Comparison of HPLC and Enzymatic Recycling Assays for the Measurement of Oxidized Glutathione in Rat Brain

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Abstract

Glutathione (reduced, GSH and oxidized, GSSG) concentrations were analysed in rat cerebellar homogenate using high performance liquid chromatography with ultraviolet detection (HPLC-UV) or enzymatic recycling assays.

GSSG levels found using the HPLC-UV assay were 200-fold higher than those obtained with the enzymatic recycling procedure. Reduction of synthetic GSSG by glutathione reductase showed total conversion to GSH as assessed by HPLC-UV analysis. In contrast, only approximately 50% of the HPLC peak for GSSG could be reduced by glutathione reductase. Increasing the period of incubation with glutathione reductase for longer than 15 min did not alter GSSG levels.

These results suggest that another substance present in brain tissue is derivatized and eluted at the same time as GSSG using the HPLC-UV assay, thus contributing to the apparently high GSSG levels found employing this technique.

The antioxidant glutathione may be measured in tissue by a variety of different techniques including high performance liquid chromatography with ultra violet detection (HPLC-UV) (Reed et al 1980), high performance liquid chromatography with electrochemical detection (Slivka et al 1987) or using a spectrophotometric technique (Tietze et al 1969). However, there has been considerable controversy over which technique should be applied for the analysis of oxidized glutathione (GSSG) levels in human post mortem brain material. Using an automated amino acid analyser Perry et al (1982) reported very high levels of oxidized glutathione in brain from control individuals (89·3% of total glutathione) and from Parkinsonian patients (100%). In contrast, Slivka and colleagues (1987) found low GSSG levels ($< 1.2\%$) in brain samples from controls analysed using an enzymatic recycling assay.

The high GSSG content in human brain reported by Perry and colleagues (1982) indicates an unusual cellular redox state of reduced glutathione (GSH):GSSG, which is not compatible with the normal oxidative state of the brain (Meister 1983). Indeed, intracellular glutathione is predominantly (98%) maintained in the reduced state by the activity of NADPH-dependent glutathione reductase (Meister 1983). Furthermore, even under conditions of severe oxidative stress GSSG is rapidly transported out of the cells, possibly by membrane-bound γ-glutamyl transpeptidase, to protect from cytotoxic events associated with its intracellular accumulation (Meister 1983; Griffith & Meister 1985). Thus, the high brain GSSG content reported by Perry and colleagues (1982) may have been due to methodological problems, or to an artefact resulting from post-mortem delay. Since Perry et al (1971) also found unusually high GSSG levels in human biopsy specimens

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frozen within 30 s after removal from brain, it is likely that the analysis procedure rather than post-mortem effects were responsible for their findings.

To clarify the discrepancy over the measurement of brain GSSG levels, we have compared analysis using HPLC-UV detection with an enzymatic recycling assay. The HPLC technique produced apparent GSSG levels considerably greater than those measured with the enzymatic recycling assay. We therefore determined the composition of the HPLC peak obtained at the retention time of GSSG by treatment of brain tissue with glutathione reductase.

Materials and Methods

Tissue extraction

Male Wistar rats, 200–300 g, were killed by cervical dislocation, brain removed and the cerebellum was dissected on ice. The cerebellum was then homogenized in 6 vol ice-cold 0·4 M perchloric acid containing 0·1 mM diethylenetriamine penta-acetic acid using a microprobe sonicator. The samples were centrifuged at 4000 rev min^{-1} for 15 min at 4°C. The supernatant was analysed for GSSG and GSH using two techniques, the enzymatic conversion assay and HPLC analysis.

Enzymatic conversion assay for GSSG measurement

This sensitive assay is based on the enzymatic recycling procedure originally described by Tietze (1969) but subsequently modified by Cooper et al (1980). It utilizes the conversion of GSSG to GSH by glutathione reductase. A sample (0·15 mL) of the acidified supernatant was added to 1·35 mL 11 mM N-ethylmaleimide (NEM) in 100 mM potassium phosphate buffer containing 5 mM ethylenediaminetetraacetic acid, pH 7·5. After a 20-min incubation period at room temperature, the reaction mixture was passed through Sep-Pak C-18 cartridges

(Millipore, Water Associates, Watford) to remove unreacted NEM. Before this the cartridges had been rinsed in equal volumes of methanol and 100 mM potassium phosphate buffer. The retention of NEM by the Sep-Pak cartridges was verified in a separate experiment, by measuring the absorbance of NEM solutions spectrophotometrically at 315 nm before and after elution through the cartridges (Akerboom & Sies 1981). These experiments showed 99·6% (n = 6) retention of unreacted NEM.

