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Review

Recent advances in separation and detection methods for thiol compounds in biological samples☆

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ABSTRACT

Biological aminothiols, such as cysteine, homocysteine, and glutathione, widely occur in animal tissues and fluids. The altered levels of the thiols (reduced forms) and their disulfides (oxidized forms) in physiological liquids have been linked to specific pathological conditions and closely associated with several human diseases. Therefore, it is well recognized that the determination of thiols and disufides is important in order to understand their physiological roles. The derivatization utilizing a suitable labeling reagent followed by chromatographic separation and detection is the most reliable means for sensitive and selective assays. Many reagents have typically been synthesized and successfully used for the determination of thiols and disulfides in biological specimens. The development of new reagents for highly sensitive detection is still continuing. This review describes the approaches for the separation assay of various thiol compounds, obtained through the analytical papers published in 2000–2008. The derivatization reagents are categorized with each type of chromophore and fluorophore and evaluated in terms of their reactivity, stability, detection wavelength, handling, sensitivity and selectivity. Application examples of the reagents for bioanalysis are also described in the text.

Abbreviations: ABD-F, 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole; AcABD-F, 4-(*N*-acetylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole; AcCySH, *N*-acetylcysteine; AIDS, acquired immunodeficiency syndrome; AMT, 3-amino-5-mercapto-1,2,4-triazole; APCI, atmospheric pressure chemical ionization; dBBr, dibromobimane; mBBr, monobromobimane; mBCl, monochlorobimane; BIPM, *N*-[*p*-(2-benzimidazolyl)phenyl]maleimide; BMA, *S*-benzylmercapturic acid; 5-BMF, 5-bromomethylfluorescein; *p*-BPB, *p*-bromophenacyl bromide; CE, capillary electrophresis; CMQT, 2-chloro-1-methylquinolinium tetrafluoroborate; CSF, cerebrospinal fluid; CySH, cysteine; CySS, cystine; CZE, capillary zone electrophoresis; DAABD-Cl, 7-chloro-*N*-[2-(dimethylamino)ethyl]-2,1,3-benzoxadiazole-4-sulfonamide; DBD-F, 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole; DBPM, *N*-(*p*-(2-(6-dimethylamino)benzofuranylphenyl)maleimide; DHLA, dihydrolipoic acid (reduced form of LA); DMPS, dimercaptopropanesulfonate; DPDS, 2,2′-Dipyridyldisulfide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent); DTT, dithiothreitol; EC, electrochemical; ECD, electrochemical detection; EDTA, ethylenediamine tetraacetic acid; FID, flame photometric detection; FL, fluorescence; FM, fluorescein-5-maleimide; GC, gas chromatography; GNPs, gold nanoparticles; GSH, reduced glutathione (L-γ-glutamyl-L-cysteinylglycine); GSSG, oxidized glutathione; HCySH, homocysteine; HFBCF, heptafluorobutyl chloroformate; HPLC, high-performance liquid chromatography; HTL, homocysteine-thiolactone; IAB, 3-iodoacetylaminobenzanthrone; 1,5-I-AEDANS, (5-({2-[iodoacetyl]amino}ethyl}amino)naphthalene-1-sulfonic acid); 5- or 6-IAF, (5 or 6-iodoacetamidofluorescein); IPBT, 2-(*p*-iodoacetylaminophenyl)benzothiazole; LA, α-lipoic acid; LC, liquid chromatography; LIF, laser induced fluorescence; LLys, lipoyllysine; MA, methyl acrylate; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MASCOT, database searching; 3-MB, 3-maleimidylbenzanthrone; 2-ME, 2-mercaptoethanol; MEKC, micellar electrokinetic chromatography; MESNA, 2-mercaptoethane sulfonate; MIAC, *N*-(2-acridonyl)maleimide; MIPBO, 5-methyl(2-(*m*-iodoacetylaminophenyl)benzoxazole; MMPB, 5-maleimidyl-2-(*m*-methylphenyl)benzoxazole; MS, mass spectrometry; MT, metallothionein; N_2O, nitrous oxide; $NaBH_4$, sodium borohydride; NADH, nicotinamide adenine dinucleotide, reduced form; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; NAM, *N*-(9-acridinyl)maleimide; NEM, *N*-ethylmaleimide; OPA, ortho-phthalaldehyde; PC, phytochelatine; Pen, D-penicillamine; PK, pharmacokinetic; PMA, *S*-phenylmercapturic acid; PMSF, phenylmethylsulfonyl fluoride; R-VX, *O*-isobutyl *S*-2-diethylaminoethyl methylphosphonothioate; SBD-F, ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate; scFv, single-chain antibody fragment; TBP, tributhylphosphine; TCAcABD-F, 4-(*N*-trichloroacetylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole; TCDI, 1,1′-thiocarbonyldiimidazole; TCEP, tris-(2-carboxyethyl)-phosphine; ThioGlo™3, (9-acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)pyeny)-3-oxo-3H-naphtho[2,1-b]pyran); TP, tiopronin, *N*-(2-mercaptopropionyl)-glycine; UV, ultraviolet; UV–vis, ultraviolet-visible; VX, *O*-ethyl *S*-diisopropylaminoethyl methylphosphonothioate.

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Contents

1. Introduction

The biological aminothiols, such as cysteine (CySH), *N*-acetylcysteine (AcCySH), homocysteine (HCySH), and glutathione (GSH), are critical physiological components and widely occur in animal tissues and fluids. The altered levels of the thiols (reduced forms) and their disulfides (oxidized forms) in physiological liquids have been linked to specific pathological conditions and closely associated with several human diseases, especially premature arteriosclerosis, occlusive vascular and neurodegenerative disorders, leukemia, diabetes, and acquired immunodeficiency syndrome (AIDS) [1,2]. Furthermore, cysteine-rich peptides, such as metallothioneins (MTs) and phytochelatines (PCs), are important components for understanding their physiological roles. Several sulfhydryl-containing compounds, e.g., D-penicillamine (Pen) and tiopronin (TP), are also well known as therapeutic pharmaceuticals.

CySH, which is an amino acid synthesized by the liver, is involved in a variety of important cellular functions, including protein synthesis, detoxification, and a metabolic process [3,4]. Its biological significance is well established, but the level of CySH in physiological fluids, such as plasma and urine, has recently been recognized as an important indicator for several clinical disorders [5–7]. Cystinosis and cystinuria are the typical examples. Nephropathic cystinosis is an autosomal-recessive lysosomal storage disease caused by defective transport of cystine (CySS) out of the lysosomes [8,9]. The stored CySS is poorly soluble and crystallizes within the lysosomes of many cell types, leading to widespread tissue and organ damage.

AcCySH also exclusively presents in human as a result of acetylation in the kidney [10]. The sulfhydryl group in AcCySH is also active and interacts with the disulfide bonds of mucous glycoproteins, thereby breaking the protein network into less viscous strands [11]. AcCySH is commonly used mucolytic agent for administration into respiratory tracts to loosen secretions. Furthermore, AcCySH is widely used for the treatment acetoaminophen overdose. Administration of AcCySH is also beneficial in systemic sclerosis, HIV infection and septic shock [12–14].

HCySH is one of the endogenous sulfhydryl-containing amino acids, which is generated through the demethylation of methionine (Met). Under normal conditions, the intracellular concentration of HCySH is kept low as the result of the re-methylation reactions to Met and catabolism via the trans-sulfuration pathway. These transformations are controlled by enzyme reactions. Although 15–20 mmol HCySH are synthesized in humans each day [15], most of them are converted to CySH or Met under the enzymatic control of cystathionine β-synthase or methionine synthase and methylenetetrafolate reductase. The total homocysteine concentration in plasma of a normal subject is between 5 and 15 μM [16]. Thus, an elevated plasma concentration, greater than 15 μM, may be the result of inherited disorders respective metabolism [17–20]. However, following reasons must also be considered: (1) nutritional deficiencies of vitamin co-factors (B6, B12 and folates), (2) several diseases (e.g., chronic renal failure, malignancies, acute lymphoblastic leukemia, anemia, hypothyroidism and diabetes), (3) physiological factors (e.g., sex and age), (4) medical treatments (e.g., methotrexate, phenytoin, carbamazepine, nitrous oxide (N_2O), theophylline, metformin, colestipol, niacin, penicillamin and thiazide diuretics), and (5) lifestyle determinants (e.g., smoking, alcohol intake, coffee consumption) [21]. Interest in the determination of HCySH in physiological fluids has rapidly grown during the last decade [15,22]. Its elevated concentration in plasma (hyperhomocysteinemia) is considered an important risk factor or marker for several diseases, in particular, for cardiovascular disease [15].

