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Simultaneous determination of reduced glutathione, glutathione disulphide and glutathione sulphonamide in cells and physiological fluids by isotope dilution liquid chromatography–tandem mass spectrometry☆

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ABSTRACT

A stable isotope dilution liquid chromatography tandem mass spectrometry (LC–MS/MS) method was developed and validated for simultaneously quantifying glutathione (GSH), glutathione disulphide (GSSG) and glutathione sulphonamide (GSA) from biological samples. GSA is a selective product of the reaction of GSH with hypochlorous acid and a potential biomarker of myeloperoxidase activity. GSH was detected as the *N*-ethylmaleimide alkylated adduct, as formation of this species prevented GSH oxidation occurring during sample processing. Synthesised stable isotope analogues were used as internal standards to accurately quantify each target species. The limit of quantification was determined as being 0.1 pmol for each species and excellent linearity was observed over relevant concentration ranges for biological samples. Relative standard deviations were <5% for within-day variation and <10% for between-day variation, except at the lower limit of quantification where they remained <20%. Accuracy was between 82% and 113%. We could detect GSA in neutrophils and endothelial cells treated with hypochlorous acid and in bronchoalveolar lavage fluid from children with cystic fibrosis. This is the first time GSA has been quantified in clinical material and suggests it is formed *in vivo*. The assay can now be used for investigating GSA as a biomarker of myeloperoxidase activity in inflammatory conditions, and is also applicable to measuring GSH:GSSG molar ratios as a general index of oxidative stress.

1. Introduction

Reduced glutathione (GSH, gamma-L-glutamyl-L-cysteinyl-glycine) is the major low molecular weight thiol in cells, with intracellular concentrations typically in the millimolar range [1,2]. GSH acts as a recyclable antioxidant through the formation of GSSG and subsequent enzymatic reduction through the action of glutathione reductase. GSH is a likely target of oxidants generated *in vivo* due to its relatively high concentration within cells and favourable rates of reaction [3].

Hypochlorous acid (HOCl) is produced by myeloperoxidase-catalysed oxidation of chloride and is regarded as the major strong oxidant generated by neutrophils [4,5]. It reacts extremely favourably with thiols [6]. The production of myeloperoxidase-derived oxidants, including hypochlorous acid, is associated with the development of the pathologies associated with chronic inflammation [7]. However, even though production of this oxidant has been implicated in the tissue damage observed, it has proven difficult to categorically demonstrate it plays a crucial role in any pathology. This is largely due to hypochlorous acid being an extremely reactive and short-lived species. It is important to have biomarkers that are able to quantify its production as their levels could be related to disease severity, thereby linking oxidant formation with the damage observed at inflammatory sites. 3-Chlorotyrosine is frequently measured in clinical samples as a molecular fingerprint for myeloperoxidase-derived hypochlorous acid formation *in vivo*, and is the only biomarker currently being used specifically for this oxidant [8]. Although frequently used, there is still an ongoing need for the development of specific markers that are able to detect hypochlorous acid production *in vivo*.

Glutathione sulphonamide (GSA) has recently been identified as a potential biomarker of hypochlorous acid formation and myeloperoxidase activity *in vivo*. It is formed as an additional oxidation product to GSSG when GSH is oxidised by hypochlorous acid [9]. Importantly, GSA has been shown to be sufficiently selective for hypochlorous acid to be used as a biomarker of this oxidant [10]. The molecular structure of this species has recently been elucidated [11], and consists of a nine-membered heterocycle with a covalent

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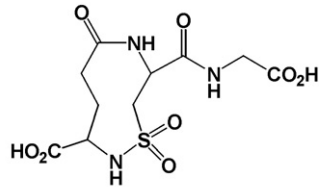

Glutathione Sulphonamide

Structure 1.

linkage between the γ-glutamyl amine and the cysteinyl sulphur (Structure 1).

Changes in redox state are commonly used as an index of oxidative stress within biological systems. Numerous methods have been established to quantify GSH and GSSG for this purpose, with the majority using reverse-phase chromatography for separation with detection by UV absorbance or as a fluorescent adduct [12–14]. Although these measurements have proved useful, no information can be obtained as to the oxidant responsible for the GSH oxidation. It would be useful to quantify GSA in addition to GSH and GSSG as this would indicate whether hypochlorous acid was involved in GSH oxidation. Unfortunately the methods traditionally used for GSH and GSSG quantification are unable to be adapted to include GSA because it is a polar species that is poorly retained on reverse-phase columns. Also, GSA cannot easily be detected by UV as it lacks a distinctive chromophore and cannot be probed with fluorescent labels as it lacks derivatisable thiol and amine groups.