GSSG levels were then detected spectrophotometrically using 1·5 mL of eluate to which 0·4 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 0·17 mM β-NADPH and 16 μg mL^{-1} GSSG reductase was added. The reaction was initiated by the addition of GSSG reductase. The final assay volume was 2·0 mL. The rate of reduction of DTNB to 5-thio-2-nitrobenzoate (TNB) was followed using a Shimadzu dual beam spectrophotometer (UV-2101 PC) at 412 nm, at ambient temperature for 5 mins. Rates of colour formation were corrected for the blank rate (rate of DTNB reduction by GSSG reduction in the absence of brain homogenate) compared with a standard curve obtained using known amounts of synthetic GSSG (10–100 ng). Standard curves to GSSG were linear (r = 0·994) over this concentration range. In separate experiments, GSSG recovery (> 90%) was calculated using known concentrations of GSSG (100 μg) added to cerebellar homogenates.

HPLC with UV detection assay for measurement of GSH and GSSG

GSH and GSSG were measured using a minor modification of the method described by Reed et al (1980). This method of extraction is based on the derivatization reaction between glutathione and iodoacetic acid to form 5-carboxymethyl glutathione which complexes with dinitrofluorobenzene and is subsequently detected by high-pressure liquid chromatography with ultraviolet detection.

A sample (0·17 mL) of the acidified supernatant was added to the internal standard (1 mM cysteic acid) and 0·88 M iodoacetic acid. Excess sodium hydrogen carbonate was added to the reaction mixture to precipitate sodium perchlorate. The samples were incubated at room temperature in the dark for an hour. Subsequently, 0·5 mL of an alcoholic solution of 1·5% (v/v) 2,4-dinitrofluorobenzene was added to the samples and incubated for 4 h. Diethyl ether (1·0 mL) was added and the samples were shaken and centrifuged at 2000 rev min^{-1} for 20 min at room temperature. The residual aqueous phase containing derivatized glutathione was separated and analysed by HPLC. GSH recoveries were found to be > 90% using synthetic GSH added to cerebellar homogenates.

Separation of 5-carboxymethyl glutathione was carried out at room temperature with a flow rate of 1·0–1·25 mL min^{-1} and a corresponding pressure of about 2700 psi. Chromatographic separation of derivatives was performed by injecting samples (10 μL) of the aqueous phase onto a Spherisorb S-5 amino ODS column (25 cm × 4·6 mm, Phase Separations). The use of a commercial Spherisorb column contrast with Reed's initial method which utilized a hand packed 3-amino-propyl-Spherisorb column. Derivatives were eluted using an ammonium acetate gradient in glacial acetic acid, methanol and water at pH 5·05 (Table 1).

Glutathione was subsequently detected using an ultraviolet detector at a wavelength of 365 nm. Chromatographic peaks

Table 1. Ammonium acetate gradient utilized for measuring derivatives of GSH and GSSG.

Time (min)	Flow rate (mL min^{-1})	% Buffer A methanol/water 4 : 1	% Buffer B ammonium acetate/ acetic acid pH 5.05
Initial–15	1·0	85	15
15–20	1·0	60	40
20–22	1·0	15	85
22–30	1·25	0	100

were integrated by a Waters 645 data module. Glutathione derivatives (GSH and GSSG) were quantified in relation to the internal standard (cysteic acid).

Reduction of brain GSSG with glutathione reductase

Neutralized rat cerebellar supernatant samples (0·5 mL) were incubated with 0·17 or 0·34 mM NADPH and 16 or 32 μg mL^{-1} glutathione reductase (pH 7·5) at 25°C for 30 min. After this 0·88 M iodoacetic and 1 mM cysteic acid (internal standard) were added to the samples, which were then derivatized and analysed for glutathione content by HPLC with UV detection. Control samples consisted of aliquots (0·5 mL) of neutralized cerebellar supernatant incubated with 0·17 or 0·34 mM NADPH at 25°C for 30 min. The samples were added to iodoacetic acid and cysteic acid and subsequently derivatized.

