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Simultaneous quantitation of oxidised and reduced glutathione in equine biological fluids by reversed-phase high-performance liquid chromatography using electrochemical detection

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Abstract

A change in the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) can be used to indicate oxidative stress in vivo. A rapid and highly sensitive isocratic reversed-phase high-performance liquid chromatographic method using coulometric electrochemical detection (LCEC) has been developed to simultaneously detect GSH and GSSG in equine biological fluids. Perchloric acid was used to extract GSH and GSSG from equine plasma and haemolysates, and methanol was used to deproteinise bronchoalveolar lavage fluid samples. Injection of extracts onto a Hypersil ODS HPLC column produced well resolved peaks corresponding to GSH and GSSG. The concentrations of GSH and GSSG found in equine haemolysates were similar to those previously found in humans and laboratory animals, although, to the authors' knowledge, previous attempts to measure GSH and GSSG in bronchoalveolar lavage fluid using LCEC have been unsuccessful. This method can be used to measure the GSH redox ratio in biological fluids during physiological conditions that may induce oxidative stress, such as exercise and disease.

1. Introduction

The tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine) is found in vivo as both the reduced thiol (GSH) and oxidised disulphide (GSSG) forms. GSH is a potent antioxidant and forms GSSG in vivo in the presence of pro-oxidants [1]. GSSG is rapidly converted back to GSH by the action of the enzyme glutathione reductase and, therefore, much less GSSG, compared to GSH, is detected in the normal individual. A reduction in the ratio of GSH to GSSG (glutathione redox ratio) has been used as an indicator of oxidative stress and/or disease in man and laboratory animals [2–4], although few studies have investigated this parameter in the horse.

Previous methods reported for the measurement of GSH and GSSG in biological samples, including enzymatic [5,6], fluorimetric [7] and colorimetric [8] assays, often require separate assays for GSH or GSSG and have inadequate detection limits and low reproducibility. HPLC methods using UV [9,10] and fluorimetric [11] detection have also been described, but have the disadvantage of requiring pre- or post-column derivatization. HPLC methods using electrochemical detection (LCEC) have been reported,

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although inability to detect GSSG [12], complicated sample preparation and cumbersome maintenance of amperometric detectors [13–16] reduce the practicality of such methods.

The present method utilises a sensitive LCEC technique for the simultaneous measurement of GSH and GSSG concentrations in equine biological fluids and reports the glutathione redox ratio in equine haemolysate. GSH and GSSG concentrations have been measured for the first time in bronchoalveolar lavage fluid (BALF) using LCEC.

2. Experimental

2.1. Materials

Sodium dihydrogen phosphate (NaH_2PO_4; 99.999%) and 85% (w/v) phosphoric acid (99.999%) were purchased from Aldrich (Gillingham, UK). GSH, GSSG and disodium EDTA (Na_2EDTA) were from Sigma (Poole, UK). Sodium chloride, perchloric acid and potassium hydroxide were purchased from BDH (Poole, UK). Methanol (99.9%, HPLC grade super purity solvent) was from Romil (Loughborough, UK) and water was purified by reverse osmosis and deionisation.

2.2. Instrumentation

The liquid chromatographic system consisted of a LDC/Milton Roy Constametric 1 metering pump (Thermoseparations, Stone, UK) followed immediately by a pulse damper (ESA Analytical, Bedford, MA, USA), a Perkin-Elmer LC-420B autosampler (Perkin-Elmer, CT, USA) with a 10-μl sample loop and an ESA Coulochem Model 5100A electrochemical detector (ESA Analytical). The electrochemical detector was equipped with a Model 5011 dual analytical cell and a Model 5020 guard cell, both with in-line filters. The guard cell was positioned between the pulse damper and the injector, and acts as a scrubber for the mobile phase. A Perkin-Elmer LCI-100 Laboratory Computing Integrator was employed for data acquisition.

