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## Short Communication

# Fluorimetric determination of oxidised and reduced glutathione in cells and tissues by high-performance liquid chromatography following derivatization with dansyl chloride

JANET MARTIN* and IAN N. H. WHITE

*MRC Toxicology Unit, Woodmansterne Road, Carshalton, Surrey SM5 4EF (UK)*

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ABSTRACT

A high-performance liquid chromatographic method utilising fluorimetric detection of oxidised and reduced glutathione, following derivatization with dansyl chloride is described. Dansyl derivatives are separated on an aminopropyl silica column with a methanol–sodium acetate gradient system giving detection limits (signal-to-noise ratio = 2) of 1 pmol. This is in the order of 100-fold more sensitive than established methods based on the ultraviolet detection of dinitrophenylglutathione derivatives. The present procedures have been used to determine oxidised and reduced glutathione in rat lung tissues and in alveolar macrophages.

## INTRODUCTION

A number of methods are available for the determination of reduced glutathione (GSH) in cells. These are often based on reactions with the –SH function, *e.g.* Ellman's reagent [5,5-dithio-bis(2-nitrobenzoic acid)] [1] or more recently, monobromobimane [2]. Estimation by high-performance liquid chromatography (HPLC) of both oxidised and reduced glutathione has been carried out using electrochemical detection [3] or fluorimetric methods, following derivatization with *o*-phthalaldehyde [4]. However, one of the most robust and widely used HPLC procedures for biological samples is that of Reed and coworkers [5,6], based on the reaction of the amino function of glutathione with 1-fluoro-2,4-dinitrobenzene and detection by UV absorption. Such UV detection results in limited sensitivity of the method, which can create difficulties in extrahepatic tissues or isolated cells having low glutathione levels.

Many derivatization procedures have been developed for the analysis of fluorescent derivatives of amino acids (reviewed in ref. 7). Of these, derivatization with 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride) gave

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the highest yields with sulphur amino acids such as cysteine [8]. With fluorescence detection, this method gave sensitivities in the femtomole range.

The aim of this study was to investigate if dansyl chloride could be used as a fluorescent derivatization reagent for oxidised glutathione (GSSG) and GSH while still retaining the chromatographic techniques of Reed *et al.* [5].

## EXPERIMENTAL

### *Chemicals*

GSH and GSSG, γ-glutamylglutamine (γ-glu-glu), dansyl chloride, iodoacetic acid, lithium hydroxide, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), diethylenetriaminepentaacetic acid (DEPA) and cresol red were obtained from Sigma (Poole, UK). Remaining chemicals were from FSA Supplies (Loughborough, UK). Deionised water was used throughout.

### *Reagents*

Stock solutions of iodoacetic acid in water, γ-glu-glu, GSH and GSSG in 50 m*M* HEPES buffer, pH 7.4 containing 2 m*M* DEPA, were prepared on the day of use. Perchloric acid (PCA) (5%, v/v) contained DEPA (2 m*M*), boric acid (0.2 *M*) and cresol red indicator (5 mg/l).

### *Derivatization procedures*

*Standards.* To reaction mixtures of 0.4 ml volume in 1.5-ml Eppendorf tubes containing GSSG and GSH (0–4 nmol) in PCA–DEPA–boric acid at 0–4°C was added iodoacetic acid (1 μmol) and γ-glu-glu internal standard. A previously determined volume of 2 *M* lithium hydroxide, sufficient to bring the pH value of the mixture to 8.0–8.5, was then added. This was accompanied by a colour change of yellow to mauve of the cresol red indicator. Tubes were mixed and left for 0.5 h in the dark at 25°C. An equal volume of dansyl chloride (1 mg/ml in acetonitrile) was then added. Tubes were mixed and left for 1 h at 25°C before aliquots were subjected to HPLC analysis. Care was taken to keep samples in the dark during and after derivatization.