GSH (L-γ-glutamyl-L-cysteinylglycine), a tripeptide, which occurs in various cells of animals, plants and bacteria, is the most abundant non-protein thiol in biological systems. GSH can be obtained from diet or can be synthesized *de novo* in the liver. GSH plays numerous important physiological roles in many biological processes, such as protein and DNA syntheses, transport, catabolism, and metabolism [23]. GSH can be readily oxidized to its disulfide (GSSG). Therefore, the ratio of both forms is crucial for the characterization of the oxidative stress in living systems. GSH participates in the protection against free radicals and reactive oxygen species (ROS) generated during metabolism [24–26]. As a scavenger or reducing agent, GSH protects the living cells against hypoxia, toxicity, mutagenicity or transformation by radiation and carcinogens. GSH also functions to inactivate a number of exogenous compounds such as drugs and pollutants. The altered level of the GSH concentration in the body is responsible for some diseases, such as premature arteriosclerosis, occlusive vascular, leukemia, diabetes, AIDS and cataracts [27,28].

MTs and PCs (class III MTs) are known as metal-binding thiol-rich peptides [29]. MTs and PCs are ubiquitous in various organisms and have been recognized as serving critical cellular functions including the storage of essential metals, detoxification of heavy metals, and protection against oxidative damage [30]. MTs are widely found in the animal kingdom, while PCs mainly occur in vascular plants, algae and fungi. PCs are oligo- and polypeptides with the amino acid structure (γ-glutamyl-L-cysteinyl)$_n$-glycine ((γ-Glu-Cys)$_n$-Gly), where n = 2–11. PCs are synthesized by the consecutive transfer of γ-Glu-Cys units to GSH via the action of PC synthase [31].

D-penicillamine (2-amino-3-mercapto-3-methylbutanoic acid) (Pen) and thiopronin (*N*-(2-mercaptopropionyl)-glycine, Thiola) (TP), which belong to the aminothiol family, are pharmaceutically important thiol compounds. Although the D- and L-enantiomers exist in penicillamine, only the D-isomer is clinically useful due to the excessive toxicity of the L-isomer. D-Pen is used for the treatment of Wilson's disease, an autosomal recessive disorder of copper transport. It is used as an antifibrotic agent to treat scleroderma

and as an antirheumatic drug to treat patients with active rheumatoid arthritis [32–34]. TP is a synthetic compound that is used in the treatment of rheumatoid arthritis, hepatic diseases and as a mucolytic in respiratory disorders [12,35]. Some studies [36] indicated that TP acts as a radioprotective agent by scavenging ROS produced by irradiation.

As previously described, the determination of aminothiols in physiological fluids is important for the diagnosis of several human diseases. These facts accelerate the investigation of the measurement of the aminothiols in biological specimens and the development of highly sensitive and selective methods for analysis. However, the biological thiols have no specific physicochemical properties (e.g., strong absorption in UV–vis regions or native fluorescence), which is required for a high detection sensitivity. Furthermore, the measurement of thiols has to be careful due to their instability in aqueous medium and their tendency to oxidize to disulfides. Besides, these thiols are generally highly polar and water soluble, which makes their extraction from biological matrices difficult. Consequently, a robust method is highly desired in the fields of biological and clinical sciences.

The separation methods using LC and CE are popular for selective and sensitive determinations of thiols. Various separation and detection techniques, such as GC–MS, LC–MS, LC–ECD, LC–UV, LC–FL, CE–UV and CE–LIF, can possibly be used for the determination of thiols in biological specimens. Although a number of techniques have been employed to assay the thiols, some problems still occur due to the operation of sophisticated instrumentation and/or the complexity of the procedure. Among the techniques, the most frequently used method for the thiol determination is the approach based on derivatization and separation followed by FL detection because of the high selectivity and sensitivity. During the last five decades, various types of tagging reagents, such as *N*-substituted maleimides, bimanes, halides, halogenobenzofurazans, and ortho-phthalaldehyde (OPA), have been developed for the determination of sulfhydryl-containing compounds. The development of new reagents for the highly sensitive detection of thiols is still continuing. The present review describes recent approaches for the separation assay of various thiol compounds, obtained from analytical papers published in 2000–2008. The labeling reagents, described herein, have been used for the analysis of thiols in biological specimens. The application examples to bioanalysis are also listed in Table 1 . The approaches are categorized with the labeling reagents and are discussed in the following sections.

2. Labeling methods by UV reagents

Cysteamine (mercaptamine or β-mercaptoethylamine) is a sulfhydryl-containing compound which appears from the decarboxylation of CySH or the breakdown of pantetheine. Cysteamine is therapeutically used as a radio-protective agent [37] and prevents severe liver damage after paracetamol poisoning [38]. It is also widely used as a drug for the treatment of nephropathic cystinosis, a rare autosomal recessive disease characterized by poor growth, renal Fanconi syndrome and renal glomerular failure [39,40]. The determination of cysteamine has been directly carried out, without chemical modification, by LC with ECD [41,42]. However, the majority of the analyses involved the derivatization process with some FL tagging reagents [43,44].

As a derivatization reagent for UV detection, 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT) is used for the determination of the total cysteamine (reduced and oxidized forms) in human urine. The oxidized cysteamine is reduced with sodium borohydride ($NaBH_4$) to the reduced cysteamine before labeling with CMQT. This method is based on the tagging of the SH group in cysteamine with CMQT to its *S*-quinolinium derivative, followed by ion-pairing reversed-phase LC separation and UV detection at 355 nm. The tagging reaction conditions are very mild and the detection wavelength is favorable. The mild labeling conditions (i.e., room temperature for a few minutes in neutral medium) and the absorption property (around 355 nm) seem to be suitable for the determination of thiols. Of course, the method using CMQT is applicable not only for cysteamine, but also various thiol compounds such as HCySH and GSH [45–50]. Some sulfhydryl-containing drugs, i.e., Pen, TP and captopril, in human urine are also determined by LC with UV detection after derivatization with CMQT [51–54]. The reaction scheme of CMQT with TP is shown in Fig. 1. Fig. 2 also shows the typical chromatograms of biological thiols in plasma. The total homocysteine and cysteamine in human plasma are determined by CZE with UV detection after derivatization with CMQT [55,56]. This method includes the reduction of disulfides (oxidized form) to thiols (reduced form) with tris 2-carboxyethylphosphine (TCEP) [55].

Aminothiols (e.g., CySH and HCySH) and thiol drugs (e.g., Pen) in plasma, urine and cerebrospinal fluid (CSF) are analyzed after derivatization with 1,1′-thiocarbonyldiimidazole (TCDI) [57]. The cyclic dithiocarbamate derivatives were separated by LC and detected at the maximized wavelengths (250–300 nm). Because TCDI reacts with both the amino and thiol groups to form cyclic dithiocarbamates, the existence of both functional groups is essential for the method.

Well-known *p*-bromophenacyl bromide (*p*-BPB) is also used as a UV label. TP in human plasma was determined by LC–UV after derivatization with *p*-BPB [58,59]. Since the detection wavelength at 263 nm is fairly short, the interference of endogenous substances should be considered in this method.

As a unique method, Lu et al. [60] developed a post-column LC–UV detection method for the determination of small biothiols based on the analyte-induced aggregation of gold nanoparticles (GNPs). Brij35-capped GNPs selectivity respond toward low molecular mass biothiols, and the colorimetric evolution of the colloidal solution rapidly occurred. The determination of CySH in human urine could be achieved using the colloid as the post-column reagent.