2.3. Chromatographic conditions

Chromatography was performed on a Hypersil ODS (150 × 4.6 mm I.D.; 3 μm particle size) column (Jones Chromatography, Mid Glamorgan, UK). Isocratic elution at ambient temperature was performed using a mobile phase consisting of 10 m*M* NaH_2PO_4 (1.998 g/l) adjusted to pH 2.7 with 85% (w/v) phosphoric acid, and containing 5% methanol (v/v). The mobile phase was prepared daily, filtered (0.22-μm filter, Millipore, Bedford, MA, USA) and vacuum degassed prior to use, and also sparged with helium during use. Separations were performed with a flow-rate of 1.0 ml/min. The applied electrode potentials of detector 1, detector 2 and guard cell working electrodes were set at 0.35 V, 0.85 V and 0.90 V, respectively. The system was washed daily with water followed by methanol, and stored in 100% methanol overnight and while not in use.

2.4. Current–voltage curves

The potentials applied at each detector were determined by generating a hydrodynamic voltammogram for both GSH and GSSG. A constant mass (10 ng each) of GSH and GSSG was injected at increasing potentials applied at detector 1 and a current–voltage curve was plotted of response (peak height in cm) against potential (volts) for each analyte.

2.5. Standard preparation

GSH and GSSG stock solutions (1 mg/ml) were prepared weekly by dissolving in HPLC mobile phase containing 2 m*M* Na_2EDTA and stored at 4°C. Working standard solutions were prepared daily in mobile phase. Standard curves for GSH and GSSG were produced using a range of concentrations: for GSH 1.0, 2.0, 3.0, 5.0, 8.0 and 10.0 μg/ml; for GSSG 0.1, 0.5, 1.0, 1.5, 2.0, 3.0 μg/ml.

2.6. Detection limits

The detection limits for the analysis of GSH and GSSG were determined by injecting serial

dilutions of GSH (100, 50, 10, 5 and 1 ng/ml) and GSSG (500, 100, 50, 10 and 5 ng/ml) onto the HPLC column. The lower limit of detection was defined by a peak height to baseline noise ratio of 3:1 or greater.

2.7. Sample collection and preparation

Equine blood was collected via jugular venipuncture into tubes containing lithium heparin as anticoagulant (12.5 IU/ml) and immediately transferred to ice. Plasma and red blood cells (rbc) were separated by centrifugation (500 *g* for 5 min) within 30 min of collection and the plasma was snap frozen and stored in liquid nitrogen until analysis. The rbc (0.5 ml) were added to an equal volume of 0.9% NaCl (containing 2 m*M* Na_2EDTA) and snap frozen in liquid nitrogen until analysis.

Haemolysates were prepared immediately prior to analysis by thawing the rbc–saline pellet in a 25°C water bath. Perchloric acid (5% w/v; 800 μl) was added to 200 μl of haemolysate and the mixture was vortex-mixed then left to stand at room temperature for 15 min. The sample was then centrifuged at 13 000 *g* for 5 min and the supernatant was removed and neutralized with 10 *M* potassium hydroxide (40 μl). The extract was vortex-mixed again and centrifuged for 2 min at 13000 *g*. The supernatant was diluted with mobile phase (1:9) and 10 μl injected onto the HPLC column.

Plasma was thawed immediately before analysis at room temperature. Plasma (0.5 ml) was deproteinised and extracted by adding 5% perchloric acid (0.5 ml), vortex-mixing and centrifuging as for haemolysate. The supernatant was diluted with mobile phase (1:1) and 10 μl injected for analysis.

BALF was collected using standard techniques [17]. The BALF (1.0 ml) was immediately deproteinised with an equal volume of methanol, followed by vortex-mixing and centrifuging at 13 000 *g* for 2 min. The supernatant was snap frozen in liquid nitrogen, freeze-dried and reconstituted in mobile phase (200 μl) prior to analysis.

2.8. Recovery study

Blank haemolysates and plasma were prepared as described previously. Spiked samples were prepared by adding 50 μg GSH and 10 μg GSSG to 1.0 ml of blank haemolysates or plasma. Blank and spiked samples were extracted as described above and recoveries were calculated by subtracting GSH and GSSG concentrations in blank samples from spiked samples and comparing with spike concentrations. Spiked BALF samples were prepared by adding 1.0 μg GSH and 0.4 μg GSSG to 1.0 ml BALF immediately before deproteinising with methanol. Recoveries were calculated as for haemolysates.