There are many methods that use liquid chromatography coupled with mass spectrometric detection for quantifying GSH and GSSG from a variety of biological sources [15–26]. GSA has also been detected using mass spectrometry [27,28]. However, no LC–MS method is available for the quantification of all three analytes in biological systems. In the current study we have developed a chromatographic method for separating these species using a column capable of binding polar compounds and used tandem mass spectrometry for detection and quantification purposes. The thiol groups of GSH have been blocked in samples at the time of collection, both to prevent oxidation during processing and improve chromatography. Internal standards containing stable isotopes have been included for each species to allow accurate quantification. This method has been validated and has potential to assess the influence of myeloperoxidase-derived hypochlorous acid on GSH oxidation *in vivo*. GSA has been detected in HOCl-treated cells and bronchoalveolar lavage fluid (BALF) from children with cystic fibrosis. This method also represents a convenient and sensitive way to quantify GSH and GSSG in cells and tissues.

2. Experimental

2.1. Chemicals

Glutathione (GSH), glutathione disulphide (GSSG), *N*-ethylmaleimide (NEM) were from Sigma (St. Louis, MO, USA). Labelled GSH ([glycine 1,2-$^{13}C_2$, ^{15}N]-GSH) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Acetonitrile and methanol were from Malinckrodt Baker (Paris, KY, USA). Propan-2-ol (IPA) and ethanol were from BDH (Poole, Dorset, UK). Sodium hypochlorite (NaOCl) was from Sarah Lee (Auckland, NZ). Water of 18 MΩ quality was prepared by a Millipore Milli-Q system (Bedford, MA, USA). Phosphate buffered saline (PBS) had a pH of 7.4 and consisted of 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4, 140 mM sodium chloride and 2.7 mM KCl. Hanks' Balanced Salt Solution, pH 7.4 (HBSS): PBS containing 1 mg/mL glucose, 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$.

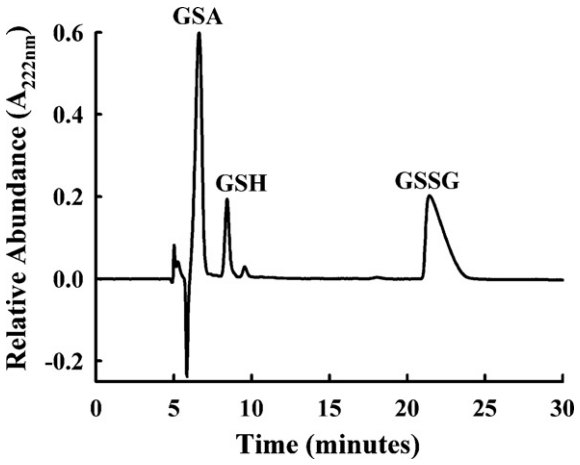

Fig. 1. HPLC separation of an undiluted equimolar reaction mixture of GSH (100 mM) and hypochlorous acid. The GSA peak, eluting at 7 min, was collected and used to generate the GSA standard.

2.2. Preparation of standards

For the analyses, GSH was detected as the GSH-NEM alkylated adduct. To form this species a 5-fold molar excess of *N*-ethylmaleimide (NEM) was added to GSH and left for 30 min. Both the NEM and GSH were dissolved PBS (pH 7.4) and the reaction gave stoichiometric conversion to the adduct [29]. GSA is not commercially available and was synthesised by gently mixing an equal volume of 100 mM GSH in phosphate buffer (100 mM, pH 7.4) with equimolar NaOCl [11]. It was separated from residual GSH and other oxidation products by reverse-phase HPLC using a Phenomenex Jupiter column (250 mm × 4.6 mm). Isocratic elution using 50 mM formic acid was used with UV detection at 222 nm. GSA eluted close to the solvent front, but before GSH and GSSG, which were well retained (Fig. 1). Fractions containing GSA were freeze-dried to give a fluffy white powder. The purity of the synthesized standards was confirmed by directly infusing the material into the mass spectrometer and monitoring a wide mass range (*m/z* 100–1000) using positive ESI. Purity was also assessed using chromatographic separation and detection of the eluting species using full scan mass spectrometry. The LC–MS method used for this purpose had been developed for a separate study to monitor oxidation products of GSH when treated with various two-electron oxidants [10]. As no additional peaks were observed the various preparations were determined to be of high purity. A concentrated GSA stock was prepared in PBS from the purified material from which a calibration curve was constructed. This solution was stable over a period of at least a year at −20 °C.

Commercially available glycine-labelled GSH ([glycine 1,2-$^{13}C_2$, ^{15}N]-GSH) was used to generate isotopically labelled GSA, GSSG and GSH-NEM for use as internal standards. Labelled oxidation products were formed by treating 50 μg of glycine-labelled GSH, dissolved in PBS, with an equal volume of equimolar NaOCl under conditions of gentle mixing. The reaction mixture was separated by reverse-phase chromatography in an identical fashion to unlabelled material. The GSSG fraction was collected in addition to that of GSA. Both fractions were freeze-dried separately. Labelled GSH-NEM was generated in an identical fashion to unlabelled GSH-NEM. Purity was again assessed by direct infusion positive ESI and LC–MS analysis.

