Journal of Chromatography B, 879 (2011) 1290–1307

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Review

Ultraviolet derivatization of low-molecular-mass thiols for high performance liquid chromatography and capillary electrophoresis analysis☆

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ARTICLE INFO

Article history:
Received 4 August 2010
Accepted 28 October 2010
Available online 9 November 2010

Keywords:
Derivatization
HPLC determination
CE determination
Cysteine
Cysteinylglycine
Glutathione
γ-Glutamylcysteine
Homocysteine
Nε-homocysteinyllysine
Drug thiols

ABSTRACT

Thiols play an important role in metabolic processes of all living creatures and their analytical control is very important in order to understand their physiological and pathological function. Among a variety of methods available to measure thiol concentrations, chemical derivatization utilizing a suitable labeling reagent followed by liquid chromatographic or electrophoretic separation is the most reliable means for sensitive and specific determination of thiol compounds in real world samples. Ultraviolet detection is, for its simplicity, commonly used technique in liquid chromatography and capillary electrophoresis, and consequently many ultraviolet derivatization reagents are in used. This review summarizes HPLC and CE ultraviolet derivatization based methods, including pre-analytical considerations, procedures for sample reduction, derivatization, and separation of the primary biological aminothiols – cysteine, homocysteine, cysteinylglycine and glutathione, and most important thiol-drugs in pharmaceutical formulations and biological samples. Cognizance of the biochemistry involved in the formation of the analytes is taken.

Contents

Abbreviations: ABD-F, 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole; BAN, 2-Bromo-2′-acetonaphthone; BCPB, 1-benzyl-2-chloropyridinium bromide; BMP, 3-bromomethyl-4-isopropylphenazone; CMPI, 2-chloro-1-methylpyridinium iodide; CMLT, 2-chloro-1-methyllepidinium tetrafluoroborate; CMQT, 2-chloro-1-methylquinolinium tetrafluoroborate; CPPI, 2-chloro-1-propylpyridinium iodide; Cys, cysteine; CysGly, cysteinylglycine; DTNB, 5,5-dithio-bis-2-nitrobenzoic acid (Ellman's reagent); 2,2′DTP, 2,2′-dithiopirydyne; 4,4′DTP, 4,4′-dithiopirydyne; EAA, Ethacrynic acid; ESB, 1,1′-[Ethenylidenebis(sulfonyl)]bis-benzene; GSH, glutathione; GSSG, oxidized glutathione; γ-GluCys, γ-glutamylcysteine; Hcy, homocysteine; mBrB, monobromobimane; MPG, 2-mercaptopropionylglycine; NAcCys, N-acetylcysteine; NEM, N-ethylmaleimide; Nε-HcyLys, Nε-homocysteinyllysine; pBPB, p-bromophenacyl bromide; SBD-F, ammonium-7-fluoro-2,1,3-benzoxadiazole-4-sulfonate; TCDI, 1,1-thiocarbonyldiimidazole; TCEP, tris(2-carboxyethyl)phosphine; TNB, 5-thio-2-nitrobenzoate; 2TP, 2-thiopyridone; 4TP, 4-thiopyridone.

☆ This paper is part of the special issue "Enhancement of Analysis by Analytical Derivatization", Jack Rosenfeld (Guest Editor).

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doi:10.1016/j.jchromb.2010.10.035

1. Introduction

1.1. Need for derivatization

Liquid phase separation techniques, including high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE), are the most frequently used techniques for determination of organic substances in various matrices. Unfortunately, many substances of interest including thiols cannot be detected because they lack the structural properties necessary for the production of signals compatible with common HPLC or CE detectors, such as UV absorbance and fluorescence. This problem can be overcome by inducing derivatization reactions that add chromophoric or fluorophoric groups to the investigated molecules. Almost all methods for determination of thiols, except those based on refractive index and evaporative light scattering detection, depend on derivatization step before or after separation (pre-column, on-column or post-column derivatization). 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 important not only for the sensitive detection but also for the stabilization of thiols, improvement of chromatographic properties and ionization responses (electrospray ionization-mass spectrometry), or introduction of a charge (CE). Several reviews on analytical methods for thiols including those with derivatization step were published in recent years [1–9]. In this report application of ultraviolet derivatization reactions in HPLC and CE analysis of hydrophilic thiols are reviewed.

