Review

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Recent advances in analysis of glutathione in biological samples by high-performance liquid chromatography: a brief overview

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ABSTRACT: Glutathione (GSH) is a tri-peptide that plays an important role in protecting cells and tissues against oxidative stress. So far a lot of analytical methods of glutathione have been reported. This brief review presents an overview of the analysis of glutathione in biological samples by high-performance liquid chromatography (HPLC) in recent five years, focusing on the sample pretreatment, derivatization and mass spectrometric detection.

Keywords: High-performance liquid chromatography, mass spectrometry, derivatization, glutathione

1. Introduction

Glutathione (GSH, γ-L-glutamyl-L-cysteinyl-glycine) is a tri-peptide that plays an important role in protecting cells and tissues against oxidative stress. Oxidative stress is to be involved in aging, and in various diseases such as cardiovascular diseases, diabetes mellitus, rheumatoid arthritis, Alzheimer's diseases, Parkinson's disease, and carcinogenesis (*1-5*). Under these conditions GSH is converted to glutathione disulfide (GSSG), the oxidized form of GSH, resulting in a decrease in GSH/GSSG ratio. Therefore, GSH/GSSG ratio is considered to be an indicator of redox status and disease risks, and it is important to measure GSH and GSSG level in the biological samples.

There are several difficulties to measure GSH and GSSG level accurately. GSH can be easily non-enzymatically oxidized to GSSG by molecular oxygen. Furthermore GSH reacts with disulfides and thiol-disulfide exchange can take place. Under normal conditions, GSH is known to be present in mammalian cells in the concentration range 1-10 mM, and the molar ratio of GSH/GSSG is maintained at about 100:1 to 1,000:1 (*6*). Therefore, oxidation of the small amount of GSH to GSSG or thiol-disulfide exchange greatly changes the GSH/GSSG ratio. To measure GSH level in plasma, the following point should be also considered. GSH level in erythrocytes is about 500-fold higher than that in plasma, thus, hemolysis can cause overestimation of GSH in plasma samples. These facts suggest that the sample pre-treatment is crucial for the exact measurement of GSH and GSSG level.

GSH and GSSG have been measured by several analytical methods (*7*). In particular, high-performance liquid chromatography (HPLC) with various detection methods such as ultraviolet absorbance (UV), fluorescence detection (FL), electrochemical detection (ECD), is widely used because of its convenience, sensitivity, and selectivity (*8-11*). Recently, liquid chromatography-mass spectrometry (LC-MS) is sometimes used for the analysis of glutathione and its related compounds (*12*). This brief review presents an overview of the analysis of glutathione in biological samples in recent five years (2009-2013), focusing on the sample pretreatment, derivatization and LC-MS.

2. Analysis of GSH by HPLC

2.1. *Sample pretreatment*

Pretreatment of GSH analysis by HPLC usually involves the following procedure, *i.e.*, sample collection, reduction of GSSG, deproteinization, and derivatization of GSH. Samples must be carefully treated for the accurate quantification of GSH and GSSG, since non-enzymatic oxidation of GSH, thiol-disulfide exchange, other side reactions, and hemolysis can occur during the pretreatment. These reactions disturb GSH/GSSG ratios in the samples. Oxidation reaction and thiol-disulfide exchange reaction (*13*) are dependent on pH and temperature, therefore lower pH and lower temperature is recommended during sample pretreatment and storage. Tubes for sampling are recommended to contain chelating agent such as ethylenediaminetetraacetic acid (EDTA) to suppress oxidation reactions. Addition of the derivatization reagent such as *N*-ethylmaleimide (NEM) to the sample was effective to protect thiol group

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from oxidation and thiol-disulfide exchange reactions. Reagents, reactions, and practical considerations for the sample pretreatment of GSH and GSSG analysis were described and discussed in detail in the previous reviews (*14,15*).