3. Labeling methods by FL reagents

To increase the specificity and sensitivity, various reagents for UV, FL and MS detections have been developed for the determination of minute amounts of sulfhydryl compounds. Among these reagents, the approach using FL detection is the most popular because of its high selectivity and sensitivity toward thiol determination. Although several types, such as *N*-substituted maleimides, bimanes and halides, have been widely accepted as FL reagents to measure thiol compounds, new FL reagents are still being developed to improve the sensitivity, selectivity and handling. The recently developed methods for thiol determination using new FL reagents are summarized in the following sections.

3.1. Maleimides

Maleimide-type reagents, e.g., *N*-(9-acridinyl)maleimide (NAM), *N*-(*p*-(2-(6-dimethylamino)benzofuranylphenyl)maleimide (DBPM), *N*-[*p*-(2-benzimidazolyl)phenyl]maleimide (BIPM) and fluorescein-5-maleimide (FM), are most frequently used for the derivatization of thiols for long periods. The maleimides possessing various fluorophors have been applied to the determination in biological specimens. Recently, the methods utilizing ThioGlo™3 (9-acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)pyeny)-3-oxo-3H-naphtho[2,1-b]pyran), *N*-(2-acridonyl)maleimide (MIAC), 5-maleimidyl-2-(*m*-methylphenyl)benzoxazole (MMPB) and 3-maleimidylbenzanthrone (3-MB) have been reported

Table 1
Bioanalysis of thiol compounds.

Analyte	Biological sample	Derivatization reagent	Procedure	Separation	Detection	LOD	Reference
Cysteamine (reduced and total)	Human urine	CMQT	Reacted in pH 7.5 at room temp for 2 min. $NaBH_4$ reduction at room temp. for 2 min	Reversed-phase LC using ODS column	UV 355 nm	0.05 μM (reduced), 0.2 μM (total)	[45]
Tiopronin (TP), D-penicillamine (Pen)	Human urine	CMQT	Reacted in pH 7.5 at room temp for 5 min. $NaBH_4$ reduction at Room temp. for 1 min	Reversed-phase LC using ODS column	UV 355 nm	0.5 μM (TP), 1.0 μM (Pen)	[51]
Homocysteine (total)	Human plasma	CMQT	Reacted in pH 7.6 at room temp for 4 min. TCEP reduction at 25 °C for 30 min	CZE using fused-silica capillary	UV 355 nm	1.0 μM (total)	[55]
Cysteamine (total)	Human plasma	CMQT	Reacted in pH 7.6 at room temp for 4 min. TBP reduction at 60 °C for 30 min	CZE using fused-silica capillary	UV 355 nm	1.0 μM (total)	[56]
Cysteine, homocysteine, glutathione, cysteinylglycine	Human plasma	CMQT	Reacted in pH 7.6 at room temp for 1 min. $NaBH_4$ reduction at room temp for 1.5 min.	Reversed-phase LC using ODS column	UV 355 nm	0.1–0.3 nmol/mL (reduced), 0.1–2 nmol/mL (total)	[47]
Cysteine, homocysteine, cysteinylglycine (total)	Human urine	CMQT	Reacted in pH 7.5 at room temp for 2 min. $NaBH_4$ reduction at room temp for 2 min	Reversed-phase LC using ODS column	UV 355 nm	0.12–0.25 nmol/mL (total)	[48]
Cysteine, cysteinylglycine (total)	Human urine	CMQT	Reacted in pH 7.5 at room temp for 5 min in the presence of TCEP	Reversed-phase LC using ODS column	UV 355 nm		[49]
N-Acetylcysteine, thioglycolic acid (total)	Human urine	CMQT	Reacted in pH 7.8 at room temp for 5 min in the presence of TCEP	Reversed-phase LC using ODS column	UV 355 nm	0.12 nmol/mL (*N*-acetylcysteine), 0.25 nmol/mL (thioglycolic acid)	[50]
Captpril (total)	Human urine	CMQT	Reacted in pH 7.8 at room temp for 2 min in the presence of TCEP	Reversed-phase LC using ODS column	UV 355 nm	0.05 nmol/mL (total)	[52]
Methimazole (total)	Human urine	CMQT	Reacted in pH 7.5 at room temp for 5 min in the presence of TCEP	Reversed-phase LC using ODS column	UV 345 nm	0.15 μg/mL (total)	[53]
Mesna (total)	Human urine	CMQT	Reacted in pH 7.3 at room temp for 5 min. TBP reduction at 60 °C for 30 min	Reversed-phase LC using ODS column	UV 350 nm	0.1 nmol/mL (total)	[54]
Cysteine, homocysteine, cysteinylglycine (total)	Human plasma, urine, CSF	TCDI	Reacted in pH 8.0 at 37 °C for 20 min. TCEP reduction at 24 °C for 30 min	Reversed-phase LC using ODS column	UV 250–300 nm	Each 2 pmol/inj	[57]
Glutathione (total)	Human plasma, erythrocyte	DPDS	On-column reaction. DTT reduction at room temp. for 30 min	CE-MEKC using capillary	UV 343 nm	5 μM (total)	[135]
Cysteine (total)	Human urine	DPDS	On-column reaction. TBP reduction at 60 °C for 30 min	CE-MEKC using capillary	UV 343 nm	2.5 μM (total)	[136]
Homocysteine (total)	Human plasma	DPDS	In-capillary reaction. TCEP reduction at 37 °C for 20 min	CE-MEKC using capillary	UV 343 nm	6 μM (total)	[137]
Cysteine, homocysteine, glutathione (reduced and total)	Rat brain, lung, liver, heart, kidney, erythrocyte, plasma	DTNB	Reacted in pH7 at room temp. for 1 min. Disulfides are no treatment	Reversed-phase LC using ODS column	ESI–MS (SIM)	3–15 pmol	[138]
Gemopatrilat (thiol drug)	Rat, mouse, dog, monkey, human, plasma	MA and PMSF	Immediate reaction	Reversed-phase LC using ODS column	APCI–MS/MS	1 ng/mL (LOQ)	[139]
Tiopronin (TP)	Human plasma	*p*-BPB	Reacted in aqueous MeOH at room temp for 30 min	Reversed-phase LC using ODS column	UV 263 nm	12 ng/mL	[59,68]

Table 1 (*Continued*)

