were weighted $1/X^2$ with quadratic curve fitting.

method was accurate and precise from 0.200–150 μg/ml GSH and 0.500–200 μg/ml GSSG (0.651–489 μM and 0.816–326 μM for GSH and GSSG, respectively). Although the sensitivity was inadequate to detect basal levels of GSSG in hepatocytes, this instrument was readily able to detect the elevated concentrations of GSSG that resulted from treatment with menadione. Generally, calibrations were weighted $1/X^2$ with a quadratic fit due to a slight curvature. The method was also validated on different column lots and different days, and results were precise and accurate on each day. Although MS^1 detection provides less specificity than tandem MS methods, no interferences were detected for any of the analytes tested.

Detection using the triple quadrupole mass spectrometer operated in SRM mode provided excellent sensitivity and specificity. The results from a series of calibrations performed on a single day are summarized in Table 2. The method was accurate and precise from 0.0500–50.0 μg/ml for both GSH and GSSG (0.163–163 μM and 0.0816–81.6 μM for GSH and GSSG, respectively).

3.4. Effects of model compounds on GSH and GSSG

3.4.1. Menadione

Menadione is a quinone that can induce oxidative stress in cells via redox cycling, or can serve as a Michael-type acceptor and form a covalent conjugate with GSH [18–24]. Rat hepatocytes were treated with menadione (30 and 300 μM) in suspension culture to determine the changes in GSH-related components with time. Hepatocyte incubation samples were first analyzed by LC-MS using the ion trap with the gradient chromatography conditions described above to investigate the presence of GS–menadione conjugates (Fig. 2). An ion was observed at m/z 478 that was consistent with the $[M + H]^+$ for a GS–menadione conjugate formed via addition of GSH to menadione and a two electron oxidation. LC-MS using an ion trap mass spectrometer facilitated the application of MS^n analyses to characterize the structure of the conjugate. The resulting product ion mass spectra for the conjugate, as well as the

proposed fragmentation scheme, are shown in Fig. 3. The data obtained from dissociating the ion sequentially using MS^n were key in assigning structures to the fragment ions and were consistent with formation of an intramolecular Schiff base intermediate during the dissociation pathway. The proposed conjugate structure is in agreement with the structure that was previously described [24].

Since the GS–menadione conjugate could be detected in the same analysis as GSH and GSSG, the concentrations of GSH, GSSG, and GS–menadione were determined as indicators of oxidative stress and cellular GSH status. The results are shown in Fig. 4. Preliminary studies confirmed that GSH was retained primarily in the hepatocytes while GSSG was rapidly secreted into the medium (data not shown). In order to assess the mass balance of total GSH products present in the incubation mixture, samples were collected from the hepatocyte incubation suspension (containing both hepatocytes and medium). GSH concentrations decreased while GSSG and GS–menadione concentrations increased concomitantly in a time- and menadione concentration-dependent manner. The basal concentration of GSSG measured in the combined hepatocyte/medium samples was approx. 0.6 μM and was readily quantifiable with the triple quadrupole instrument. The amount of GSSG formed increased approx. 2-fold with a 10-fold increase in menadione concentration, but the amount of the GS–menadione conjugate detected increased more than 20-fold with 300 μM menadione indicating a predominance of conjugation relative to redox cycling at higher menadione concentrations.

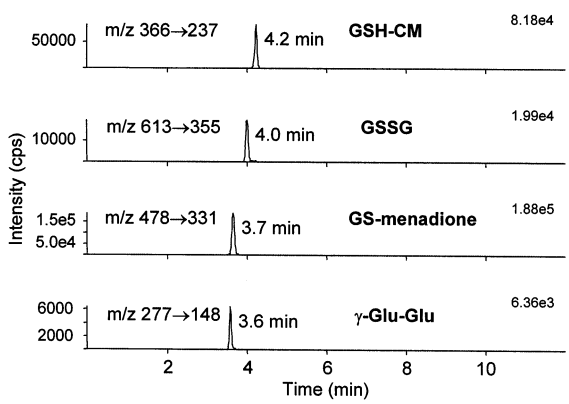

Fig. 2. Reconstructed ion chromatogram from a triple quadrupole mass spectrometer (SRM) for a 15 min hepatocyte incubation sample (300 μM menadione) showing the presence of GSH-CM (m/z 366 → 237), GSSG (m/z 613 → 355), the GSH–menadione conjugate (m/z 478 → 331), and the internal standard γ-Glu-Glu (m/z 277 → 148).