2.2. *Determination of GSH by HPLC-UV, ECD, FL*

HPLC-UV, ECD, FL methods were summarized in Table 1. During acid-induced denaturation of blood samples, oxyhemoglobin elicits the production of several oxidants, which induce a strong artifactual increase in both GSSG and PSSG (S-glutathionylated proteins) concentration. This phenomenon can be prevented by blocking the –SH groups with alkylating agents such as NEM before acidification (*16-18*). Taking into these considerations, Rossi *et al*. reported the measurement of GSH, GSSG, and PSSG in human red blood cells (*19*). Blood samples were drawn in the presence of EDTA, and red blood cells (RBCs) were prepared by centrifugation. RBCs samples were washed with phosphate buffered saline (pH 7.4) containing 6 mM glucose (PBSG) and 10 mM NEM, and acidified with 10% (w/v) trichloroacetetic acid (TCA). After deproteinization by centrifugation, the excess of NEM in the supernatants was extracted by dichloromethane. The samples were reacted with 2,4-dinitrofluorobenzene (FDNB) for 3 h at room temperature and aliquots were subjected to HPLC analysis. They examined the role of ketone bodies (β-hydroxybutyrate, acetone, acetoacetate) in generation of oxidative stress in human erythrocytes. A simple and rapid determination method of the reduced form of GSH in whole blood was reported (*20*). Blood samples collected in the tubes containing of a solution of K_3EDTA and were let to react with NEM for 1 min. The samples were acidified by TCA (12%, w/v) and proteins were spun by centrifugation, and aliquots of supernatants were loaded onto an HPLC. Though this method detects only GSH and not its oxidized form, GSSG, it can be used for large-scale clinical studies. HPLC-UV method requires a simple instrument, and can be easily applied to the routine analysis.

HPLC-ECD is a simple and sensitive detection method for thiol compounds such as GSH. In HPLC-ECD, GSH was usually analyzed without derivatization. Park *et al*. reported the determination of GSH and GSSG using LC-ECD with boron-doped diamond electrode (*21*). Liver was homogenized in ice-cold PBS containing 1,3-diamino-2-hydroxypropanetetraacetic acid (DPTA) in ice bath. The homogenate was mixed with ice-cold 10% perchloric acid (PCA) containing DTPA. The acidified supernatant was stored at −80ºC and subjected to HPLC. This method was applied to the evaluation of hepatic glutathione redox status. Khan *et al.* reported simultaneous determination of ascorbic acid and aminothiols in plasma and erythrocyte using HPLC-ECD (*22*). Blood samples were collected in EDTA tube, centrifuged, and stored at −80ºC. The plasma and 10% meta-phosphoric acid solution were mixed and vortexed. After addition of mobile phase and centrifugation at 4ºC, the supernatant was injected to HPLC. Lysed erythrocytes were similarly analyzed. HPLC-FL is widely used method for the analysis of glutathione, since it is highly sensitive and selective. Derivatization reagents such as ortho-phthaldialdehyde (OPA), monobromobimane (MBB), ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F), and fluorescent maleimide were often used for this purpose. Conlan *et al*. reported the determination of intracellular glutathione and cysteine using MMB (*23*). After deproteinization by methanesulfonic acid, the samples were reacted with MMB in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 8.5) in the dark for 30 min in the presence of diethylenetriaminopentaacetic acid (DTPA). GSSG and cysteine (CysSS) were reduced to GSH and Cys by dithiothreitol (DTT) in 1 M HEPES buffer (pH 8.5) at 37ºC for 30 min followed by the derivatization with MMB. The increase in GSSG content and the higher GSSG/GSH ratio in myofibers compared with myoblasts were reported.

2.3. *Determination of total glutathione by HPLC*

Assay methods of total glutathione or GSSG in biological samples were summarized in Table 2. In these methods, GSSG and protein bound GSH were reduced to GSH, and the total GSH (total of reduced and oxidized forms

Table 1. Determination of GSH and GSSG by HPLC-UV, FL, ECD

Detection	Analytes	Derivatization	Separation (Column)	GSH (LOQ)	GSSG (LOQ)	Matrix	*Ref.*
UV (355 nm)	GSH, GSSG, PSSG	NEM, FDNB	C18	NR	NR	Erythrocyte	*19*
UV (265 nm)	GSH	NEM	NH2	0.05 mM	NR	Human blood	*20*
ECD (1,475 mV)	GSH, GSSG	None	C18	62.5 pmol (S/N 139.6)	1.6 pmol (S/N 4.4)	Mouse liver	*21*
ECD (900 mV)	GSH, Cys, Hcy, Nac, Met, GSSG CysSS, Vitamin C	None	C18	30 ng/mL	96 ng/mL	Human plasma	*22*
FL (360/455 nm)	GSH, GSSG Cys, CysSS	MBB	C18 monolithic	0.03 μM (LOD, S/N = 3)	NR	Human muscle cell	*23*

NR, not reported; LOQ, limit of quantification; LOD, limit of detection; Hcy, homocysteine; Nac, *N*-acetylcysteine; Met, methionine. Other abbreviations are described in the text. The detection wavelength or electrode voltage was described in the parentheses of "Detection" column. The excitation and emission wavelengths were described in case of FL.