Analyte	Biological sample	Derivatization reagent	Procedure	Separation	Detection	LOD	Reference
Cysteinyl-peptides	Peptides, BSA, α-lactalbumin	mAEDANS	Tryptic digests are reacted in pH 8.5 at room temp. for 30 min. TCEP reduction in pH 8.5 at 50 °C for 30 min	Reversed-phase LC using ODS column	FL and ESI–MS and TOF–MS		[71]
Cysteine, homocysteine, glutathione, *N*-acetylcysteine	Human blood	IAB	Reacted in pH 8.3 at 35 °C for 15 min	Reversed-phase LC using ODS column	FL (ex. 420 nm, em. 540 nm)	1–2 nM	[72]
Cysteine, glutathione, *N*-acetylcysteine, 2-mercaptoethanol, penicillamine	Human urine	MIPBO	Reacted in pH 9.0 at 40 °C for 35 min	Reversed-phase LC using ODS column	FL (ex. 310 nm, em. 375 nm)	3.5–15 fmol	[73]
N-(2-Mercaptopropionyl)glycine	Male C57BL/6 mice, liver, lung, kidney, brain, plasma	ThioGlo™3	Reacted in pH 7.0 at room temp. for 25 min	Reversed-phase LC using ODS column	FL (ex. 365 nm, em. 445 nm)	5 nM	[63]
Captopril	Male Sprague–Dawley rat, liver, lung, kidney, plasma	ThioGlo™3	Reacted in pH 7.0 at room temp. for 25 min	Reversed-phase LC using ODS column	FL (ex. 365 nm, em. 445 nm)	200 fmol/inj	[65]
Thiocyl	Male C57BL/6 mice, liver, lung, kidney, brain, plasma	ThioGlo™3	Reacted in pH 7.0 at room temp. for 25 min	Reversed-phase LC using ODS column	FL (ex. 365 nm, em. 445 nm)	3 nM	[66]
Cysteine, homocysteine, glutathione (total)	Human plasma	MIAC	Reacted in pH 8.2 at room temp. for 1 min. TCEP reduction in pH 8.2 at 40 °C for 10 min	Reversed-phase LC using ODS column	FL (ex. 260 nm, em. 416 nm)	1–2 pmol	[68]
Penicillamine (reduced and total)	Human plasma	5-IAF	Reacted in pH 12.5 at room temp. for 15 min. TBP reduction in DMF at room temp. for 10 min	CE using uncoated fused-silica capillary	LIF (ex. Ar–ion 488 nm)	100–200 pM	[75]
Cysteine, homocysteine, cysteinylglycine, glutathione (reduced and total)	Human plasma	5-IAF	Reacted in pH 12.5 at room temp. for 15 min. TBP reduction in DMF at room temp. for 10 min	CE using uncoated fused-silica capillary	LIF (ex. Ar–ion 488 nm)	100–200 pM	[76]
Homocysteine (total)	Human plasma	6-IAF	Reacted in pH 9.5 at room temp. for 2 h. TCEP reduction in pH 9.5 at room temp. for 10 min	CE using uncoated fused-silica capillary	LIF (ex. Ar–ion 488 nm)	50 pM	[79]
Glutathione (reduced and total)	Probiotic bacteria	5-IAF	Reacted in pH 12.5 at 40 °C for 90 min	CE using uncoated fused-silica capillary	LIF (ex. Ar–ion 488 nm)	0.5 ng/mL	[78]
Cysteine, γ-glutamylcysteine, cysteinylglycine, glutathione photochelatin	Durum wheat seed, root tip	5-BMF	Reacted in pH 9.6 at 40 °C for 20 min	CE using uncoated fused-silica capillary	LIF (ex. Ar–ion 488 nm, 520 nm band pass filter)	25 nM	[82]
Cysteine, homocysteine, cysteinylglycine, glutathione (total)	Human plasma	SBD-F	Reacted in phosphate buffer at 60 °C for 1 h. TCEP reduction at room temperature for 30 min	Reversed-phase LC using ODS column	FL (ex. 385 nm, em. 515 nm)		[86]
Glutathione	Rat liver	SBD-F, ABD-F, DBD-F	Reacted in pH 7.2 at 37 °C for 30 min	Reversed-phase LC using ODS column	FL (ex. 380 nm, em. 530 nm)	1–3 pmol	[90]
Cysteine-bound albumin, cysteine (total) after oxidative stress	Human plasma	ABD-F	Reacted in pH 10.5 at 60 °C for 5 min in the presence of TCEP	Reversed-phase LC using ODS column	FL (ex. 380 nm, em. 510 nm)		[93]
α-Lipoic acid (reduced and oxidized)	Human plasma	ABD-F and SBD-F	Reacted with ABD-F in pH 9.3 at room temp. for 10 min. Reacted with SBD-F in pH 9.3 at 50 °C for 1 h in the presence of TCEP	Reversed-phase LC using ODS column	FL (ex. 380 nm, em. 510 nm)	0.13 pmol	[109]

Lipoyllysine	Rat tissues, spinach	ABD-F	Reacted with SBD-F in pH 9.3 at 60 °C for 1 h in the presence of TCEP	Reversed-phase LC using ODS column	FL (ex. 380 nm, em. 510 nm)	0.3 pmol	[118]
Glutathione (reduced and oxidized), cellular redox status analysis	RMoc and R15LO cells	ABD-F	Reacted with ABD-F in pH 9.3 at room temp. for 10 min. Disulfides are no treatment	Reversed-phase LC using ODS column	FL and ESI–MS/MS		[119]
Cysteinyl-peptides	Proteins from rat tissues	SBD-F	Reacted with SBD-F in pH9 at 60 °C for 3 h in the presence of TCEP	Reversed-phase 2D–LC using IEC and ODS columns	ESI–MS/MS (MRM)	0.01 nmol/mL	[91]
Low-molecular weight thiols (total)	*C. elegans*	DAABD-Cl	Reacted in pH9 at 40 °C for 10 min in the presence of TCEP	Reversed-phase LC using ODS column	FL (ex. 400 nm, em. 510 nm)	30–341 fmol	[122]
Homocysteine (total)	Human plasma	mBBr	Reacted in MeOH at room temp. for 20 min. DTT reduction in pH 7.4 at 50 °C for 10 min	Reversed-phase LC using ODS column	FL (ex. 270 nm, em. 474 nm)	2.4 pmol	[123]
AMT (total)	Fisher rat serum	mBBr	Reacted in aqueous MeOH at 4 °C over night. TBP reduction in aqueous MeOH at 50 °C for 1 h	Reversed-phase LC using ODS column	FL (ex. 380 nm, em. 480 nm)	0.05 μg/mL	[124]
Metallothionein (total)	Clam extract	mBBr	Reacted in aqueous MeOH at room temp.for 15 min. DTT reduction in pH 9.5 at 70 °C for 20 min	Reversed-phase LC using ODS column	FL (ex. 382 nm, em. 470 nm)		[126]
Phytochelatins, thiol-peptides	Marine microalga	mBBr	Reacted in pH9 at room temp. for 1 h in the presence of 2-ME and NaBH4	CZE using uncoated fused-silica capillary	UV 390 nm	2.5 μM (GSH)	[129]
Glutathione	Rat liver	mBCl	Reacted at room temp. for 30 min	Reversed-phase LC using ODS column	FL (ex. 380 nm, em. 470 nm)		[130]
Cystein in protein, cross-linking	γ-Crystallin from bovine eye lens	dBBr	Reacted in pH 7.6 at room temp. for 45 min. TCEP reduction in pH 7.6 at 50 °C for 30 min	Reversed-phase LC using ODS column	MALDI–TOF–MS		[131]
Glutathione (reduced and oxidized)	Rat liver, whole blood, human blood	OPA (pre-label)	Reacted in pH 8.0 at 25 °C for 15 min	Reversed-phase LC using ODS column	FL (ex. 350 nm, em. 420 nm)	5.6–14 fmol	[132]
Homocysteine-thiolactone (HTL), homocysteine (total)	Cultured cells (Hep G2)	OPA (post-label)	TCEP reduction in pH 6.0 at 37 °C for 10 min	Reversed-phase LC using C30 column	FL (ex. 370 nm, em. 480 nm)	100 fmol (HCySH), 200 fmol (HTL)	[133]

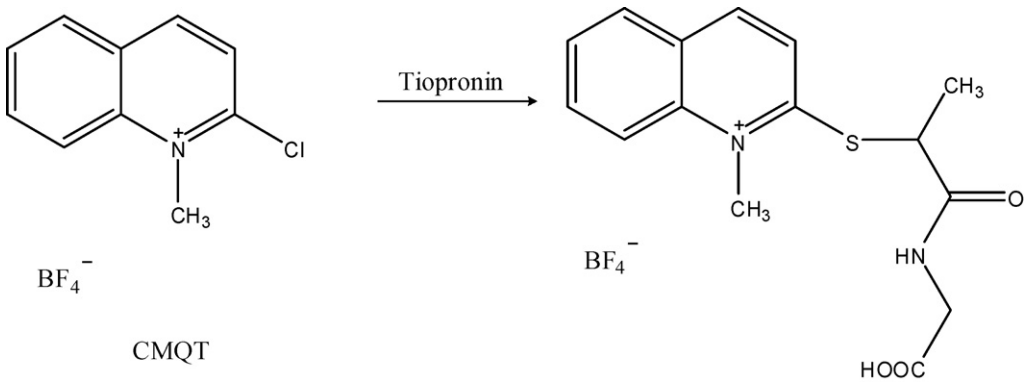

Fig. 1. Reaction scheme of CMQT with tiopronin.

for the determination of thiol compounds. ThioGlo^{TM}3 is a naphthopyranone-based fluorescent thiol probe, synthesized by Covalent Associate, Inc. [61]. Under mild reaction conditions (at room temperature for 30 min in neutral medium), various thiols are labeled with ThioGlo^{TM}3 to produce high FL derivatives (ex. 365 nm, em. 445 nm) [62]. Several drugs, i.e., TP in biological samples (plasma, lung, liver, kidney and brain in mice) [63], 2-mercaptoethane sulfonate (MESNA) in biological samples (lung, liver, kidney and brain) [64], captopril in rat plasma [65], and sodium thiosalicylate in mice plasma [66], were successfully determined by the LC–FL method, as well as biological thiols [67]. The reaction scheme of ThioGlo^{TM}3 with captopril is shown in Fig. 3. Since ThioGlo^{TM}3 is a relatively large molecule when compared with other maleimides, a high concentration of organic modifiers, such as acetonitrile, is needed for the elution of the derivatives by reversed-phase chromatography. The fluorescent hydrolysis product also appears on the chromatogram. The typical chromatograms of the derivatives are shown in Fig. 4.