*Biological sample derivatization.* The lungs of male Fischer F-344 rats (150–200 g) were perfused with Hanks buffered saline at pH 7.4 to remove the blood, and 200-mg samples were homogenised in 10 ml of 5% PCA–DEPA–boric acid using a Teflon–glass homogeniser at 0–4°C. The homogenate was centrifuged (10 000 *g* for 10 min at 4°C). To the supernatant (400 μl) was added γ-glu-glu internal standard (4 nmol). Derivatization was carried out as described above. Macrophages were lavaged from the lung through a tracheal cannula using phosphate-buffered saline (PBS). Red blood cells in the lavage fluid were lysed in 0.155 *M* ammonium chloride, 0.01 *M* potassium bicarbonate, 0.1 m*M* EDTA pH 7.4 for 5 min, centrifuged (1000 *g* for 4 min) and resuspended in PBS. No further puri-

fication was attempted. Macrophage preparations were counted with the aid of a haemocytometer. To $1 \cdot 10^6$ pelleted cells in an Eppendorf tube was added ice-cold PCA–DEPA–boric acid solution (0.1 ml). Cells were disrupted by repeated passage through an Eppendorf pipette tip. Samples were centrifuged (10 000 *g* for 10 min) and the supernatant fraction processed as described for lung tissue using proportionally reduced derivatization reagents. To further increase sensitivity, following derivatization, an equal volume of chloroform was added. Samples were vortex-mixed and centrifuged (3000 *g* for 5 min). Unreacted dansyl chloride was extracted into the lower chloroform phase together with the acetonitrile. The upper aqueous phase containing the concentrated dansyl glutathione adducts was removed and a sample subjected to HPLC.

*High-performance liquid chromatography*

The liquid chromatographic system consisted of two Waters 510 pumps, a gradient controller and a WISP 710 autoinjector. A 5-μm LiChrospher column (12.5 cm × 0.4 cm I.D., E. Merck, Darmstadt, Germany) with a 0.4 cm × 0.4 cm I.D. precolumn was used. A UV detector (Kontron 432) set to 328 nm was connected in series to a Waters 470 fluorescence detector set to the excitation and emission wavelengths of 328 and 541 nm, respectively. Peak areas were quantitated with a Kontron PC integration pack. The gradient solvent system was essentially as described by Reed *et al.* [5]. Solvent A contained methanol–water (4:1, v/v), solvent B was made by adding 50 ml of a stock solution containing sodium acetate trihydrate (272 g), glacial acetic acid (378 ml) and water (122 ml) to 450 ml of solvent A. A linear 20-min gradient at a flow-rate of 1.3 ml/min was used followed by 10 min at 100% B. Glutathione concentrations were estimated by peak-area ratio method relative to the γ-glu-glu internal standard.

*Dansylated S-carboxymethylglutathione and oxidised glutathione standards*

To 1 mmol (307 mg) of GSH dissolved in 30 ml of 0.1 *M* $KHCO_3$ was added 5 mmol (925 mg) of iodoacetate in water (10 ml). Where necessary, the pH value was adjusted to 8.0 with 1 *M* potassium hydroxide. After 0.5 h, 5 mmol (1.34 g) of dansyl chloride dissolved in acetonitrile (40 ml) was added, and the mixture was stirred for 1 h at 25°C in the dark. Unreacted dansyl chloride was then extracted into chloroform (2 × 80 ml). The aqueous phase was freeze-dried. The residue was extracted into methanol (3 × 20 ml) at room temperature and the extracts concentrated under nitrogen at room temperature to about 5 ml. Solid material was removed by centrifugation (3000 *g* for 5 min). The supernatant fraction was applied to a 30 cm × 2 cm silica gel column and eluted with ethyl acetate–methanol–acetic acid (6:2:1, v/v). The orange fluorescent fractions eluting between 150 and 200 ml were pooled, concentrated to dryness at room temperature under a stream of nitrogen and recrystallised from methanol–ethanol (4:1, v/v) to give the product (80 mg, 11% yield) of >95% purity as judged by HPLC. GSSG

of a similar purity was prepared by the same method but omitting derivatization with iodoacetate.

## RESULTS AND DISCUSSION

Fig. 1A shows the chromatographic separation of the sulphur-containing amino acids cystine and cysteine together with GSH and GSSG (retention times 14.6, 15.7, 21.7 and 26.0 min, respectively) following derivatization with dansyl chloride as described in the Experimental section. Adequate resolution was achieved using commercial $NH_2$-silica columns and solvent conditions similar to those

