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Review

Chromatographic and mass spectrometric analysis of glutathione in biological samples☆

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ABSTRACT

Biological thiol compounds are classified into high-molecular-mass protein thiols and low-molecular-mass free thiols. Endogenous low-molecular-mass thiol compounds, namely, reduced glutathione (GSH) and its corresponding disulfide, glutathione disulfide (GSSG), are very important molecules that participate in a variety of physiological and pathological processes. GSH plays an essential role in protecting cells from oxidative and nitrosative stress and GSSG can be converted into the reduced form by action of glutathione reductase. Measurement of GSH and GSSG is a useful indicator of oxidative stress and disease risk. Many publications have reported successful determination of GSH and GSSG in biological samples. In this article, we review newly developed techniques, such as liquid chromatography coupled with mass spectrometry and tandem mass spectrometry, for identifying GSH bound to proteins, or for localizing GSH in bound or free forms at specific sites in organs and in cellular locations.

Contents

1. Introduction

Reduced glutathione (GSH) is the major intracellular non-protein thiol compound, and plays a major role in the protection of cells and tissues structures from oxidative injury. It is a tripeptide composed of cysteine, glutamic acid and glycine. GSH occurs in all organs, particularly in the liver. It is present in virtually all mammalian tissues. Intracellular and blood concentrations of GSH

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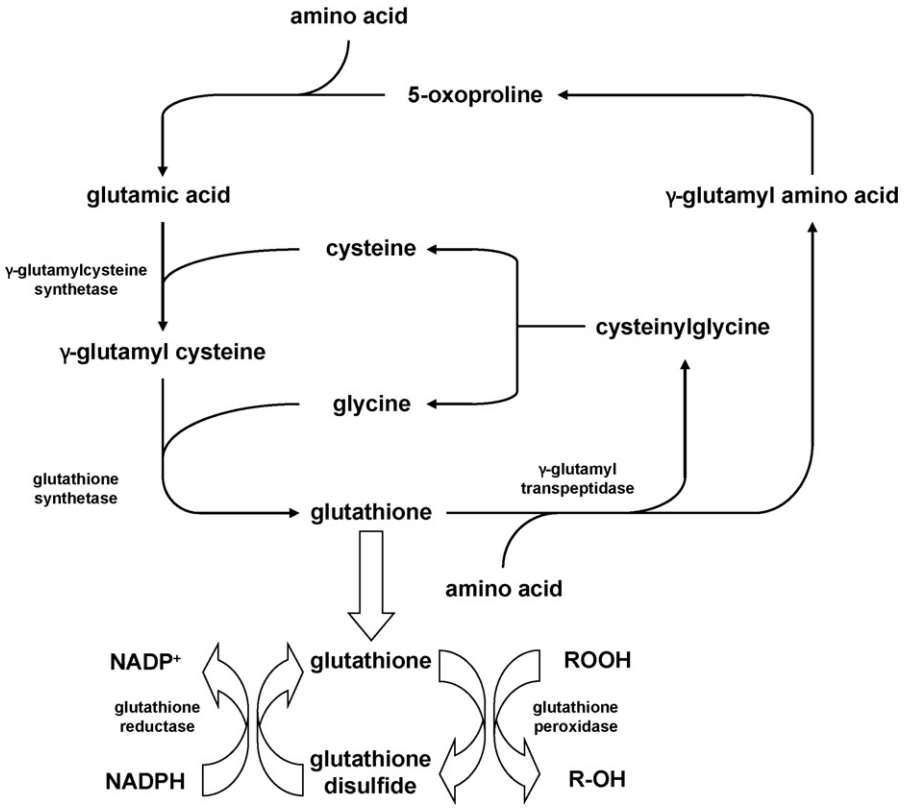

Fig. 1. Glutathione synthesis and metabolism pathway.

are in the millimolar range, while the plasma concentration is in the micromolar range and accounts for approximately 0.4% of total blood GSH [1–5].

The GSH synthesis and metabolism pathway is shown in Fig. 1 [6–8]. GSH is synthesized in the cell by γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase [9]. The γ-GCS-catalyzed formation of γ-glutamylcysteine is the first and rate-limiting step in *de novo* GSH synthesis and is feedback-inhibited by GSH, a mechanism that is central to the regulation of cellular GSH concentrations [10]. Cysteine is a rate-limiting substrate for *de novo* GSH synthesis. It is derived from the hydrolysis of extracellular GSH by γ-glutamyltranspeptidase (GGT) or from methionine-to-cysteine conversion through the cystathionine pathway [11]. While the cystathionine pathway is characteristic for liver cells, the cleavage of circulating GSH takes place at external plasma membrane surfaces of various epithelial cells such as those of the kidney, pancreas, bile duct and intestine. In the GGT-catalyzed reaction, the γ-glutamyl moiety is transferred from GSH or GSH conjugates to acceptors such as usually amino acids, often cysteine, dipeptide or GSH itself, while cysteinylglycine is cleaved by membrane-bound dipeptidases. The resultant constituent amino acids and γ-glutamyl products are taken up into cells for *de novo* GSH synthesis. Thus, the GGT reaction is a part of the γ-glutamyl cycle in intracellular GSH synthesis and GSH homeostasis [12,13].