An incubation period of 30 min with glutathione reductase was determined from separate experiments where cerebellar supernatants were incubated with 16 mg mL^{-1} glutathione reductase plus 0·17 mM NADPH (pH 7·5) at 25°C for 5, 10, 15, 30 or 45 min. A maximal decrease in GSSH levels (70%) was observed after 15 min and remained stable over the next 30 min (data not shown).

Reduction of synthetic GSSG by glutathione reductase

Samples (0·5 mL) of a solution containing 25 μL synthetic GSSG (1 mM final concentration) and 25 μL synthetic GSH (1 mM) dissolved in 100 mM potassium phosphate buffer and 5 mM EDTA (pH 7·5) were incubated with 0·17 mM NADPH and 16 mg mL^{-1} glutathione reductase (pH 7·5) at 25°C for 30 min. Subsequently, the samples were derivatized and analysed for glutathione by HPLC with UV detection. Control samples consisted of samples (0·5 mL) of a solution of synthetic GSSG (1 mM) and synthetic GSH (1 mM) incubated with 0·17 mM NADPH at 25°C for 30 min and then derivatized.

Reduction of synthetic GSSG added to cerebellar supernatant by glutathione reductase

Synthetic GSSG (1 mM) was added to neutralized rat cerebellar supernatant (0·5 mL) and incubated with 0·17 mM NADPH and 16 mg mL^{-1} glutathione reductase (pH 7·5) at 25°C for 30 min. After this, the samples were derivatized and analysed by HPLC with UV detection. Control samples consisted of synthetic GSSG (1 mM) added to neutralized cerebellar supernatant (0·5 mL) and incubated with 0·17 mM NADPH at 25°C for 30 min. Subsequently, the samples were derivatized.

Synthetic GSH added to cerebellar supernatant

Synthetic GSH (1 mM) was added to neutralized rat cerebellar supernatant (0·5 mL) derivatized and analysed by HPLC with

UV detection. Control samples consisted of neutralized cerebellar supernatant (0·5 mL) derivatized.

Chemicals and reagents

Glutathione (GSH and GSSG), cysteic acid, 2,4-dinitrofluorobenzene, iodoacetic acid, β-nicotinamide adenine dinucleotide phosphate (NADPH), glutathione reductase, N-ethylmaleimide, potassium dihydrogen phosphate, EDTA, diethylenetriamine pentaacetic acid, 5,5'-dithio-bis-(2-nitrobenzoic acid) were obtained from Sigma Chemical Co. Ammonium acetate, sodium hydrogen carbonate, methanol (HPLC grade) and glacial acetic acid were obtained from Fisons. HPLC-grade water was prepared by passing de-ionized water (Purite model RO 100) through an organic filter (Millipore Corporation).

Statistical analysis

Glutathione levels (GSH and GSSG) in samples which were and were not treated with glutathione reductase were compared using the Mann-Whitney U-test.

Results

Comparison of GSSG levels in rat cerebellar supernatant using HPLC-UV and enzymatic recycling assays

Mean cerebellar concentration of GSH and GSSG glutathione, determined from 6 animals by the HPLC derivatization assay were respectively $1·11 \pm 0·28$ and $0·45 \pm 0·09$ mmol (g wet weight of brain tissue)$^{-1}$. Consequently GSSG concentrations determined by the HPLC technique formed 22·5% of the cerebellar total glutathione content. In contrast using the enzymatic recycling assay cerebellar GSSG concentrations were determined from 6 animals to be $0·0021 \pm 0·0001$ mmol (g wet weight of tissue)$^{-1}$.

Utilizing the GSH concentrations determined by the HPLC derivatization assay the GSH : GSSG ratios for the enzymatic recycling assay was 534 : 1 compared to 2·5 : 1 for the HPLC derivatization assay, a 200-fold difference.