3.4.2. Precocene I

Precocene I is a naturally occurring insect growth regulator. It is well know that in mammals precocene I causes extensive cytochrome P-450-dependent hepatic necrosis in vivo that has been proposed to be mediated by an epoxide metabolite [25,26]. This metabolite is detoxified by conjugation with glutathione and can result in glutathione depletion. Because precocene I lacks the ability to redox cycle, it would not be expected to result in extensive formation of GSSG. Precocene I was, therefore, incubated with rat hepatocytes at 250 and 1000 μM to serve as a model compound that would deplete glutathione without concomitant formation of GSSG. The analyses for glutathione related products were conducted using an ion trap operated in scanning MS^1 mode.

Example mass chromatograms are shown in Fig. 5. Single ion chromatograms were reconstructed for GSH and GSSG and concentrations for these components were determined relative to their corresponding calibration lines. In addition, an ion was observed at m/z 514 that was consistent with the $[M + H]^+$ for the glutathione conjugate of precocene I epoxide (GS–precocene). This ion was not observed in control hepatocytes that were not exposed to precocene I, but was formed in a time-dependent manner upon treatment of hepatocytes with precocene I. CID analysis of this compound using a triple quadrupole mass spectrometer yielded a product ion mass spectrum (Fig. 6) that was consistent with the proposed structure. The ion at m/z 385 was consistent with loss of the glutamyl moiety from glutathione and the ion at m/z 308 corresponded to protonated GSH following loss of precocene. The base peak at m/z 207 resulted from elimination of GSH from the conjugate. Results for the GSH and