1.2. Endogenous thiols

Thiols have been of continuing interest for many years because of their important role in several biological and pharmacological processes. Biological thiols are products of sulfur metabolism (Fig. 1). Methionine taken with diet is converted intracellularly, via S-adenosylmethionine and S-adenosylhomocysteine, to homocysteine (Hcy) which in turn, following metabolic transsulfuration pathway, is converted to cysteine (Cys), a fundamental substrate for glutathione (GSH) biosynthesis [10]. The first step in the synthesis of GSH is production of the dipeptide γ-glutamylcysteine (γ-GluCys) from Cys and glutamate. This step traditionally has been considered rate limiting and the enzyme activity is regulated by feedback inhibition by GSH. The second synthetic step, adding glycine to the γ-GluCys dipeptide, is catalyzed by glutathione synthetase [11]. Endogenous low molecular weight thiol-containing compounds (Fig. 2), GSH, Cys, Hcy, cysteinylglycine (CysGly), γ-GluCys and their corresponding disulfides are important in a variety of physiological processes. For example, cysteine is a critical substrate for protein synthesis being the rate-limiting precursor to taurine [12,13] and plays an important role as an extracellular reducing agent [14]. GSH is a major component of the cellular antioxidant system, and it plays an important role in the detoxification of xenobiotic compounds and in the antioxidation of reactive oxygen species and free radicals [13,14]. Several studies have demonstrated that aminothiols are involved in the pathogenesis of human diseases [10,15]. When the cellular processes do not work properly, changes in the amount of thiols and disulfides in body fluids have been observed in recent studies. Disorders of cysteine metabolism include cystinosis, an autosomal recessive disease produced by a defect in lysosomal transport, and cistinuria, a common heritable disorder of the amino acids cysteine, lysine,