MIAC [68], MMPB [69] and 3-MB [70] are new maleimide-type reagents for the derivatization of sulfhydryl-containing compounds. Because the reactivity to the SH group is based on the coupling with the maleimide structure, the derivatization conditions are almost comparable for each maleimide reagent. However, the fluorescence properties significantly depend on the fluorophors connected to the maleimide. The excitation and emission maxima are 260 and 416 nm (MIAC-derivative), 300 and 356 nm (MMPM-derivative), and 401 and 496 nm (3-NB-derivative).

3.2. Halides

Because the reaction between iodoacetamide and thiols is well known, various iodoacetamide-type reagents have been synthesized and used for the determination of thiols in real samples. 1,5-I-AEDANS (5-({2-[iodoacetyl]amino}ethyl}amino)naphthalene-1-sulfonic acid) is the most popular reagent of this type. The reagent is very soluble in aqueous solution and selectively labels the CySH residue in protein. Clements et al. [71] used the reagent for fluorescence-based peptide labeling and the fractionation analysis of cysteine-containing peptides. In this study, 1,5-I-AEDANS was used as a versatile fluorescence-based peptide quantification tool and provided readily interpretable tandem mass spectra for *de novo* peptide sequencing. As the derivatization reagents containing the iodoacetamidyl moiety, 3-iodoacetylaminobenzanthrone (IAB) [72], 5-methyl(2-(*m*-iodoacetylaminophenyl)benzoxazole (MIPBO) [73] and 2-(*p*-iodoacetylaminophenyl)benzothiazole (IPBT) [74] were also developed by Zhang and co-workers. The fluorescence maximal wavelengths of the derivatives with IAB, MIPBO and IPBT were 540 (ex. 420), 375 (ex. 310) and 383 nm (ex. 330 nm), respectively. Several aminothiols were analyzed by LC–FL using these reagents under mild reaction conditions.

5- or 6-IAF (5- or 6-iodoacetamidofluorescein) is one of the aliphatic halide-containing reagents for thiol labeling [75–81]. The reaction scheme of 5-IAF with Pen is shown in Fig. 5. The reactive group is the same as those of other acetoamydyl-type reagents. The maximal wavelengths of excitation and emission of the derivative are relatively long. The excitation maximum is very close to the emission of the Ar–ion laser (488 nm). To best use the wavelength characteristics, the reagent was mainly used for the

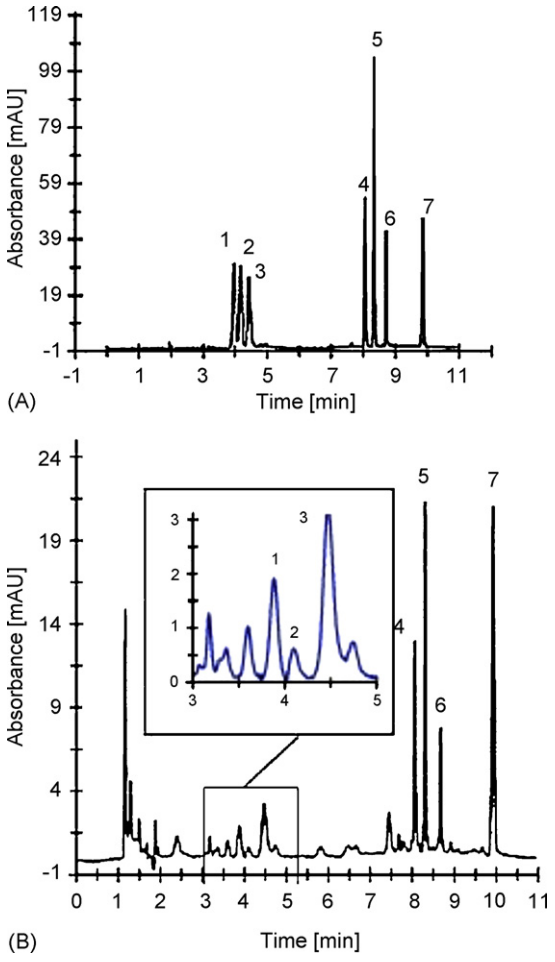

Fig. 2. Typical chromatograms of main plasma thiols labeled with CMQT. (A) Standard solution of each thiol (20 nmol/mL), (B) Thiols in plasma. Peaks: 1, GSH; 2, HCySH; 3, 2-mercaptopropionic acid; 4, 3-mercaptopropionic acid; 5, CySH; 6, cysteinylglycine; 7, CMQT. UV detection: 355 nm. Reproduced from Fig. 1 in Ref. [47].

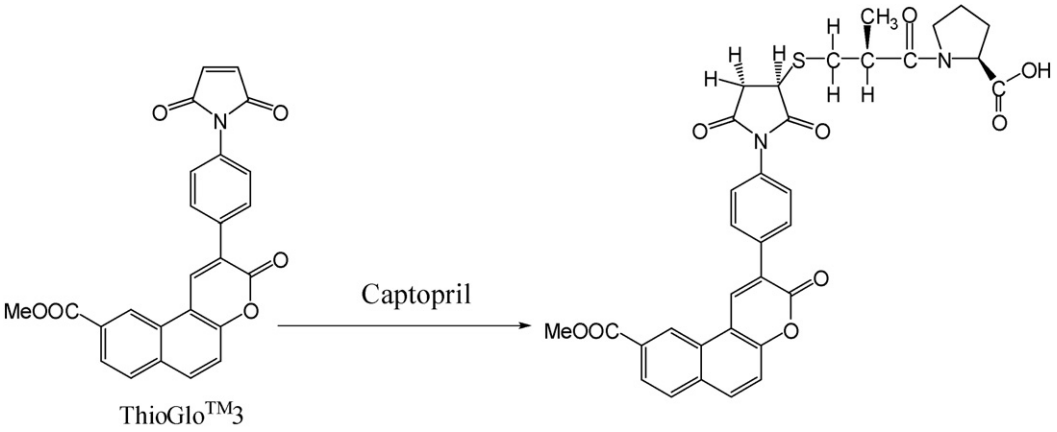

Fig. 3. Reaction scheme of ThioGlo™3 with captopril.

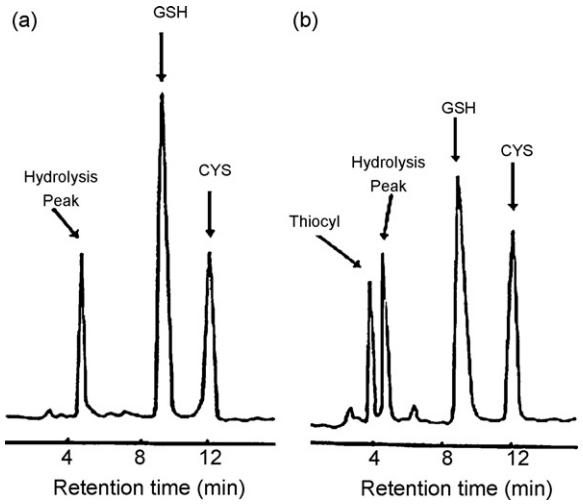

Fig. 4. Chromatograms of liver obtained from C57BL/6 mice. (a) Control liver sample, (b) Liver sample after i.p. administration of thiocyl (100 mg/kg body weight). FL detection: ex. 365 nm; em. 445 nm. Reproduced from Fig. 8 in Ref. [138].

determination of thiols by CE-LIF. The electropherograms of 5-IAF derivatives, obtained from CE-LIF, are shown in Fig. 6. Although iodine (I) in these reagents reacts with thiol compounds to form thioether derivatives, the reagent having bromide (Br) as the active site was also developed by Hart et al. [82]. The reagent, i.e., 5-bromomethylfluorescein (5-BMF), was used for the measurement of phytochelatin (PC2) by CE-LIF at 488 nm with an Ar–ion laser. The derivatization conditions are essentially similar to those of the iodoacetamide-type reagents.

