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An Improved Simultaneous Measurement of Oxidized and Reduced Glutathione in Biological Samples by High-Performance Liquid Chromatography Following Derivatization with Dansyl Chloride

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Reduced (GSH) and oxidized glutathione (GSSG) concentrations in biological samples can be determined simultaneously utilizing high-performance liquid chromatography (HPLC) following derivatization with dansyl chloride. In the present study, two points were investigated in detail to improve upon their method : 1) pH in the derivatization reaction and 2) the method of deproteinization prior to dansylation. Dansylation of standard GSH after S-carboxymethylation and of GSSG both gave the highest relative fluorescence at pH 8.5. Degradation of GSSG to GSH was not detected when GSSG was dansylated at pH 8.5 or more acidic pHs but was apparent at pH 9.0 and even more obvious at pH 10.0. The highest fluorescence values for dansylated GSH and GSSG were also obtained in tissue homogenates following the derivatization at pH 8.5. Although the value for dansylated GSH in the homogenates was also high at pH 9.0, this probably was due to degradation of intrinsic GSSG to GSH. Deproteinization with trichloroacetic acid (TCA) was found to give considerably higher relative fluorescence of dansylated GSSG than that with perchloric acid (PCA), whereas the dansylated-GSH concentrations obtained with the two acids were similar. Moreover, dansylated GSSG was more stable in TCA than in PCA, although there was no difference between these acids in relation to stability of dansylated GSH. In the livers and kidneys of rats, the GSH and GSSG concentrations obtained by the present method were consistent with those obtained by the recycling enzymatic method.

Key words —— glutathione, reduced glutathione, oxidized glutathione, HPLC, dansyl chloride, rat

INTRODUCTION

Reduced glutathione (GSH), an essential thiol ubiquitous in mammalian cells, and oxidized glutathione (GSSG) are involved in various physiologically important functions. In particular, GSH acts as a major antioxidant that protects cells from oxidative damage,[1] and alterations in the ratio of GSH to GSSG have been used as an indicator of oxidative stress and/or disease in humans and laboratory animals.[2,3] Therefore, to ascertain the presence of oxidative stress, it is important to have an accurate method for the determination of GSH and GSSG concentrations. Although they have been measured using enzymatic,[4] fluorimetric,[5] and colorimetric assays,[6] all these methods have some disadvantages including inadequate detection limits, low reproducibility, and inability to conveniently measure both GSH and GSSG simultaneously in biological samples. To determine both compounds in biological samples, a high-performance liquid chromatography (HPLC) procedure proposed by Reed *et al.*,[7] with analysis based on ultraviolet absorptive derivatives of the amino function, has been used widely. Subsequently, many derivatization analyses using fluorimetric detection have been developed, and of these, derivatization with 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride) provided a high sensitivity and yield for sulfur-containing oligopeptides such as GSH and GSSG.[8] In this method, biological samples must be treated with a deproteinizing reagent, such as perchloric acid (PCA), and be adjusted to pH 8.0 to 8.5 prior to dansylation because the reaction can be markedly influenced by pH. However, an optimum pH of this reaction has not been determined, nor is it known which deproteinizing reagent is suitable

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for detecting GSH and GSSG.

The purpose of this study was to investigate the optimum conditions for deproteinzation and dansylation using the HPLC method for simultaneous measurement of GSH and GSSG which was first proposed by Martin and White.[8]

MATERIAL AND METHODS

Chemicals——GSH, GSSG, γ-glutamylcysteine, and dansyl chloride were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Animals——Male Sprague-Dawley rats weighing 160 to 200 g (purchased from Nippon SLC, Shizuoka, Japan) were used as the experimental animals. Rats were killed under sodium pentobarbital anesthesia, and the kidney and liver were removed and immediately frozen in liquid nitrogen. The tissues were stored in −80℃ until analysis.

Preparation of Homogenate and Deproteinization——With a Physcotron (Microtec Co., Tokyo), the kidney and liver tissues were homogenized in 0.5 M boric acid–LiOH containing 5 mM EDTA (pH 7.0) to yield a 10% (w/v) homogenate at 0—4℃ for 1 min and then heated at 100℃ for 5 min. The homogenate was centrifuged at 10000 × *g* for 10 min at 4℃, and the resulting supernatant was subjected to GSH and GSSG analyses as heated sample.