Within cells total GSH exists free and bound to proteins. Free glutathione is present mainly in its reduced form (GSH) which can be oxidized to glutathione disulfide (GSSG) under certain conditions including oxidative stress. GSSG can be reduced to GSH by the action of glutathione reductase (GR). The redox status depends on the relative amounts of the reduced and disulfide forms of glutathione, i.e. of the GSH:GSSG molar ratio. Under normal conditions, the GSH redox couple is well known to be present in mammalian cells in the concentration range of 1–10 mM. In a resting cell, the molar GSH:GSSG ratio exceeds 100:1, whereas in various models of oxidative stress, this ratio has been reported to decrease to values between 10:1 and even 1:1 [14].

Several studies have supported the fundamental role of *S*-glutathionylation in some pathophysiological processes [15–18]. *S*-Glutathionylated proteins have been investigated as possible biomarkers of oxidative stress in human diseases. Among *S*-glutathionylated proteins the biological relevance of S-glutathionyl-hemoglobin is growing because of its potential use as a clinical marker of oxidative stress in human blood [19–25].

GSH has two characteristic structural features: a γ-glutamyl linkage and a sulfhydryl (SH) group. These structures of the tripeptide facilitate its participation in a variety of functions. GSH functions as a hydrogen donor in ribonucleotide reductase-catalyzed reduction of ribonucleotides to deoxyribonucleotides and thus plays a contributory role in DNA synthesis [26–29]. On the other hand, GSH has a detoxification ability towards hydrogen peroxide [30–32], other peroxides [33,34], free radicals [35–38] and various electrophiles. Thus, GSH plays a role in the detoxification of a variety of xenobiotics and/or their metabolites, which are ultimately excreted in the urine or feces in the form of mercapturic acids. Analogous derivatives of GSH are formed with endogenous metabolites, for example, in the metabolism of leukotrienes [39,40], prostaglandins [41,42] and steroids [43].

GSH has long been measured by several analytical methods. In particular, high performance liquid chromatography (HPLC) with various detection techniques, such as ultraviolet (UV) absorbance and fluorescence (FL) detection, is widely used because of its convenience, specificity and satisfactory sensitivity. This article reviews the methodologies currently available for measuring GSH in human biological samples and their feasibility as routine methods in the clinical chemistry laboratory. Due to the great advances in liquid chromatography-tandem mass spectrometry (LC–MS/MS) techniques in recent years and their potential for routine use in clin-

Table 1
Blood concentrations of GSH and GSSG (expressed in μM) reported in the literature.

Control	Diseases and conditions					Diseases and condition	Reference
GSH	GSSG	GSH/GSSG	GSH	GSSG	GSH/GSSG		
420	150	2.8	150	400	0.4	Physical exercise	[73]
800	29.2	27.4	800	50	16.0	Physical exercise	[74]
1100	90	12.2	180	520	0.3	AIDS	[75]
930	26	35.8	995	25	39.8	Breast cancer	[76]
930	26	35.8	1037	26	39.9	Lung cancer	[76]
950	90	10.6	1000	110	9.1	Elderly	[45]
950	90	10.6	700	140	5.0	Diabetes	[45]
1110	18	61.7	1095	60	18.3	Breast cancer (initial)	[77]
1110	18	61.7	1005	170	5.9	Breast cancer (advanced)	[77]
1110	18	61.7	1065	26	41.0	Colon cancer (initial)	[77]
1110	18	61.7	1035	99	10.5	Colon cancer (advanced)	[77]
1380	2.7	511.1	900	4.5	200.0	Retinopathy of prematurity	[78]
1035	56	18.5	760	51.7	14.7	Chronic diseases	[79]
916	43.1	21.3	880	41.1	21.4	Atherosclerosis	[80]
916	43.1	21.3	902	44.5	20.3	NASCV	[80]

Chronic diseases: cancer and genitourinary, gastrointestinal, cardiovascular, and musculoskeletal diseases. NASCV: nonatherosclerotic cardiovascular disease.

ical chemistry, these particular techniques are in the focus of this review.

2. Oxidative stress

Oxidative stress is manifested by excessive reactive oxygen species (ROS) production in the face of insufficient or defective antioxidant defense systems. Oxidative stress causes profound alterations of various biological structures including cellular membranes, lipids, proteins and nucleic acids. Oxidative stress is considered to be involved in aging [44–51] and in various diseases such as diabetes mellitus [52–54], atherosclerosis [55,56], rheumatoid arthritis [57–60], Alzheimer's disease [61–63], Parkinson's disease [64–66] and cancer [67–71]. There is an increasingly growing interest in identifying biomarkers for diseases in which oxidative stress is involved [72].

GSH is the most abundant intracellular thiol. It exists in millimolar concentrations in most cell types. Moreover, GSH plays an essential role in maintaining the intracellular redox environment that is critical for the functioning of various cellular proteins. Many studies have indicated that the concentrations of GSH and GSSG are influenced by various diseases, with a tendency to decrease the ratio GSH/GSSG (see Table 1). Nevertheless it is evident that the measured concentrations for both GSH and GSSG are highly divergent among different research groups, so these findings should be reconsidered once obtained a general agreement about the physiological levels of these compounds [45,73–80].