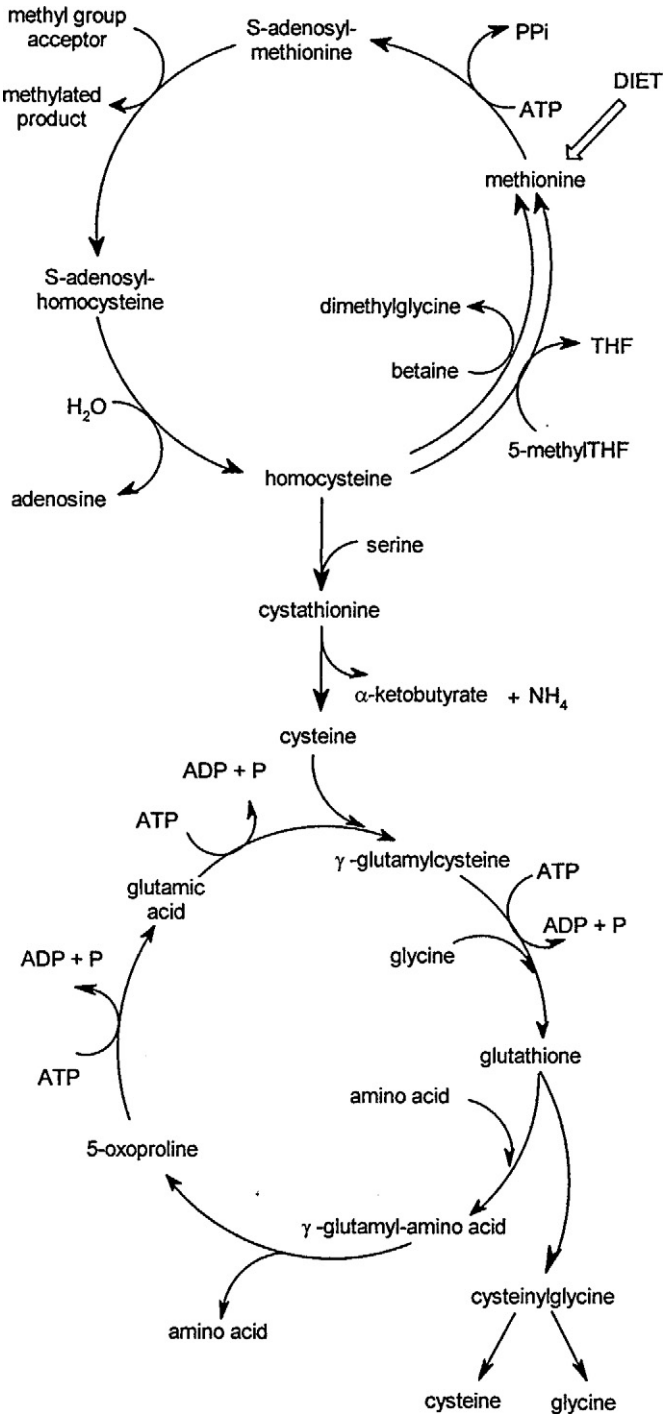

Fig. 1. Metabolism involved in the formation of aminothiols. ATP, adenosine triphosphate; THF, tetrahydrofolate; 5-methyl-THF, 5-methyltetrahydrofolate.

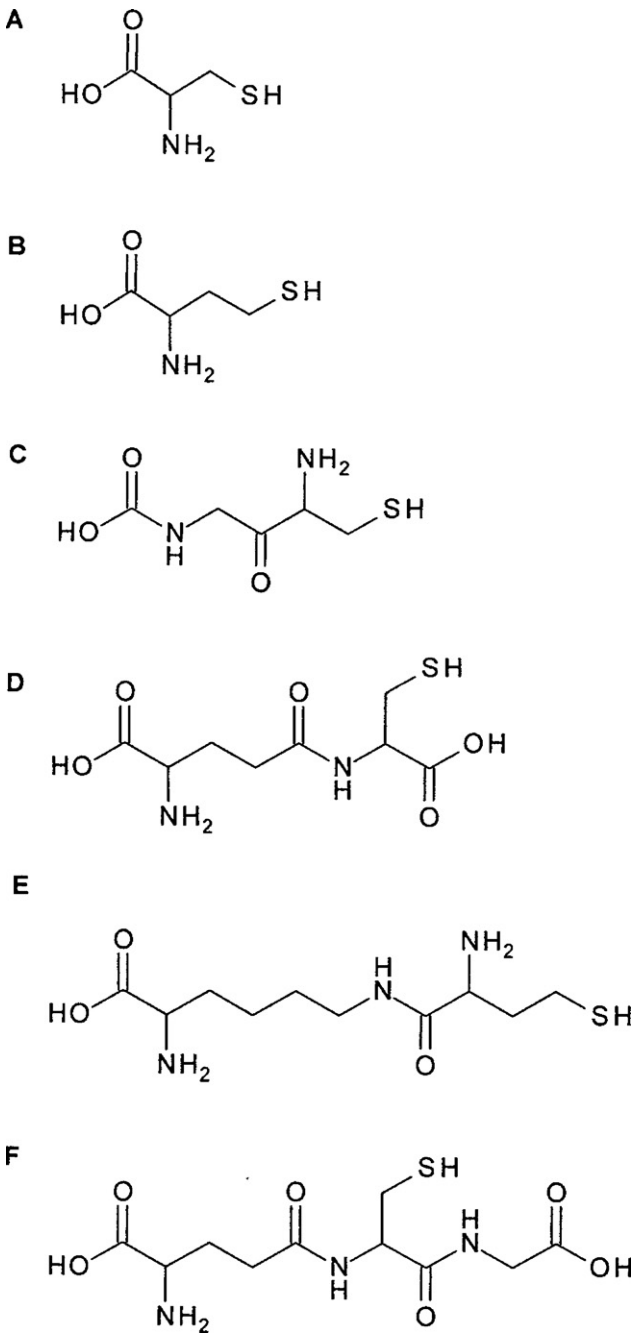

Fig. 2. Structures of some endogenous thiols: A, Cys; B, Hcy; C, CysGly; D, γ-GluCys; E, Nε-HcyLys; F, GSH.

