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Short communication

A stable-isotope HPLC–MS/MS method to simplify storage of human whole blood samples for glutathione assay

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ABSTRACT

Background: Glutathione is the principal non-protein tripeptide thiol present in most mammalian cells and plays an important role in the redox status of biological systems. The accurate assessment of reduced glutathione (GSH) status as a reliable index of oxidative stress is of research and clinical significance. GSH undergoes rapid oxidation after sample collection and this presents a challenge.
Methods: Validation of an HPLC–MS/MS assay is reported. Storage stability using four variants of a methanolic precipitation with addition of stable isotope internal standard at collection is compared to L-serine borate/EDTA with perchloric acid precipitation (SBPE).
Results: Precipitation with methanol and addition of stable isotope on sample collection, combined with storage in solution at −70 °C showed superior storage stability to SBPE and other variants of the methanolic precipitation method up to 99 days.
Conclusions: The combination of stable isotope with methanolic precipitation at collection, with assay by HPLC–MS/MS provides superior results after storage of whole blood samples for at least 99 days.

1. Introduction

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine, reduced glutathione or GSH) is the principal non-protein tripeptide thiol present in mammalian cells [1]. GSH plays an important role in biological systems and is readily oxidised to its disulfide (GSSG). It is gaining increasing clinical importance [2]. The accurate assessment of GSH status is a reliable index of oxidative stress. Of concern in the assay of GSH is its storage stability in biological samples [3]. Rapid autooxidation to GSH disulphide (GSSG) has been shown to occur in samples at pH values above 7, hence acidification is often used [4]. Acid also conveniently precipitates proteins in samples, which facilitates sample cleanup for HPLC.

The current pretreatment of samples prior to storage is complex [4]. The aim of the current study was to develop an improved and simplified approach by simultaneously considering storage and preparation of samples for assay, thereby proposing immediate methanolic precipitation with/without addition of stable isotope as internal standard at the time of collection. Given its reported suitability for long-term storage, we included the method of Michelet et al. [5] in the comparison.

There are a plethora of methods for GSH assay in biological samples, including whole blood and plasma [4]. Spectrophotometry [6], fluorimetry [7], and high performance liquid chromatography (HPLC) [8], or HPLC coupled to tandem mass spectrometry (HPLC–MS/MS) [9] all used for analysis of GSH. Among these methods, assays which involve derivatisation followed by HPLC, using ultra-violet (UV) [8], fluorescence [10] or electrochemical (EC) [11] detection are widely reported.

2. Materials and methods

2.1. Chemicals and reagents

GSH and its stable isotope GSH* (glycine 13C2, 15N) were obtained from Sigma–Aldrich (Castle Hill, NSW, Australia) and Novachem (Collingwood, Vic, Australia) respectively. Oxidised glutathione (GSSG) reference material was obtained from

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Table 1
Optimum parameters for the tandem mass spectrometer.

Parameter	Optimum value
Curtain gas	25.0 psi
Probe temperature	650 °C
GS1 (ion source gas 1)	60 psi
GS2 (ion source gas 2)	70 psi
CAD gas	6.00 psi
Nebuliser current	3.00 μA
Collision cell exit potential	4.00 V
Collision energy	21 V
Declustering potential	27 V (GSH); 21 V (GSH*)
Entrance potential	7 V (GSH); 9 V (GSH*)

Sigma–Aldrich (Castle Hill, NSW, Australia). Acetonitrile and methanol were HPLC grade and formic acid was analytical grade (Sigma–Aldrich, Castle Hill, NSW, Australia). Deionised water of at least 18 MΩ cm quality was obtained using a Millipore Milli-Q Elix 3 water purification system (Millipore Australia Pty Ltd., North Ryde, NSW, Australia) linked to an Aquacure Labmate high purity demineraliser (Aquacure Water Treatment Pty Ltd., Salisbury, QLD, Australia). All solvents were HPLC grade or better, and all other reagents were analytical reagent grade or better.