The antioxidant function of GSH is primarily due to its involvement in enzymatic pathways that cells have developed against ROS. The most important pathway involves glutathione peroxidase (GPx) and GR. GPx catalyzes the reduction of hydrogen peroxide, which is produced by superoxide dismutase (SOD) through the dismutation of superoxide anion or of organic hydroperoxides. GSH and GSH-dependent enzymes act in cooperation to scavenge ROS and/or neutralize their toxic oxidizing effect. These systems act at the same time and in cooperation to protect human body from ROS. Under oxidative stress conditions, GSH is oxidized to GSSG. Therefore, the GSH to GSSG ratio is altered.

In mammalian cells, there are three mechanisms of maintaining GSH homeostasis, namely: (1) *de novo* synthesis; (2) uptake from exogenous sources across plasma membrane transport of GSH such as basolateral and brush-border plasma membranes [81]; and (3) GR-catalyzed reduction of GSSG to GSH [9,11]. Actually, GSH is not entered cells as it is required its enzymatic decomposition into its amino acid components by activity of GGT and dipeptidases [81]. These mechanisms appear to be important in cellular GSH control under physiological and stress conditions [82–85]. In recent years, redox signaling and redox regulation of cell function have been extensively investigated and the existence of well-defined compartments of redox systems within a cell has been recognized. Intracellular GSH is compartmentalized within the mitochondrion, nucleus and endoplasmic reticulum, all of which constitute separate redox pools [86].

3. General preparation methods for the determination of glutathione in biological samples

3.1. Deproteinization

GSH analysis usually involves protein precipitation by acidification using, for example, trichloroacetic acid (TCA), perchloric acid, metaphosphoric acid, or sulfosalicylic acid, which yields a clear, protein-free supernatant after centrifugation. Rossi et al. discussed the best precipitant. They concluded that TCA seems to be the most useful deproteinization acid in that GSH oxidation with time is minimal [87]. Thus, it has been observed that only 3–4% of GSH are oxidized within 20 h at 0 °C after the addition of TCA to the sample [87]. Artefactual oxidation of thiols during sample treatment may represent a major problem because it may lead to an overestimation of disulfide concentration. This may be avoided by alkylating SH groups before acidification by addition of *N*-ethylmaleimide (NEM).

3.2. Reduction

In the determination of total GSH (GSH, GSSG and protein bound GSH) in biological samples, the reduction of the disulfide bond into the GSSG molecule and between GSH and other thiol compounds such as free cysteine or cysteinyl residues in proteins is required. Previous studies have revealed many reductants: dithioerythritol [88,89], dithiothreitol (DTT) [90–101], mercaptoethanol [102–107] and tris-(2-carboxyethyl)-phosphine (TCEP) [108–113]. In particular, TCEP has become commercially available in recent years; it is highly soluble in water and nonvolatile [114,115]. Getz et al. compared the properties of DTT and TCEP, which are generally important in protein biochemistry, using the motor enzyme myosin as an example protein. They concluded that with regard to TCEP have advantages over DTT, although the choice of reductant may be application-specific [116]. GSH formed in situ during the reduction step can be oxidized before derivatization, thus leading to incorrect results. Thiol oxidation is promoted by many transition metals such as iron and copper. Therefore, it is recommended to include ethylenediaminetetraacetic acid (EDTA) [117,118], deferox-

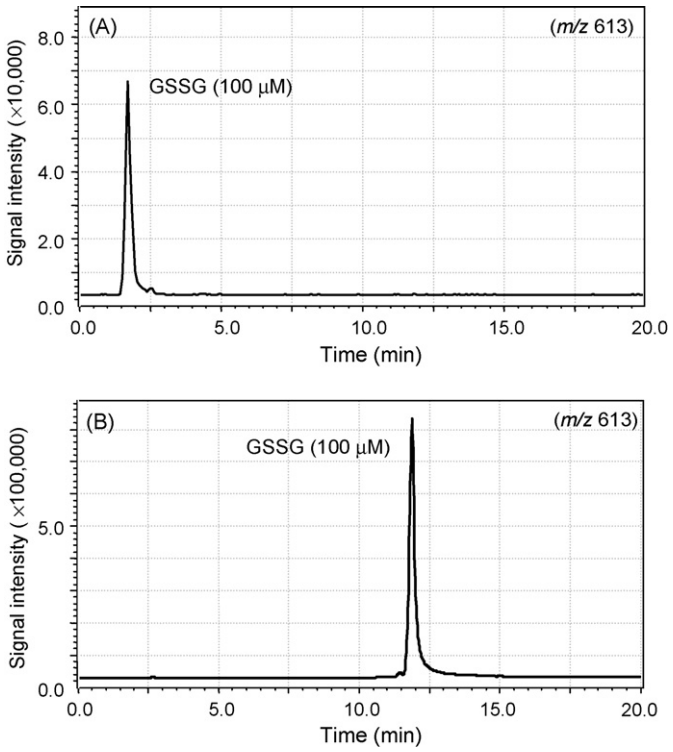

Fig. 2. Chromatograms of glutathione disulfide (100 μM) as obtained by (A) LC–MS and (B) HILIC–MS methods. (A) CAPCELL PAK (150 × 2.1 mm, 5 μm; SHISEIDO, Tokyo, Japan), (B) Atlantis™ HILIC silica column (150 × 2.1 mm, 5 μm; Waters, Tokyo, Japan). The mobile phase of (A) was 0.5 mM formate buffer (pH 4.0)/acetonitrile (95:5, v/v). The mobile phase of (B) was 0.5 mM formate buffer (pH 4.0)/acetonitrile (10:90, v/v).

amine [119] or 1,10-phenanthroline [120] in the reaction mixture to chelate divalent metals and inhibit autooxidation.