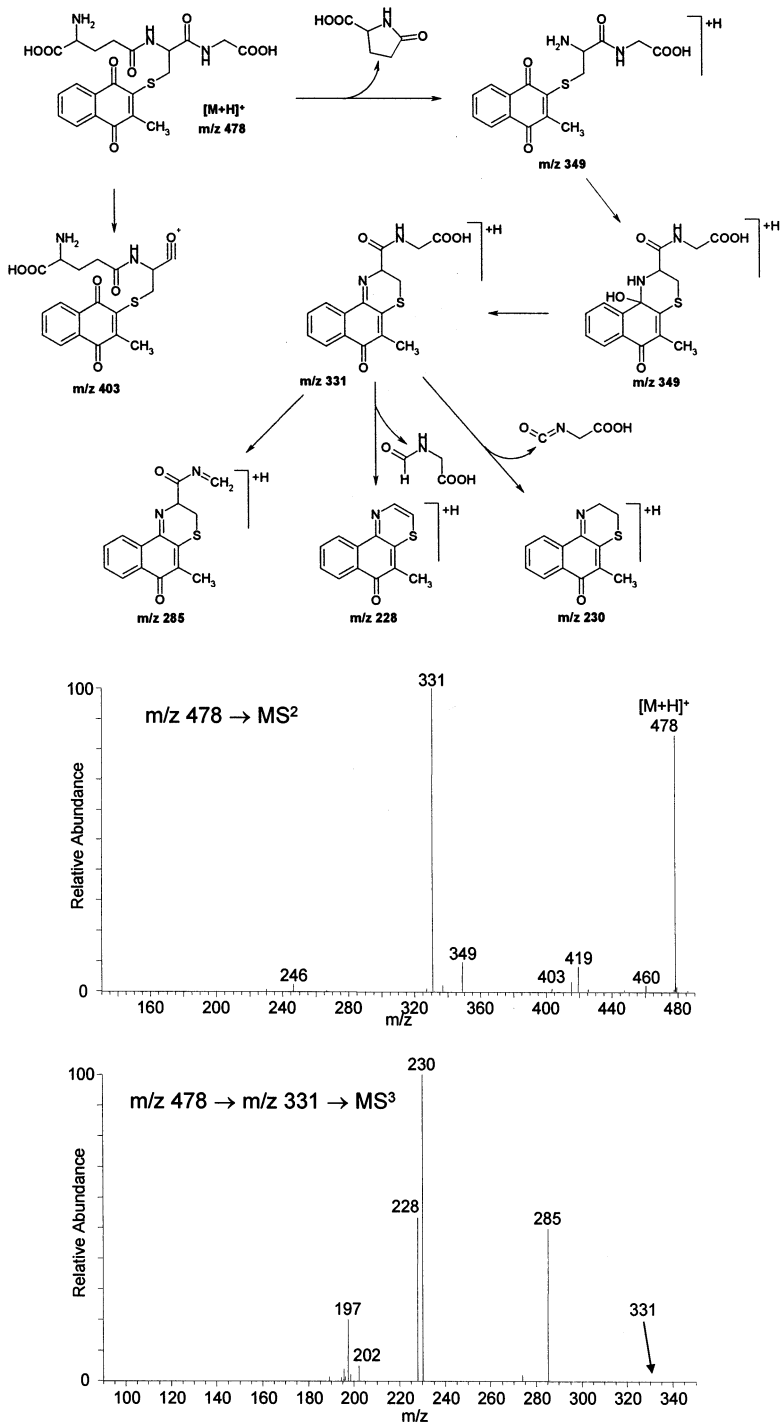

Fig. 3. Product ion mass spectra (MS^2 and MS^3) obtained using an ion trap mass spectrometer and fragmentation scheme for the glutathione conjugate of menadione ($[M+H]^+$ at m/z 478) that was formed by hepatocytes.

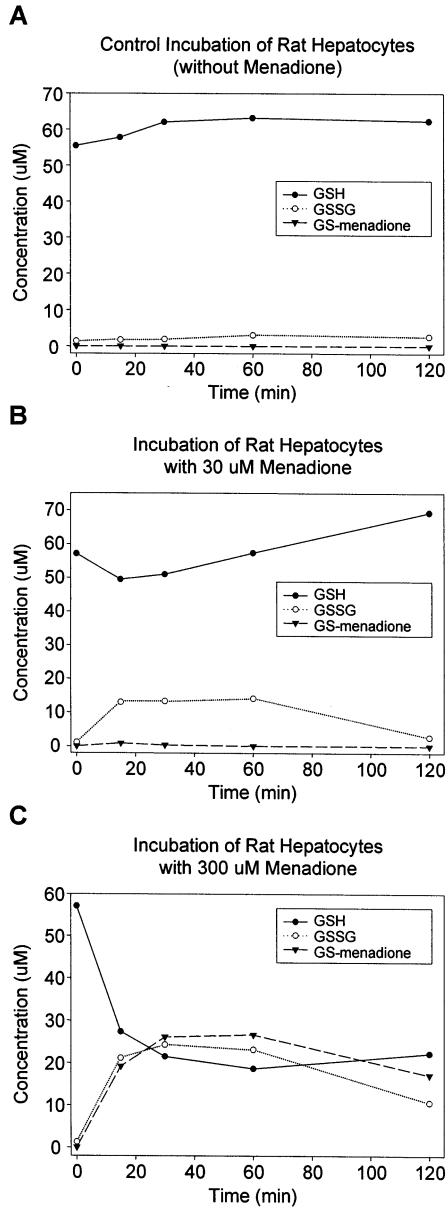

Fig. 4. Concentrations of GSH, GSSG and the GS–menadione conjugate in control hepatocyte incubations (a) and following treatment with menadione at 30 μM (b) and 300 μM (c) determined by LC-MS/MS using a triple quadrupole instrument operated in SRM mode.

GSSG concentrations in the hepatocyte incubations are summarized in Fig. 7. The GS–precocene conjugate could not be quantified due the lack of an authentic standard to generate calibration samples. The results for GS–precocene (also shown in Fig. 7) are based on relative peak area values. In the presence of 250 μM precocene I, GSH concentrations decreased with time while the amounts of the conjugated metabolite increased over the 3 h incubation. GSSG concentrations remained below the lower limit of quantification consistent with the lack of redox activity with precocene. Concentrations of GSH decreased more quickly over time with 1000 μM precocene I. Concentrations of the conjugated metabolite increased over 50 min, but plateaued

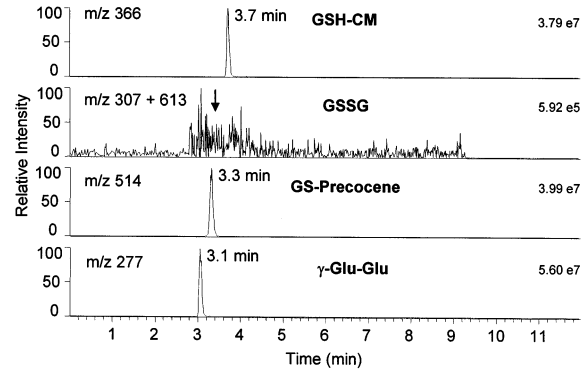

Fig. 5. (Top) Reconstructed ion chromatogram from an ion trap mass spectrometer for a 60 min hepatocyte/medium sample (250 uM precocene) showing the presence of GSH-CM (m/z 366), GSSG (m/z 307 + m/z 613), a GS–precocene conjugate (m/z 514), and the internal standard γ-Glu-Glu (m/z 277).