2.2. HPLC–MS/MS

The HPLC system was a Shimadzu LC 20 Prominence with two LC-20AD pumps, a CBM-20A communications module and a SIL-20AC autosampler (Shimadzu Australia, Rydalmere, NSW, Australia). Chromatography used a Phenomenex Luna C18 (2) (150 mm × 2.0 mm; 3 μm particle size) (Phenomenex Australia, Sydney, Australia) column. The mobile phase flow rate was 0.25 mL/min (Table 1), and comprised component A of 0.1% (v/v) formic acid in water, and component B of 15% (v/v) methanol in acetonitrile. A gradient from 0% to 75% mobile phase B over 7 min was used, holding for 30 s, followed by a return to 0% B at 8.2 min, and holding for 1.8 min. Detection used an API 3200 triple quadrupole mass spectrometer (Applied Biosystems Australia and New Zealand, Scoresby, VIC, Australia), with an electrospray ion source (heated nebuliser) in positive ion mode and Applied Biosystems Analyst (v.4.1) software. To monitor GSH, the precursor ion 308.1 *m*/*z* was chosen and the product ion at 179.1, using multiple reactant monitoring (MRM). For GSH* the precursor ion at 311.2 *m*/*z* was chosen and the product ion at 182.0 *m*/*z* again using MRM. Optimum MS/MS parameters were determined and utilised.

2.3. Assay validation

To establish the linearity of the assay, standards were prepared in mobile phase A from 10 to 400 μg/mL (32.5–1300 μM). Standard curves were constructed by regressing the peak area ratio (GSH/GSH*) on GSH concentration, using reciprocal weighting of the concentration. To demonstrate reproducibility and accuracy, whole blood was obtained from a healthy volunteer and allowed to stand at 4 °C for several hours to deplete the GSH. This was then used to prepare a seeded control (SC) with standard addition of 350 μg/mL (1138 μM) of GSH. Using both protocol 1 (SBPE method) and methanol precipitation protocol 2b (see below), both the GSH-depleted whole blood (DWB) and SC samples were assayed in singlicate on 10 separate occasions. Assay accuracy was estimated using the formula;

$$\frac{\text{Assayed [SC]} - \text{Assayed [DWB]}}{\text{seeded concentration}} \times 100\%$$

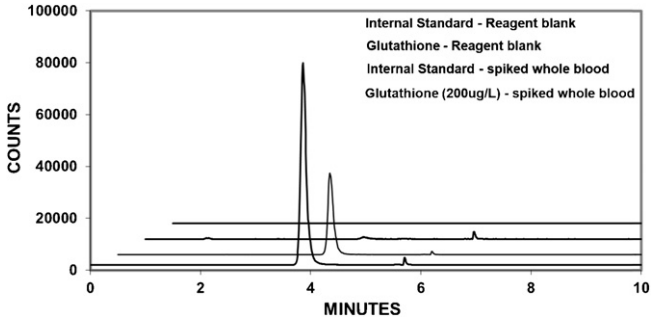

Fig. 1. Chromatograms (from lowest to highest) of (a) 200 μg/mL glutathione (GSH – transition from 308.1 to 179.1 *m*/*z*) standard, (b) stable-isotope GSH (GSH* – transition from 311.2 to 182.0 *m*/*z*) prepared in mobile phase A, (c) glutathione (GSH) and (d) stable-isotope GSH (GSH*) prepared in depleted whole blood (without GSH or GSH* addition).

Limit of detection was determined as the concentration producing a signal three times the height of the baseline noise.

2.4. Stability study

A single well-mixed whole blood sample was used for all protocols, immediately after collection into ethylenediaminetetraacetic acid (EDTA). In all protocols, a standard (200 μg/mL (650 μM)) in mobile phase A and controls (prepared by standard addition to DWB) were also batched with the test whole blood sample during sample preparation. A standard curve was prepared on the day of assay using single point calibration forced through the origin.

2.4.1. Stability study – protocol 1: L-serine borate/perchloric acid/EDTA (SBPE) method [5]

40 μL of 100 mM L-serine borate was added to 2 mL of whole blood, standard or control in respective 12 mL glass culture tubes. 1 mL of 35% perchloric acid in EDTA was added. Samples were centrifuged at 6000 × *g* for 3 min. 200 μL aliquots of the supernatants were pipetted into 1.5 mL plastic disposable tubes. The test whole blood, standard and control samples were then frozen and stored at −70 °C, with the exception of the zero time batch. On the day of assay, 50 μL of the samples were diluted to 20 mL with mobile phase A. 100 μL was pipetted into 1.5 mL tubes. 100 μL of the internal standard solution (IS) (0.5 μg/mL GSH* in methanol) was added, and duplicate 5 μL volumes were injected.

