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Determination of reduced and oxidized glutathione in erythrocytes by high-performance liquid chromatography with ultraviolet absorbance detection

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Abstract

A method is described for simultaneous quantitation of reduced (GSH) and oxidized (GSSG) glutathione in erythrocytes by HPLC. They were determined by standard addition method. Blood samples were collected in tubes containing 1,10-phenanthroline. The separated erythrocytes were hemolyzed with water containing standard. After deproteinization, GSH and GSSG were converted to *N*-(2,4-dinitrophenyl) derivatives and analyzed by HPLC with UV detection. The coefficients of variation of GSH and GSSG on replicate assays were 6% and 8%, respectively. The stabilities of GSH and GSSG and of the derivatives were also examined. The present method appears to be satisfactory for determination of these physiological concentrations in erythrocytes.

Keywords: Glutathione; Peptides; Erythrocytes

1. Introduction

Glutathione, which is a tripeptide (Glu–Cys–Gly), is the most abundant non-protein thiol, and exists in most tissues of the human body. It is particularly abundant in erythrocytes and the lens, and is present principally as the reduced form (GSH) with only little small of the glutathione disulfide (oxidized form, GSSG). The role of GSH in the detoxication of xenobiotics has been well established [1]. It also plays an essential protective role against reactive oxygen species that are generated during the metabolism of many xenobiotics. This protective mechanism results in increased formation of GSSG [2,3]. Therefore, analysis of changes in GSH status produced by toxic compounds can provide important information about the toxicity mechanism of a compound.

Recently, numerous methods have been developed using HPLC for the quantification of glutathione in biological samples, based on a variety of detection and separation techniques. These methods include UV absorbance detection with pre-column derivatization by 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman reagent) [4] or by 2,4-dinitrofluorobenzene (Sanger's reagent) [5,6] and reversed-phase column, recycling post-column reaction on an anion-exchange column [7], fluorometric detection of monobromobimane derivatives [8], [9] or of 1-dimethylaminonaphthalene-5-sulphonyl (dansyl) derivative [10] separated on a reversed-phase column, fluorometric detection with pre- [11] or post-column [12] de-

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rivatization by *o*-phthalaldehyde and reversed-phase column, and HPLC on cation-exchange [13] or reversed-phase column [14–17] with electrochemical detection (ED).

While many methods have been reported for the determination of glutathione, there are few reports concerning the simultaneous determination of GSH and GSSG, except for HPLC methods with ED. Analytical methods with UV absorbance detection are inferior in terms of sensitivity and selectivity but simple compared with electrochemical or fluorometric determination. Reed et al. [5] developed an HPLC analysis with UV absorbance detection for simultaneous determination of GSH and GSSG in lymphoma cells and rat hepatocytes. For sample preparation, the *S*-carboxymethyl derivative of GSH is formed with iodoacetic acid to prevent artifactual auto-oxidation of GSH to GSSG, then the free amino group of the derivative and GSSG are converted to *N*-(2,4-dinitrophenyl) derivatives by reaction with 2,4-dinitrofluorobenzene. This pre-column derivatization method permits nanomole-level analysis of GSH and GSSG in biological samples, though the absorbance intensities of the parent compounds were weak in the UV wavelength region. This method was used to determine GSH and GSSG in plasma by Lash and Jones [18]. The glutathione levels are much higher in erythrocytes than in plasma. However, they are difficult to measure in erythrocytes because of the presence of ferric ions (Fe^{3+}), which accelerate oxidation of GSH. Roberts and Ager [19] described an analytical method only for GSH in erythrocytes by colorimetry using an autoanalyzer.

To our knowledge, the present paper is the first which presents an analytical method for simultaneous determination of GSH and GSSG in erythrocytes. We describe a method based on HPLC, which is a modification of the method of Reed et al. [5].

2. Experimental

2.1. Chemicals

GSH and GSSG were purchased from Sigma (St. Louis, MO, USA). Heparin sodium salt, 1,10-phenanthroline monohydrate, metaphosphoric acid, monoiodoacetic acid and 2,4-dinitrofluorobenzene were obtained from Wako (Osaka, Japan). All other chemicals used were purchased from Ishizu Pharmaceutical (Osaka, Japan). Methanol was of liquid chromatographic reagent grade. All other chemicals were of reagent grade and used without further purification. Water used during the experiments had been deionized and distilled.