ornithine and arginine transport [10,16]. The defect leads to a high concentration of these compounds in urine because their reabsorption mechanism in kidneys does not function. An elevated cystine concentration in the urinary tract is responsible for the formation of kidney stones. Homozygous homocystinuria, a rare genetic disorder usually caused by cystathionine β-synthase deficiency, leads to severe increase of plasma homocysteine to concentrations higher than 100 μM [17] and is associated with venous thrombosis and premature atherosclerosis. Mildly elevated plasma homocysteine levels have been associated with early pregnancy loss [18], pregnancy complication [19], neural tube defects, mental disorders [20] and some tumors [21]. Moderate hyperhomocysteinemia is associated with an increased risk of coronary artery and cerebrovascular diseases [22], including atherosclerosis [23] and thrombosis [24]. Several research groups report successful homocysteine-lowering therapies with the use of vitamins B_6, B_{12} and folate, but the consequences in terms of attenuation of development of cerebrovascular diseases remain controversial. One of the recent reports [25] after 5 years of follow up of 5522 randomly assigned patients, concludes that supplementation combining folic acid and vitamins B_6 and B_{12} did not reduce the risk of major cardiovascular events. In another work [26] with 646 screened individuals was found that mild cognitive impairment could be slowed by treatment with homocysteine lowering B vitamins. Elevated plasma or urinary levels of cysteinylglycine are observed in patients with rheumatoid arthritis [27,28] and may be associated with the extend of inflammation. Decrease of glutathione concentration may be associated with aging [29] and the pathogenesis of many diseases, including AIDS [30], Alzheimer's disease [31], alcoholic liver disease [32] and pulmonary diseases [33,34], e.g. chronic obstructive pulmonary disease and asthma. γ-GluCys forms complexes with As(III) resulting in inactivation of As in the plants [35].

Glutathione, cysteine, homocysteine, cysteinylglycine and γ-glutamylcysteine are the most important endogenous aminothiols in human biofluids. Moreover, the presence of N-acetylcysteine and thioglycolic acid in urine has been confirmed and these compounds are assumed to be endogenous constituents of human urine [36,37].

Nε-homocysteinyllysine (Fig. 2E) was recently identified [38] as a novel metabolite in human and mouse plasma. This isopeptide is generated by proteolytic degradation of N-homocysteinylated protein in a liver, and may be considered as an important pathology related component of homocysteine metabolism in human and mice.

1.3. Thiol drugs

Cysteamine, N-acetylcysteine, captopril, mesna, 2-mercaptopropionylglycine, D-penicillamine, thyreostats and thiopurines are thiols commonly used as drugs in the treatment of many diseases. Severe adverse reactions to oral thiol-drugs use have been described in subjects in which abrupt incremental dosing of the drugs were started. This suggests that monitoring the concentrations of these compounds in biological fluids, over the course of therapy, is warranted and consequently a number of different methods for quantification of these thiols have been described.

Cysteamine (mercaptamine) is used therapeutically as a radioprotective agent [39] and prevents severe liver damage after paracetamol poisoning [40]. First of all it is a drug widely used for the treatment of nephropathic cystinosis, a rare autosomal recessive disease characterized by poor growth, renal Fanconi syndrome and renal glomerular failure [41].

N-acetylcysteine (NAcCys) is an endogenous product of cysteine metabolism. Moreover, it is a drug widely used for the treatment of paracetamol overdose [42] and as mucolytic agent for administration into respiratory tracts to loosen secretions [43]. NAcCys reduces the risk of re-hospitalisation among patients with chronic obstructive pulmonary disease [44] and prevents the reduction in renal function induced by iopromide, a nonionic, low-osmolality contrast agent, in patients with chronic renal insufficiency [45]. NAcCys administration has been reported to be beneficial in systemic sclerosis, HIV infection and septic shock [14]. The in vitro incubation of erythrocytes with NAcCys [46] shows the putative biological mechanism for replenishing intracellular GSH level during oxidative insult favoring GSH regeneration via a reversible thiol exchange reaction ($^{\text{NAcCys + GSSG} \leftrightarrows \text{GSH + GS-NAcCys}}$) while rising level of GSSG. This hypothesis for supporting GSH level while attenuating the rate of GSSG formation during acute oxidative stress is clearly different from the earlier discussed mechanism of NAcCys action involving deacetylation for increasing intracellular

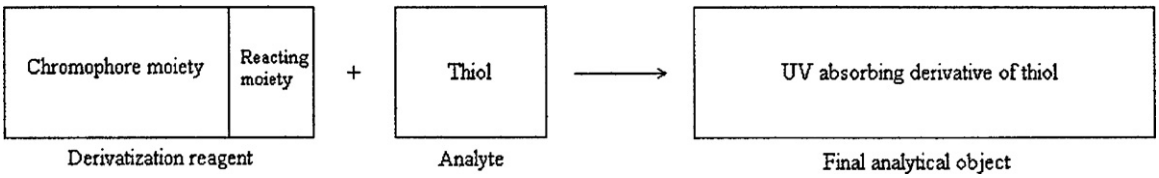

Fig. 3. Universal scheme of UV derivatization reaction of thiols.