The effect of any delay in processing time on GSH and GSSG concentrations was investigated by analysing haemolysates and BALF immediately after collection and at 30 min and 2 h after collection from samples stored on ice.

3. Results

3.1. Chromatography

The current–voltage curves constructed for GSH and GSSG are shown in Fig. 1. The detector 1 potential was set at the base of the current–voltage curve for GSH to decrease background currents and prevent unwanted peaks

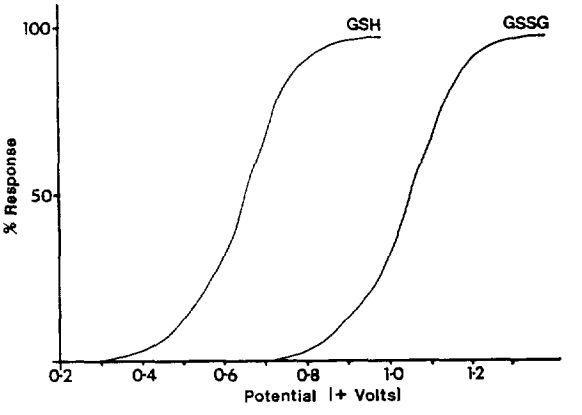

Fig. 1. Hydrodynamic voltammograms for GSH and GSSG at various applied potentials.

which may result from eluents that oxidise at lower potentials than GSH or GSSG. The detector 2 potential was set 0.05 V higher than the potential at which the current–voltage curve for GSH begins to plateau so that compounds which oxidise at even higher potentials will not produce chromatographic peaks. This is known as an oxidative screen mode of operation. The guard cell potential was set 0.05 V more positive than detector 2 so that the mobile phase is pre-oxidised and the concentration of electroactive contaminants in the mobile phase is eliminated, thereby decreasing the background current measured at the analytical cell.

A typical chromatogram for reduced and oxidised glutathione is shown in Fig. 2. The retention times of GSH and GSSG are 2.9 and 5.3 min, respectively. There was no interference from other thiols including cystine, cysteine, homocystine, homocysteine, cystamine, cysteamine, cystathionine, glutathione sulphonic acid and methionine (Fig. 3).

Representative chromatograms of GSH and GSSG in haemolysate and BALF are shown in Fig. 4.

Concentrations of GSH and GSSG measured in equine biological fluids are listed in Table 1. There was no detectable GSH or GSSG in equine plasma.

3.2. Assay feasibility

The standard curves for GSH ($y = 2.54x + 0.86$) and GSSG ($y = 2.56x + 5.82$) showed a good linear relationship, with correlation coeffi-

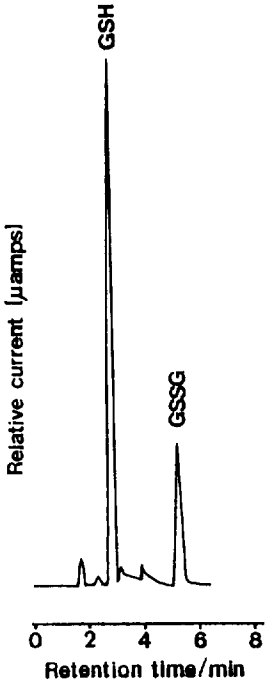

Fig. 2. Chromatogram of 5 μg/ml GSH and 1 μg/ml GSSG. Mobile phase was 10 m*M* sodium dihydrogen phosphate adjusted to pH 2.7 with 85% phosphoric acid and containing 5% methanol. Flow-rate was 1.0 ml/min. Attenuation of the integrator was initially 1024 and decreased to 128 at 4 min. Gain of electrochemical detector set to 10 × 1 = 10. Response time 10 s.