Calibration curves were generated for each analyte, ranging from 2 nM to 10 μM for GSH-NEM (0.9 μg/L–4.3 mg/L), and 2 nM–2 μM for both GSSG (1.2 μg/L–1.2 mg/L) and GSA (0.7 μg/L–0.7 mg/L). With a 50 μL injection volume, this equated to 0.1–500 pmol GSH-NEM and 0.1–100 pmol of GSSG and GSA on the column. The amount

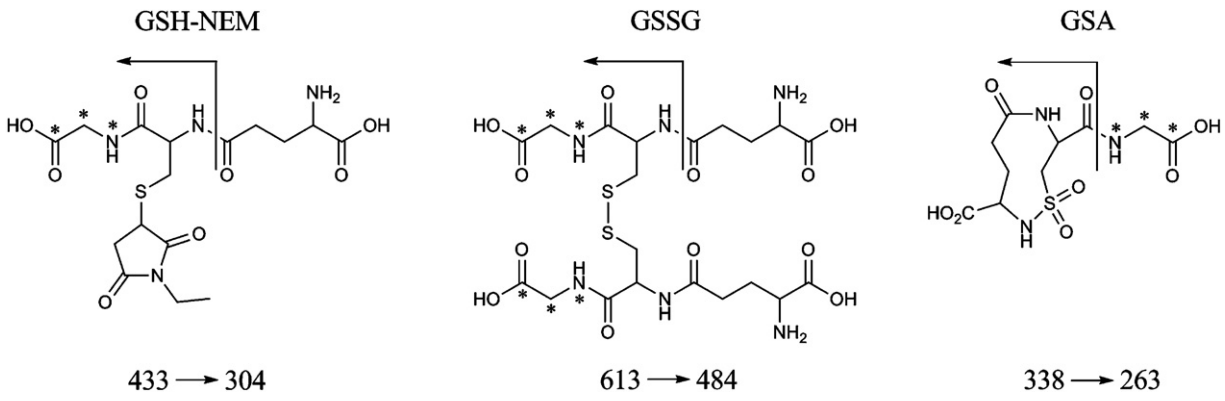

Fig. 2. Structures and collision-induced dissociation (CID) fragmentation sites of the species monitored. Arrows associated with structures designate bond cleavage sites when undergoing fragmentation and portion of the molecule monitored during quantification (the ionising proton is not shown). The stars associated with the various glutathione species (*) represent those atoms labelled when synthesised using $^{13}C_2$,$^{15}N_1$-GSH. Masses associated with each species represent the $[M+H]^+$ ion and the protonated mass of the fragment ion monitored from the unlabelled target analyte.

of isotopically labelled internal standard in the various calibrants equated to 200 nM GSH-NEM, 100 nM GSSG and 100 nM GSA. The levels of GSH, and the two oxidation products monitored, varied considerably depending on which sample type was being investigated. As such, the calibration curves for each analyte covered a wide concentration range. Calibration curves were plotted with the *y*-axis representing the peak area ratio observed between the sample and the isotopically labelled internal standard and the *x*-axis representing the amount of analyte.

2.3. Chromatographic conditions

The analytes were separated by liquid chromatography using a Thermo Hypercarb column (100 × 2.1 mm) held at 40 °C. Eluent A was H_2O containing formic acid (0.5%, v/v) and eluent B was acetonitrile/propan-2-ol (50/50, v/v) containing formic acid (0.5%, v/v). A linear gradient from 100% eluent A to 30% eluent B over 15 min was used, followed by a 5 min wash with 100% eluent B then returned to the initial conditions for equilibration. The flow rate was 0.2 mL/min and the injection volume 50 μL. The eluent from the column was introduced into the mass spectrometer without splitting.

2.4. Mass spectrometry conditions

Mass spectrometry analyses were performed with a Thermo Finnigan LCQ Deca XP Plus ion trap mass spectrometer (San Jose, CA, USA). The electrospray needle was held at +4.5 kV. Nitrogen, the sheath gas, was set at 45 units. The collision gas was helium and the temperature of the heated capillary was 275 °C. Quantification of GSH-NEM, GSSG and GSA was by selective reaction monitoring mode (SRM) using positive electrospray ionisation mass spectrometry. In each case, the largest fragment ion generated by collision-induced dissociation of the $[M+H]^+$ ion was used for quantification (Fig. 2). Settings for the target analytes were (parent ion → fragment ion); GSH-NEM *m*/*z* 433 → 304, GSSG *m*/*z* 613 → 484, GSA *m*/*z* 338 → 263. For the isotopically labelled internal standards; GSH-NEM *m*/*z* 436 → 307, GSSG *m*/*z* 619 → 490, GSA *m*/*z* 341 → 263. As each species was baseline resolved, the run was divided into three segments. Each segment monitored one analyte with two scan events, one for the naturally abundant species and the other for its corresponding isotopically labelled internal standard. The number of ions entering the ion trap for each scan event was regulated by the automatic gain control (AGC) function, the settings of which were the same as the default. The maximum time allowed for each scan event was set to 50 ms. Data acquisition was performed with XCalibur™ software version 1.3.