3.3. Derivatization

GSH and other thiols and their disulfides are measured by several methods. For instance, HPLC determination allows the simultaneous detection of GSH and other low-molecular-mass thiols and disulfides, but requires sample derivatization. In any case, neutral-alkaline pH must be restored before derivatization by agents such as iodoacetic acid (IAA) or monobromobimane (MBB). However, after neutralization, rapid oxidation of thiols may occur at a pH-dependent rate. Derivatization of GSH usually increases the method specificity and sensitivity. Recently, several HPLC methods utilizing pre-column derivatization with colorimetric reagents, such as Ellman's [121–124] or Sanger's [125] reagents, have been described. Ellman's reagent is widely used for the analysis of thiols in biological samples via the determination of the liberated thionitrobenzoate anion [126]. HPLC with pre-column derivatization with fluorogenic reagents, such as ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F) has become the most popular method for thiol analysis [127–130].

4. HPLC methods

4.1. Separation methods of GSH and GSSG

Non-derivatized GSH and GSSG are very highly polar compounds and difficult to determine by reversed-phase HPLC. Recent studies suggested that GSH and GSSG can be separated using a highly polar stationary phase, for example, amino [131] and diol [132] columns. Hydrophilic interaction chromatography (HILIC) is characterized by the presence of a high initial organic modifier concentration to favor hydrophilic interactions between solutes and the hydrophilic stationary phase [133]. HILIC, based on silica columns, is an alternative to normal-phase chromatography, but it utilizes

Table 2
Conventional UV method for GSH and GSSG.

Instrument	Derivatization	Wavelength	Procedure	GSH (LOQ)	GSSG (LOQ)	Sample	Reference
HPLC-UV	5,5′-Dithio-bis-nitrobenzoicacid (DTNB)	280 nm	Reacted in pH 8.0 Dithioerythritol reduction	N.D.	N.D.	Rat liver	[140]
HPLC-UV	Iodoacetic acid (IAA) and 1-fluoro-2,4-dinitrobenzene	N.D.	Reacted in excess sodium bicarbonate at room temperature for 15 min (IAA) Reacted at room temperature for 4 h in the dark (1-fluoro-2,4-dinitrobenzene)	N.D.	N.D.	Hepatocyte cell	[143]
HPLC-UV	5,5′-Dithio-bis-nitrobenzoicacid (DTNB)	330 nm	Reacted in pH 7.2 for 5 min Dithiothreitol reduction	0.5 nM	N.D.	Human plasma and cell	[141]
HPLC-UV	2-Chloro-1-methylquinolinium tetrafluoroborate	355 nm	Reacted in pH 7.6 at room temperature for 1 min $NaBH_4$ reduction at room temperature for 1.5 min	0.3 nM	N.D.	Human plasma	[145]
HPLC-UV	2-Chloro-1-methylquinolinium tetrafluoroborate	355 nm	TBP reduction at 60 °C for 30 min	0.5 nM	N.D.	Human saliva	[146]
HPLC-UV	1-Chloro-2,4-dinitrobenzene	340 nm	Reacted in Dulbecco's phosphate-buffer Saline (DPBS) (pH 7.4) at 10 °C for 10 min	0.1 nM	N.D.	Human placental tissue	[148]
HPLC-UV	5,5′-Dithio-bis-nitrobenzoicacid (DTNB)	330 nm	Reacted in pH 8.0 for 5 min in an ice bath	135 nM	N.D.	Red blood cell and whole blood	[142]

N.D.: not described.

traditional reversed-phase mobile phases. Thus, the retention times of highly polar compounds are increased with the increase of the hydrophilicity of the solutes. This method is advantageous in that it is easy to use coupled with a mass spectrometer since HILIC may be described as a variant of normal-phase chromatography. In HILIC, a hydrophilic stationary phase is used in combination with a mostly organic mobile phase, and elution is usually performed by increasing the water concentration. Recently, HILIC–MS was employed to separate and quantify highly polar compounds in biological samples [134–139]. Fig. 2 shows chromatograms of GSSG that was separated with octadecyl silica (ODS) and HILIC columns. GSSG could be retained on a HILIC column.