2.2. Equipment

The liquid chromatograph was assembled from components primarily from Hitachi (Tokyo, Japan), which included a Model L-6200 intelligent pump, a Model 655A-52 column oven, a Model L-4000 UV detector and a Model D-2500 chromato-integrator. The injector was a Rheodyne (Cotati, CA, USA) Model 7125 chromatographic injector with a 20-μl injection loop. A type Transsonic T570 supersonic bath obtained from Elma (Singen, Germany) was used to degas the mobile phase. A Hitachi Model Himac CR 15D centrifuge was used. Vortex mixer was purchased from Iuchi (Osaka, Japan). A Thermonics (Tokyo, Japan) Model WR-100 wave rotor was used to stir the sample solution for a long time. A Kubota (Tokyo, Japan) Model hematocrit KH-120A was used to measure the hematocrit value of blood.

2.3. Chromatographic conditions

The analytical conditions for HPLC were slightly modified from the method described for determination of GSH and GSSG in plasma by Lash and Jones [20]. GSH and GSSG were separated from erythrocytes using a LiChrospher 100 NH_2 analytical column (250 × 4 mm I.D., 5 μm particle diameter, Merck, Darmstadt, Germany) protected by the same NH_2 guard column (4 × 4 mm^2 I.D.). The water for the mobile phase was filtered through an Advantec cellulose nitrate membrane filter (0.45 μm pore size, Toyo Roshi Kaisha, Tokyo, Japan). The following gradient solvent system was used. Solvent A was water–methanol (1:4, v/v), and solvent B was 2 *M* sodium acetate (pH 4.6)–methanol (36:64, v/v). Chromatographic runs were performed at a flow-rate of 1.2 ml/min, starting at 25% B followed by a 30 min linear gradient to 95% B and a 15 min isocratic period at 95% B. The column was then re-equili-

brated to the initial conditions for 15 min. The column temperature was maintained at 40°C. The UV detector was set at 365 nm for absorption measurements. A 20-μl sample was injected into the chromatographic system using a Rheodyne injection valve.

2.4. Standard solutions of GSH and GSSG

To prepare the standard solutions, 200 mg of GSH and 80.0 mg of GSSG were dissolved separately in 20 m*M* 1,10-phenanthroline solution in 100-ml volumetric flasks. Equal volumes of these solutions were combined, and standard solutions of the two compounds were prepared by diluting the mixed solution with 20 m*M* 1,10-phenanthroline solution to yield concentrations of 0, 62.5, 125.0 and 250.0 μg/ml for GSH and of 0, 25.0, 50.0 and 100.0 μg/ml for GSSG. Each standard solution was freshly prepared on the day of analysis.

2.5. Sample preparation

Blood samples (about 3 ml) were collected in heparinized tubes containing 30 mg of 1,10-phenanthroline powder and transferred in 0.5-ml portions into four polypropylene 1.5-ml snap-cap conical-bottom centrifuge tubes (Bio Plastics, Osaka, Japan). The samples were immediately centrifuged (7000 *g* for 10 min) at 0–5°C in order to separate the erythrocytes. The supernatants were removed and then the remaining erythrocyte samples were hemolyzed by adding 0.4 ml of each concentration of the standard solutions. Next, 0.3 ml of 10% metaphosphoric acid was added to each hemolyzate as a precipitant of protein. The mixtures were vortex-mixed and centrifuged (15 000 *g* for 15 min). The supernatants (0.4 ml) were decanted into other centrifuge tubes, and treated with 0.1 ml of a fresh aqueous solution of iodoacetic acid (400 m*M*), and then neutralized with an excess of sodium bicarbonate (dry powder, about 20 mg). The mixtures were allowed to react using a wave rotor for 1 h in the dark at room temperature (about 20°C) to form the *S*-carboxymethyl derivative of GSH. A 0.5-ml volume of 5% 2,4-dinitrofluorobenzene diluted in ethanol was added to each reaction solution, and the mixtures were allowed to react for 20 h in the dark at room temperature to form the *N*-(2,4-dinitrophenyl) derivatives of *S*-carboxymethyl-GSH and GSSG. The mixtures were centrifuged (15 000 *g* for 15 min) before analysis and aliquots of the supernatants were used for the determination of GSH and GSSG.