2.5. Biological samples

The assay was applied to the analysis of a selection of biological samples, including extracts from human erythrocytes, neutrophils, cultured umbilical vein endothelial cells and lung lavage fluid. The number of cells used for each experiment gave a quantifiable response for both GSH and GSSG using the calibration curves generated. This equated to 10^7 erythrocytes, 10^6 neutrophils and 1.2×10^5 endothelial cells. For the analysis of lung lavage fluid, 200 μL was used from samples that had an average protein concentration of 0.2 mg/mL. All samples, regardless of their source, were prepared in a similar fashion for LC–MS/MS analysis. Initially an excess of NEM was added (final concentration 10 mM) and the sample left for 20 min at room temperature to ensure complete GSH alkylation. This was followed by addition of isotopically labelled internal standards to give a final concentration of 200 nM GSH-NEM, 100 nM GSSG and 100 nM GSA. Cold ethanol (volume to give 80% ethanol, v/v) was then added to lyse cells and/or precipitate protein. Samples were left at 4 °C for an additional 30 min then centrifuged at 16,100 × *g* for 5 min to pellet lysed cellular remnants and precipitated protein. The colourless supernatant was removed and taken to dryness under vacuum using a Savant SpeedVac concentrator system (model SPD131DDA). Samples were reconstituted in 200 μL of H_2O and 50 μL was injected onto the column for analysis.

Erythrocytes and neutrophils were isolated using standard methods from human blood obtained from healthy donors with informed consent [30,31]. Cells were prepared at a concentration of 2×10^7 and 4×10^6 cells/mL respectively in fresh HBSS. Human umbilical vein endothelial cells were harvested from umbilical cords obtained with informed consent and grown under standard conditions [32]. The cells were grown in M-199 media supplemented with 15% foetal calf serum and growth supplements. Cells were prepared in a 24-well plate and when confluent, there were approximately 1.2×10^5 cells/well. Endothelial cells were washed twice with HBSS to remove the culture media before oxidant treatment. Ethical approval for donation of blood and umbilical cords was provided by the Upper South A Ethics Committee, Christchurch, New Zealand. Untreated cells (HBSS buffer treated) and those exposed to hypochlorous acid were examined. The amount of hypochlorous acid (in HBSS) added to the cells gave substantial, but not total, loss of intracellular GSH and left the majority of the cells still viable. This equated to 30 nmol/10^6 neutrophils (120 μM HOCl final concentration); 5 nmol/10^7 erythrocytes (5 μM HOCl) and 50 nmol/1.2×10^5 endothelial cells (100 μM HOCl). After 10 min at room temperature, cells were treated with 10 μL of a concentrated methionine solution to give a final concentration of 500 μM. This stopped the reaction by scavenging unreacted hypochlorous

acid and chloramines. Intact cells were isolated without washing by aspirating the supernatant from pelleted erythrocytes and neutrophils (13,000 × *g*) or confluent endothelial cells. Intact cells were then processed for LC–MS/MS analysis using the protocol described above.

Bronchoalveolar lavage fluid was obtained from 25 children with cystic fibrosis. They were enrolled at Princess Margaret Hospital, Perth, Western Australia and written consent was obtained from the children's parents. Bronchoalveolar lavage was typically performed at a time when the children were clinically stable, but not necessarily asymptomatic. Aliquots of saline were instilled into the middle lobe or right lower lobe when the child was under general anaesthesia and retrieved using low-pressure suction. Aliquots were pooled, centrifuged (13,000 × *g*, 5 min) and the supernatant was frozen at −80 °C. Frozen samples were transported to Christchurch on dry ice.

2.6. Assay validation

Sets of standards and quality control (QC) samples were prepared and analysed on five different days to assess linearity, accuracy and precision. Three QC levels were used for each of the analytes quantified, with the concentrations covering low, medium and high points on the respective calibration curves. Method validation was undertaken using the criteria described in the article titled 'Bioanalytical Method Validation' that was produced by the U.S. Food and Drug Administration [33]. Recovery was assessed by spiking neutrophil extracts with known amounts of the three analytes before being processed for LC–MS/MS analysis; GSA (2 pmol), GSSG (10 pmol) and GSH (200 pmol). As endogenous GSSG and GSH were present in the sample, recoveries were calculated by subtracting the amounts measured in the unspiked samples from those in the spiked sample.