3.3. Halogeno-benzofurazans

Halogeno-benzofurazans, such as ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F) [83–86], 4-(aminosulfonyl)-

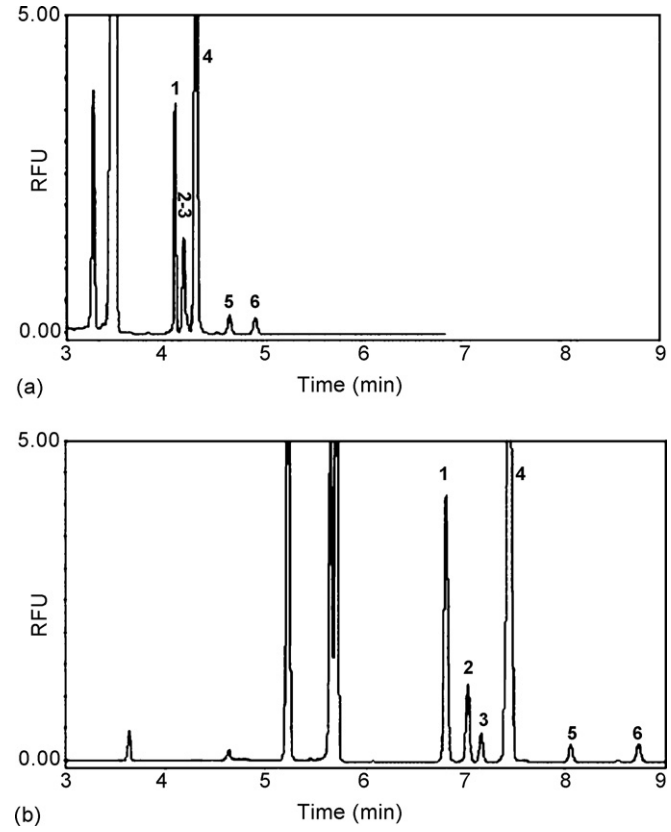

Fig. 6. Electropherograms of standard thiols. Run buffers: (a) pH 11 (5 mM sodium phosphate, 4 mM boric acid, and 75 mM *N*-methyl-D-glucosamine); (b) pH 11.4 (18 mM sodium phosphate, 14.5 mM boric acid, and 75 mM *N*-methyl-D-glucosamine). Peaks: 1, cysteinylglycine; 2, penicillamine; 3, HCySH; 4, CySH; 5, GSH; 6, glutamylcysteine. CE, uncoated fused-silica capillary (57 cm × 75 μm, i.d.); LIF, Ar–ion at 488 nm. Reproduced from Fig. 2 in Ref. [67].

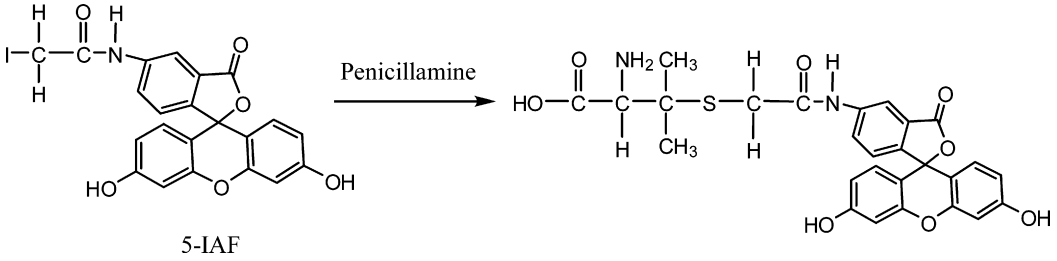

Fig. 5. Reaction scheme of 5-IAF with penicillamine.

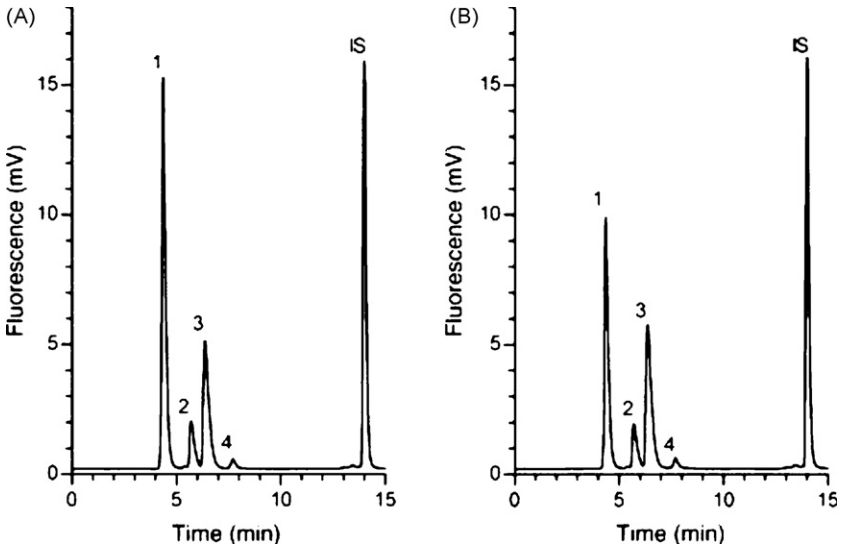

Fig. 7. Representative chromatograms of plasma obtained from a chronic kidney disease patient. (A) Before administration, (B) After orally administration of α-lipoic acid (600 mg three times/day for 2 days). Peaks: 1, CySH; 2, HCySH; 3, cysteinylglycine; 4, GSH; IS, TP. FL detection: ex. 385 nm; em. 515 nm. Reproduced from Fig. 4 in Ref. [86].

7-fluoro-2,1,3-benzoxadiazole (ABD-F) [87,88] and 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) [89] have been used as reliable labeling reagents for thiol compounds. The reactive site of these reagents to thiols is the same, and the activity is in this order, DBD-F > ABD-F > SBD-F. On the other hand, both the selectivity to thiols and the solubility in aqueous solution are opposite, SBD-F > ABD-F > DBD-F. Therefore, SBD-F or ABD-F is usually employed for the labeling of aminothiols and the CySH residue in peptides. The determination of hepatocellular GSH and the cytotoxicity evaluation of environmental pollutants (volatile organic compounds and endocrine disrupting chemicals) were carried out by a method using these reagents [90]. Fig. 7 shows the typical chromatograms of biological thiols labeled with SBD-F in the presence of TCEP (a reducing reagent). The resolution of the cellular redox status based on stable isotope dilution LC–MS was also performed by Zhu et al. [91]. In this case, both the ABD-labeled GSH and GSSG without labeling are separated by LC and detected by MS/MS. The total amounts of reduced and oxidized compounds in human plasma were also determined by LC–FL after derivatization with ABD-F in the presence of TCEP [92]. The influence of oxidative stress based on the determination of the relative amounts of various albumin-bound thiols in human plasma was also analyzed after labeling with ABD-F [93].

Since the excitation and emission maxima of the derivatives obtained from SBD-F, ABD-F and DBD-F are almost the same (approximate to ex. 380 nm, em. 540 nm), the simultaneous determination of thiols and disulfides is possible. In this method, only the thiols in the sample are first labeled with ABD-F, and then the excess amounts of unreacted ABD-F are extracted out by ethyl acetate. The disulfides in the sample are then labeled with SBD-F in the presence of a reducing agent (e.g., TBP or TCEP) to produce a two-fold excess of SBD-thiols. Finally, the ABD-thiols, derived from reduced thiols, and SBD-thiols, derived from disulfides, are simultaneously separated by reversed-phase LC and detected by FL at around 540 nm (ex. around 380 nm) [94,95].