The hematocrit value (%) was measured by centrifuging (10 000 *g* for 5 min) a blood sample in a capillary tube.

2.6. Calibration

GSH and GSSG in the erythrocyte sample were determined by standard addition method. The concentrations were calculated by extrapolation from the linear least-squares regression line of the calibration plot of peak areas of GSH and GSSG versus these concentrations added to the sample. The concentrations of GSH and GSSG determined were corrected by the hematocrit value.

2.7. Stability

Stabilities of GSH and GSSG in metaphosphoric acid solution

Erythrocytes prepared from 10 ml of blood were hemolyzed by adding 8 ml of 20 m*M* 1,10-phenanthroline solution free of GSH and GSSG, and then were deproteinized with metaphosphoric acid according to the present method. After centrifugation, about 8 ml of supernatant were obtained. The solution was transferred in 0.4-ml portions into seven polypropylene 1.5-ml centrifuge tubes. GSH and GSSG from one centrifuge tube were analyzed immediately after preparation. The concentrations determined in this tube were considered to be those of GSH and GSSG at time zero. The remaining six tubes were stored at −80°C until analysis, and the contents of each were analyzed periodically.

Stabilities of S-carboxymethyl-N-(2,4-dinitrophenyl) derivative of GSH and N-(2,4-dinitrophenyl) derivative of GSSG

A 4-ml portion of the above supernatant was treated with iodoacetic acid and 2,4-dinitrofluorobenzene according to the present method. The resulting solution was transferred in 1-ml portions into seven centrifuge tubes. GSH and GSSG from one tube were analyzed immediately after centrifugation. The

concentrations determined in this tube were considered to be those of GSH and GSSG at time zero. The remaining six tubes were stored at 4°C until analysis, and the contents of each were analyzed periodically after centrifugation.

3. Results and discussion

3.1. Sample preparation

Sample collection

In sample collection, care is needed to prevent auto-oxidation of GSH, resulting in GSSG, due to the presence of Fe^{3+} in erythrocytes. This can be done by using chelating agents such as EDTA and 1,10-phenanthroline. Lash and Jones [18] employed 1,10-phenanthroline for determination of GSH and GSSG in plasma because EDTA damages the aminopropylsilane particles packed in HPLC columns. In the present study, blood (0.5 ml) was also collected in heparinized tubes, each containing 5 mg of 1,10-phenanthroline (powder) or 0.5 ml of normal saline in which 1,10-phenanthroline (10 or 20 m*M*) had been dissolved. GSH and GSSG in these blood samples were determined, and the ratio of GSSG to GSH (GSSG/GSH) was calculated (Table 1). Better inhibition of GSH auto-oxidation was observed with the 1,10-phenanthroline powder rather than when it was dissolved in normal saline. The GSSG/GSH ratio further decreased by the addition of more than 5 mg of 1,10-phenanthroline powder, but hemolysis occurred. The 20 m*M* 1,10-phenanthroline solution was nearly saturated at room temperature. We finally decided to collect blood samples (3 ml) in heparinized tubes containing 30 mg of 1,10-phenanthroline powder, i.e. the final concentration of 1,10-phenanthroline was 50 m*M* in blood samples.

Table 1
Inhibition of GSH auto-oxidation by 1,10-phenanthroline added to collected blood

Sample	GSSG/GSH (mol/mol, %)
Normal saline[a]	15.8
10 m*M* Solution[b]	9.7
20 m*M* Solution[b]	9.3
Powder[c]	7.5

[a]0.5 ml of normal saline without 1,10-phenanthroline was added to 0.5 ml of blood.
[b]0.5 ml of 1,10-phenanthroline normal saline solution was added to 0.5 ml of blood.
[c]5 mg of 1,10-phenanthroline were added to 0.5 ml of blood.

Solution for hemolysis

We tried to hemolyze erythrocytes with 1,10-phenanthroline solution to minimize the auto-oxidation of GSH to GSSG, though hemolysis of erythrocytes is generally achieved by addition of water. As shown in Table 2, compared with the results with water, GSSG/GSH ratio after hemolysis with 20 m*M* 1,10-phenanthroline solution was lower and therefore used in our method.