2.4.2. Stability study – protocol 2a (methanol precipitation with stable isotope added prior to storage and stored dry)

Standards, test whole blood samples, and controls (100 μL) were diluted to10 mL with methanol. After mixing and centrifuging the samples for 5 min (6000 × *g* at room temperature). To 2.70 mL of the supernatant 150 μL of IS in methanol (5.0 μg/mL) was added. After mixing, 200 μL was pipetted into respective tubes and evaporated to dryness under nitrogen gas at 45 °C and capped immediately. All tubes were immediately placed at −70 °C until required for assay.

On the day of assay, one batch containing standard, controls and test whole blood was reconstituted in 400 μL of mobile phase A per tube. After mixing, 5 μL volumes were injected.

2.4.3. Stability study – protocol 2b (methanol precipitation with stable isotope added prior to storage and stored in solution)

As per protocol 2a, except samples were placed under nitrogen gas for 1 min only, instead of evaporating to dryness prior to capping and storage. Samples were later evaporated to dryness on the day of assay and treated subsequently as per the samples in 2a.

Table 2
Assay accuracy and reproducibility: $n = 10$ (on separate occasions).

Sample	Mean assayed concentration (μg/mL)	Accuracy (%)	Reproducibility (CV (%))
Unspiked DWB preparation using protocol 1	14.9	N/A	17.5
DWB with 350 μg/mL added. Preparation using protocol 1	375	103	4.7
Unspiked DWB preparation using protocol 2b	22.3	N/A	4.5
DWB with 350 μg/mL added. Preparation using protocol 2b	383.9	103	6.5

Abbreviations: CV, co-efficient of variation; DWB, depleted whole blood.