3. Results and discussion

3.1. Method development

Chromatographic conditions were optimised for baseline separation and quantification of GSA, GSSG and GSH using a Thermo Hypercarb column. This column offers different retention and selectivity to silica- and polymer-based phases and is typically used for the retention of very polar compounds, including GSA [10,28]. GSH was detected as the GSH-NEM adduct for several reasons; unalkylated GSH displayed poor chromatographic properties on the Hypercarb column compared to the alkylated form and the NEM-adduct was more easily detected by positive electrospray ionisation. Also, it is essential to block the thiol group of GSH to prevent artifactual oxidation during sample processing [25,34,35]. Although GSSG showed poor separation characteristics on the Hypercarb column under the elution conditions originally described for the separation of GSA [28], its peak shape improved substantially by elevating the column temperature (25 → 40 °C) and increasing the formic acid concentration of solvents (0.1 → 0.5%, v/v). These modifications also made small but beneficial differences to the retention and peak shape of both GSA and GSH-NEM.

3.2. Assay validation

The assay was selective for each analyte due to a combination of liquid chromatography separation with an isotopically labelled internal standard and selective monitoring of specific fragment ions of the target analytes by SRM. For a contaminant species to interfere with the analysis of each species it would have to elute at an identical retention time and also exhibit the same fragmentation pattern.

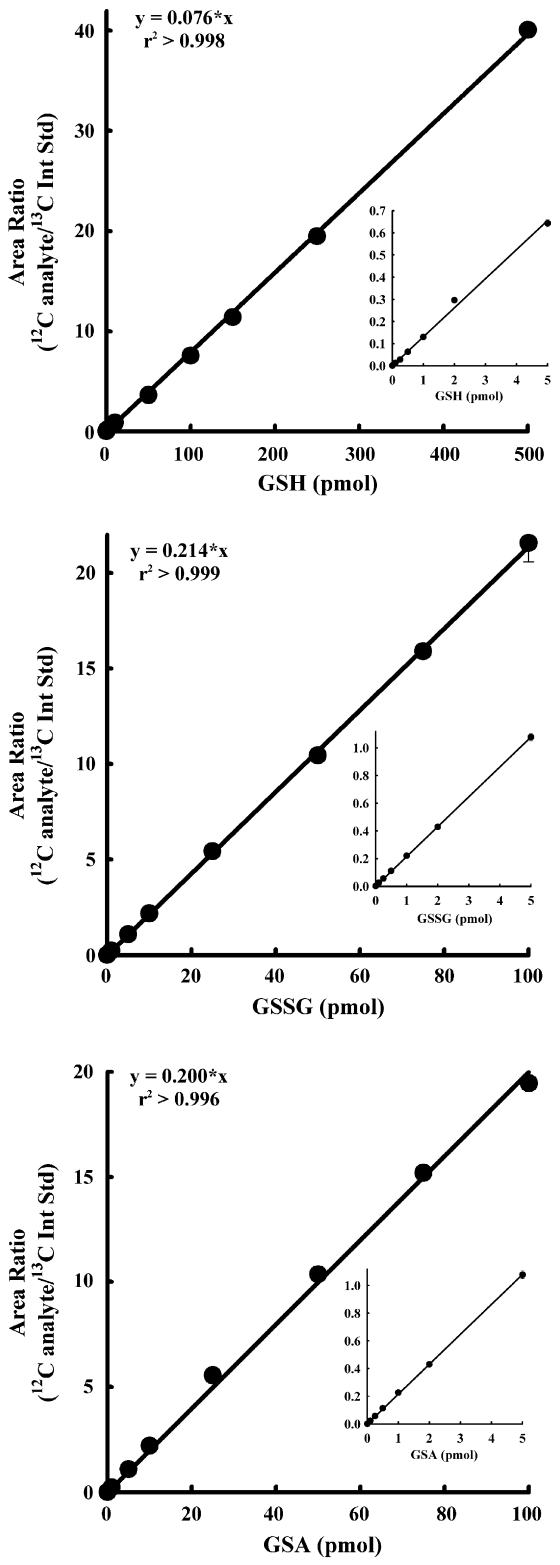

Fig. 3. Calibration curves were generated for the GSH-NEM conjugate (0.1–500 pmol), GSSG (0.1–100 pmol) and GSA (0.1–100 pmol) using 50 μL injections of calibration standards containing varying amounts of analyte and a fixed amount of corresponding isotopically labelled internal standard (10 pmol GSH-NEM, 5 pmol GSSG and 5 pmol GSA). Each data point represents the mean area ratio ± standard deviation from at least three calibration curves plotted on different days. Where error bars are not visible, they fall within the symbol size. The insets for each analyte show the linear response at the low end of the respective calibration curves (0.1–5 pmol).

