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Effects of Storage Time and Temperature on the Stability of Glutathione in Deproteinized Blood Sample

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ABSTRACT

Glutathione is ubiquitous in eucaryotic cells and is implicated in many cellular functions. Release of reduced and oxidized glutathione (GSH and GSSG) into the plasma and the ratio of GSH/GSSG are considered a reliable index to evaluate the status of oxidative stress in biological systems. As storage temperatures and time periods have not been studied and discussed, we evaluated the effects of storage period and temperatures on the stability of GSH in blood samples by an automated capillary electropherograph system. After two-way ANOVA analysis, the result indicated that there were statistical significance between the GSH status and the storage period, and the GSH status and the preserved temperature as well. However, there was no statistical significance between samples with EDTA or with EDTA-NaF added as anticoagulant. Furthermore, no matter which freezing temperature (-20°C or -80°C) was used for storing the samples, the concentration of total GSH was quite consistent over the course of the study. We suggest that the alteration of the GSH status at room temperature should be due to not only the autoxidation of GSH but also the enzyme-catalyzed degradation of GSH and GSSG.

Key words: glutathione, blood, stability, autoxidation

INTRODUCTION

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH) is a tripeptide that plays an important role in the detoxification processes of electrophilic metabolites of xenobiotics and oxygen free radicals. Release of reduced and oxidized glutathione (GSH and GSSG) into the plasma is considered to be a reliable index of oxidative stress. Alterations of the glutathione status have been found in a variety of diseases, including diabetes mellitus[(1)], human immunodeficiency virus (HIV) infection[(2)], cystic fibrosis[(3)], acute respiratory distress syndrome[(4)], chronic renal failure[(5)], sepsis[(6)], and liver disease[(7)]. GSH is also important for the maintenance of α-tocopherol and ascorbic acid in the reduced state. Therefore, the ratio of GSH/GSSG is used to evaluate the status of oxidative stress in biological systems.

GSH not only protects cell membranes from oxidative damage but also helps maintain the sulphydryl groups of many proteins in the reduced form. Irreversible cell damage supervenes when the cell is no longer able to maintain its GSH content[(8)]. Therefore, the measurements of various forms of GSH concentrations in biological samples are important for the understanding of GSH homeostasis in clinic applications.

A variety of analytical methods have been described for the determination of GSH and GSSG in the blood and other biologic samples, including enzymatic[(9)], spectrophotometric and spectrofluorimetric[(10)], high performance liquid chromatography[(11)] with electrochemical detectors[(12)] and by capillary electrophoresis[(13,14)] methods although no reference method has yet been defined[(15)]; nonetheless, few of these could detect both GSH and GSSG simultaneously. The separation of GSH and GSSG by capillary zone electrophoresis (CZE) with direct ultraviolet detection (200 nm filter) in whole blood, red blood cells, tissue, mitochondria[(15-18)], and by micellar electrokinetic capillary electrophoresis (MEKC) in plasma has also been reported[(19)].

The temperature of -80°C is suggested for preserving and storing glutathione in biological samples[(20)]. But different storage temperatures and time periods for CZE method have not been studied and discussed. To evaluate the effects of storage periods and temperatures on the stability of GSH and GSSG in blood, we adopted the washed red blood cells (RBC) from the blood with EDTA or EDTA-NaF as anticoagulants.

MATERIALS AND METHODS

I. *Reagents and Calibrators*

All chemicals were of an analytical grade and

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solutions were prepared with water from the Millipore RiOs System. Boric acid, Metaphosphoric Acid (MPA), GSH, and GSSG were obtained from Sigma Chemical (St. Louis, MO, USA). Stock solutions containing 1 mM of GSH and GSSG were prepared in 1% (w/v) MPA, aliquoted, and stored at -80°C. Working standard solutions of GSH and GSSG were prepared by diluting the stock solutions appropriately with 1% (w/v) MPA (seven concentrations including 100 μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM, 3.12 μM, and 1.56 μM). The linearity was up to 100 μM GSH and GSSG, because there was a dilution of 20× in the b1ood sample, the concentration equivalent to 2000 μM. Calibration curves were constructed by liner regression of the corrected peak area of GSH and GSSG ($R > 0.999$).

II. *Within-run, Between-run and Recovery Study*

The within- (n =10) and between-run (n = 10) assay for GSH and GSSG were studied at higher concentrations (1000 μM for GSH and 100 μM for GSSG) and lower concentrations (500 μM for GSH and 50 μM for GSSG). Recoveries of GSH and GSSG from spiked calibrators and washed RBC (n = 5) were also studied.