Deproteinization

To analyze blood samples by HPLC, the proteins must be removed. Lash and Jones [18] used 5-sulfosalicylic acid to remove protein in plasma; however, Srivastava and Beutlar [21] reported that GSH oxidized in solutions of 5-sulfosalicylic acid, perchloric acid or trichloroacetic acid but, in contrast, was stable in metaphosphoric acid. Thus, in the present study, deproteinization with 10% metaphosphoric acid was chosen to prepare the analytical sample.

S-Carboxymethylation of GSH

GSH in erythrocytes was converted to its *S*-carboxymethyl derivative by reaction with iodoacetic acid to inhibit its artifactual auto-oxidation to GSSG. The effects of iodoacetic acid concentration and of reaction time on formation of *S*-carboxymethyl-GSH were examined, and the results are shown in Fig. 1. The amount of product from GSH increased with time on reaction with 200 m*M* iodoacetic acid at

Table 2
Inhibition of GSH auto-oxidation by water and 1,10-phenanthroline solution used for hemolysis

Sample	GSSG/GSH (mol/mol, %)
Water[a]	8.6
20 m*M* 1,10-Phenanthroline solution[a]	7.4

[a]0.4 ml of each was added to erythrocytes prepared from 0.5 ml of blood.

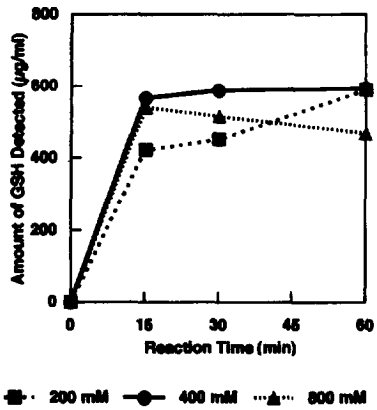

Fig. 1. Effects of iodoacetic acid concentration and reaction time on formation of the *S*-carboxymethyl derivative of GSH. A 0.1-ml portion of each concentration of iodoacetic acid was added to the sample solution prepared from 0.5 ml of blood, and the mixture was allowed to react under neutral conditions at room temperature (about 20°C) in the dark.

room temperature, while for that with 800 m*M* iodoacetic acid, the yield decreased after reaction times of more than 15 min. For the reaction with 400 m*M* iodoacetic acid, the amount of product was approximately constant for reaction times between 15 and 60 min. The reaction was considered to be essentially complete in the presence of 400 m*M* iodoacetic acid for 60 min. Therefore, 0.1 ml of 400 m*M* iodoacetic acid was added to the sample solution prepared from 0.5 ml of blood, and the mixture was allowed react under neutral conditions for 1 h in the dark at room temperature.

N-Dinitrophenylation of S-carboxymethyl-GSH and GSSG

The free amino groups of *S*-carboxymethyl-GSH and GSSG were converted to their *N*-(2,4-dinitrophenyl) derivatives, which were detectable by UV absorbance detector, by reaction with 2,4-dinitrofluorobenzene. The effects of the 2,4-dinitrofluorobenzene concentration and of reaction time on the formation of *S*-carboxymethyl-*N*-(2,4-dinitrophenyl)-GSH and *N*-(2,4-dinitrophenyl)-GSSG were examined, and the results are shown in Fig. 2. The amounts of reaction products from both GSH and GSSG increased with time at each concentration of 2,4-dinitrofluorobenzene. After 20 h of the reaction, about the same amounts of products were detected for all concentrations of 2,4-dinitrofluorobenzene. In the present study, 0.5 ml of 5% 2,4-dinitrofluorobenzene was added to the sample solution prepared from 0.5 ml of blood, and the mixture was allowed to react for 20 h in the dark at room temperature.