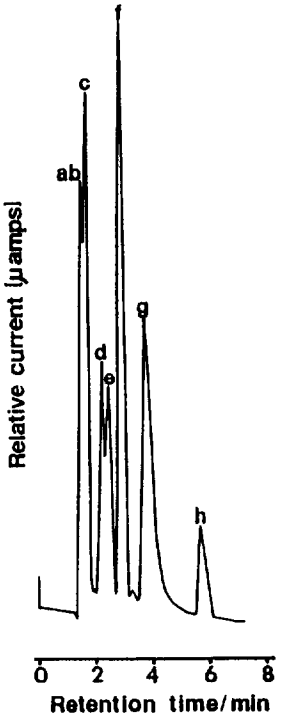

Fig. 3. Chromatogram of a mixed standard containing 1 μg/ml each of: a = cystathionine, b = cystine, c = cysteine, d = homocysteine, e = homocystine, f = GSH, g = methionine, h = GSSG; cystamine and cysteamine did not produce peaks. Conditions as for Fig. 2, except that attenuation was 128.

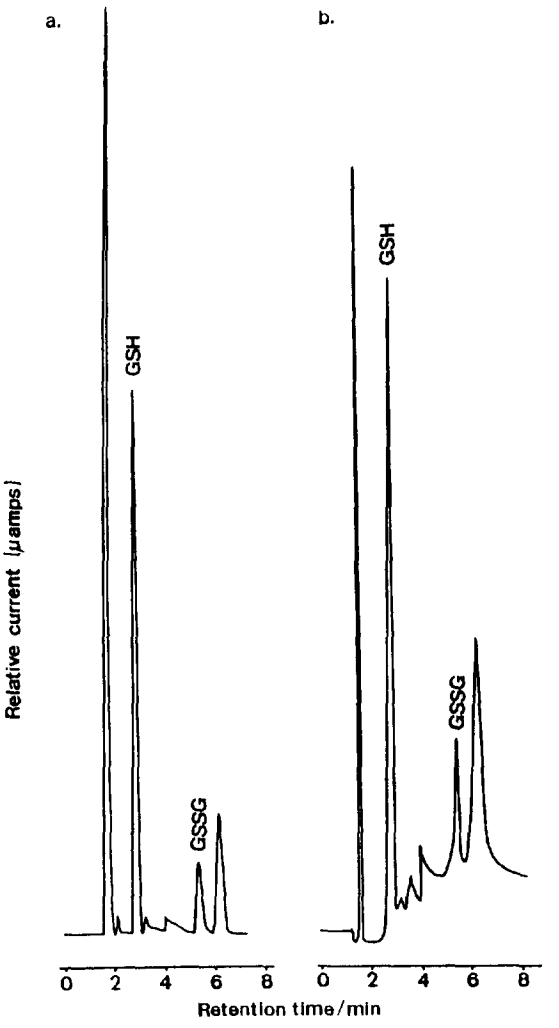

Fig. 4. Chromatograms of (a) an equine haemolysate extract, and (b) a deproteinised equine BALF sample. Sample preparation as described in text. HPLC conditions as described in Fig. 2 except for BALF (attenuation of integrator was 256 then changed to 64 at 4 min).

cients of 0.9996 and 0.9998, respectively. The lower limit of detection of the LCEC method was 5 ng/ml and 10 ng/ml for GSH and GSSG, respectively. The inter- and intra-assay coefficients of variation (C.V.) for the LCEC analysis of GSH in equine haemolysate were 8.8% (n = 6) and 2.0% (n = 6), respectively, and for GSSG were 10.8% (n = 6) and 5.1% (n = 6), respectively.

3.3. Recovery study

Recoveries of GSH and GSSG were 99.05% and 99.10%, respectively, from plasma or haemolysate, and 91.4% and 117.6%, respectively, from BALF. These recoveries were obtained from samples that were processed immediately after collection. Concentrations of GSH and GSSG in haemolysates were relatively stable after 30 min from blood stored on ice, although the concentration of GSH decreased by 1.6% and the concentration of GSSG increased by 10.8% after 2 h (Table 2). However, GSH and GSSG concentrations were altered by approximately 10% in BALF samples after 30 min storage on ice.

4. Discussion

A sensitive method for the measurement of GSH and GSSG in biological samples has been presented which can be used to indicate oxidative stress or disease in the individual or in a specific organ system. For example, a decrease in the concentration of total glutathione levels has been associated with lung diseases, such as cystic fibrosis and adult respiratory distress syndrome [18–20].