cysteine concentration as the rate-determining step in GSH synthesis. Very recent study [47] has proved the positive impact of high dose oral NAcCys pretreatment on reducing exercise-induced oxidative stress in erythrocytes. The authors postulate a causal link between NAcCys pretreatment, global metabolomic down regulation, oxidative stress attenuation, and fatigue delay during prolonged exercise. Besides GSH and GSSG they show evidences for four unknown biomarkers of oxidative stress 3-methylhistidine, carnitine, creatine, and O-acetyl-carnitine.

Captopril (1-[3-mercapto-2-(S)-methyl-1-oxopropyl]-(S)-proline) is widely used in the treatment of essential hypertension [48] and to reduce mortality in patients with acute myocardial infraction [49]. As a chelating agent, has been proposed to complex cysteine in the treatment of cystinuria, an autosomal recessive genetic defect of the transepithelial transport of cystine and other dibasic amino acids in the kidney [16].

Mesna (sodium-2-mercaptoethanesulfonate) is an important thiol compound that prevents hemorrhagic cystitis in patients who receive oxazaphosphorine treatment, such as ifosfamide or cyclophosphamide, by neutralizing the highly reactive urotoxic metabolites of oxazaphosphorines locally in the urine [50].

2-Mercaptopropionylglycine (MPG, Tiopronin) is used in the treatment of rheumatoid arthritis [51], hepatic diseases, heavy metal poisoning [51,52] and as a mucolytic in respiratory disorders [14,51]. It has been shown to protect against ischemic/reperfusion-mediated injury [53] and could also be effective against radiation-induced damages, even at low doses [54]. MPG is frequently used for the treatment of cystinuria [16,55]. It prevents the formation of cystine stones in kidney when there is too much cystine in the urine. MPG transforms cystine into a mixed disulfide, 50 times more soluble than cystine itself [16].

D-penicillamine (2-amino-3-mercapto-3-methylbutanoic acid) is a thiol drug used in the treatment of heavy metal poisoning [51,52] cystinuria [16,55], and Wilson's disease, an autosomal recessive disorder of copper transport [56]. It is also used as antifibrotic agent to treat scleroderma [57] and as antirheumatic drug to treat patients with active rheumatoid arthritis [58].

Methimazole (1-methyl-2-mercaptoimidazole) and propylthiouracil (6-propyl-2-thiouracil) are drugs widely used for the treatment of hyperthyroidism in humans [59] and against Grave's disease [60].

2. Ultraviolet derivatization reagents for thiols

Ultraviolet detection is commonly used technique in high performance liquid chromatography and capillary electrophoresis. Derivatization reagent reacts with thiol functional group to produce final analytical object – UV absorbing derivative (Fig. 3). There is a number of thiol-reactive reagents, commercially available or in-laboratory made, that allow UV-absorbance detection. The reagents can be classified by type of the reactive moiety into four categories: activated halogen compounds, activated double bound compounds (enons), disulfides, and the others. An ideal reagent should react rapidly and specifically with thiol at lowest possible temperature and weakly acidic pH to prevent oxidation of the analytes leading to artefactual results of analysis. As can be seen from the data gathered in Table 1 the reagents BCPB, CMQT and NEM are approaching to fulfill these criteria. Experimental details for particular UV-derivatization reactions of thiols are shown in Table 1.

2.1. Compounds with activated halides

Compounds with activated halides (Fig. 4A–G) are very important derivatization reagents commonly used for determination of endogenous and exogenous thiols in various real world samples. The halide leaving group is easily displaced by the sulfur atom from the analyte sulfhydryl, leading to formation of a stable thioether with well defined absorption maximum.