III. *Blood Sample Preparation and Storage*

Venous blood samples (~10 mL) from 2 healthy and nonfasting adult volunteers were collected in EDTA and EDTA-NaF anticoagulated tubes and immediately placed on ice. To minimize the autoxidation of thiols or the enzymatic reduction of the disulfides, the processing of the sample was carried out at 4°C. Red blood cells were separated from plasma by centrifugation (3,000 rpm for 5 min) followed by three times washing with 0.9% w/v NaCl. Red blood cells 100μl aliquots of washed RBC were added to 300 μL of ice-cold 5% MPA. To precipitate proteins completely, the samples were vortexed and incubated on ice for 10 min. The acidic suspensions were centrifuged at 12,000 rpm for 10 min at 4°C; the supernatants were then filtered through a 0.2-μm filter, divided into parts and stored at three different temperatures [room temperature (RT), -20°C, and -80°C]. For each condition, triplicate measurements for each sample were made on each of the following days: 0, 1st, 2nd, 7th, 14th, 21st, and 28th. A 20 μL aliquot was diluted with 80 μL of deionized water before injection (final dilution, 1:20).

IV. *Instrument and Method of the Capillary Electrophoresis System*

All experiments were carried out using an automated capillary electropherograph P/ACE-MDQ system equipped with a fixed wavelength UV detector with an interference filter at 200 nm (Beckman Coulter, Fullerton, CA, USA). Throughout all the experiments, uncoated fused-silica capillaries (75 μm I.D., 50 cm effective length) were used. Injection of the sample was by the application of 0.5-psi pressure for 15 sec. The capillary was thermostated at 28°C. Before each analytical run, the capillary was rinsed and filled with the running buffer [300 mM boric acid (pH 7.8)] for 2 min at 25 psi. The electrophoresis was performed with a constant voltage of 25 kV (~62 μA) for 8 min. Between analytical runs, the capillary was rinsed with 0.1 N NaOH and deionized water at 25 psi for 2 min in each. The Beckman P/ACE System 32 KaratTM software was used for the instrument control; data were quantified on the basis of corrected peak areas with migration times.

V. *Statistical Analysis*

Two-way ANOVA with one factor repeated-measurement was used in this study.

RESULTS

The imprecision study for the analysis of GSH and GSSG by CZE was evaluated in this study. The within-run CVs for GSH and GSSG were 0.9% and 0.8%, and 3.8% and 4.2% respectively at the study concentrations. The between-run CVs for both GSH and GSSG were less than 10% (Table 1). The percentage of recovery in studies, spiked with GSH and GSSG into calibrators and washed RBC samples respectively, are depicted in Table 1.

Table 2 summarizes the percent change (gain or loss) of GSH, GSSG, total GSH (expressed as equivalent GSH + 2GSSG) and the ratio of GSH/GSSG in washed RBC from the blood with EDTA or EDTA-NaF added as anticoagulant. Moreover, the GSH, GSSG, and the total GSH concentrations (at day 0) were respectively adopted to be the mean baseline concentrations. Concentration of GSH and GSSG was determined with the washed RBC in triplicate, and total GSH and the ratio of GSH/GSSG were calculated. The mean values from the blood with EDTA added as anticoagulant were 1703.5 ± 74.0 μM for GSH, 46.5 ± 4.1 μM for GSSG, 1796.7 ± 67.7 μM for total GSH, and 36.6 ± 4.6 for the GSH/GSSG ratio; the mean values from the blood with EDTA-NaF added as anticoagulant were 1787.5 ± 67.4 μM for GSH, 62.0 ± 5.2 μM for GSSG, 1911.2 ± 77.5 μM for total GSH, and 28.7 ± 1.4 for the GSH/GSSG ratio. The statistical analysis (two-way ANOVA), indicated there were significant differences with storage period and in the preserved temperature ($p < 0.001$). However, there was no statistical significance for the percent change of GSH, GSSG, and total GSH between the use of anticoagulants EDTA or EDTA-NaF.