The chromatograms from equine haemolysate, plasma and BALF had minimal interference using this method, while good resolution of GSH and GSSG peaks was achieved. Mean total GSH (TGSH) concentrations (GSH + GSSG) in equine haemolysates (314.68 μg/ml, n = 7) was equivalent to those reported in man (312.22 μg/ml [21]). Similarly, the GSH redox ratio (GSSG/TGSH × 100) was approximately 6% in the horse and man. No glutathione (reduced or oxidised) was detected in the equine plasma samples analysed from rested or exercised horses. This corresponds to the results of Gohil et al [2] who found undetectable levels of GSH and GSSG in human plasma during exercise.

A comparison between GSH and GSSG concentrations in BALF from horses and from other species required correction for the concentration of saline used to perform the BAL and was not

Table 1
The concentrations of GSH and GSSG in haemolysates, plasma and bronchoalveolar lavage fluid of the horse

Sample type	Sample number	GSH (μg/ml)		GSSG (μg/ml)	
		Mean ± S.D.	Range	Mean ± S.D.	Range
Haemolysate	6	295.41 ± 56.48	219.93–366.61	38.59 ± 16.72	20.28–69.47
Plasma	6	–[a]	–	–	–
BALF[b]	4	0.36 ± 0.01	0.28–0.37	0.04 ± 0.01	0.04–0.06

[a] Not detected.
[b] Bronchoalveolar lavage.

attempted at this stage. However, a high analyte recovery and low limit of detection would make the LCEC method suitable to measure GSH and GSSG in BALF collected from any species. Oxidation of GSH appeared to occur rapidly in BALF despite strict attention to processing and possibly accounted for the lower recovery of GSH (91.4%) compared to GSSG (117.6%). Instability of GSH in BALF was also obvious if samples were stored on ice and emphasized the requirement of rapid sample processing and then immediate transfer to liquid nitrogen.

Several methods are currently available to measure GSH and/or GSSG in biological samples. In particular, a recycling enzymatic method [6] is frequently used to measure total glutathione, then GSSG in a second step. Since the concentration of GSSG is usually below 10% of GSH and oxidative stress is assessed by relatively minor changes in the ratio of GSH to GSSG [2–4,21], intra-assay variation between separate assays for total glutathione and GSSG may falsely contribute to the diagnosis of oxidative stress. Furthermore, no attempt was made to distinguish between GSH, GSSG and other thiols and mixed disulphides in the biological samples analysed, or to determine a detection limit specifically for GSH and GSSG. Liquid chromatography permits specific measurement of GSH and GSSG in biological samples, while electrochemical detection can increase the analytical sensitivity by 1000 fold, compared to ultraviolet or fluorescence detectors [9,11,13–16]. The coulometric detector with porous graphite electrodes that was used in this investigation requires minimal maintenance, providing strict attention to sample and mobile-phase preparation is followed. Amperometric electrochemical detectors, with glassy carbon or mercury-based electrodes, are difficult to maintain and have inconsistent electrode decay [13–15].

Table 2
The effect of a delay in sample processing time on the concentration of GSH and GSSG in equine haemolysate stored on ice

Processing time (min)	GSH (μg/ml)	GSSG (μg/ml)
0 (immediate)	315.83	35.30
30	317.47	35.16
120	310.74	39.11

A number of studies investigating oxidative stress in vivo have indicated the necessity of rapid sample processing to prevent iatrogenic oxidation of GSH to GSSG [21,22]. Storage of blood samples on ice for up to 30 min maintained the relative proportions of GSH and GSSG in plasma and haemolysates when compared to samples analysed immediately. However, oxidation of GSH to GSSG occurred rapidly in BALF even when stored on ice. An advantage of the method of sample processing described in this study is the use of liquid nitrogen to both prepare haemolysates and BALF and to store all samples prior to chromatography. We found no change in the concentrations of GSH and GSSG in samples snap frozen in liquid nitrogen and analysed immediately and samples that had been stored in liquid nitrogen for up to 28 days.

In summary, we have developed a sensitive and specific method to measure GSH and GSSG in biological samples, including, for the first time, BALF samples. The sample processing described in this method produced good analyte recovery, while the use of liquid nitrogen permitted storage of samples and subsequent bulk analysis, in turn both reducing between assay variation and eliminating the requirement for immediate sample analysis.

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