α-Lipoic acid (LA) and dihydrolipoic acid (DHLA: reduced form of LA) are well known as excellent antioxidants [96–100]. Their simultaneous determination is thus important in order to understand the function of LA and DHLA. LA is easily determined by several methods, such as GC [101,102] and HPLC [103–108]; however, the simultaneous assay was quite difficult. Satoh et al. [109] demonstrated the simultaneous determination of LA and DHLA by the above method using both ABD-F and SBD-F. LA is naturally found in mitochondria as the coenzyme for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase [110–113]. It is found in most prokaryotic and eukaryotic microorganisms as well as in many plant and animal tissues [114]. According to previous studies [115–117], LA is covalently bound to the ε-NH_2 group in the lysine residue(s) of proteins and plays important roles in biological systems. The protein-bound LA was determined as lipoyllysine (LLys) by LC–FL after enzymatic hydrolysis, the reduction with TCEP and the derivatization with ABD-F [118].

SBD-F and ABD-F were also used for the identification of protein in tissues [119] and the labeling of CySH in single-chain antibody fragments (scFv) [120]. The proteins labeled with SBD-F in the presence of TCEP were digested with trypsin, and the FL-peptide fragments were separated by two-dimension (2D) LC. The FL-peptides were analyzed by LC–MS/MS. The original proteins were identified by database searching (MASCOT) of the MS/MS data of the peptide fragments [119]. On the other hand, the scFv antibody fragments reduced with TBP were labeled with ABD-F. The labeled-proteins were separated by SDS-PAGE and detected by fluorometry [120].

Imai et al. synthesized similar reagents having the benzofurazan structure, i.e., 4-(*N*-acetylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (AcABD-F), 4-(*N*-trichloroacetylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (TCAcABD-F) [121] and 7-chloro-*N*-[2-(dimethylamino)ethyl]-2,1,3-benzoxadiazole-4-sulfonamide (DAABD-Cl) [122]. Both the reactivity to the thiol and the fluorescence property of the derivative are almost equivalent to ABD-F. DAABD-Cl was used for the determination of thiols in *Caenorhabditis elegans* [122].

As shown in the reports described herein, the benzofurazans, such as SBD-F and ABD-F, are excellent labeling reagents for thiols. Since the reagents do not react with phosphine-type agents such as TBP and TCEP, the reagents can be used for the labeling of disulfides in the presence of this type of reducing agent. This is one of the excellent features of these reagents.

3.4. Bimanes

Monobromobimane (mBBr) is an another promising reagent for thiol labeling. Many papers about this reagent have been published by various research groups. Aminothiols, such as CySH,

HCySH and GSH, are determined at the pmol level by LC–FL after derivatization with mBBr [123]. The total homocysteine in human plasma in several diseases (i.e., occluded coronary artery disease and hemodialysis patients) was labeled with mBBr in the presence of DTT and determined by LC–FL [123]. The method using mBBr was also used for the determination of the total 3-amino-5-mercapto-1,2,4-triazole (AMT) in rat serum, which is a chemical used in a number of commercial applications including pesticide synthesis and the processing of silver halide photographic materials [124]. Buratti et al. [125] reported the method using mBBr for the determination of *S*-phenylmercapturic acid (PMA) and *S*-benzylmercapturic acid (BMA) in human urine, which are the responsible metabolites of benzene and toluene, respectively. According to the method, PMA and BMA are hydrolyzed by alkaline to thiophenol and benzylmercaptan. The resulting thiols were labeled with mBBr and determined by LC–FL. The derivatization reaction proceeds under mild conditions (for example at room temperature in neutral pH) and the resulting derivative emits light in the range of 470–480 nm.

mBBr is also used for the labeling of cysteine in proteins. Alhama et al. [126] successfully determined the content of metallothionein (MT) in the digestive gland of clams from southern Spanish coastal sites with different metal levels by LC–FL. mBBr is classically used for the sensing of the cysteine residue in proteins [127,128]. Fan et al. [128] used it for the SDS-PAGE electrophoretic profiling of thiol-rich phytochelatins (PCs) and MTs. The analysis of PCs and the precursors (CySH, GSH and γ-glutamyl-cysteine) labeled with mBBr in the biological samples of marine microalga was also carried out by CZE separation and detection at UV 390 nm [129]. Various lengths of the PCs were well separated by the CZE using 150 mM phosphate buffer at pH 1.6. Monochloro-bimane (mBCl), of which the reactive site is chloride (Cl) instead of bromide (Br) in mBBr, is also used for the labeling of GSH in tissue homogenate [130]. The reactivity of mBCl to thiol compounds seems to be slightly lower than that of mBBr. Of course, the fluorescence maxima of the derivatives are very close to the derivatives of mBBr. Dibromobimane (dBBr) possessing two methylene bromide functional groups is used as a cross-linking reagent for CySH residues in protein [131]. Because dBBr has two active Br in the structure, the reagent covalently binds with a pair of CySH within the distance of 3–6 Å. Sinz and Wang [131] described the method for identifying spatially close CySH residues in γ-crystallin F. The cross-linking product between Cys-18 and Cys-78 was identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF–MS).

3.5. OPA

OPA is well known as a labeling reagent for primary amines. OPA reacts with the primary amino functional group ($R\text{-}NH_2$) in the presence of a thiol compound, such as 2-mercaptoethanol (2-ME) and AcCySH, to produce isoindole-type fluorescent derivatives [132]. Aminothiols, such as HCySH and GSH, also form the isoindol derivatives without the thiol, which are fluorescent tricyclic derivatives. Mukai et al. [133] determined HCySH and homocysteine-thiolactone (HTL) in cultured cells with OPA in the absence of the thiol. HCySH and HTL were separated on a C30 reversed-phase LC column, and then derivatized with OPA by a post-column labeling method. Because HTL itself is not labeled with OPA, HTL was cleaved with 0.5 M NaOH contained in the OPA solution, followed by labeling with OPA. GSH and GSSG in plasma, whole blood and rat hepatocytes were also determined by OPA and *N*-ethylmaleimide (NEM) [132]. For the measurement of GSSG, GSH was labeled with NEM in advance. 2-Dimethylaminoethanethiol and 2-diethylaminoethanethiol, each of which are the hydrolysis products or hydrolysis product simulants of the chemical warfare agents, *O*-ethyl *S*-diisopropylaminoethyl methylphosphonothioate (VX) and *O*-isobutyl *S*-2-diethylaminoethyl methylphosphonothioate (R-VX), were separated by CE and detected by LIF at 325 nm using a He-Cd laser after derivatization with OPA/taurine [134]. Other similar thiols, i.e., 1-pentanethiol and 2-mercaptoethanol, were simultaneously resolved by this method. The detection limits of 2-dimethylaminoethanethiol, 2-diethylaminoethanethiol, 2-mercaptoethanol, and 1-pentanethiol were 35, 1.8, 15 and 89 μg/L, respectively.

4. MS and miscellaneous detection

2,2′-Dipyridyldisulfide (DPDS) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) are generally used as UV labels for thiols. The derivatization mechanism is based on the thiol-disulfide exchange reaction. When thiol (RSH) is allowed to react with excess amounts of disulfide R'SSR' (correspond to DPDS or DTNB), the mixed-disulfide (R'SSR) and the corresponding thiol (R'SH) are produced by the reaction. The reaction is rapid at room temperature and the resulting R'SH is stabilized either by resonance or by thiol–thione tautomerization. Glatz and Maslanova [135,136] used the on-column reaction of DPDS for the specific detection of biological thiols during micellar electrokinetic chromatography (MEKC). The mixed-disulfides separated by MEKC were selectively detected at UV 343 nm. Ellman's reagent is also used for the thiol exchange reaction of biological thiols in rat blood [137]. According to this paper, the resulting mixed-disulfides were separated by reversed-phase LC and detected by MS. An excess amount of Ellman's reagent is necessary to ensure the complete derivatization of all the thiols in the samples, because for low concentrations of the reagent, the mixed-disulfides produced from the exchange reaction can possibly react with residual thiols (e.g., protein thiols) to form an undesirable disulfide. Therefore, the concentration of the reagent in the thiol-exchange reaction is important for obtaining reliable results. Although disulfides, such as GSSG, cannot react with Ellman's reagent, the simultaneous determination of biological thiols and disulfides was carried out by the MS detection [138].