4.2. HPLC-UV

The first HPLC-UV method using pre-column derivatization with Ellman's reagent has been described [140]. Katrusiak et al. [141] developed a technique for the simultaneous determination of GSH and other related thiols in plasma and cell extracts. Recently, Garcia et al. reported a DTNB-based HPLC-UV method for erythrocytes [142]. Reed et al. [143] developed a new method which allows the simultaneous determination of GSH and GSSG by blocking free thiols with IAA and derivatizing amino groups by 2,4-dinitrofluorobenzene (FDNB). Giustarini et al. reported an improved HPLC-UV method using NEM and FDNB derivatization reagents for the measurement of GSH and GSSG in human blood [144]. The advantages of this method are simplicity, reproducibility and the simultaneous detection of GSH and GSSG in blood samples. There are many other derivatization reagents, such as 2-chloro-1-methylquinolinium [145,146] and 1-chloro-2,4-dinitrobenzene [147,148], for the determination of GSH and other thiol compounds by HPLC-UV. The derivatization reagents reported for HPLC-UV absorbance detection are listed in Table 2. HPLC-UV methods fulfill the experimental needs for the determination of GSH in most laboratories, i.e. use of cheap derivatization reagents and convenient HPLC-UV systems. HPLC-UV methods can be applied to the routine assessment of the biological thiol redox status under physiological and pathological conditions.

Table 3
Conventional FL method for GSH and GSSG.

Instrument	Derivatization	Excitation and emission	Procedure	GSH (LOQ)	GSSG (LOQ)	Sample	Reference
FL	SBD-F	Ex: 380 nm, Em: 515 nm	Reacted in 0.1 M borate buffer (pH 9.5) at 60 °C	100 pmol/ml (LOD)	N.D.	N.D.	[153]
HPLC-FL	ABD-F	Ex: 381 nm, Em: 512 nm	Reacted in 0.1 M borate buffer (pH 8.0) at 50 °C for 5 min	0.4 pmol	N.D. (LOD)	N.D.	[157]
HPLC-FL	DBD-F	Ex: 390 nm, Em: 520 nm	Reacted in 0.1 M borate solution (pH 8.0) at 50 °C for 10 min	0.16 pmol (LOD)	N.D.	Rat tissues	[161]
HPLC-FL	SBD-F	Ex: 380 nm, Em: 510 nm	Reacted in borate buffer (pH 9.5) at 60 °C for 60 min	150 pg (LOD)	N.D.	Human blood	[155]
HPLC-FL	ABD-F	Ex: 265 nm, Em: 418 nm	Reacted in 0.1 M tris buffer (pH 8.0) at 50 °C for 30–60 min TBP reduction	N.D.	N.D.	Calffetuin and rabbit muscle enolase	[160]
HPLC-FL	SBD-F	Ex: 386 nm, Em: 516 nm	Reacted in pH 8.5 at 60 °C for 60 min TBP reduction	20 pmol	N.D.	Urine	[127]
FL	Monochlorobimane	Ex: 380 nm, Em: 470 nm	Reacted at room temperature for 30 min	N.D.	N.D.	Rat liver	[152]
HPLC-FL	SDB-F	Ex: 385 nm, Em: 515 nm	Reacted in borate buffer (pH 9.5) at 60 °C for 30 min TCEP reduction at room temperature for 30 min	N.D.	N.D.	Plasma	[109]
HPLC-FL	SDB-F, ABD-F and DBD-F	Ex: 380 nm, Em: 530 nm	Reacted in pH 7.2 at 37 °C for 30 min	1–3 pmol	N.D.	Rat liver	[156]
HPLC-FL	SBD-F	Ex: 385 nm, Em: 515 nm	Reacted 0.125 M borate buffer (pH 9.5) at 60 °C for 60 min TCEP reduction at room temperature for 30 min	15 pmol	N.D.	Human plasma	[115]
HPLC-FL	SDB-F and ABD-F	Ex: 390 nm, Em: 510 nm	Reacted in the basic medium (pH 8.5–9.5) at 60 °C (SBD-F) Reacted in 0.5 M borate buffer (pH 9.3) at 60 °C for 5 min (ABD-F)	N.D.	N.D.	N.D.	[158]
HPLC-FL	SBD-F	Ex: 385 nm, Em: 515 nm	Reacted in phosphate buffer at 60 °C for 60 min TCEP reduction at room temperature for 30 min	N.D.	N.D.	Human plasma	[112]
HPLC-FL	OPA and MBB	Ex: 390 nm, Em: 478 nm (GSH); Ex: 340 nm, Em: 425 nm (GSSG)	GSH was reacted by MBB in sodium hydroxide solution for 2 min GSSG was reacted after on-line postcolumn derivatization with OPA in pH 12.4	140 nM (LOD)	11 nM (LOD)	Plasma	[151]
HPLC-FL	*N*-(2-Acridonyl)-maleimide	Ex: 260 nm, Em: 416 nm	Reacted in pH 8.2 for 1 min TCEP reduction at 40 °C for 10 min	2.0 pmol (LOD)	N.D.	Human plasma	[163]
HPLC-FL	*N*-(1-Pyrenyl)-maleimide	Ex: 330 nm, Em: 376 nm	Reacted in borate buffer (pH 8.3) at room temperature for 5 min DTT reduction in borate buffer (pH 8.3) at room temperature for 30 min	10 nM	10 nM	Liver, lung and brain	[162]

N.D.: not described.