DISCUSSION

Measurement of blood GSH statuses (GSH & GSSG) can be used as a marker for the change of oxidative status

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in various pathological conditions. The GSH/GSSG ratio provides a physiologic indicator of the activity of intracellar defense system against the reactive oxygen species[21]. Analysis of GSH and GSSG with one single run requires a simple, quick, sensitive, and reproducible analytic method such as CZE. In this study, we used a CZE

Table 1. Recovery of GSH and GSSG from the spiked (A) calibrator and (B) washed RBC sample (n = 5) & imprecision tests of reduced and oxidized glutathione in calibrators*

		Recovery		
		Calibrator added (μM)	Measured concentration (μM ± S.D.)	Recovery (%)
(A)	GSH	0	498.6 ± 6.8	—
		100	597.5 ± 7.2	98.9
	GSSG	0	53.5 ± 3.2	—
		10	63.9 ± 3.4	104.1
(B)	GSH	0	1305.2 ± 75.8	—
		200	1488.8 ± 80.5	91.8
	GSSG	0	87.5 ± 25.6	—
		20	109.2 ± 26.3	108.5

	Imprecision					
	Within-run variation (n = 10)			Between-run variation (n = 10)		
	Mean (μM)	S.D. (μM)	%CV	Mean (μM)	S.D. (μM)	%CV
GSH (μM)						
500	495.6	4.3	0.9	492.6	7.2	1.3
1000	998.9	10.6.1	0.8	991.8	5.5	0.6
GSSG (μM)						
50	47.2	2.5	3.8	47.6	2.9	5.0
100	103.1	3.8	4.2	97.2	4.7	5.5

*CV, coefficient of variation; S.D., standard deviation; GSH, reduced glutathione; GSSG, oxidized glutathione.

Table 2. The percentage changes (gain or loss) of reduced glutathione, oxidized glutathione, total glutathione, and the GSH/GSSG ratio in the washed RBC of the two kinds of anticoagulants

		GSH (% change)		GSSG (% change)		Total GSH (% change)		GSH/GSSG (% change)	
Temperatures	Days	EDTA	EDTA-NaF	EDTA	EDTA-NaF	EDTA	EDTA-NaF	EDTA	EDTA-NaF
R.T.	0	0	0	0	0	0	0	0	0
	1	-9.8	-4.4	97.1	91.0	-4.2	1.9	-54.2	-49.9
	2	-14.1	-14.4	139.1	125.9	-6.2	-5.2	-64.1	-62.1
	7	-38.0	-39.3	176.5	168.9	-26.9	-25.8	-77.6	-77.4
	14	-51.5	-56.0	191.3	194.2	-38.9	-39.7	-83.3	-85.1
	21	-61.5	-64.3	219.1	217.1	-47.0	-46.0	-87.9	-88.7
	28	-73.1	-77.7	214.0	203.6	-58.2	-59.4	-91.4	-92.7
-20°C	0	0	0	0	0	0	0	0	0
	1	-7.1	-7.5	62.2	36.1	-3.5	-4.6	-42.7	-32.0
	2	-3.9	-3.2	111.4	74.5	2.1	1.9	-54.6	-44.5
	7	-5.3	-5.2	232.3	135.0	7.0	4.0	-71.5	-59.6
	14	-15.6	-15.7	266.8	176.2	-1.0	-3.2	-77.0	-69.5
	21	-16.5	-15.7	335.6	225.6	1.7	0.0	-80.8	-74.1
	28	-19.7	-10.5	416.8	253.7	2.9	6.7	-84.5	-74.7
-80°C	0	0	0	0	0	0	0	0	0
	1	3.4	-2.5	9.3	7.8	3.7	-1.8	-5.4	-9.6
	2	1.6	-1.9	-0.9	1.3	1.4	-1.7	2.5	-3.2
	7	0.3	-1.2	3.4	2.3	0.5	-0.9	-3.0	-3.4
	14	-0.4	-2.4	8.1	5.5	0.0	-1.9	-7.9	-7.5
	21	1.4	-1.5	8.4	4.0	1.8	-1.1	-6.5	-5.2
	28	3.5	-2.8	7.9	1.8	3.8	-2.5	-4.1	-4.6

method modified by Serru *et al*.[15] and Ciriaco *et al*.[16]. In Serru's study, the CZE was performed with a buffer containing boric acid 75 mM and Bis-Tris 25 mM at pH 8.4. We found the peak of GSH was decreased gradually in the within-run assay but not so for the GSSG peak which was not increased or decreased in concentration. The confusing problem has been solved after we changed the buffer of the Ciriaco's formula (300 mM boric acid, pH 7.8). To make the CZE results more stable and correct, rapid acidification of the sample is critical because it prevents both autoxidation and enzyme-catalyzed degradation via the γ-glutamyl transfersae reaction with GSH and GSSG. We chose Meta-phosphoric acid (MPA) to deproteinize the whole blood and the washed RBC samples, which was described as one of the most reliable agents for glutathione storage[20,22].