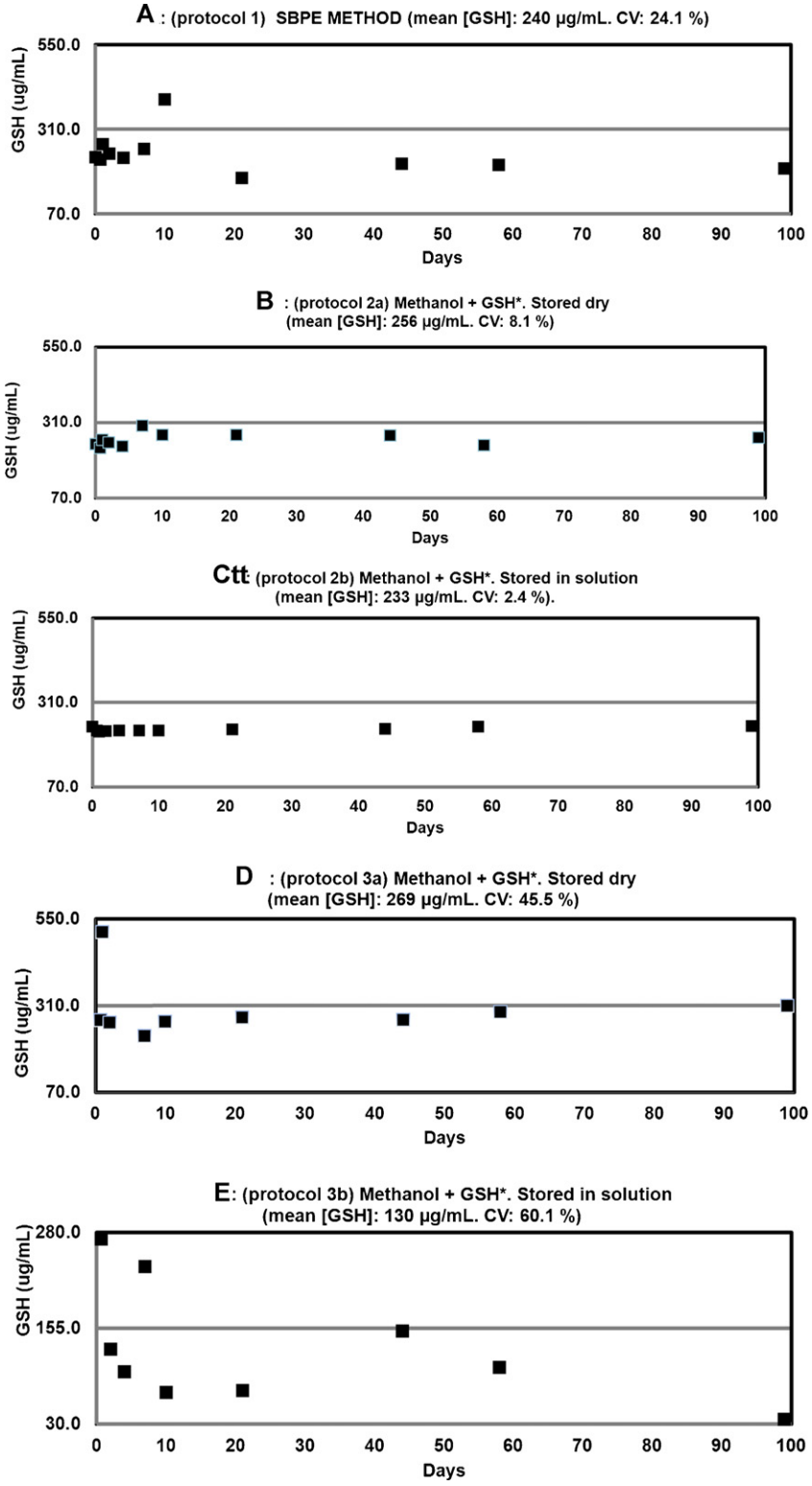

Fig. 2. Comparison of assayed concentrations after storage using various treatments. (CV = co-efficient of variation). Horizontal line shows mid-point of *y* axis.

2.4.4. Stability study – protocol 3a (methanol, stored dry + GSH)*

Standard, blood samples, and controls (100 μL) were each diluted to 10 mL with methanol. The samples were centrifuged for 5 min at 6000 × *g*. After mixing, 100 μL was pipetted into respective tubes. The contents of each tube was evaporated to dryness under a stream of nitrogen at 45 °C. The tubes were capped immediately and placed at −70 °C,until required for assay.

On the day of assay, one set each of standard, test whole blood and controls were reconstituted in 200 μL of 0.3 μg/mL GSH* in mobile phase A. Duplicate 20 μL volumes were injected.

2.4.5. Stability study – protocol 3b (methanol, stored in solution + GSH)*

As per protocol 3a, except samples were placed under a stream of nitrogen gas for 1 min only, instead of evaporating to dryness, prior to capping. Samples were later evaporated to dryness on the day of assay and treated as per protocol 3a.

3. Results

Optimum MS/MS parameters for the assay are listed in Table 1. Fig. 1 shows chromatograms for (a) 200 μg/mL (650 μM) GSH standard (retention time 3.80 min); (b) GSH* in standard (retention time 3.85 min); (c) GSH in unspiked DWB, (without internal standard); and (d) GSH* in DWB (without internal standard).

The limit of detection, (3 × height of baseline noise) was 0.56 μg/mL (1.8 μM). Linearity was confirmed over the concentration range 10–400 μg/mL ($y = 0.0014x + 0.0002$, $r^2 = 0.9997$). Reproducibility and accuracy are reported in Table 2.

Storage stability data obtained using the different sample preparation protocols are shown in Fig. 2. Mean concentrations determined over 99 days and the co-efficient of variation for these results are also included.

4. Discussion

The HPLC–MS/MS method demonstrated acceptable specificity in that DWB produces a peak less than 1% of that of actual samples at the same retention time as GSH. This small peak is likely to be remaining traces of GSH, given that it is endogenous to this matrix. To identify the small later-eluting peak with a retention time of 5.8 min, solutions of oxidised glutathione (GSSG) were prepared and injected. A peak was apparent at 5.8 min (monitoring precursor ion at 308.1 *m*/*z* and product ion at 179.1, as for GSH). Therefore, the small peak observed at that time is almost certainly GSSG after its reduction to GSH during assay.

Since GSH is an endogenous substance, it is not possible to determine assay accuracy by preparation of seeded "blank" blood. We therefore used the approach of 'standard addition' to DWB by subtracting the background concentration from the standard addition to estimate the accuracy, which was found to be satisfactory. Total Imprecision was less than 7% at concentrations most likely to be encountered in clinical samples. These data also indicate the lack of impact of ion suppression, as the accuracy of seeded controls in DWB was acceptable after quantitation against standards in mobile phase A, which is a simple matrix and unlikely to exhibit ion suppression.