For PCA and trichloroacetic acid (TCA) deproteinization, tissues were homogenized in 5% ice-cold PCA or TCA to yield a 10% (w/v) homogenate. The homogenate was immediately centrifuged as described above to obtain the supernatant as PCA or TCA sample.

Dansylation and HPLC Assay——The method of Martin and White[8] was modified, and standards and homogenate samples were allowed to dansylate under the same conditions: briefly, 200 μl of 0.5 M boric acid–LiOH (pH 8.5) and 4 μl of 1 mM iodoacetic acid were added to 200 μl of 5% TCA–LiOH (pH 7.0) containing GSH and GSSG (0.1—15 μM) in an Eppendorf centrifuge tube. This mixture was mixed and left in the dark for 0.5 h at 25℃. Four hundred microliters of dansyl chloride (1 mg/ml acetonitorile) was then added, and the mixtures was left in the dark at 25℃ again, this time for 1 h. An equal volume of chloroform (400 μl) was added, and the unreacted dansyl chloride was extracted into a lower organic solvent phase. The upper phase was centrifuged at 10000 × *g* for 5 min. The clear supernatant was transferred to another tube and wrapped with aluminum foil.

To the heated tissue sample (10 μl) was added 190 μl of 0.5 M boric acid–LiOH (pH 7.0). One microliter of the 2 M LiOH and 189 μl of TCA–LiOH (pH 7.0) or PCA–LiOH (pH 7.0) was added to 10 μl of the TCA or PCA tissue sample. Dansylation of samples was carried out as described above.

The liquid chromatographic system consisted of two LC-10AT pumps (Shimadzu, Kyoto, Japan), a gradient controller, and an RF-10A fluorescence detector (Shimadzu). A 5-μm LiChrospher-NH_2 column (12.5 cm × 0.4 cm, Merck, Germany) with a precolumn was used with the column temperature maintained at 35℃ for separation. The fluorescence detector was set to excitation and emission wavelengths of 328 and 541 nm, respectively. The following elution system was based on the method of Reed *et al.*[7] Solvent A consisted of methanol–water (80 : 20, v/v), and solvent B was prepared 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. Linear gradient elution at a flow-rate of 1.0 ml/min was carried out with linear increases from 0 to 100 % of solvent B for 26 min. The column was then maintained with solvent B for 10 min and reequilibrated with solvent A for 10 min prior to the next injection. Fifty microliters of liver or kidney samples was injected into the HPLC system for analysis.

Enzymatic assay——In some cases, GSH and GSSG concentrations in samples deproteinized by TCA were also determined according to the 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)-enzymatic recycling method described by Tietze *et al.*[4]

RESULTS AND DISCUSSION

Chromatography of GSH and GSSG

Typical chromatograms of various standards, *i.e.* GSH, GSSG, cysteine, cystine, cysteinylglycine, and γ-glutamylcysteine, and of renal and liver samples are shown on Fig. 1. The retention times of GSH and GSSG were 26.9 and 31.8 min, respectively, and there was no interference with other standards (Fig. 1A). The chromatograms of GSH and GSSG in renal and liver samples also showed no interference signals (Fig. 1B, 1C).