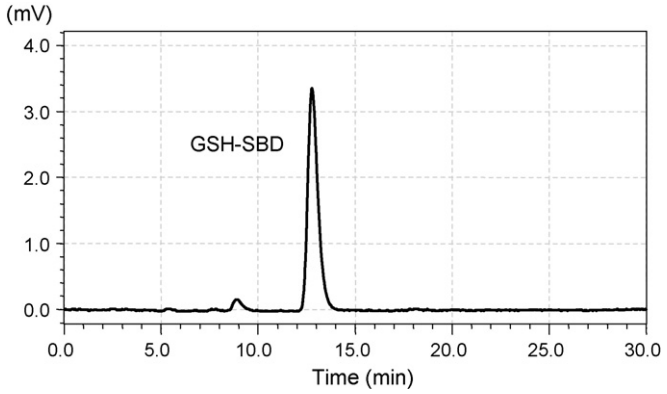

Fig. 3. Representative chromatogram of human blood labeled with SBD-F using HPLC-FL. Reproduced from Fig. 3 in Ref. [112].

4.3. HPLC-FL

HPLC-FL is the most widely used method for thiols because of its high sensitivity and specificity. On the basis of the reaction between ortho-phthaldialdehyde (OPA) and the amino groups of amino acids, HPLC-FL assays for GSH and GSSG have been described [149,150]. OPA reacts with the primary amino group in the presence of a thiol compound, such as 2-mercaptoethanol, to produce fluorescent isoindole derivatives.

Bimanes such as MBB react rapidly with thiols at room temperature at neutral or alkaline pH [151]. Kamencic et al. reported a method for the determination of GSH in tissue homogenates using monochlorobimane (MBCl) [152]. MBCl, of which the reactive site is chloride instead of bromide, is used for the labeling of GSH. The reactivity of MBCl to thiol compounds seems to be slightly lower than that of MBB.

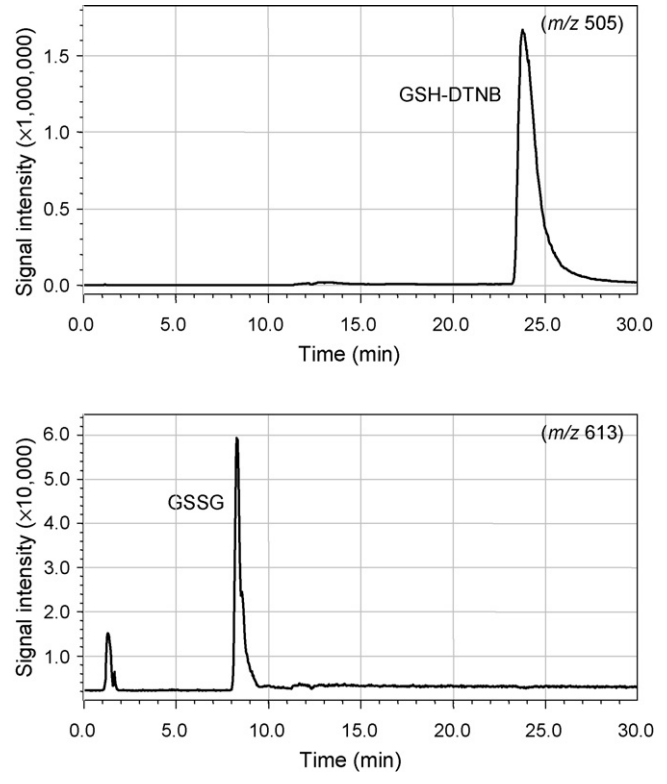

Fig. 4. Representative chromatograms of human blood labeled with DTNB using LC–MS. Reproduced from Fig. 4 in Ref. [172].

SBD-F was first synthesized as a fluorogenic reagent for thiol compounds [153]. A frequently used method for total plasma aminothiol measurement is reversed-phase HPLC with fluorescence detection after derivatization of plasma aminothiols with SBD-F [109,112,127,149,153–156] (Fig. 3). SBD-F has excellent features with regard to sensitivity, fluorescence characteristics, fluorophor stability and solubility in water. However, the conditions for the completion of the reaction are rather drastic, usually heating at 60 °C for 1 h in an alkaline medium. Toyo'oka and Imai [157] reported the synthesis of 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) through the intermediate 4-(chlorosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (CBD-F), which is also a bifunctional reagent. ABD-F is a preferred derivatization reagent for the accurate determination of reduced form of thiols in samples containing disulfide form of thiols [158]. Endogenous thiols in biological samples have been simultaneously determined by HPLC-FL using SBD-F, ABD-F [154,156,158–160] or 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) [156,161].

Maleimide-type reagents are frequently used for the derivatization of thiols [162]. Benkova et al. described the design, synthesis and properties of a new thiol derivatizing reagent, *N*-(2-acridonyl)maleimide, and its application to the quantification of aminothiols in human plasma [163].

Derivatization reagents available for use in HPLC-FL analysis of thiols are listed in Table 3. The use of these reagents is not without limitations. Some of these reagents may generate decomposition fluorescent products which interfere to determination of thiols, and may require long derivatization time and elevated temperatures. Thus, the development of sensitive, selective, and reactive derivatization reagents is still needed.

