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Short Communication

Thermal decomposition of reduced glutathione in solution for organ preservation

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Introduction

Reduced glutathione (GSH) is *N*-(*N*-L-γ-glutamyl-L cysteinyl) glycine, a physiological tripeptide which is associated with detoxification mechanisms in the body; it is involved in conjugation reactions catalysed by glutathione-*S*-transferase. GSH is claimed to be an essential component of solutions for organ preservation. GSH plays an important role in protecting cells from injury produced by a variety of reactive metabolites and has been suggested to be involved in the cellular response to oxygen deprivation and reoxygenation. It plays this central role through actions to maintain protein sulphydryl groups in the reduced state, to limit lipid peroxidation and to combine directly with toxic reactive metabolites.

Glutathione is metabolized in the cell by glutathione peroxidase, which catalyses the conversion of hydrogen peroxide to oxygen and water. Hydrogen peroxide can react with transition metals in the cell to yield the highly cytotoxic hydroxyl radical; the metabolism of hydrogen peroxide is important in suppressing oxidative stress. One end-product of this reaction is oxidized glutathione (GSSG), which is converted back to GSH by glutathione reductase. Oxidized glutathione is not effective in the metabolism of hydrogen peroxide; increased cellular concentrations of GSSG are thought to indicate oxidative stress.

GSH is known to be unstable in aerated water solution. Astier and Paul [1] showed an oxidative mechanism in the production of the disulphide product from GSH in an organ preservative solution. This finding is consistent with the reported role of transition metals and high pO_2 in generating the radical that reduces O_2 and initiating the chain oxidative process of thiols.

The Central Pharmacy for Paris Hospitals manufactures aqueous solutions of GSH (92.2 mg ml^{-1}) for organ preservation. For this purpose, the solutions have to be sterilized by a method that maintains the integrity of the tripeptide structure. The principal objective of the present work was to determine the effect of temperature of sterilization on the stability of GSH in such solutions.

Experimental

Reagents

Solution for organ preservation. Glutathione solution contained GSH 92.2 mg ml^{-1}.

Titrimetric method. 0.05 M iodine Titrisol® (Merck, Darmstadt, Germany); demineralized water with ion-exchange resins (Aquadem, France).

Chromatographic method. Heptane sulphonic acid sodium salt (Sigma, St Louis, MO, USA); anhydrous sodium sulphate (Merck); methanol (chromatographic quality) (Merck); sulphuric acid (for analysis) (U.C.B., Belgium); demineralized water with ion-exchange resins.

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Sterilization

Five process of sterilization were tested: heating at 120°C for 20 min; heating at 110°C for 30 min; heating at 100°C for 45 min; tyndalization at 70°C for 3 × 1 h; and filtration with a 0.22 μm porosity membrane.

HPLC method

The liquid chromatographic system comprised a Shimadzu (Kyoto, Japan) LC 6 A pump and a 150 mm × 4.6 mm i.d. column packed with 5-μm Rosil C_{18} (Altech, Eke, Belgium). Samples were injected using a Rheodyne injection valve 7125 with a 20-μl loop. The UV spectrophotometer detector was an Applied Systems 783 A (USA) model set at 210 nm. Chromatograms were recorded with a CR 3A Shimadzu computer integrator (Kyoto, Japan).

The mobile phase was methanol–water (5:95, v/v) with 0.3% w/w of heptane sulphonic acid sodium salt and 0.5% w/w of anhydrous sodium sulphate and adjusted to pH = 2.5 with 1 M sulphuric acid; the flow rate was 1 ml min^{-1} at 25°C.

Standard solutions were prepared from reduced glutathione (Sigma) and oxidized glutathione (Sigma). Histidine hydrochloride (Sigma) was used as the internal standard.

Calibration curves were constructed for concentrations of 15–55 μg ml^{-1} of reduced glutathione. The linearity of the response was verified (r = 0.9998).

Results and Discussion

GSH and GSSG could be identified and measured simultaneously by HPLC analysis. A titrimetric method was carried out at the same time to evaluate the percentage of non-oxidized thiol groups (SH).

Results are listed in Table 1 and are expressed as percentage concentrations of GSH and GSSG in relation to the theoretical concentration of GSH in aqueous solution.

Titrimetric results indicate no oxidation of SH in any solution. This result was confirmed by the chromatographic determination of oxidized glutathione. Moreover reduced glutathione concentrations were decreased in every solution whichever sterilization method was used. This decrease is correlated to the sterilization temperature and is associated with a degradation mechanism of tripeptide with the liberation of amino acids (glutamic acid, glycine, cysteine).

The degradation of GSH was examined by HPLC. First liberation of glutamic acid (Gln) was observed. The cysteinyl glycine (Cys–Gly) was undegraded and the thiol group of cysteine was in the reduced form (SH) (Fig. 1). Secondly, glutamic acid, the most unstable amino acid in acidic solution (pH = 3), was degraded. Glycine (Gly) and cysteine (Cys) were also liberated (Fig. 2).

The degradation mechanism deduced from observations during 1 month's storage of solutions is independent of the oxidation mechanism. Thus, cysteine dissolved in water is not oxidized to cystine. The degradation is outlined in Scheme 1.