GSSG reduction with glutathione reductase

Incubation of the rat cerebellar supernatant with glutathione reductase ($16 \mu g$ mL^{-1}) resulted in a marked increase in GSH levels (by 150%) but only a reduction in GSSG content, 43% as measured using HPLC derivatization assay (Table 2). Incubation with twice the concentration of glutathione reductase ($32 \mu g$ mL^{-1}), failed to stimulate any further significant increase in GSH levels or a further reduction in GSSG content (Table 2). The GSH:GSSG ratio remained low (8 : 1) in cerebellar supernatant after incubation with glutathione reductase. The chromatograms of the rat cerebellar supernatant showed a peak with a retention time corresponding to GSSG. This peak was still present at the same retention time following treatment of samples with glutathione reductase (data not shown).

Synthetic GSSG reduction by glutathione reductase

Incubation of synthetic GSH (1 mM) and GSSG (1 mM) with glutathione reductase ($16 \mu g$ mL^{-1}) showed complete conversion to GSH as measured using HPLC and UV detection (Table 3).

Reduction of synthetic GSSG added to cerebellar supernatant by glutathione reductase

In rat cerebellar supernatant samples incubated with synthetic GSSG and glutathione reductase, the GSH levels measured

Table 2. Reduced (GSH) and oxidized (GSSG) glutathione levels from rat cerebellar supernatant samples incubated with NADPH (0·17 or 0·34 mM) and glutathione reductase (16 or 32 mg mL^{-1}) at 25°C for 30 min.

Glutathione reductase (μg mL^{-1})	Glutathione concn (mmol g^{-1})	
	GSH	GSSG
0	$1·0 \pm 0·4$	$0·45 \pm 0·09$
16	$2·5 \pm 0·2*$	$0·21 \pm 0·01*$
32	$2·6 \pm 0·4*$	$0·21 \pm 0·11*$

Glutathione concentrations were measured by HPLC-UV. Values are expressed as mean $\pm$ s.e.m., $n = 3-6$ for each group. $*P < 0·05$ compared with glutathione levels in the control group.

Table 3. Synthetic reduced (1 mM GSH) and oxidized (1 mM GSSG) glutathione incubated with NADPH (0·17 mM) and glutathione reductase (16 mg mL^{-1}) at 25°C for 30 min. GSH and GSSG levels measured by HPLC-UV are expressed as μmol mL^{-1}.

	Glutathione concn (μmol mL^{-1})	
	GSH	GSSG
Control	$50 \pm 5·6$	$50 \pm 5·6$
Glutathione reductase	$126 \pm 13*$	$0·0$

Values are expressed as mean $\pm$ s.e.m. No GSSG was detected after incubation. $*P < 0·01$ compared with control values, Mann-Whitney U-test, $n = 3$ for each group.

Table 4. Synthetic oxidized glutathione (1 mM GSSG) added to rat cerebellar supernatant and incubated with NADPH (0·17 mM) and glutathione reductase (16 mg mL^{-1}) at 25°C for 30 min. Glutathione concentrations measured by HPLC-UV are expressed as μmol (g wet weight tissue)$^{-1}$.

	Glutathione concn (μmol g^{-1})	
	GSH	GSSG
Control	$0·4$	$3·38 \pm 0·56$
Glutathione reductase	$3·87 \pm 0·92*$	$1·13 \pm 0·42*$

Values are expressed as mean $\pm$ s.e.m. (The s.e.m. for control GSH levels was very small and is not indicated). $*P < 0·05$ compared with control values, Mann-Whitney U-test, $n = 3$ for each group.

with HPLC and UV detection increased by 8-fold as a result of reduction of both synthetic GSSG and endogenous GSSG (present in homogenate) (Table 4). However, 32% of the original peak height was still present with a retention time corresponding to GSSG after incubation with glutathione reductase.

Synthetic GSH added to cerebellar supernatant

In rat cerebellar supernatant samples incubated with synthetic GSH, the GSH content measured with HPLC and UV detection increased by 80% (Table 5). In contrast, GSSG levels were unaltered.

Discussion

High-pressure liquid chromatography with ultraviolet detection (HPLC-UV) as described by Reed & colleagues (1980) for the analysis of both GSH and GSSG levels involves a deri-

Table 5. Synthetic reduced glutathione (1 mM GSH) added to rat cerebellar supernatant and analysed by HPLC with UV detection. Glutathione concentrations measured by HPLC-UV are expressed as μmol (g wet weight tissue)$^{-1}$.