of GSH) was analyzed. DTT (*24,25*), tributylphosphine (TBP) (*26,27*) and tris-(2-carboxyethtyl)phosphine (TCEP) (*28,29*) were used as a reducing agent. The generated GSH was derivatized with OPA (*24*), SBD-F (*26,29*), 5-bromo-7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-BF) (*28*), *N*-1-(pyrenyl)maleimide (NPM) (*25*), 1,3,5,7-tetramethyl-8-phenyl-(4-iodoacetamido)difluoroboradiaza-s-indane (TMPAB-I) (*30*), 1,3,5,7-tetramethyl-8-bromomethyl-difluoroboradiaza-s-indacene (TMMB-Br) (*31*). GSH, GSSG and their derivatives are usually separated on the reversed-phase column. Hydrophilic interaction chromatography (HILIC) is also useful for the separation of these highly polar compounds. Isokawa *et al.* reported the improvement of the sensitivities of SBD-thiol compounds using HILIC column. The content of organic solvent in HILIC is rather high and the fluorescence intensities of SBD-thiols are greatly increased in the solution containing high percentage of organic solvent (*29*). Yoshitake *et al.* reported a sensitive analysis of aminothiols using intramolecular fluorescence resonance energy transfer (FRET) between the amine-derivatized and thiol-derivatized fluorophores (*27*).

2.4. *Determination of GSH by LC-MS*

Recently, LC-MS has been widely used for the determination of biological compounds, because of its sensitivity and selectivity. This method is also applied to the analysis of GSH, GSSG, and related compounds (Table 3). Iwasaki *et al*. used *N*-benzylmaleimide (NBenzM) and *N*-cyclohexylmaleimide (NCycloM) for

Table 2. Determination of total GSH by HPLC

Detection	Analytes	Derivatization	Separation (Column)	GSH (LOQ)	GSSG (LOQ)	Matrix	*Ref.*
FL (350/420 nm)	GSSG, GSH	OPA	C18	1.0 μM	0.2 μM	Human blood, plasma	*24*
FL (330/376 nm)	GSH	NPM	C18	10 nM	NR	Rat brain, liver, lung	*25*
FL (385/515 nm)	GSH, Cys, Hcy, CysGly,	SBD-F	C18	0.5 μM	NR	Human plasma	*26*
FL (390/540 nm)	GSH, Cys, Hcy	DCIA, NBD-F	C18	150 fmol	NR	Human plasma	*27*
FL (385/515 nm)	GSH, Cys, Hcy, CysGly	SBD-BF	C18	0.05 μM	NR	Human plasma	*28*
FL (375/510 nm)	GSH, Cys, Hcy, CysGly,γ-GluCys, Nac, CA	SBD-F	HILIC	2.7 nM	NR	Human plasma	*29*
FL (500/510 nm)	CoA, GSH, Nac, Cys, Hcy, 6-MP	TMPAB-I	C18	0.3 nM (LOD S/N = 3)	NR	Human plasma	*30*
FL (505/525 nm)	GSH, Cys, Hcy, Nac	TMMB-Br	C8	0.2 nM (LOD S/N = 3)	NR	Human blood	*31*

NR, not reported; LOQ, limit of quantification; LOD, limit of detection; Hcy, homocysteine; Nac, *N*-acetylcysteine; Met, methionine; CA: cysteamine, 6-MP: 6-mercaptopurine; DCIA, 7-diethylamino-3-[{4'-(iodoacetyl)amino}phenyl]-4-methylcoumarin; NBD-F, 4-fluoro-7-nitro-2,1,3-benzoxadiazole. Other abbreviations are described in the text. The excitation and emission wavelengths were described in the parentheses of "Detection" column.

Table 3. Determination of GSH and GSSG by LC-MS

Detection	Analytes	Derivatization	Separation (Column)	GSH (LOQ)	GSSG (LOQ)	Matrix	*Ref.*
MS	GSH, Cys, Hcy GSSG, CysSS, HcySS	NBenzM, NCycloM	HILIC	10 μM	10 μM	Mouse serum	*32*
MS/MS	GSH, Cys, Hcy, CysGly, and their disulfids	IAM, IPCF	C18	10 nM	5 nM	Human blood	*35*
MS/MS	GSH, GSSG	NEM	Porous graphitic carbon	1.5 μM	0.1 μM	Human blood	*36*
MS/MS	GSH, GSSG, GSA	NEM	Porous graphitic carbon	0.1 pmol	0.1 pmol	Human erythorocyte *et al.*	*37*
MS/MS	GSH, Cys, Hcy, Nac and their disulfides	BQB	HILIC	3.27 nmol/L (LOD S/N = 3)	NR	Human urine	*34*
MS/MS	GSH, GSSG	none	Pentafluorophenyl	0.5 μM	0.0625 μM	Human blood	*38*
MS/MS	GSH	none	C18	1.8 μM (LOD S/N = 3)	NR	Human blood	*39*
MS	GSH, GSSG	none	C18	0.4 ng/mL	0.5 ng/mL	Microdialysate	*40*
MS/MS	GSH, GSSG	none	C18 high strength silica	0.40 μM	0.80 μM	Rat and mouse bile	*41*
MS/MS	22 reduced thiols and 7 disulfides	cICAT	C18	NR	NR	Endothelial cells	*42*