Table 1
Intra- and inter-day precision (RSD) and accuracy for GSH-NEM, GSSG and GSA.

	GSH-NEM			GSSG			GSA		
	Low QC	Med QC	High QC	Low QC	Med QC	High QC	Low QC	Med QC	High QC
Accuracy									
Nominal (pmol)	0.10	2.0	200	0.10	2.0	75	0.10	2.0	75
Mean (pmol)	0.11	2.1	200.4	0.12	2.1	74.8	0.08	1.8	74.9
Bias (%)	7.3	5.0	0.2	13	4.5	−0.3	−17	−9.3	−0.2
Precision									
Intra-day RSD (%) $n = 5$	5.7	3.8	2.7	3.0	2.0	1.4	9.5	1.7	0.2
Inter-day RSD (%) $n = 6$	18.7	2.4	1.0	15.6	3.1	2.0	13.4	6.0	0.7

Quality controls were prepared (Low, Med and High QC) covering the expected concentration range in biological samples. To assess accuracy, a nominal amount of analyte was injected and quantified using the LC–MS/MS method described. The same quality controls were used to determine precision.

Table 2
Cross-validation of LC–MS/MS assay using the DTNB assay.

HOCl (μM)	[GSH] (μM)		RSD (%)
	LC–MS/MS	DTNB	
0	99	102	2
25	82	77	4
50	67	65	2
100	47	47	1
200	13	11	10
400	0	0	0

GSH (100 μM) was reacted with stated concentrations of hypochlorous acid in PBS (pH 7.4) for 30 min at room temperature. Samples were then reacted either with DTNB and the absorbance measured at 412 nm (ε_{412} 14,100 M^{-1} cm^{-1}), or alkylated with a 5-fold molar excess of NEM and analysed using LC–MS/MS. Variation between the assays is shown as RSD (%).

Table 3
Effect of sample matrix on the recovery of internal standards.

Sample	Recovery		
	$^{13}C_2$,$^{15}N_1$-GSH-NEM	$^{13}C_4$,$^{15}N_2$-GSSG	$^{13}C_2$,$^{15}N_1$-GSA
Neutrophil	94 ± 5%	102 ± 6%	89 ± 9%
HUVEC	96 ± 4%	100 ± 4%	100 ± 5%
Erythrocytes	103 ± 5%	98 ± 2%	83 ± 3%
BALF	96 ± 5%	96 ± 4%	91 ± 4%

The average peak areas of the three isotopically labelled internal standards were compared to those observed when no biological matrix was present (i.e. from buffer). The percentages indicate the effects the biological matrix had on signal intensity. HUVEC = human umbilical vein endothelial cells, BALF = brochoalveoloar lavage fluid.

Linear calibration curves were obtained for GSH, GSSG and GSA over the concentration ranges tested (Fig. 3). Results for accuracy and precision for all QCs are summarised in Table 1. Accuracy was within the range of 83–113% for all three target species and relative standard deviations were <10% for intra-day precision and <20% for inter-day precision. The limit of quantification (LOQ) was 0.1 pmol for each of the three species. At this level, the signal-to-noise (S/N) ratios for the respective peaks was greater than 10 and the values obtained for accuracy and precision were within an acceptable range. The limit of detection (LOD; S/N ratio > 3) was not determined but was lower than the LOQ, especially for GSH-NEM, where the signal-to-noise ratio was considerably greater than 10 at the 0.1 pmol level. Cross-validation of the LC–MS/MS method with the DTNB assay [36], using GSH that had been treated with varying amounts of hypochlorous acid, showed no discernable differences between the two methods, with variation between the data sets being ≤10% RSD (Table 2).

3.3. Analysis of biological samples

The method was applied to various biological samples, which included those taken directly from an inflammatory site (BALF from the cystic fibrosis lung) and extracts from human neutrophils, erythrocytes and endothelial cells. It was first established that all three analytes could be recovered without undue losses or matrix effects from these samples. Recoveries from control neutrophil extract spiked with GSA (2 pmol), GSSG (10 pmol) and GSH-NEM (200 pmol) were between 91% and 96% (data not shown). Recoveries of the internal standards from each of the cell types and BALF were typically greater than 90% compared to that observed from the calibration standards (Table 3). An acceptable level of recovery of the internal standard confirmed that the extraction process was satisfactory and that the samples gave minimal matrix effects during analysis.