	Glutathione concn (μmol g^{-1})	
	GSH	GSSG
Control	$1 \cdot 0 \pm 0 \cdot 4$	$0 \cdot 14$
Synthetic reduced glutathione	$1 \cdot 86 \pm 0 \cdot 18$	$0 \cdot 18$

Values are expressed as mean $\pm$ s.e.m. (The s.e.m. for GSSG level was very small and is not shown). $*P < 0 \cdot 05$ compared with control values, Mann-Whitney U-test, n = 3 for each group.

vatization procedure which yields high non-physiological GSSG levels. The enzymatic recycling assay for GSSG analysis produces realistic low GSSG (< 1·2%) levels in agreement with those reported in literature (Slivka et al 1987). The high non-physiological GSSG levels detected in brain using the HPLC-derivatization technique contrast markedly with the physiological GSSG levels detected by Reed (1980) in lymphoma cells using the same technique. One reason for the unusually high GSSG levels obtained using the HPLC assay may be that it detects another compound which elutes at the same time as GSSG.

To investigate whether the chromatographic peak for GSSG in the HPLC-derivatization assay represents the true GSSG levels in brain or whether the HPLC peak represents a mixture of compounds which elute at the same time a series of experiments were conducted on the effects of glutathione reductase on synthetic and endogenous GSSG.

Incubation of cerebellar supernatants with glutathione reductase failed to remove the GSSG peak by its conversion to GSH. Whereas synthetic GSSG was totally reduced to GSH after incubation with glutathione reductase. This would suggest that some other thiol is present in the brain which is capable of being derivatized and is eluted at the same time as GSSG in the HPLC-derivatization assay. Alternately glutathione reductase employed in these studies was not able to totally reduce the brain GSSG to GSH. The latter is unlikely since doubling the concentration of glutathione reductase or increasing the incubation time failed to further reduce the apparent GSSG content in the cerebellar supernatant.

Incubation of the cerebellar supernatant with glutathione reductase resulted in a large increase in GSH levels (by 150%). Such an increase cannot be explained simply by the conversion of part of the GSSG peak to GSH. Consequently, glutathione reductase must have been acting on other glutathione conjugates to form GSH, thus adding to the GSH formed from the conversion of GSSG. Indeed, Reed and colleagues (1980), using the HPLC-derivatization assay, demonstrated that other glutathione conjugates like cysteine glutathione-mixed disulphide were present in large concentrations (equivalent to 46% of the GSH content) in L5178Y lymphoma cells. Similarly, a comparison of published studies by Perry and colleagues (1982; 1986) on total glutathione levels (GSH + 2 × GSSG + cysteine glutathione-mixed disulphides) and that of GSH and GSSG levels alone in human brain suggests that cysteine glutathione-mixed disulphide levels may account for up to 30% of the total glutathione content in human brain.

Our studies using the HPLC-derivatization technique on brain would indicate that the GSSG may consist of a mixture of compounds which elute at the same time. A portion of the GSSG HPLC peak may correspond to a related non-protein thiol. The nature of this is unknown but again one candidate would be cysteine glutathione-mixed disulphides. In the initial description of the HPLC technique by Reed and colleagues (1980) a separate HPLC peak was detected for cysteine glutathione-mixed disulphides which eluted very close to the GSH and GSSG peaks. Even with extensive modification of the HPLC gradient the peak for cysteine glutathione-mixed disulphides could not be resolved when brain tissue was analysed. Whether the cysteine glutathione-mixed disulphide present in brain differs slightly to that in the periphery, such that its derivative elutes at the same time as GSSG is not known.

However, the presence of cysteine glutathione-mixed disulphide as a contaminant in the GSSG peak can only partially explain the results obtained. The actions of glutathione reductase on the cerebellar supernatant caused a 43% reduction in the GSSG peak. If all of this decrease was assumed to be GSSG, the HPLC-derivatization assay would still give GSSG levels higher than expected and a low GSH-GSSG ratio. Hence, only a small portion of the reducible component of the GSSG peak may actually be accounted for by authentic GSSG. The remainder may include cysteine glutathione-mixed disulphide but there remains a glutathione reductase resistant substance which may make up a significant portion of this peak.

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