Small quantities of hydrogen sulphide were generated in all heated solutions, especially at 120°C for 20 min and 70°C for 3 × 1 h. These results can be correlated with the titrimetric results and correspond to the breakdown of the C—S bond [2].

Much recent interest has focused on the major, direct protective effect of GSH on the response of rabbit renal proximal tubules to hypoxia [3]. But the data indicate that this protection is unrelated to cell GSH or reactive oxygen metabolites. Instead, it appears to be mediated by glycine produced from GSH metabolism. Both GSH and glycine provide

Table 1
Study of glutathione degradation after sterilization

Sterilization process	Chromatographic method (n = 5)		Titrimetric method (n = 5) expressed as reduced glutathione (%)
	Reduced glutathione (%)	Oxidized glutathione (%)	
120°C for 20 min	23	< limit of detection	97.0
110°C for 30 min	28	< limit of detection	99.4
100°C for 45 min	40	< limit of detection	99.2
70°C for 1 h × 3	59	< limit of detection	96.3
Filtration	88	< limit of detection	99.6

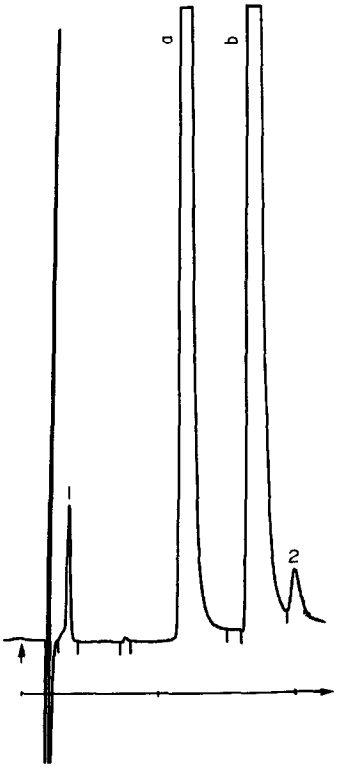


Figure 1
Identification of the degradation fragments of reduced glutathione at Day 0. 1, Gln; 2, Cys–Gly; a, GSH; b, internal standard.

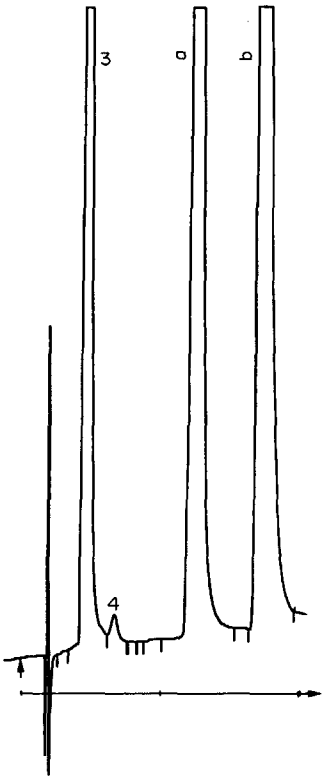
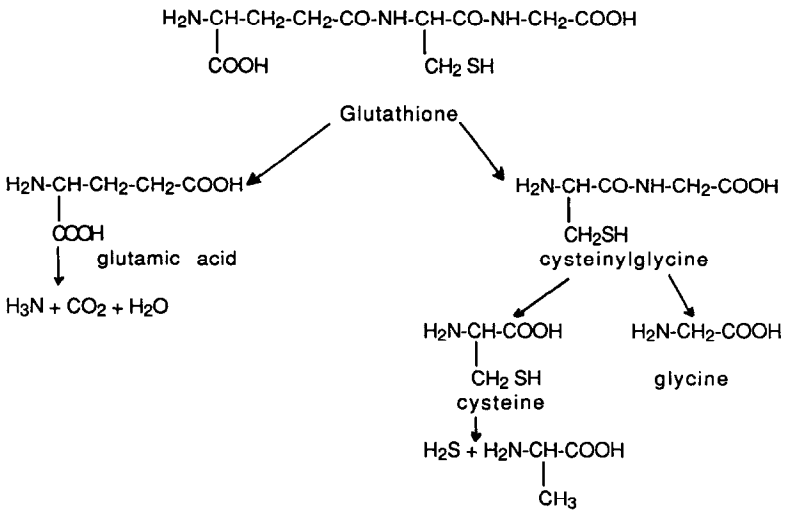


Figure 2
Identification of the degradation fragments of reduced glutathione at Day 30. 3, Gly; 4, Cys; a, GSH; b, internal standard.



Scheme 1
Degradation kinetics of reduced glutathione.

substantial protection against hypoxic injury to isolated kidney tubule and the major protective factor appears to be the glycine.

Conclusion

As expected, the results show that the diminution in reduced glutathione concentration is a function of sterilization temperature. The thermodegradation is not always explained by an oxidative process.

In addition to the oxidation mechanism described for reduced glutathione in solutions for organ preservation, another thermodegradation mechanism is now proposed; this comprises the progressive liberation of amino acids. Because of this unstability, it is impossible to sterilize the solution by autoclaving. Sterilization by filtration with a 0.22 μm membrane seems to be the best method.

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