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A sensitive and specific assay for glutathione with potential application to glutathione disulphide, using high-performance liquid chromatography–tandem mass spectrometry

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Abstract

We have utilised the combination of sensitivity and specificity afforded by coupling high-performance liquid chromatography (HPLC) to a tandem mass spectrometer (MS–MS) to produce an assay which is suitable for assaying glutathione (GSH) concentrations in liver tissue. The sensitivity suggests it may also be suitable for extrahepatic tissues. The method has been validated for GSH using mouse liver samples and also allows the assay of GSSG. The stability of GSH under conditions relevant to the assay has been determined. A 20-μl amount of a diluted methanol extract of tissue is injected with detection limits of 0.2 pmol for GSH and 2 pmol for GSSG. The HPLC uses an Altima C_{18} (150×4.6 mm, 5 μm) column at 35°C. Chromatography utilises a linear gradient from 0 to 10% methanol in 0.1% formic acid over 5 min, with a final isocratic stage holding at 10% methanol for 5 min. Total flow rate is 0.8 ml/min. The transition from the M+H ion (308.1 *m/z* for GSH, and 613.3 *m/z* for GSSG) to the 162.0 *m/z* (GSH) and 355.3 *m/z* (GSSG) fragments are monitored.

Keywords: Glutathione; Glutathione disulphide

1. Introduction

In recent years the problems of dealing with very large solvent volumes, compared to analyte volumes, from HPLC effluents have been overcome using interfaces such as the electrospray interface, enabling the coupling of HPLC to mass spectrometers. Tandem mass spectrometers offer several advantages over single stage instruments. Tandem mass analysers can be used to target specific ions in the first mass analyser and a characteristic fragment in the second mass analyser offering greater specificity. Although absolute signal is reduced, the reduction in noise offers substantial improvement in signal-to-noise ratio. The overall effect is instrumentation capable of accepting HPLC eluent and having excellent specificity and sensitivity.

Glutathione (GSH) is recognised as playing an important role in maintaining intracellular redox status and preventing and/or minimising the damage

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caused by reactive electrophiles especially in the liver [1]. Glutathione disulphide (GSSG), produced by enzymatic oxidation of two GSH molecules, is also present in tissues, usually in amounts at least one order of magnitude less than glutathione. GSSG therefore represents a substantial sensitivity challenge to quantitation. Sensitive and specific assays for GSH and GSSG are therefore essential for continued research into xenobiotic metabolism and activation.

Assay methods for GSH and/or GSSG can be divided into four main groups. These are the enzymatic [2], spectrophotometric [3], fluorometric [4] and HPLC based methods. HPLC methods can further be subdivided based on the use of either ultra-violet [5], fluorometric [6] or electrochemical detection [7,8]. In all of the assays quoted here derivitisation of GSH and/or GSSG are required. The specificity of assays which do not utilise the separation capacity of HPLC must be questioned especially when assaying low concentrations of GSH and/or GSSG. The enzymatic method as well as several others use Ellmans' reagent [3]. The stability of GSH where this reagent is used has been questioned [9]. Few of these reports provide detailed validation of the methods for quantitative use.

Both the spectrophotometric and HPLC–UV methods lack sensitivity for assay of many extrahepatic tissues where sample size and concentrations are both limited. Doubts also exist concerning the specificity of GSSG determinations using HPLC–UV [10]. Martin and White [6] have reported an HPLC method using fluorometric detection after derivatisation with dansyl chloride. This method has a detection limit of 1 pmol of GSH making it two orders of magnitude more sensitive than the spectrophotometric and/or HPLC–UV methods, but no validation data was provided. Lakritz et al. [11] have discussed the limitations of the HPLC-electrochemical methods in some detail.

We present here a method for the analysis of GSH and GSSG which utilises the sensitivity and specificity afforded by coupling high-performance liquid chromatography (HPLC) to a tandem mass spectrometer (MS–MS) to produce an assay which is suitable for monitoring low GSH concentrations in tissues. The sensitivity and specificity of this method is such that it is suitable for application to extrahepatic tissues containing low concentrations of GSH. We have also demonstrated the suitability of this method for the assay of GSSG. Considerable data is also presented on the stability of GSH under various conditions relevant to the assay.

2. Materials and methods

Unless otherwise specified all solvents were of HPLC grade and water was of 18 MΩ quality or better. All reagents were of AR grade.