NR, not reported; LOQ, limit of quantification; LOD, limit of detection; Hcy, homocysteine; CysSS, cystine; HcySS, homocystine; GSA, glutathione sulfoneamide; Nac, *N*-acetylcysteine; Met, methionine; CA, cysteamine; cICAT, cleavable isotope coded affinity tag. Other abbreviations are described in the text.

the determination of GSH and GSSG (*32*). At first blood samples were collected into plastic tubes containing NBenzM (10 mM) to react with reduced form of GSH in the presence of deferoxamine mesylate. After reduction of GSSG with DTT, the samples were reacted with NCycloM (50 mM) in the presence of deferoxamine mesylate. After deproteinization using 5-sulfosalicylic acid (5-SSA), the samples were subjected to LC-MS equipped with column-switching system (on-line solid phase extraction and separation). HILIC column was used for separation. The content of organic solvent in HILIC was rather high and it was suitable for electrospray ionization (ESI)-MS (*32-34*). This method was applied to the investigation of the roles of thiol compounds in lipopolysaccharide (LPS)-induced acute inflammation. Serum glutathione disulfide and cysteine levels were significantly decreased after LPS treatment. Suh *et al.* used iodoacetamide (IAM) for the quenching of GSH and reduced form of thiols (*35*). The collected blood samples in the tubes containing EDTA were immediately mixed with IAM in Tris-HCl (pH 8) buffer. After deproteinization and solid phase extraction, samples were reacted with isopropyl chloroformate (IPCF) for the derivatization of amino and carboxylic groups, and analyzed with LC-MS/MS. The method was used to assess thiol redox states in plasma and erythrocytes isolated from healthy subjects and thalassemia patients. Moore *et al.* reported LC-MS/MS method for the clinical determination of reduced and oxidized glutathione from whole blood (*36*). Blood was collected in heparin or EDTA containing tube. The blood samples were mixed with solution containing NEM, 5-SSA and EDTA in 15% methanol. After deproteinization, GSSG and GSH-NEM were analyzed by LC-MS/MS. Harwood *et al* .used NEM to quench GSH (*37*). An excess of NEM (10 mM) was added, and the samples were left for 20 min at room temperature. Cold ethanol was added to lyse cells and precipitate proteins. After centrifugation the supernatant was removed. Samples were reconstituted in water and subjected to LC-MS/MS. GSH-NEM, GSSG and glutathione sulfonamide in cells were analyzed. Huang *et al.* synthesized ω-bromoacetonylquinolinium bromide (BQB) for derivatization of thiol group and applied to the determination of aminothiols and their oxidized forms in urine (*34*). Urine samples were divided into two parts equally in the presence of EDTA. One part was mixed with Gly-HCl buffer solution (pH 3.5) containing d7-BQB. The labeling was completed within 3 min with the aid of microwave. The other part was treated with TCEP to reduce the oxidized forms of thiols, and reacted similarly with BQB. Finally two solutions were mixed and subjected to LC-MS/MS.

LC-MS/MS methods without derivatization were reported. Blood sample was drawn into tubes containing EDTA and kept on ice to minimize the GSH oxidation and enzymatic degradation, and whole blood was immediately mixed with 10% TCA containing EDTA. The comparison between LC-MS/MS and HPLC-ECD was discussed (*38*). Norris *et a.l* reported that the combination of stable isotope with methanolic precipitation at sample collection provided superior storage stability of whole blood samples in LC-MS/MS analysis (*39*). Robin *et al*. reported GSH and GSSG in the microdialysis samples from human dermis (*40*). Samples were analyzed without any pre-treatment. To improve the sensitivity, silver nitrate (100 μM) was introduced into LC effluent after column separation. Cao *et al.* reported GSH and GSSG analysis in bile samples. After acidification, dilution, and centrifugation, the samples were subjected to LC-MS/MS analysis (*41*). Drug-induced changes in the biliary GSSG/GSH ratios were observed.

3. Conclusion

In this brief review, determination methods of GSH by HPLC in recent years are summarized. In these methods, separation and detectability were considerably improved compared with the previous papers, and the adoption of mass spectrometric detection greatly improved the selectivity. For the accurate quantification of GSH and GSSG, oxidation, thiol-disulfide exchange, and other side reactions of GSH must be suppressed during the sample pretreatment and storage. The simple and appropriate sample pretreatment methods are still required for determination of GSH.

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