GSH and GSSG were readily detected in the cell extracts, at molar ratios of approximately 1000:1 (Table 4). The control GSH concentrations are within the ranges reported for these cell types when using alternative quantification methods [27,31,37,38]. No GSA was detected in any of the control cells. Treatment with hypochlorous acid resulted in increases in GSSG and/or GSA. The relative amounts of GSSG and GSA formed varied between cell types, with neutrophils generating more GSA than GSSG, and treatment of erythrocytes producing no detectable GSA. The neutrophil and endothelial cell results represent the first time that GSA has been quantified unequivocally in oxidant-treated cells and substantiate previous suggestive evidence for its formation [27,38]. Our quantitative analysis indicates that GSA is a minor but significant product. Conversion of virtually all of the GSH lost to GSSG in HOCl-treated erythrocytes was also observed by Vissers and Winterbourn [31], and further investigation is needed

Table 4
GSH, GSSG and GSA content of control and HOCl-treated cells.

	Control			HOCl-treated		
	GSH	GSSG	GSA	GSH	GSSG	GSA
Neutrophils (pmol/10^6 cells)	1569 ± 131	1.1 ± 0.3	nd	874 ± 95	2.4 ± 0.2	85 ± 12
Erythrocytes (pmol/10^7 cells)	1549 ± 133	0.8 ± 0.4	nd	945 ± 155	313 ± 31	nd
HUVECs (pmol/1.2×10^5 cells)	1687 ± 164	1.9 ± 0.8	nd	472 ± 50	44.1 ± 3.0	9.2 ± 1.8

The levels were quantified from both control and HOCl-treated neutrophils (10^6 cells/sample, ±30 nmol HOCl), erythrocytes (10^7 cells/sample, ±5 nmol HOCl) and HUVECs (~1.2×10^5 cells/sample, ±50 nmol HOCl). At these concentrations the HOCl did not cause cell lysis. Results are expressed as means and standard deviations from at least 3 experiments. nd = Not detectable (for GSA the LOQ was determined as 0.1 pmol).

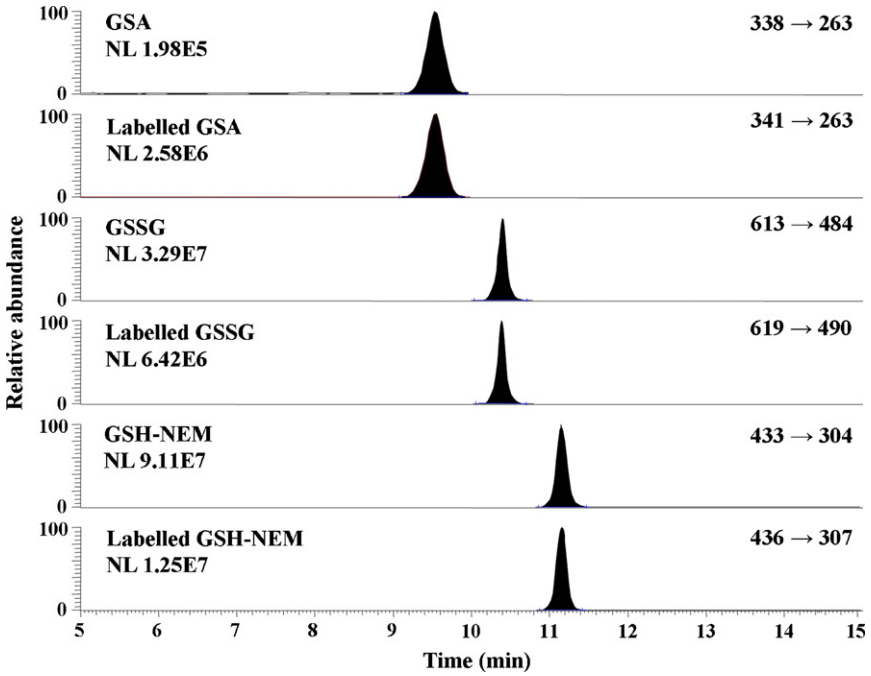

Fig. 4. Extracted chromatograms of the three target analytes and their isotopically labelled internal standards from a NEM-alkylated cystic fibrosis bronchoalveolar lavage sample using the optimised LC–MS/MS method. Also shown are the mass transitions used for each species and the signal intensity observed (NL) from the sample.

to explain why there is stoichiometric conversion to the disulphide in these cells and why no GSA was formed. In contrast, a large portion of the GSH lost from neutrophil and endothelial cells remains unaccounted for. This observation is not unique to this study [27,39] and suggests there is an alternative fate for GSH when oxidised within these cells. It is likely that some GSH would form mixed disulphides. A previous study found approximately 20–30% of neutrophil GSH becomes protein bound when treated with hypochlorous acid [38]. Alternatively, GSH could form alternative oxidation products to GSSG and GSA that are not detectable using this method. Plausible species would include the sulphinic acid (GSO_2H), sulphonic acid (GSO_3H) and thiosulphonate (GSO_2SG) of GSH. Additionally, GSH and its oxidation products could be exported from the cell post oxidant treatment. GSH metabolism via the γ-glutamyl cycle requires GSH and GSSG movement across the cellular membrane into the extracellular space and subsequent action of extracellularly bound peptidase enzymes to cleave them into their constituent amino acids [40,41]. Whether it occurs within the timeframe of these investigations is unclear. Further investigations into all of these possibilities are warranted.