The HPLC–MS–MS consisted of an LC-200 series pump, series 200 autosampler and API 300 MS–MS with turbo ionspray interface (Perkin-Elmer Sciex Instruments, Thornhill, Ont., Canada). An Altima C_{18} (150×4.6 mm, 5 μm) column (Alltech Associates, Baulkam Hills, NSW, Australia) at 35°C was used. Chromatography consisted of a linear gradient from 0 to 10% methanol in 0.1% formic acid over 5 min, with a final isocratic stage holding at 10% methanol for 5 min. Total flow rate was 0.8 ml/min. Post-column splitting was used to submit 20% of the column effluent only to the MS–MS interface. Injection volumes of 20 μl were used. The transitions from the M+H ion (308.1 *m/z* for GSH, and 613.3 *m/z* for GSSG) to the 162.0 *m/z* (GSH) and 355.3 *m/z* (GSSG) fragments were monitored for quantitation using multiple reactant monitoring (MRM) mode allowing assay of both analytes from one injection. GSH and GSSG used as analytical standards were obtained from ICN Biomedicals (Seven Hills, NSW, Australia). The interface ionisation potential was set to 5200 V with a temperature of 400°C, orifice potential set to 25 V and ring potential at 240 V. Instrument settings for the nebuliser, curtain and collision gasses were 11, 15 and 3, respectively.

Mouse livers were removed immediately after euthanasia in a carbon dioxide chamber and placed immediately in a glass beaker held in ice. The livers were then macerated and 0.1–0.2 g of the tissue was taken, weighed accurately, and 1 ml of methanol (kept in ice also) was added immediately and the tissue homogenised. This homogenate was then centrifuged at 800 *g* for 5 min. A 100-μl sample of the supernatant was evaporated to dryness under a stream of nitrogen gas at room temperature and

reconstituted in 1.5 ml of deionised water immediately prior to assay. Concentrations were then determined by comparison to known standards and the concentration expressed as μmol/g after correcting for the mass of liver tissue used and applying the appropriate mass to molar conversion factors for the analytes.

Linearity was tested using six standards ranging in concentration from 0.064 to 21.3 mg/l for GSH and four standards from 0.077 to 0.77 mg/l for GSSG. Co-efficients of variation (C.V.s) were determined using mouse liver extracts which had been diluted with water to produce four different concentrations. These samples were assayed in triplicate on each of 3 days using single point calibration at 6.4 mg/l (GSH) and 0.77 mg/l (GSSG). Determination of within, between and total C.V.s was by analysis of variance [12].

Given that GSH is known to be labile during storage of tissues, substantial assessment of its stability under various conditions relating to preparation and handling of standards, obtaining extracts from mouse livers, storage of extracts and assay of prepared extracts has been determined as follows.

Stability of stock solutions of GSH (6.4 mg/ml) in 50% methanol at −18°C was determined after 2 months by assay against a GSH standard freshly prepared from dry powdered GSH (3.3 mg/ml).

Stability of GSH during extraction was determined by taking three successive subsamples of the macerated liver tissue kept on ice from each of 38 mice under the extraction conditions described below. There was ~10 min between preparation of each successive subsample (i.e. the first subsample was taken at time zero, subsample two was therefore taken at 10 min and subsample three at 20 min). These samples were assayed individually and the mean concentration for all 38 mouse livers at each of the three times was determined.

Stability of GSH in methanol extracts of mouse liver, when stored at −18°C, was determined by re-assay of extracts of subsamples from two mouse livers against fresh standards after 25 days.

Stability of standards at room temperature during assay was determined by comparing the interpolated concentrations of standards assayed at the beginning and end of batches over a 10-h period of time. Data from five such batches were obtained and the mean interpolated concentration at the beginning (i.e. time = 0) and the end (i.e. time = 10 h) of these batches was determined for each of three standards (i.e. 0.64, 6.4 and 16.0 mg/l).

Stability of extracted samples while awaiting assay at room temperature (22°C) was determined by comparing mean assayed concentrations obtained from samples of mouse livers on initial assay and after 10 h at room temperature. Results were obtained for liver extracts from 30 individual mice.

3. Results and discussion

We set out to develop a reference method for the determination of GSH. To do this we have utilised not only the selectivity of HPLC but also used the additional selectivity of tandem mass spectrometry detection. The cost of HPLC–MS–MS instrumentation is often regarded as being prohibitive. Our experience however suggests that the higher specificity afforded by this instrumentation allows its additional cost to be offset at least in part by utilising simpler, less labour intensive sample preparation.