Effect of pH on Derivatization of GSH and GSSG with Dansyl Chloride

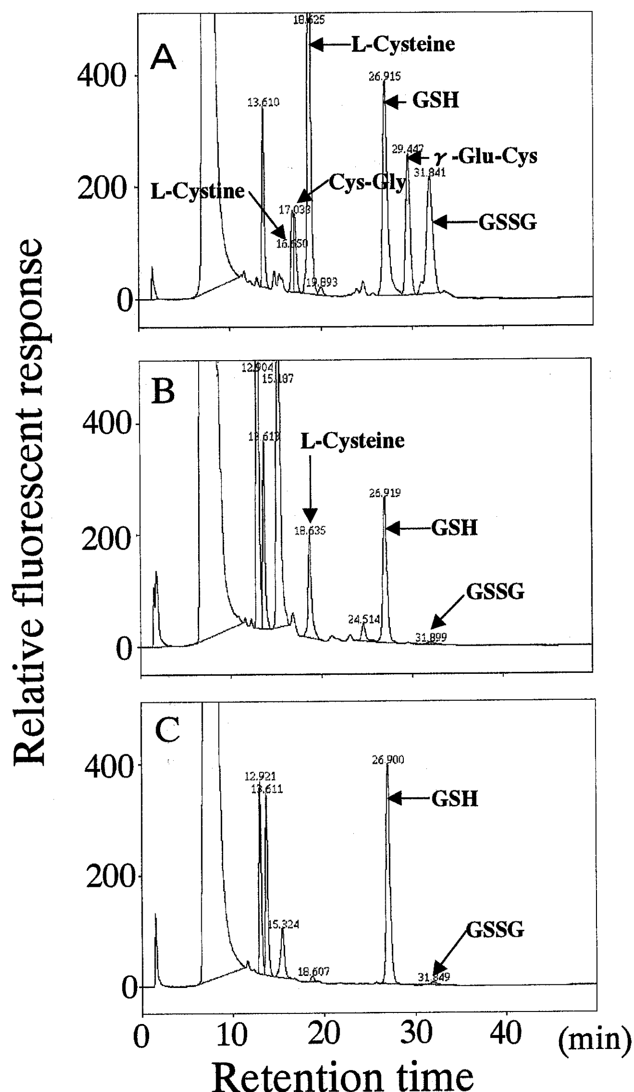

Fig. 1. Chromatographic Separation of Dansylated Standards and Homogenate Samples

All samples were deproteinized with TCA and dansylated at pH 8.5.

A, standards; B, rat kidney homogenate; C, rat liver homogenate. L-Cysteine, *S*-carboxymethylcysteine; Cys–Gly, *S*-carboxymethyl cysteinylglycine; GSH, *S*-carboxymethylglutathione; γ-Glu–Cys, *S*-carboxymethyl γ-glutamylcysteine.

The yields of GSH and GSSG dansylated at a range of pH are shown in Fig. 2. When dansylated below pH 7.0, no peak of dansylated GSH or GSSG was detected. With either standard, the highest fluorescence value was obtained following dansylation at pH 8.5. Degradation of standard GSSG to GSH was not detected at pH 8.5 or more acidic pHs but was apparent at pH 9.0 and was much more obvious at pH 10.0, judging from the appearance of a new peak that had the same retention time as carboxymethylated and dansylated GSH.

The highest fluorescence value for dansylated GSSG was also obtained in rat kidney homogenates following dansylation at pH 8.5. However, the value for dansylated GSH was highest at pH 9.0 although it also was high at pH 8.5. Thus, the results for homogenates differed somewhat from those for standard GSH. The highest value was most likely the sum of intrinsic GSH and GSH newly formed from GSSG in the homogenates during dansylation at pH 9.0. These results indicate the optimum pH for simultaneous derivatization of both GSH and GSSG with dansyl chloride to be 8.5.

Effect of Deproteinization by Heating, TCA, and PCA on GSH and GSSG Assay

The relative fluorescence of GSH and GSSG in samples of the liver and kidney treated with heating, TCA, or PCA is shown in Table 1. In the kidney sample treated with heating, no peak of GSH or GSSG was detected, and the increased peaks of cysteine and cysteinylglycine were observed. Activity of γ-glutamyltranspeptidase (γ-GTP) is known to be markedly higher in the kidney than in other organs. Therefore, GSH in the kidney is thought to be decomposed to cysteine and cysteinylglycine by γ-GTP for a short time during homogenization followed by heating. In liver samples, the heating method resulted in lower GSH and higher GSSG concentrations, suggesting that a part of GSH was oxidized to GSSG. These results suggest that although the heating procedure is very convenient for neutralization prior to dansylation, GSH is decomposed or oxidized during homogenization and/or heating.

In contrast to the heating method, the TCA and PCA methods gave substantial amounts of GSH and GSSG even in kidney homogenates. In both kidney and liver homogenates, no difference was observed between the relative fluorescence of GSH deproteinized with TCA or PCA, but the relative fluorescence of GSSG deproteinized with TCA was higher than that deproteinized with PCA.