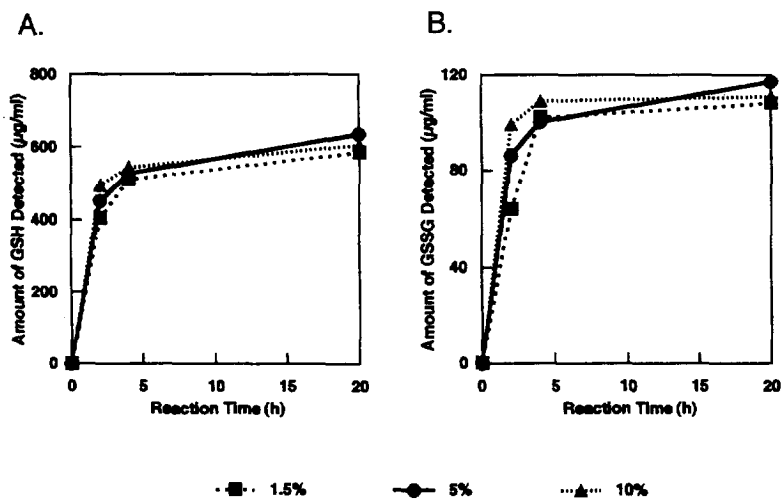

Fig. 2. Effects of 2,4-dinitrofluorobenzene concentration and reaction time on formation of the *N*-(2,4-dinitrophenyl) derivatives of *S*-carboxymethyl-GSH (A) and GSSG (B). A 0.5-ml portion of each concentration of 2,4-dinitrofluorobenzene was added to the sample solution prepared from 0.5 ml of blood, and the mixture was allowed to react at room temperature (about 20°C) in the dark.

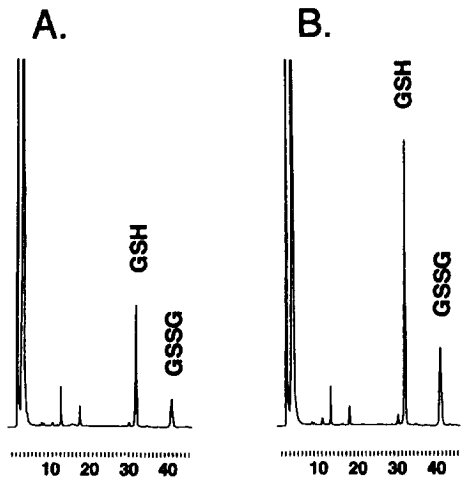

Fig. 3. Typical chromatograms of a sample prepared from human erythrocytes (A) and a human erythrocyte sample spiked with GSH (100 μg) and GSSG (40 μg) (B). Chromatographic conditions are given in the text.

3.2. Chromatography

Typical chromatograms of a sample prepared from human erythrocytes and of a human erythrocyte sample spiked with GSH (100 μg) and GSSG (40 μg) are shown in Fig. 3A and Fig. 3B, respectively. The retention times of GSH and GSSG were 31.7 and 40.6 min, respectively (Fig. 3A). Fig. 3B shows large sharp peaks with the spiked GSH and GSSG, which reveal identical retention times as signals found in the chromatograms of unspiked erythrocyte samples. This shows that the present method can be used to determine physiological concentrations of GSH and GSSG in erythrocytes.

3.3. Calibration

A 0.4-ml aliquot of each standard solution containing GSH (0–250 μg/ml) and GSSG (0–100 μg/ml) was combined with 0.2 ml of water or erythrocytes prepared from 0.5 ml of blood (hematocrit value: about 40%). Each mixture was treated with 0.3 ml of 10% metaphosphoric acid, and the GSH and GSSG content in each mixture was measured with the present method. The calibration curves plotting peak areas of GSH and GSSG versus these concentrations are shown in Fig. 4A and Fig. 4B, respectively. The slopes of the calibration graphs obtained after combining the standard solutions of GSH with water or erythrocytes were different. The slope of the graph obtained from GSH standard solutions combined with erythrocytes ($y = 2272x +$