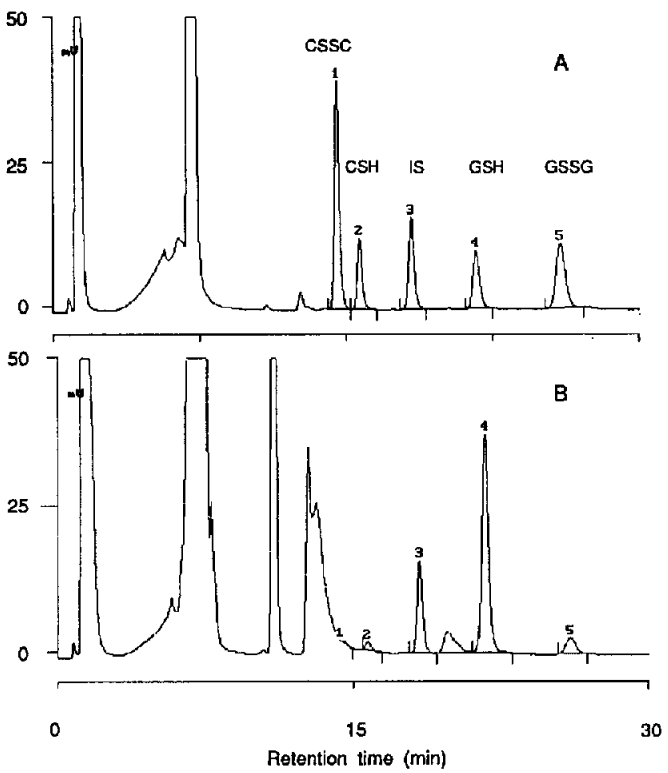


Fig. 1. Chromatographic separation of dansylated cystine, cysteine and glutathione. (A) Separation of standards: 60 pmol each of cystine (CSSC, 1), S-carboxymethylcysteine (CSH, 2), γ-glu-glu internal standard (IS, 3), S-carboxymethylglutathione (GSH, 4) and oxidised glutathione (GSSG, 5) were injected onto a 12.5 cm × 0.4 cm I.D. LiChrospher $NH_2$-silica column. A 20-min linear gradient of sodium acetate–acetic acid in methanol–water (4:1, v/v) was used as described in the Experimental section. Ordinates represent fluorescence with excitation and emission wavelengths of 328 and 541 nm, respectively. Fluorimeter gain was set at 1000. (B) Separation of a rat lung extract following precipitation of proteins with perchloric acid and dansylation. Conditions were the same as for (A).

previously employed by Reed *et al.* [5] for the separation of 2,4-dinitrophenylglutathione adducts. The limit of fluorimetric detection for GSH and GSSG (signal-to-noise ratio 2) was 1 pmol. However, in biological samples, iron- or copper-catalysed oxidation of GSH may determine practical lower limits of detection. Conditions for protecting the –SH function of GSH, by reaction with iodoacetate to minimise oxidation to GSSG, were the same as previously described [6]. Reproducibility expressed as coefficient of variation was 3.8 and 6.9% for GSH and GSSG, respectively (twenty standard analyses). Detection limits are an order of magnitude better than for *o*-phthalaldehyde derivatives of glutathione [4] and in the order of 100 times better than for the UV detection of 2,4-dinitrophenylglutathione adducts [5].

No loss of the dansylglutathione products was detected after 24 h storage at 4°C in the dark. Longer-term stability has not been assessed. Dansyl derivatives of amino acids are reportedly not as stable as those derivatized with 9-fluoroenylmethylchloroformate (FMOC) [9]. FMOC derivatives of glutathione are resolved in a similar manner to the dansyl ones, using the present HPLC procedures (unpublished results). However, FMOC derivatization procedures were not pursued due to the relatively poor conversion efficiencies with sulphur amino acids [9].

*Dansylglutathione characterization*

Fast atom bombardment mass spectrometry of S-carboxymethyldansylglutathione gave a weak $MH^+$ ion, *m/z* 599 (7% of base peak), with additional peaks at *m/z* 637 (40% of base peak), *m/z* 675 (72% of base peak), *m/z* 713 (47% of base peak) and *m/z* 751 (16% of base peak) consistent with $[M+K]^+$, $[M+2K]^+$, $[M+3K]^+$ and $[M+4K]^+$, respectively. This result reflects the abundance of $K^+$ ions during derivatization, since for the large-scale preparations $KOH–K_2CO_3$ buffers were used instead of the lithium buffers subsequently employed for analytical procedures (see below). Dansylated GSSG showed a weak $MH^+$ peak, *m/z* 1079 (7% of base peak), consistent with a didansylated product. $[M+K]^+$ peaks were seen at *m/z* 1117 ($[M+K]^+$, 10% of base peak), *m/z* 1155 ($[M+2K]^+$, 15% of base peak), *m/z* 1194 ($[M+3K]^+$, 16% of base peak) and *m/z* 1233 ($[M+4K]^+$, 10% of base peak).