The mass spectrometry parameters were optimised for GSH using flow injection analysis and adjusting ionisation and extracting voltages to achieve maximum response for the molecular ion $(M+H)^+$. Once these parameters were established, fragmentation of this ion to give product ions of acceptable intensity was achieved by adjusting collision energy, that is, ion energy and nitrogen collision gas pressure. The molecular ion to product ion transitions which gave optimal signal-to-noise ratio were chosen. Formic acid was shown empirically to enhance formation of the molecular ion. This particular acid was used as it is volatile and leaves no residue in the interface. While monitoring these transitions, injections of the analytes were made onto the HPLC column and methanol concentration adjusted to give good peak separation and shape, with the gradient being optimised to shorten run time.

Parent (M + H) ions are 308.1 and 613.3 m/z for GSH and GSSG, respectively. The major product ions, chosen for quantitation using MRM mode are at 162.0 and 355.3 m/z, respectively. The linearity of the GSH assay is demonstrated by regression parameters ($n = 6$) of: slope = 52487 area units/mg per l;

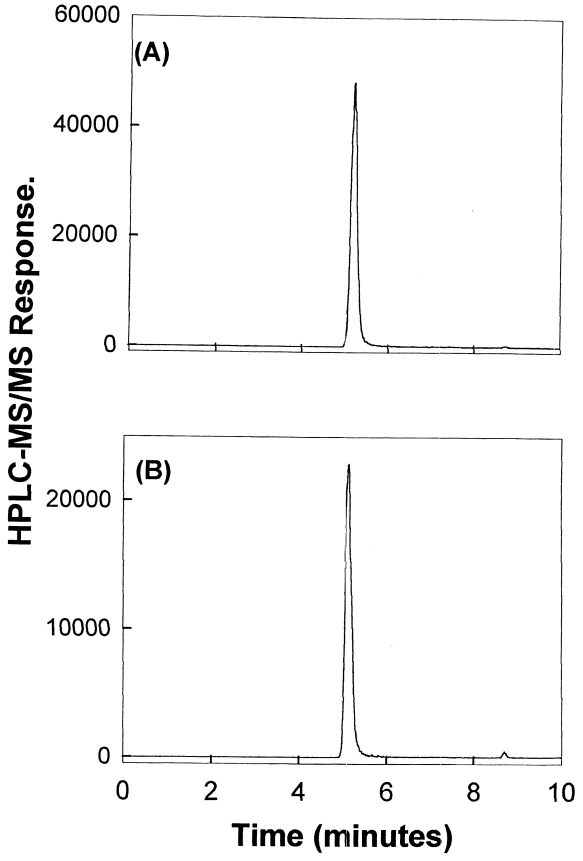

Fig. 1. Chromatogram of a pure standard of GSH (A) containing 12.8 mg/l and of a mouse liver extract (B) containing 6.8 mg/l (i.e. 2.4 μmol/g in original liver).

intercept = −1713 area units and correlation co-efficient of 1.000. For GSSG ($n=4$) regression parameters were: slope = 3580 area units/mg per l; intercept = −87.9 area units and correlation co-efficient of 0.999.

Fig. 1 demonstrates the specificity of HPLC–MS–MS as not even a solvent front appears in the chromatogram of either the pure standard or liver extract containing 2.4 μmol/g GSH. No smoothing routines have been applied to these chromatograms and they are typical of what is seen routinely for both standards and mouse liver extracts, not only for GSH, but also for GSSG. The retention time of GSSG is ~8.4 min. Given that instrument parameters were set for optimum sensitivity of GSH and used without modification for GSSG, several fold sensitivity gains could be achieved for GSSG.

Reproducibility of the assay is presented as between-day, within-day and total C.V.s in Table 1. Svardal et al. [13] reported within-day co-efficients of variation of <7.0% for plasma samples spiked with 5 and 2.5 μ*M* (16 and 8 mg/l) of GSH and GSSG, respectively, using a monobromobimane derivitisation procedure with fluorescence detection. C.V.s of 1.15% were reported for the derivitisation and analysis of standards containing 1.2 mg/l of GSH [14]. Given that our data include the variability inherent in extraction from liver samples, our method compares favourably with these reports at similar concentrations. No other reports included in our review of the literature have included assay repro-

Table 1
Reproducibility of the HPLC–MS–MS assay

Mean assayed glutathione (GSH)	0.06	0.68	5.0	18.9
Between-day C.V. (%)	14.1	3.1	4.6	5.7
Within-day C.V. (%)	24.5	6.0	4.7	4.2
Total C.V. (%)	28.3	6.8	6.5	7.1
Mean assayed glutathione disulphide (GSSG)		0.23	2.3	9.6
Between-day C.V. (%)		9.2	15.2	13.9
Within-day C.V. (%)		19.7	9.0	4.4
Total C.V. (%)		21.8	17.7	14.6