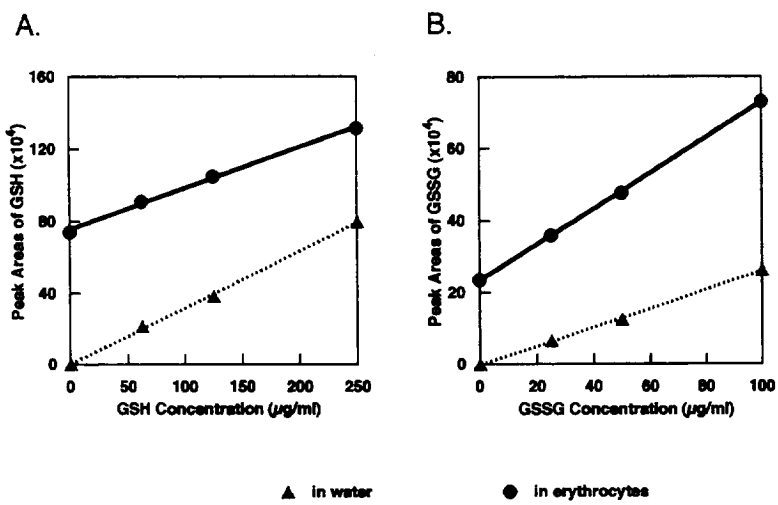

Fig. 4. Calibration curves of GSH (A) and GSSG (B). A 0.4-ml portion of each standard solution containing GSH (0–250 μg/ml) and GSSG (0–100 μg/ml) was added to 0.2 ml of water or erythrocytes prepared from 0.5 ml of blood (hematocrit: about 40%), and the mixtures were measured as described in the text. The normal linear regression line: GSH added to water, $y = 3160x + 2600$ ($r = 0.9993$); GSH added to erythrocytes, $y = 2272x + 755584$ ($r = 0.9985$); GSSG added to water, $y = 2618x - 1140$ ($r = 0.9997$); GSSG added to erythrocytes, $y = 4957x + 233940$ ($r = 0.9999$).

755584) was gentler than that with water ($y = 3160x + 2600$) (Fig. 4A). On the other hand, in the calibration graph for GSSG, the slope with erythrocytes ($y = 4957x + 233940$) was steeper than that with water ($y = 2618x - 1140$) (Fig. 4B). Consequently, in the present study, GSH and GSSG concentrations in the erythrocyte samples were determined by the standard addition method.

3.4. Precision

The precision of this method was demonstrated by repeated analysis ($n = 10$) of pooled blood samples and the results are presented in Table 3. The precision, evaluated by the coefficients of variation (C.V.), gave values for GSH and GSSG of 6% and 8%, respectively, which indicate good reproducibility.

3.5. Storage stability

The stabilities of GSH and GSSG in metaphosphoric acid solution at −80°C and of *S*-carboxymethyl-*N*-(2,4-dinitrophenyl)-GSH and *N*-(2,4-dinitrophenyl)-GSSG prepared for the HPLC analysis at 4°C were evaluated, and the results are shown in Fig. 5 and Fig. 6, respectively. As shown in Fig. 5, no GSH degradation was observed over 3 weeks although GSSG, the oxidation product of GSH, increased slowly with time, reaching 108% at 20 days of storage. Therefore, GSH and GSSG were considered to be relatively stable at −80°C in metaphosphoric acid solution for 3 weeks.

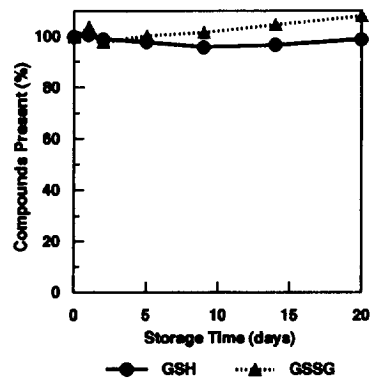

Fig. 5. Stabilities of GSH and GSSG in metaphosphoric acid solution at −80°C. The concentration of each compound just before storage (time zero) was set at 100% (GSH, about 610 μg/ml; GSSG, about 110 μg/ml).

On the other hand, the concentration of the derivatives of both GSH and GSSG decreased slowly with time, although the losses were only about 10% over 3 weeks, as shown in Fig. 6. This shows that the derivatives of both GSH and GSSG are relatively stable at 4°C.