4.4. HPLC-ECD

HPLC with electrochemical detection represents an important tool for the analysis of redox-reactive compounds, such as thiols and disulfides, including GSH. Initially, Hg pool electrodes were used for the determination of GSH and other thiols [164,165]. This method was later simplified and expanded by the development of dual-electrode electrochemical detection for the simultaneous determination of GSH and GSSG [166]. The use of HPLC-ECD has increased because of its high sensitivity. Amperometry is widely used for the detection of electrochemically oxidizable and reducible substances in HPLC [167–169]. In a recent study, a direct electrochemical method using an electrode modified with functionalized carbon nanotubes was developed as a method of ECD for HPLC [170]. The method was sensitive enough to detect difference in concentration of GSH and GSSG in hepatocytes from animals.

4.5. LC–MS, GC–MS and LC–MS/MS

Liquid chromatography–mass spectrometry (LC–MS) has been utilized for the determination of GSH and GSSG, owing to the high sensitivity of detection. Because of ion suppression and ion enhancement matrix effects, use stable-isotope labelled analogs as internal standards may be required and should be preferably used for accurate quantification by LC–MS/MS. The originally reported LC–MS methods enable the assay of thiols in tissues [171–174] and fluid samples [134,135,138,175]. LC–MS is a powerful technique for the analysis of low-molecular-mass thiols including GSH [176]. Most reported LC–MS methods include derivatization with DTNB [172] (Fig. 4), NEM [132,134] (Fig. 5) or iodoacetic acid [177], which prevents GSH autooxidation. Glutathione ethyl ester [172], thiosalicylic acid [132] or γ-glutamyl glutamic acid [134,177] have been used as internal standards.

Two publications have been reported for the determination of GSH using GC–MS [178,179]. This approach has been reported to

Table 4
Conventional MS method for GSH and GSSG.

Instrument	Derivatization	Procedure	GSH (LOQ)	GSSG (LOQ)	Sample	I.S.	Reference
LC–MS	Iodoacetic acid (IAA); dansyl chloride	Reacted in pH 8.6–8.8 at room temp, for 30 min (iodoacetic acid); reacted in acetonitrile at 4 °C for 12 h (dansyl chloride)	25 nM	25 nM	Hepatic cells	γ-Glutamyl glutamic acid	[177]
LC–MS	*N*-Ethylmaleimide	Reacted on ice for 20 min	50 nM	200 nM	Human lymphocytes	Thiosalicylic acid	[132]
LC–MS	5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB)	Reacted in phosphate buffer (pH 7) for 1 min	500 nM	500 nM	Rat blood, lung, liver, heart, kidney and brain	Glutathione ethyl ester	[172]
LC–MS	*N*-Ethylmaleimide	Reacted in 2% SSA at room temp, for 30 min	8.9 pmol	0.78 pmol	Human whole blood and RBC	γ-Glutamyl glutamic acid	[175]
LC–MS	*N*-Ethylmaleimide	Reacted in 1 mM formic acid at room temp, for 30 min	100 nM	1000 nM	Saliva	γ-Glutamyl glutamic acid	[134]
GC–MS	*N*,*S*-Ethoxycarbonyl methyl ester	Reacted in pH 7.5 at room temperature for 15 min DTT reduction at room temperature for 15 min	N.D.	N.D.	Rat liver and human blood	Enrichment isotope	[178]
GC–MS	*N*,*S*-Ethoxycarbonyl methyl ester	N.D. DTT reduction at room temperature for 15 min	N.D.	N.D.	Human RBC	γ-Glutamyl cysteinyl alanine	[179]
LC–MS/MS	N.D.	N.D.	0.2 pmol (LOD)	2 pmol (LOD)	Mice liver	N.D.	[184]
LC–MS/MS	Iodoacetic acid (IAA)	Reacted in ammonium bicarbonate (pH 10) in the dark for 60 min	163 nM	82 nM	Rat hepatocytes	γ-Glutamyl glutamic acid	[171]
LC–MS/MS	N.D.	N.D.	300 nM	N.D.	Spruce needles	GSH-$^{15}N_1$ (glycine-$^{15}N_1$)	[182]
LC–MS/MS	Iodoacetic acid (IAA)	Reacted in ammonium bicarbonate (pH 9.5) 325 nM at room temperature for 15 min	163 nM	Mice liver	Glutathione ethyl ester	[183]	
LC–MS/MS	4-Fluoro-7-sulfamoylbenzofurazan	Reacted in sodium borate buffer	5000 nM	500 nM	RAW 267.4 cell (RMock and R15LO cell)	GSH-$^{13}C_2$, $^{15}N_1$ GSSG-$^{13}C_4$, $^{15}N_2$	[174]
LC–MS/MS	N.D.	N.D.	16.3 nM	N.D.	Rat liver and mice lymphoma cell	GSH-$^{13}C_2$, $^{15}N_1$	[185]
LC–MS/MS	N.D.	N.D.	1 ng/ml (LOD)	N.D.	Human plasma	*N*-(2-Mercaptopropionyl)-glycine	[186]

N.D.: not described.