2-Halopyridinium and 2-haloquinolinium salts (Fig. 4B and C), widely used for UV derivatization of hydrophilic thiols in their HPLC and CE analysis in physiological fluids [37,38,61–91] react rapidly and quantitatively with hydrophilic thiols in slightly alkaline water solution to form stable S-pyridinium or S-quinolinium derivatives, respectively (Fig. 5A and B). The derivatization scheme takes advantage of great susceptibility of the pyridinium/quinolinium molecule at 2 position to nucleophilic displeacement and the high nucleophiliciy of the thiol group. The reaction is completed within 1–15 min at room temperature, enabling full automation of the procedure (Fig. 6) for multi thiol measurement in plasma [75]. The reaction is accompanied by analitycally advantageous bathochromic shift (Fig. 7) from reagent maximum to that of derivative (Table 1). Among the reagents with different substituents shown in Fig. 4B and C the biggest shift was observed in the case of benzyl substituent (Fig. 7) at quaternary nitrogen atom (BCPB). This phenomenon allows the use of a large reagent excess in order to push reaction to the completion without excess reagent peak on the chromatogram [66]. Of different functionalities of aminothiols (e.g. –COOH, $–NH_2$, –SH) potentially able to undergo nucleophilic attack at the 2-position of quinoline/pyridine molecule, only the –SH group reacts [63,68]. The thiol selectivity of this group of derivatization reagents was proved in the presence of other sample components such as different protein amino acids and monosacharides [63,68].

The pyridinium/quinolinium derivatives of thiols are the convenient objects for CE study for that they possess positive charge within the whole pH range [86–88]. This is a result of permanent positive charge on quaternary nitrogen atom regardless of pH of the background buffer (Fig. 8). The net positive charge of the derivative (for cysteine and metabolically related sulfur amino acids) as a whole approaches +2 in strong acidic environment due to protonation of the amino group and ion suppression of the carboxylic group.

Benzofurazan reagents 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F) and ammonium-7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F), and monobromobimane (mBrB) (Fig. 4A and D) are very popular fluorogenic reagents, however, their thiol derivatives show substantial UV absorption with well defined maxima what makes UV detection possible [92–97]. ABD-F, SBD-F have much higher selectivity to thiols than bimane. The drawbacks of using benzofurazans are a long derivatization time and elevated temperature requirements (Table 1). Monobromobimane reacts rapidly, but not specifically with thiols. The reaction scheme of

Table 1
Characteristic of thiol UV-derivatization reactions.

Reagent	Absorption maximum [nm]		Derivatization conditions				References
	Reagent	Derivative	Environment	Temp. [°C]	Time [min]	Others	
Compounds with activated halides							
ABD-F	330	220, 385	Sodium borate buffer, pH 8	50	5–10		[92,93]
BAN	NR	246	Phosphate buffer, pH 7	Room	30	BAN in acetone	[102]
BCPB	276	315	TRIS buffer, pH 7	Room	10		[66,67]
			TRIS or phosphate buffer, pH 8–8.2	Room	15		[61,62,74]
BMP	NR	254	Acetone + anhydrous K_2CO_3	105 ± 5	30		[103]
CMPI	293	312	TRIS buffer, pH 8.2	Room	15–30		[64,65,73]
CMLT	325	350	Phosphate buffer, pH 7.4	Room	3		[89]
CMQT	325	348	Ammonium acetate buffer, pH 6.2	Room	2		[68]
			TRIS or phosphate buffer, pH 7.2–8.2	Room	1–10		[37,63,69–72,75–88,90]
CPPI	293	314	TRIS buffer, pH 8.2	Room	30		[73]
mBrB	NR	210, 234, 250	pH ~8.5 (addition NaOH)	Room	5	In darkness	[94–96]
		390	Sodium borate buffer, pH 9	Room	60		[97]
pBPB	NR	263	Derivatization in blood	Room	15		[99]
			Alkaline solution	Room	30		[100,101]
SBD-F	NR	220	Sodium borate buffer, pH 9.5	60	60		[92]
Enones							
EAA	NR	266	Phosphate buffer, pH 7.4	Room	20		[106]
NEM	NR	214	TRIS buffer, pH 7	Room	5	NEM in methanol	[8,105]
Disulfides							
DTNB	325	326–357	TRIS or phosphate buffer, pH 7.4-8.9	Room	5–20		[109–113]
2,2′DTP	NR	282	Phosphate buffer, pH 7.5	20-25	NR	Determine 2TP at 343 nm	[149–151]
4,4′DTP	NR	NR	Phosphate buffer, pH 7–7.5	Room	5	Determine 4TP at 324 nm	[115–117]
Miscellaneous							
TCDI	<240	260–290	Phosphate buffer, pH 8	37	20		[118,119]
ESB	NR	254	Borate buffer, pH 7.5	Room	2	ESB in methanol	[107]

NR, not reported.

mBrB with Hcy is shown in Fig. 5C. It should be noted that mBrB and its hydrolysis products give multiple peaks on the chromatograms [96].