4. Conclusions

In Fig. 4, the slope of the calibration graph obtained from GSH standard solutions combined with erythrocytes was gentler than that with water. On the other hand, in the calibration graph for

Table 3
Precision of the GSH and GSSG analysis ($n = 10$)

Sample No.	GSH (μg/ml)	GSSG (μg/ml)
1	658	112
2	587	110
3	599	108
4	581	110
5	572	130
6	618	120
7	661	115
8	618	119
9	569	104
10	559	132
Mean ± S.D.	602±36	116±9
Coefficient of variation (%)	6.0	8.0

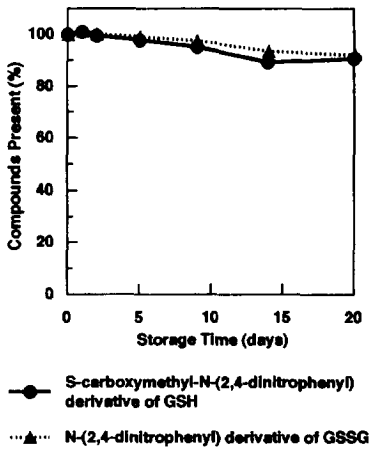

Fig. 6. Stabilities of the *S*-carboxymethyl-*N*-(2,4-dinitrophenyl) derivative of GSH and the *N*-(2,4-dinitrophenyl) derivative of GSSG at 4°C. The concentration of each compound just before storage (time zero) was set at 100% (GSH, about 610 μg/ml; GSSG, about 110 μg/ml).

GSSG, the slope with erythrocytes was steeper than that with water. The findings indicate that some of the GSH in the erythrocytes was oxidized to GSSG during sample preparation. Fe^{3+}, which is abundant in erythrocytes and accelerates this oxidation, can be removed by chelating it with 1,10-phenanthroline or EDTA. As EDTA damages aminopropylsilane particles in the HPLC column, 1,10-phenanthroline was chosen. Though oxidation of GSH to GSSG decreased with an increasing amount of added 1,10-phenanthroline, hemolysis of the erythrocytes occurred if too much was added. It is difficult to be completely suppress GSH oxidation during sample preparation. However, the oxidation was considered to proceed quantitatively under a definite analytical condition on the basis of the results in Fig. 4, which shows linear calibration curves of GSH and GSSG added to the erythrocytes as the percentage of the oxidized GSH to GSSG in the erythrocyte was constant. The present analytical method appears to be satisfactory to examine the effects of oxidizing agents on glutathione levels in erythrocytes.

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References

[1] R.T. Williams, Detoxication Mechanisms, Chapman and Hall, London, 1959.
[2] K. Nishiki, D. Jamieson, N. Oshino and B. Chance, Biochem. J., 160 (1976) 343.
[3] J.R. Dunbar, A.J. Delucia and L.R. Bryant, Res. Commun. Chem. Pathol. Pharmacol., 34 (1981) 485.
[4] J. Reeve, J. Kuhlenkamp and N. Kaplowitz, J. Chromatogr., 194 (1980) 424.
[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] K. Mertens, V. Rogiers, W. Sonck and A. Vercruysse, J. Chromatogr., 565 (1991) 149.
[7] A.J. Alpert and H.F. Gilbert, Anal.Biochem., 144 (1985) 553.
[8] A.M. Svardal, M.A. Mansoon and P.M. Ueland, Anal. Biochem., 184 (1990) 338.
[9] G.L. Newton, R. Dorian and R.C. Fahey, Anal. Biochem., 144 (1981) 383.
[10] J. Martin and I.N.H. White, J. Chromatogr., 568 (1991) 219.
[11] K. Mopper and D. Delmas, Anal. Chem., 56 (1984) 2557.
[12] J. Martensson, J. Chromatogr., 420 (1987) 152.
[13] E.G. Demaster, F.N. Shirota, B. Redfern, D.J.W. Goon and H.T. Nagasawa, J. Chromatogr., 308 (1984) 83.
[14] A.F. Stein, R.L. Dills and C.D. Klaassen, J. Chromatogr., 381 (1986) 259.
[15] L.A. Allison and R.E. Shoup, Anal. Chem., 55 (1983) 8.
[16] S.M. Lunte and P.T. Kissinger, J. Liq. Chromatogr., 8 (1985) 691.
[17] D.L. Rabenstein and R. Saetre, Anal.Chem., 49 (1977) 1036.
[18] L.H. Lash and D.P. Jones, Arch. Biochem. Biophys., 240 (1985) 583.
[19] J. Roberts and N.S. Agar, Clin. Chim. Acta, 34 (1971) 475.
[20] L.H. Lash and D.P. Jones, Arch. Biochem. Biophys., 225 (1983) 344.
[21] S.K. Srivastava and E. Beutler, Anal. Biochem., 25 (1968) 70.