*Derivatization conditions*

Lithium carbonate was originally employed as the base for dansylation of amino acids. It was not used in the present experiments due to its limited solubility. However, the use of lithium hydroxide to neutralise PCA consistently gave (*ca.* 10%) better yields of dansylated glutathione than when $KOH–K_2CO_3$ buffers were used. Dansylglutathione adducts may be stabilised by $Li^+$ pairing with the carboxylate anions [8]. In order to control the pH value of derivatization, borate buffer was used together with cresol red as a pH indicator. These components did not interfere with the subsequent HPLC analysis. The time course for

the reaction of glutathione with dansyl chloride was similar to that previously reported for amino acids [10] (results not shown). After 5 min, with GSSG, a transient additional peak (retention time 22 min) was seen. This probably represented the monodansylated product although it was not characterized further. After 1 h the reaction appeared complete. A comparison of peak areas of the authentic standards following HPLC with dansylated carboxymethylglutathione and GSSG gave conversion efficiencies of 97.2 ± 11.4 and 90.5 ± 19.3%, respectively (mean ± S.D. for ten experiments).

*Estimation of reduced and oxidised glutathione in rat lung and alveolar macrophages*

Fig. 1B shows the separation of GSH and GSSG from rat lung following reaction with dansyl chloride and HPLC. Quantitation of these gave values of 1.31 ± 0.6 and 0.04 ± 0.02 μmol/mg of tissue, respectively (mean ± S.D. for four experiments). These values are similar to those previously reported for lung, or lung slices using *o*-phthalaldehyde derivatization [9,11]. Only small peaks with retention times corresponding to cysteine and cystine were seen. Longer gradient times (30 min) were required for the adequate resolution of cystine from a contaminating peak. In the present experiments these amino acids were not quantitated. For lung macrophages, only relatively small numbers of cells ($1 \cdot 10^6$) could be obtained from a single animal. The current procedures were able to give satisfactory results for GSH and GSSG concentrations of 0.89 ± 0.29 and 0.022 ± 0.020 nmol per $10^6$ cells, respectively (mean ± S.D. for six experiments).

For the estimation of glutathione, lungs were subjected to vascular perfusion prior to alveolar lavage or taking tissue samples. Whole blood has a GSH concentration in the range of 800 nmol/ml [12], and its presence could affect apparent cellular values.

The present results show that derivatization of GSSG and GSH using dansyl chloride represents a convenient alternative to existing methods where high sensitivities, as afforded by fluorescence detection, are required.

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REFERENCES

1 I. N. H. White, *Chem. Biol. Interact.*, 13 (1976) 333.
2 R. C. Fahey and G. L. Newton, *Methods Enzymol.*, 143 (1987) 85.
3 J. P. Richie and C. A. Lang, *Anal. Biochem.*, 163 (1987) 9.
4 D. A. Keller and D. B. Menzel, *Anal. Biochem.*, 151 (1985) 418.
5 D. J. Reed, J. R. Babson, P. W. Beatty, A. E. Brodie, W. W. Ellis and D. W. Potter, *Anal. Biochem.*, 106 (1980) 55.

6 M. W. Fariss and D. J. Reed, in R. A. Harris and N. W. Cornell (Editors), *Isolation Characterization and Use of Hepatocytes*, Elsevier, New York, 1983, p. 349.
7 G. C. Barret, in J. H. Jones, G. C. Barrett, J. Brennan, J. S. Davies, I. J. Galpin, P. M. Hardy, R. W. Hay, K. B. Nolan and A. Sheppard (Editors), *Amino Acids and Peptides*, Vol. 18, Royal Society of Chemistry, London, 1987, p. 1.
8 P. Fürst, L. Pollack, T. A. Graser, H. Godel and P. Stehle, *J. Chromatogr.*, 499 (1990) 557.
9 M. F. Malmer and L. A. Schroeder, *J. Chromatogr.*, 514 (1990) 227.
10 Y. Tapuhi, D. E. Schmidt, W. Lindner and B. L. Karger, *Anal. Biochem.*, 115 (1981) 123.
11 S. J. Hardwick, A. Adam, L. L. Smith and G. M. Cohen, *Biochem. Pharmacol.*, 39 (1990) 581.
12 B. J. Mills, J. P. Richie and C. A. Lang, *Anal. Biochem.*, 184 (1990) 263.