We encountered poor reproducibility and sensitivity of the MS/MS after injecting samples containing PCA. To redress this problem we regularly disassembled and cleaned Q0. This requirement was not necessary when using methanol treated samples.

Of the sample preparation/storage methods using methanol precipitation (Fig. 2B–E), addition of the internal standard in methanol (with storage on methanol i.e. protocol 2b), was superior to the other methods in that percent co-efficient of variation for assays over 99 days was only 2.4% and the concentration determined was consistent with other reports for human whole blood samples (6). The results for similar preparation with storage *after* methanol evaporation were also satisfactory. Sample preparation using methanol precipitation without addition of GSH* as the internal standard immediately after sample collection (protocol 3b) produced poor results, with a mean concentration of 130 μg/mL, substantially lower than all other protocols, and that reported in the literature [6].

Results obtained using the SBPE method (Fig. 2A) indicate comparable mean results to the other protocols with the exception of protocol 3b. Reproducibility over 99 days however is considerably less satisfactory than our proposed method. The mean assayed concentration was 238 μg/mL (774 μM) and the CV for these data was 24.1%. This is outside the expected CV based on our validation data for re-assay of the same sample and also substantially greater than the results for methanol precipitation with GSH* added on collection (CV = 2.4%). The data point after 10 days of storage could, however, be regarded as an outlier. If this assumption is made, the CV reduces to 12.3%. Our proposed method is still to be preferred. Furthermore, the SBPE method is tedious, requiring preparation of numerous reagents and handling of the corrosive and potentially dangerous PCA.

It is common practice in bioanalytical assays of homogeneous fluids to add a fixed amount of internal standard to a standard volume of each calibrator, sample, and/or control in a batch, usually in the first step of the sample workup. It is also recommended practice to use a stable isotope of the analyte as an internal standard in quantitative HPLC–MS/MS to minimise the risk of ion suppression effects on the assay [12]. We have capitalised on the ability of a stable isotope internal standard, combined with an HPLC–MS/MS assay to normalise for oxidation of GSH during sample storage. This has resulted in an assay that combines the simplicity of methanol precipitation of proteins immediately on sample collection, with the ability of a stable isotope to normalise for any residual oxidation (or other degradation) between sample collection and storage.

5. Conclusions

The precipitation of proteins using methanol containing the internal standard GSH* immediately after drawing whole blood samples, combined with the use of an HPLC–MS/MS assay, simplifies sample preparation for storage of human whole blood samples for GSH assay. This obviates the need to use potentially harmful reagents such as PCA, and minimises the risk of damage to the HPLC–MS/MS system. Our data confirm that protein precipitation using methanol rather than the L-serine borate/EDTA/PCA method, when combined with the addition of GSH* and an HPLC–MS/MS assay, is rapid, simple and produces accurate results after storage up to 99 days.

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References

[1] C. Cereser, J. Guichard, J. Drai, E. Bannier, I. Garcia, S. Boget, P. Parvaz, A. Revol, J. Chromatogr. B: Biomed. Sci. Appl. 752 (2001) 123.
[2] C.C. McIlwain, D.M. Townsend, K.D. Tew, Oncogene 25 (2006) 1639.
[3] A. Larsson, Functions of Glutathione: Biochemical, Physiological, Toxicological and Clinical Aspects, Raven Press, New York, 1983.
[4] P. Monostori, G. Wittmann, E. Karg, S. Turi, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 877 (2009) 3331.

[5] F. Michelet, R. Gueguen, P. Leroy, M. Wellman, A. Nicolas, G. Siest, Clin. Chem. 41 (1995) 1509.
[6] F. Teitze, Anal. Biochem. 27 (1969) 502.
[7] V.H. Cohn, J. Lyle, Anal. Biochem. 14 (1966) 434.
[8] M. Asensi, J. Sastre, F.V. Pallardo, J. Garcia de la Asuncion, J.M. Estrela, J. Vina, Anal. Biochem. 217 (1994) 323.
[9] E. Camera, M. Rinaldi, S. Briganti, M. Picardo, S. Fanali, J. Chromatogr. B: Biomed. Sci. Appl. 757 (2001) 69.
[10] A.M. Svardal, M.A. Mansoor, P.M. Ueland, Anal. Biochem. 184 (1990) 338.
[11] D.L. Rabenstein, R. Saetre, Clin. Chem. 24 (1978) 1140.
[12] P.J. Taylor, Clin. Biochem. 38 (2005) 328.