EDTA was added as an anticoagulant according to Serru's method[15]. Another anticoagulant, EDTA-NaF, was also used due to the fact that it could inhibit enolase during glycolysis presumably by reducing the activity of glutathione peroxidase (GPx). Our data indicated that samples with EDTA-NaF added seemed to be more stable than those with EDTA (Figure 2).

The GSH electrophoregram pattern of samples from the whole blood and the washed RBC were depicted in Figure 1. As the sample from whole blood was assayed, a broad and unknown peak appeared in front of the GSH peak, which led to the interference of the identification of GSH. The same phenomenon was also reported by Serru[15]. We thus chose the washed RBC samples for the determination of GSH status.

After the 28-day storage at -80°C, the concentration of GSSG in blood samples with EDTA-NaF added as an anticoagulant seemed to be more stable than those with EDTA added as an anticoagulant (1.8% gain vs. 7.9% gain), especially in the prevention of GSSG formation although there was no statistical significance. However, when the samples were stored at -20°C, the GSH status changed obviously in both groups with either anticoagulant added from the 2nd day. In other words, the concentration of GSSG gradually increased indicating the GSH in the assay samples stored at -20°C is not stable. In this condition (-20°C), the GSH/GSSG ratio decreased gradually which would influence the validation of the

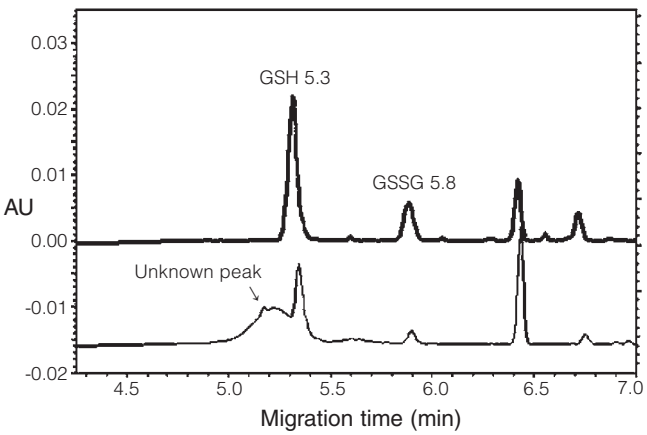

Figure 1. Electropherograms of GSH and GSSG in washed RBC (—) and whole blood (—) sample deproteinized with 5% (w/v) MPA. A broad and unknown peak was just in front of the GSH peak that interfered with the identification of GSH when we used whole blood as the sample in the same CZE system condition.