The GSH and GSSG concentrations in kidney and liver homogenates treated with TCA were stable for at least 2 weeks at −80℃. In contrast, the GSSG concentration in samples treated with PCA increased gradually during storage at −80℃ (data not shown) indicating that autooxidation of GSH occurred in the PCA solution.

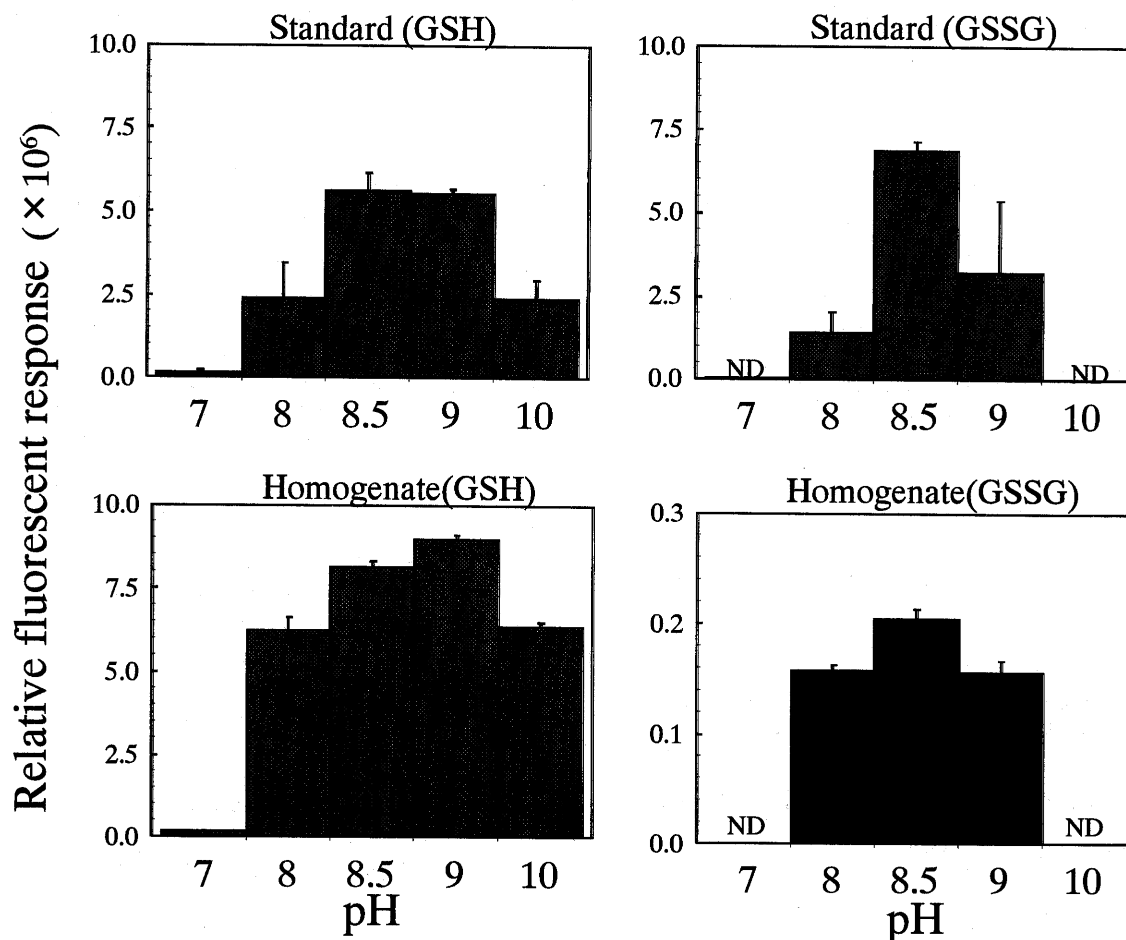

Fig. 2. Effect of pH on the Derivatization Reaction of GSH and GSSG with Dansyl Chloride for Standards and Kidney Homogenates

The homogenates were deproteinized with TCA. The boric acid–LiOH buffer was used for adjustment of pH. Data are presented as mean ± S.D. of five measurements.
ND : not detected.