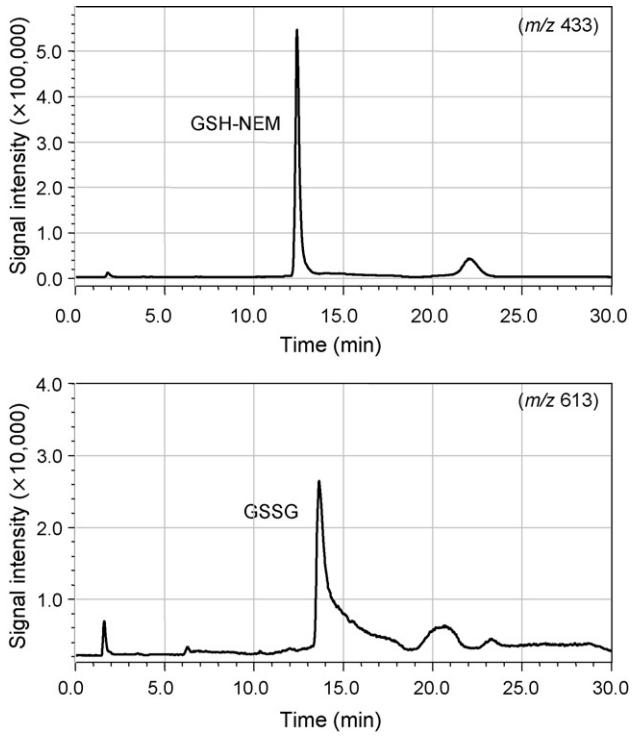

Fig. 5. Representative chromatograms of human blood labeled with NEM using LC–MS. Reproduced from Fig. 5 in Ref. [134].

be reliable for determining GSH synthesis rate in vivo and to avoid time-consuming and tedious sample treatment. Capitan et al. have successfully determined the GSH synthesis rate in human and rat liver tissues [178].

During the past two decades, LC–MS/MS has proven to be one of the most effective tools in biological research, particularly for the analysis of very low amounts of compounds in complex biological matrices. LC–MS/MS based methods are generally characterized by high specificity, sensitivity and high-throughput potential [180]. The commercially available electrospray ionization (ESI) source coupled with a triple quadrupole mass spectrometer [181] has been critical factors behind the increasing impact this technology is having in laboratory medicine. MS/MS has already emerged as a

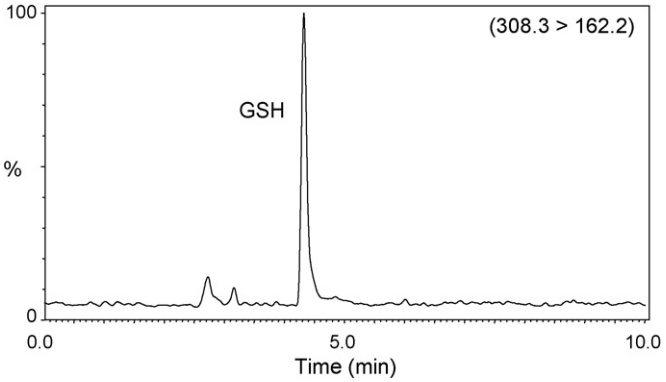

Fig. 6. Representative chromatogram of human blood using LC–MS/MS. Reproduced from Fig. 6 in Ref. [184].

powerful analytical tool in clinical biochemical genetics. The simultaneous detection of thiols and disulfides using LC–MS/MS has been published by many groups [135,182,183]. Reported LC–MS/MS methods and reagents are listed in Table 4. Norris et al. reported a LC–MS/MS method without derivatization for quantifying GSH in mouse liver by methanol extraction [184] (Fig. 6). LC–MS/MS is very sensitive and selective for GSH. For LC–MS/MS analysis of GSSG, derivatization of GSH present in the matrix is required to avoid overestimation of GSSG, analogous to other techniques (see above). Zhang et al. reported a LC–MS/MS method for the separation and quantitation of GSH in cultured cells treated with diethyl maleate (DEM, a GSH-depletion agent) and in rat liver tissues [185]. Jiang et al. developed a method for the determination of sulfur amino acids [186]. Although the prohibitive instrumentation cost and the complexity of operation have been major obstacles to their routine application to laboratory medicine, LC–MS/MS is, even now, best suited for routine analysis in clinical chemistry.

5. Conclusion

GSH is a fundamental low-molecular-mass antioxidant that plays a key role in cellular defense against oxidative damage. GSH and GSH-dependent enzymes act in cooperation to scavenge ROS and/neutralize their toxic oxidizing effect. Upon enzymatic and nonenzymatic oxidation, GSH oxidizes to GSSG and, under particular conditions, to other oxidative products. Determination of GSH is useful in studying oxidative stress. Today, GSH and GSSG in biological fluids are analyzed preferably by HPLC with UV or FL detection. Newly developed techniques, namely LC–ESI-MS/MS, are expected to become a reference tool for GSH and a powerful approach to analyze GSH, notably GSH bound to proteins, or to localize GSH in bound or free forms at specific sites in organs and in cellular locations. The quantification of GSH and GSSG in biological samples not only enables assessment of oxidative stress, but it also may provide an early diagnostic method for human diseases in the future.

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