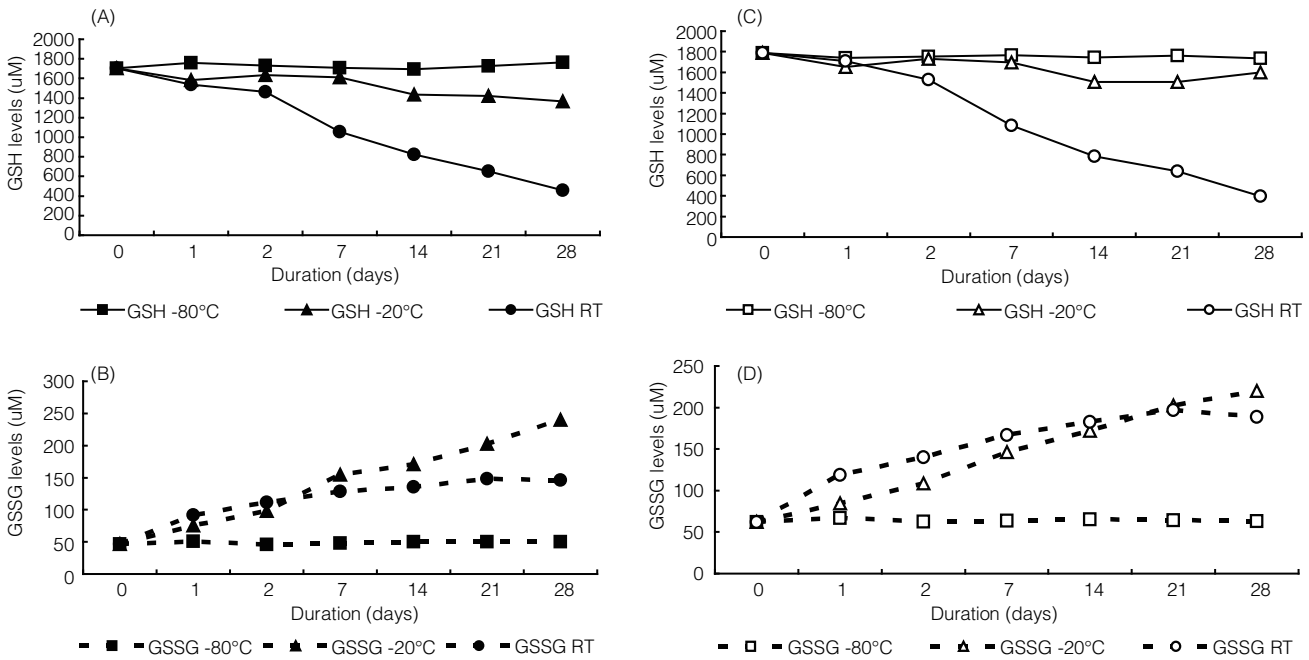

Figure 2. Changes of GSH status in EDTA-blood (A, B) and in EDTA-NaF blood (C, D) at three different temperatures (Room temperature, -20°C, and -80°C) for 7 different periods of time.

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Table 3. The reference values of the whole blood samples and the washed RBC samples for GSH statuses of our laboratory.

	Washed RBC (n* = 137)	Whole blood (n = 57)
GSH (μM)	1294.5 ± 258.2**	873.4 ± 164.3
GSSG (μM)	81.7 ± 45.7	58.2 ± 24.9
Total GSH (μM)	1457.8 ± 277.5	989.4 ± 170.6
GSH/GSSG	20.3 ± 10.2	17.7 ± 7.9

*n: sample number.
**Values are presented as mean ± S.D.

GSH status results, indicating that storage of GSH samples at this condition is not suitable. Interestingly, no matter which freezing temperature (-20°C or -80°C) we chose for storing the samples, the concentration of the total GSH was quite stable with either of these two anticoagulants over the course of the study. We suggest that alteration of GSH and GSSG concentrations at -20°C should be due to the autoxidation of the GSH.

In comparison to samples with EDTA added and stored at -20°C for 7 days and samples with EDTA-NaF and stored at -20°C for 21 days, the GSSG concentration obviously decreased when stored at RT and the percentage change of total GSH concentration decreased approximately 26% from the 7th day of the study. We suggest that alteration of GSH, GSSG, and total GSH concentrations over the course of the study at RT should be due to not only the autoxidation of the GSH but also the enzyme-catalyzed degradation of GSH and GSSG.

The reference values for GSH status of our laboratory were shown in Table 3. The mean values of the washed RBC samples from 137 volunteers, including 65 males and 72 females and average 51.5 years in age (from 10 to 76 years), were 1294.5 ± 258.2 μM for GSH, 81.7 ± 45.7 μM for GSSG, 1457.8 ± 277.5 μM for total GSH, and 20.3 ± 10.2 for the GSH/GSSG ratio, respectively. The reference values of the whole blood samples from 57 volunteers, including 27 males and 30 females and average 52.2 years in age (from 26 to 76 years), were 873.4 ± 164.3 μM for GSH, 58.2 ± 24.9 μM for GSSG, 989.4 ± 170.6 μM for total GSH, and 17.7 ± 7.9 for the GSH/GSSG ratio, respectively.

The reference values for total, free and reduced glutathione in plasma and in whole blood differ somewhat from one laboratory to another. The variability may be related to different methodology, difference in sample processing and/or storage or the selection of subjects who are under the influence of various factors affecting the plasma/blood glutathione concentration. Whole blood, washed RBC, and plasma samples were chosen for glutathione status determination in different laboratories for various clinical purposes. RBC contains approximately 500 times higher GSH concentration than plasma; therefore, minor hemolysis (0.1% to 1%) can result in erroneously high plasma values[23]. Alternatively, GSH can be lost by autoxidation which occurs with a half-time of about 5 min in plasma at room temperature[24]. Human plasma also contains γ-glutamyltranspeptidase, an enzyme that degrades GSH[25] and can be present at high activities in human plasma in association with liver disease.

Griffith's study[26] showed the stability of total glutathione in the acidic, protein-free supernatants to be fair at 0°C or -20°C (5 to 20 % loss per 24 hr) and good at -78°C to -190°C (0 to 5% loss even with long-term storage). In this study, we showed the different result in total GSH stored at -20°C. RT is the prefer temperature in clinical sample collection, but the GSH statuses at RT was very unstable. Therefore, the freezing temperature is still in need to maintain GSH statuses. In summary, the data from the present study showed that (1) GSH and GSSG are more stable at -80°C than those at RT or -20°C, and (2) GSH stored at -80°C possesses good and prolonged stability for at least one month.

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