As an another MS detection method, Wang et al. [139] report the determination of a thiol drug, gemapatrilat (hexahydro-6(*S*)-[2(*S*)-mercapto-1-oxo-3-phenylpropyl]amino-2,2-dimethyl-7-oxo-1H-azepine-1-acetic acid), in the plasma of beagle dogs by LC–APCI–MS/MS. In this method, methyl acrylate (MA) was employed for the coupling of the sulfhydryl group in the drug. The resulting derivative was stabilized with phenylmethylsulfonyl fluoride (PMSF), a potent inhibitor of carboxylesterase [140]. The method was used for pharmacokinetic (PK) studies in plasma.

Simek et al. [141] reported the determination of biomarkers related to the folate and cobalamine status in human serum after reduction with dimercaptopropanesulfonate (DMPS) and derivatization with heptafluorobutyl chloroformate (HFBCF). The *N*(*S*,*O*)-heptafluorobutoxycarbonyl heptafluorobutyl ester derivatives, including aminothiols and thiol-containing dipeptides, were analyzed by GC–MS. Because the method is of course not specific for thiol compounds, the method seems to be suitable for the study of biomarker discovery. As another GC method, cysteamine is determined by GC–FID (flame photometric detection) after conversion into the *N*,*S*-diisobutoxycarbonyl derivative for the volatilization [142].

5. Bioanalysis of sulfhydryl-containing compounds

The sulfhydryl group (–SH) in thiol compounds is very reactive and can be readily converted to disulfides (–SS–) via an oxidative

process such as chemical and enzymatic oxidations. The biological system has devised an extraordinary mechanism to defend itself from highly reactive intermediate(s) and/or free radicals. The defense mechanism involves the participation of endogenous scavengers like GSH and CySH. These thiols readily react with other thiols to form the disulfides in the presence of ROS and several chemicals. The disulfides thus produced in biological systems are easily reduced to the corresponding thiols via several mechanisms, such as the thiol-disulfide interchange and reductase enzyme requiring NADH or NADPH. Therefore, the stabilization of thiol and disulfide in biological samples is very important to obtain accurate and precise results.

The pH of the sample is also important in order to stabilize the thiols because the SH group is more reactive in alkaline medium. As another option of the stabilization, the addition of EDTA is effective to prevent the oxidation. EDTA may prevent the catalytic oxidation of the SH group by trace heavy metals in the samples, because EDTA easily forms a chelate with heavy metals. The utility has been successfully shown in several examples [143,144].

The derivatization with a suitable reagent is another effective means for the stabilization of thiol compounds. The derivatization step is categorized to before and after chromatographic separations (i.e., pre-column and post-column labeling). The pre-column method seems to be recommended for the labeling because thiols might be decomposed during the separation in the analytical column. The choice of the derivatization reagent is also important not only for the sensitive detection but also for the stabilization of thiols. The reagent that can instantaneously and quantitatively reacts under mild conditions is more suitable for the thiol labeling. Furthermore, the derivatization provides good separation efficiency in reversed-phase chromatography, because the labeling usually increases the hydrophobicity of the thiols. Of course, immediate extraction of the target thiols, isolation of the derivatives, suitable chromatographic separation, and effective detection are required for the sensitive and reliable determination in real samples.

A number of *N*-substituted maleimides has been reported and used for the determination of thiol compounds in real samples. However, these types of reagents possess some essential problems in the Michael addition of the thiols to the C=C bond of the reagent. The resulting adduct has an asymmetric carbon and is thus changed to a chiral compound. When the target thiols are achiral compounds, the determination might be practically no problem. However, if a chiral center already exists in the thiol compound, a pair of diastereomeric derivatives is produced during the reaction of the reagent, and thus appears on the reversed-phase chromatography. Therefore, care should be taken using the *N*-substituted maleimides in such compounds.

mBBr and its analogues are other important reagents for the labeling of thiol compounds. These reagents are highly reactive and sensitive toward thiols, and have been utilized in the determination or characterization of intracellular low molecular mass thiols. However, bimane-type reagents are not specific for thiols because they react with other functional groups such as amines. Recently, the use of these reagents is mainly for the detection of cysteine residue(s) in proteins [131].

As another class of labeling reagents, various fluorobenzofurazans (fluoro-benzoxadiazoles) have been developed and used for the determination of thiols in real samples. This family of reagents have the following advantages: (i) the reagents themselves have no fluorescence, (ii) the reactivity of these reagents to thiols are relatively high, although each reactivity is different in the reagent, (iii) the resulting derivatives show a strong fluorescence intensity in the rather long wavelength region, and (iv) the solubility of the reagents in water and water-miscible solvents (e.g., MeOH, EtOH and acetonitrile) is relatively high. This property is rather important for the determination in biological specimens because many target thiols are not only water soluble, but also exist in water solution.

As described in this review, the determination of thiols (–SH) is relatively easy. The derivatization conditions to various biological thiols (i.e., CySH, AcCySH, HCySH, GSH and cysteamine) are essentially the same in each reagent. The labeling of AcCySH is quite easy because the reactive functional group in the structure is only sulfhydryl (–SH). In the case of the labeling of aminothiols, however, the amino group ($-NH_2$) is also reactive with several reagents. Consequently, care should be taken for the reaction conditions and the structures of the derivatives. The reagents could not react with disulfides. Therefore, the S–S bond should be cleaved in advance for the labeling of disulfides with the reagents.

The determination of disulfide species (–SS–) is important in several cases. Although most disulfides (–SS–) lack a definitive analytical signature in the presence of other endogenous constituents in real samples, the direct determination of disulfides is fairly difficult. Based on this information, the values of the total thiols involving both disulfides and mixed-disulfides are determined in many reports. In these cases, disulfide and mixed-disulfides are first treated with suitable reducing agents, such as dithiothreitol (DTT) or tributhylphosphine (TBP), to liberate the corresponding thiols, which is then labeled with a suitable labeling reagent. According to this procedure, the determination of the exact contents of disulfides and mixed-disulfides is difficult because the determination is performed by the subtraction from total thiol amounts to free thiol amounts. As the reducing agents, the thiol-type reducing agents, such as DTT, are not recommended because excess amounts of DTT still exist along with the thiols, produced from the disulfides, in spite of the completion of the labeling reaction. The excess reducing reagents which are added to the reaction solution to avoid the production of mixed-disulfide (DTT-thiol) also react with the labeling reagent. Thus, the phosphine-type reducing agents, e.g., TBP and TCEP, are more suitable for the reduction of the disulfides. In many cases, the disulfide amounts are calculated from subtraction of the free thiol determined in advance. However, the simultaneous determination of the thiol and disulfide without the subtraction are required for the detailed functional resolution of the thiol and disulfide. Toyo'oka et al. [94,95] reported the simultaneous determination of the thiol and disulfide using different fluoro-benzofurazan labeling reagents (i.e., ABD-F and SBD-F) by a single chromatographic run.

Overall, the selection of a thiol labeling reagent should address the aspects of the stability of the reagent itself and the derivative, solubility of the reagent itself and the derivative in an aqueous solution, selectivity and reactivity toward the sulfhydryl group, and promotion of the hydrophobicity of the derivative, and increase in the detection sensitivity of the derivative.

6. Conclusion and prospect

As described in this review, in most cases, pre-column derivatization utilizing a suitable labeling reagent has been employed for the determination of thiol compounds in real samples [145,146]. Of course, the direct quantification based on on-line oxidation is also logically possible by ECD because the SH group is easily oxidized [147]. Since the easy oxidation means the low stability of thiol compounds, it may be very hard to obtain exact results.

Because various thiol compounds in real samples are easily determined by the in-direct derivatization method using the FL labeling reagent, followed by the separation and detection, this trend will be continued. The detection using LC–MS and CE–MS are excellent methods and the reports have gradually increased. Therefore, the MS detection of various thiols after labeling with a suitable reagent will became another important detection means in the near future.

Acknowledgment

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