These data are based on triplicate assays on each of 3 days of dilutions of mouse liver extracts as described in the text. C.V., co-efficient of variation. C.V. was determined as the standard deviation/mean and expressed as a percentage. Standard deviations were obtained by analysis of variance. Units are mg/l.

ducibility data. The data in Table 1 also show that reproducibility below ~0.2 mg/l is unacceptable for GSH and below ~0.5 mg/l for GSSG. It should be noted however that the C.V.s were determined under the worst case scenario where they were incorporated throughout batches of samples requiring ~10-h run time and utilising a single batch of standards. The within-day C.V.s therefore attest to both the assay precision and instrument stability given that no internal standard was used. For our purposes, variability has been minimised by replicating samples and duplicating injections using samples assayed. The use of a suitable internal standard would undoubtedly improve the assay performance even further if required.

The limit of detection (LOD), determined as three times the height of the background signal, is 0.2 pmol for GSH and 2 pmol for GSSG using the conditions described. This is comparable to the enzymatic recycling method of Tietze [2] who reported a LOD of 0.1 pmol and is an improvement over most other reported HPLC procedures. HPLC–UV assays after derivitisation with 2,4-dinitrophenol have reported limits of detection of 25 pmol for GSH and 50 pmol for GSSG [15], while the use of fluorescent derivatives improves this limit of detection to 10 pmol for *O*-phthalaldehyde [16] and 0.5–2.0 pmol for monobromobimane, dansyl and other derivatives [6,13,14,17]. The use of electrochemical detection produces LOD of 1–2 pmol [11].

Upon assay in triplicate the freshly prepared stock solution of GSH in 50% methanol and the stock stored at −18°C for 2 months agreed within 2%. Stock solutions of GSH in 50% methanol were stable at −18°C for up to 2 months. Stability of standards at room temperature during assay was determined by comparing the interpolated concentrations of standards assayed at the beginning and end of batches over a 10-h period of time. Mean data from five such batches are presented in Table 2. The mean assayed concentration at the beginning (i.e. time = 0) and the end (i.e. time = 10 h) of these batches was determined for each of three standards (i.e. 0.64, 6.4 and 16.0 mg/l). These data suggest no differences between the treatments. As this situation represents an extreme case where extracts are left for up to 10 h at room temperature while awaiting assay, this is not likely to pose a problem.

Table 2
Mean (SD) of assayed concentrations (mg/l) of pure standards of glutathione at time zero and after 10 h at room temperature in the HPLC autosampler ($n = 5$)

Spiked concentration (mg/l)	0.64	6.4	16.0
0 Time	0.71 (0.14)	6.3 (0.83)	13.6 (1.2)
10 h	0.63 (0.14)	7.0 (0.84)	15.3 (2.9)

Successive subsamples at 10-min intervals of the minced liver tissue kept on ice from each of 38 mice under the extraction conditions described below gave mean (SD) GSH concentrations of 2.17 (1.27), 2.45 (1.41) and 2.40 (1.33) μmol/g, respectively. This demonstrates a change in GSH content of less than 13% during 20 min after collecting the liver under our extraction conditions. Given the magnitude of the changes, they are most likely explained by experimental error and do not suggest the need for more rigorous extraction procedures. Stability of GSH in methanol extracts of liver when stored at −18°C was determined by re-assay of livers from each of two mice against fresh standards after 25 days' storage. Contrary to expectations, the mean assayed concentration decreased from 6.93 to 1.95 μmol/g in this time. As the storage of pure solutions at the same temperature in 50% methanol demonstrated less than 2% change in concentration, it is difficult to explain these data other than the possibility of some residual enzymatic activity in the extracts despite the presence of 80–90% methanol and low temperatures. Degradation of GSH by gamma-glutamyltranspeptidase has been reported [9]. Significantly better storage of liver extracts may result from transferring the supernatant to a separate container and evaporating to dryness prior to storage.

Stability of extracted samples while awaiting assay at room temperature (22°C) was determined by comparing results obtained from re-injecting 30 samples from mouse livers ~10 h after initial assay. The mean concentration at time = 0 was 4.30 mg/l and 4.02 at time = 10 h. This represents a 6.3% reduction in concentration over 10 h. Sample to sample variation in this data is shown in Fig. 2. Given the overall agreement between the two treatments and the regression parameter values from Fig. 2