Table 1. Effect of Deproteinization on Measurement of Dansylated Derivatives of GSH and GSSG in Biological Samples

Tissue	Compound	Relative strength of fluorescence (%)		
		TCA	PCA	Heating
Kidney	GSH	102.6	100.0	ND
	GSSG	115.5	100.0	ND
Liver	GSH	100.8	100.0	79.7
	GSSG	151.3	100.0	156.8

The relative values of TCA and heating methods were compared with the value of PCA as control (100%). ND : not detected.

Accuracy, Recovery, and Reproducibility of GSH and GSSG Assay

Unless otherwise specified, all the following results were obtained with deproteinization with TCA when tissue homogenates were used as samples and with dansylation at pH 8.5 as described under Material and Methods. The standard curves for GSH (1—15 μM) and GSSG (0.1—5.0 μM) showed a good linear relation, both with a correlation coefficient of 0.999 (data not shown). The detection limits for GSH and GSSG were 20.2 and 27.4 pmol/50 μl injected, respectively, based on a signal-to-noise ratio of 3.

The recovery of GSH and GSSG added to a renal deproteinized sample ranged from 98.4% to 100.2% and 94.9% to 106.0%, respectively (Table 2). The results imply quantitative derivatization of GSH and GSSG with dansyl chloride. The reproducibility of the assay for GSH and GSSG was determined using a renal homogenate and authentic GSH and GSSG solutions (Table 3). Intra-assay variability was assessed and expressed as a coefficient of variation (CV). The CVs of GSH and GSSG were 0.61% and 1.19%, respectively, in the standard solutions, and 2.88% and

Table 2. Recovery of GSH and GSSG

	Amount of added (μM)	Theoretical value	Observed value	Recovery (%)
GSH	1	7813339	7690481	98.4
	10	16483912	16511830	100.2
GSSG	1	2264078	2149418	94.9
	5	6692345	7104182	106.0

GSH or GSSG was added to a renal deproteinized sample.

Table 3. Reproducibility of the Assay for GSH and GSSG

	Standard		Homogenate sample	
	10μM GSH	5μM GSSG	GSH	GSSG
Sample number	10	10	10	10
Mean	9.97	5.06	7.3	0.43
S.D.	0.06	0.06	0.21	0.02
CV (%)	0.61	1.19	2.88	4.65

4.65% in the homogenate samples. These results demonstrate the precision of this assay.

GSH and GSSG Concentrations in the Liver and Kidney of Rats

Table 4 shows the GSH and GSSG concentrations determined in extracts from rat liver and kidney using the present method. The GSH/GSSG ratio was approximately 34.19 for the liver and 47.00 for the kidney.

The GSH/GSSG ratio in the liver and kidney determined by a recycling enzymatic method has been reported to usually be over about 12—15[4,9,10] which is comparable with the values obtained in the present study using HPLC after dansylation.

Measurement of GSH and GSSG concentrations is essential for studies aimed at ascertaining oxidative stress produced by drugs or pathological conditions. Therefore, it is particularly important to have a reliable, easy, and sensitive method for quantifying these compounds. In general, the recycling enzymatic method[4] is used to measure GSH and GSSG concentrations in biological samples. The total GSH (GSH + GSSG) is determined first, and then GSSG is measured in the second step. In this present study, we showed that the HPLC method following derivatization with dansyl chloride makes it possible to determine GSH and GSSG simultaneously in biological sample. Moreover, this HPLC method is more convenient, specific, and sensitive than the enzymatic method.

Table 4. Concentrations of GSH and GSSG in Tissues of Adult Rat

Tissue	Methods	GSH (μmol/g)	GSSG (μmol/g)	GSH/GSSG
Kidney	HPLC	2.33±0.198	0.056±0.020	47.00±8.81
	DTNB-recycling	1.78±0.100	0.125±0.011	14.30±0.80
Liver	HPLC	5.97±0.655	0.176±0.010	34.19±5.08
	DTNB-recycling	5.70±0.520	0.372±0.041	15.4 ±1.00

Data are presented as mean ± S.D. of five measurements.

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