All three glutathione species were quantifiable in alkylated BALF samples (Fig. 4). The 25 samples analysed had median concentrations of 1714 nM GSH (interquartile range [IQR] 900–3000 nM) and 137 nM GSSG (IQR 40–450 nM). GSA was detected in all but one sample, with a median concentration of 3.3 nM (IQR 1–6 nM). These findings provide the first evidence that GSA is formed *in vivo*. The assay was sufficiently sensitive to detect GSA in samples collected by a lavage process that typically gives ∼100-fold dilution of the epithelial lining fluid [42]. Based on this dilution factor, actual GSA concentrations could be several hundred nanomolar. The cystic fibrosis lung represents a likely place for GSA to form as chronic inflammation and high neutrophil infiltration arise from an early age, even in the absence of a pathogenic challenge [43]. Elevated levels of myeloperoxidase, and the HOCl-specific product 3-chlorotyrosine, have been measured in BALF from cystic fibrosis patients [44]. The current assay will allow further investigation of how GSH oxidation relates to clinical outcomes and the importance of neutrophils, myeloperoxidase and hypochlorous acid in the process.

Analysis of BALF for GSA should have application for a number of other inflammatory lung diseases in which oxidative stress is implicated. As demonstrated with cystic fibrosis, the fluid can be analysed by the LC–MS/MS method directly. The assay should have wider application for analysing more accessible fluids such as plasma or urine. However, for greater sensitivity, an initial concentration step using solid phase extraction may be necessary. The method possesses sufficient sensitivity for detecting plasma GSH and GSSG, which are typically in the micromolar or submicromolar range respectively [45].

A variety of assays for GSH and GSSG have been applied to biological fluids and tissues. Reported values vary widely. Oxidation of GSH to GSSG during processing is a major problem, and unless a blocking step is included to prevent this, GSSG concentrations can be artifactually high by at least one order of magnitude [46,47]. Therefore, lower GSSG:GSH molar ratios are considered to be more reliable. In our assay, addition of NEM to the cells before extraction or to BALF samples immediately after collection gave molar ratios in an acceptably low range. For example, our GSSG:GSH ratio of 0.08% for erythrocytes, compares with 0.22% reported for whole blood by Rossi et al. [47] (the GSH in blood comes almost entirely from erythrocytes). The molar ratios measured for neutrophils and endothelial cells were also low. GSA is less likely to be generated during processing as it requires a strong oxidant such as hypochlorous acid, but blocking is still preferable as a precaution and so that GSA can be related to other glutathione species.

4. Conclusion

A LC–MS/MS method was developed and validated for the simultaneous quantification of GSH, GSSG and GSA within a single run. Naturally abundant GSA and isotopically labelled standards were synthesised using straightforward procedures and the assay is sufficiently sensitive to measure all of these species in biological samples even after substantial dilution. The method provides sensitive measurement of tissue concentrations of GSH and GSSG. However,

its main application will be for measuring GSA in biological material. Although a variation of the current method has been used for *in vitro* systems [10], the inclusion of internal standards is essential for accurate quantification in biological material. GSH is converted to GSA in much higher yields with hypochlorous acid than with other oxidants. It is chemically stable and now that it has been shown to form *in vivo*, it must be considered as a promising selective biomarker of hypochlorous acid production. As myeloperoxidase activity is the only physiological source of hypochlorous acid, GSA measurements should have wide application in clinical studies of diseases where oxidative injury by myeloperoxidase or neutrophils is implicated. GSA could provide an alternative to 3-chlorotyrosine, which is also specific to hypochlorous acid [8,48], especially as thiols such as GSH are much more favourable targets than tyrosine. However, the use of any biomarker is complicated by factors such as substrate accessibility and its physiological fate, and greater insight is likely to come from complementary measurements of both.

There is considerable interest in the role played by myeloperoxidase in numerous inflammatory conditions including cardiovascular disease. Our finding that GSA is produced in the airways of children with cystic fibrosis demonstrates that it is produced *in vivo* under conditions where neutrophils are activated. Furthermore, its formation in cells exposed to hypochlorous acid implies that it should be formed at additional sites where neutrophils are stimulated to release hypochlorous acid. Whether myeloperoxidase acts as more than an inflammatory marker or contributes to the disease pathology is still debatable. Analysis of GSA will allow such relationships to be explored. The assay also provides a reliable method for quantifying GSSG:GSH molar ratios as a general marker of oxidative stress.

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