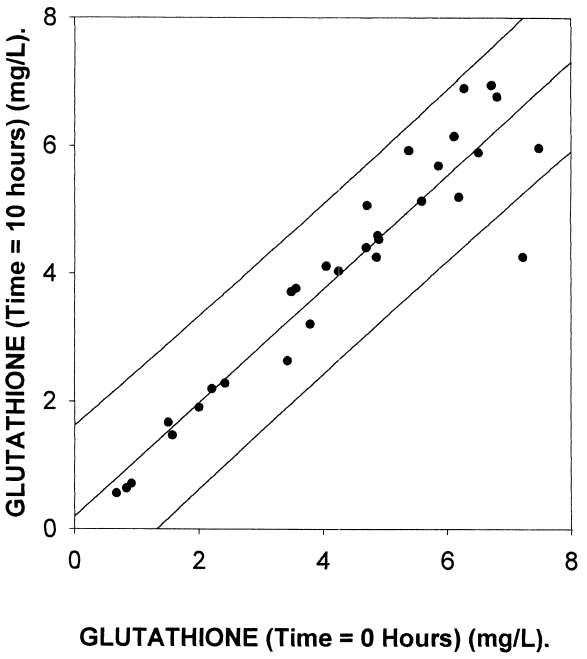

Fig. 2. Stability of mouse liver extracts at room temperature for up to 10 h during assay. Regression parameters ($n=30$) are; slope = 0.889, intercept = 0.200 and correlation co-efficient = 0.890. 95% Confidence intervals shown.

the extracts of liver samples demonstrate adequate stability during assay.

The assay of GSH has been complicated by autooxidation of GSH to GSSG during sample preparation and storage. This problem has largely been dealt with by the inclusion of acids such as perchloric [18], trichloroacetic [19] or sulphosalicylic acids [9] during extraction. As these acids are largely incompatible with the use of HPLC–MS–MS a completely different approach has been used for sample preparation here. As previously indicated, degradation due to the enzyme gamma-glutamyltranspeptidase has also been shown to occur [9]. We have therefore chosen an extraction process which is rapid and undertaken at reduced temperature to minimise both autooxidation and enzymatic degradation. Methanol has been used to precipitate proteins in place of acids in previous assays, to provide extracts compatible with HPLC–MS–MS. Given these changes, we have extensively assessed the stability of GSH during the various stages of sample preparation and analysis using our procedure and demonstrated autooxidation and degradation are not excessive if extended storage times are not required.

4. Summary

In summary, we have developed a rapid, sensitive, reproducible, and specific assay for glutathione which is suitable for assay of GSH and GSSG at concentrations found in mouse liver samples and, given its sensitivity, should readily adapt to assay of GSH and GSSG in extrahepatic tissues.

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References

[1] P. Josephy, B. Mannervik, P. De Montellano, in: P. Josephy (Ed.), Molecular Toxicology, Oxford University Press, New York, 1997, p. 152.
[2] F. Tietze, Anal. Biochem. 27 (1969) 502.
[3] G. Ellman, Arch. Biochem. Biophys. 82 (1959) 70.
[4] P. Hissin, R. Hilf, Anal. Biochem. 74 (1976) 214.
[5] J. Russell, J. McKeown, C. Hensman, W. Smith, J. Reglinski, J. Pharm. Biomed. Anal. 15 (1997) 1757.
[6] J. Martin, I. White, J. Chromatogr. 568 (1991) 219.
[7] G. Carro-Ciampi, P. Hunt, C. Turner, P. Wells, Pharmacol. Methods 19 (1988) 75.
[8] P. Harvey, R. Ilson, S. Strasberg, Clin. Chim. Acta 180 (1989) 203.
[9] J. Roberts, D. Francetic, Anal. Biochem. 211 (1993) 183.
[10] J. Sian, D. Dexter, G. Cohen, P. Jenner, C. Marsden, J. Pharm. Pharmacol. 49 (1997) 332.
[11] J. Lakritz, C. Plopper, A. Buckpitt, Anal. Biochem. 247 (1997) 63.
[12] J. Krouwer, R. Rabinowitz, Clin. Chem. 30 (1984) 290.
[13] A. Svardal, M. Mansoor, P. Ueland, Anal. Biochem. 184 (1990) 338.

[14] R. Gotti, V. Andrisano, R. Gatti, V. Cavrini, S. Candeletti, Biomed. Chromatogr. 8 (1994) 306.
[15] M. Fariss, D. Reed, Methods Enzymol. 143 (1987) 101.
[16] D. Keller, D. Menzel, Anal. Biochem. 151 (1985) 418.
[17] R. Fahey, G. Newton, Methods Enzymol. 143 (1987) 85.
[18] C. Thioudellet, T. Oster, P. Leroy, A. Nicolas, M. Wellman, Cell Biol. Toxicol. 11 (1995) 103.
[19] M. Asensi, J. Sastre, F. Pallardo, J. de la Asuncion, J. Estrela, J. Vina, Anal Biochem. 217 (1994) 323.