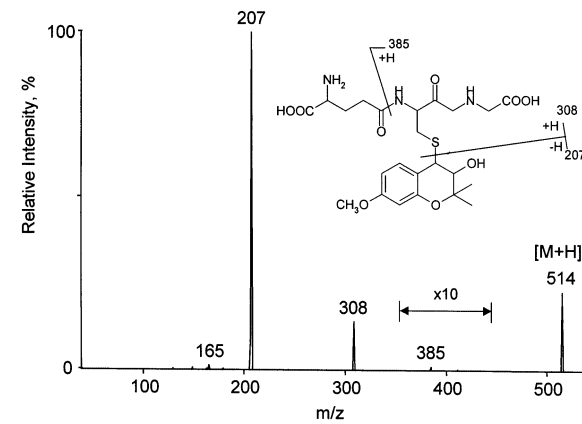

Fig. 6. Triple quadrupole product ion mass spectra and fragmentation scheme for the glutathione conjugate of precocene I ($[M + H]^+$ at m/z 514) that was formed by hepatocytes.

thereafter possibly due to frank toxicity from this high concentration of precocene I. The relatively high concentration of GSSG observed only at 180 min may also reflect general toxicity.

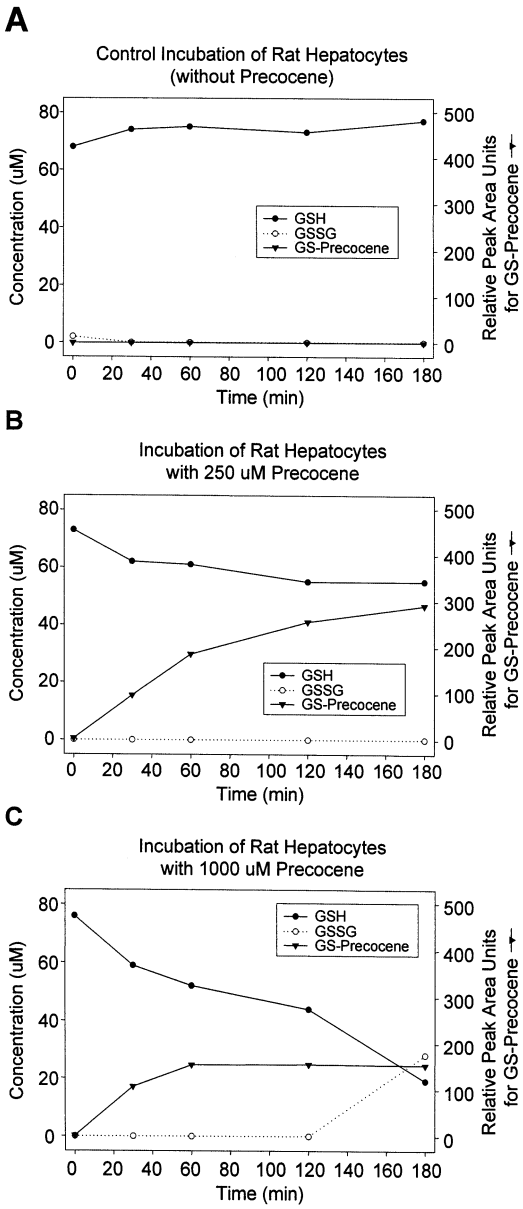

Fig. 7. Concentrations of GSH and GSSG in control hepatocyte incubations (a) and following treatment with precocene I at 300 μM (b) and 1000 μM (c). Relative levels of the major GS–precocene conjugate were also estimated from the peak areas in the corresponding reconstructed ion chromatograms.

4. Conclusions

A simple and convenient LC-MS method was developed to monitor the glutathione status of hepatocytes as an indicator of oxidative stress. Ion exchange HPLC conditions were developed that can be directly interfaced with atmospheric pressure ionization mass spectrometers. Maximum sensitivity was obtained using a triple quadrupole instrument operated in SRM mode. An ion trap mass spectrometer can, however, also provide adequate sensitivity for many experiments, as well as the ability to provide quantitative and qualitative data from a single analysis. GSH-conjugates were found to elute under the same HPLC conditions as GSH and GSSG making the method generally applicable to qualitative and quantitative analyses.

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