For derivatization of thiols, represented by captopril and 2-mercaptopropionylglycine, p-bromophenacyl bromide (pBPB) was used [98–101]. pBPB reacts with captopril to form a product that shows ultraviolet-absorbing properties [98,99]. Captopril derivative was detected at 260 nm. Optimal reaction pH of MPG with pBPB occurred at 14 and was complete at 20 °C after 30 min [100]. A sufficiently high concentration of pBPB was required for the reaction to proceed efficiently. p-Bromophenacyl bromide may also react with carboxylic groups but in anhydrous environment and at elevated temperature. Moreover, the sensitivity is not high enough for the analysis of plasma samples.

2-Bromo-2′-acetonaphthone (BAN) is another derivatization reagent with activated halide [102]. This reagent was rapidly reacted with captopril and the derivative showed a high molar absorptivity value (34,000 M^{-1} cm^{-1} at 246 nm). Complete derivatization reaction occurs after 30 min.

3-Bromomethyl-4-isopropylphenazone (BMP) is a nonselective derivatization reagent reacting with sulfhydryl and amino groups [103]. Meanwhile for captopril, having only one reactive centre (–SH group), no reaction byproducts appeared and fully derivatized product was achieved after heating for 30 min at 105 °C. The derivatized compound has shown maximum UV-absorption at 243 nm.

2.2. Compounds with activated double bound, disulfides and the others

Maleimide-type reagents, to which N-ethylmaleimide (NEM, Fig. 4I) is belonging, are frequently used for the determination of thiols with fluorescent detection. The drivatization reaction involves the addition of thiol across the double bond of maleimide (Fig. 9A). UV absorption of NEM derivatives of thiols were also reported to be the base of absorption detection HPLC methods [8,104,105]. It should be noted that NEM also binds amino groups at pH >7.5, even if more slowly as compared with thiol functionalities. If titration procedures require long incubations at alkaline pH, the excess of NEM must be removed before the alkalization of the medium [8]. The maleimide-thiol conjugates are unstable and may undergo further rearrangement, leading to the formation of two ring-cleaved products yielding multiple chromatographic peaks.

The ability of ethacrynic acid (EAA, Fig. 4H) to react with thiol groups has been described by Cavrini et al. [106]. EAA reacts with thiol to produce stable adduct with well-defined absorption maximum at 266 nm. The reagent was applied to determination of NAcCys and captopril in pharmaceutical formulations.

1,1′-[Ethenylidenebis(sulfonyl)]bis-benzene (ESB, Fig. 4M) contains a conjugated double bond reactive toward nucleophiles [107]. Nucleophile addition to the double bond does not produce a new chiral centre and single chromatographic peaks are also obtained with chiral thiols, e.g. glutathione. The reagent reacts rapidly (2 min) under mild conditions.

Among chromophor-introducing disulfide reagents (Fig. 4J–L), invented by Ellman in 1959 [108], 5,5-dithio-bis-2-nitrobenzoic acid (DTNB, Fig. 4L) is still in use in liquid phase separation methods [109–112]. This symmetric aryl disulfide, reacts with reduced thiol to produce a mixed disulfide (RS-TNB) and one equivalent of 5-thio-2-nitrobenzoate (TNB) anion (Fig. 9B), which is quantified by its strong visible absorbance at 412 nm. However, utilizing a chromatographic methods, it becomes possible to separate the mixed disulfide produced in the reaction [110,112–114].

As an alternative, 4,4′-dithiopyridine (4,4′DTP, Fig. 4J) or 2,2′-ditiopyridine (2,2′DTP, Fig. 4K) were used in place of Ellman's reagent [115]. Dithiopyridine reacts with sulfhydryl compounds via thiol-disulfide exchange. The intrinsic reactivity of thiol for 4,4′DTP is much higher than for Ellman's reagent, thus the reaction can be carried out at pH ≥ 4.5 instead of at pH 8.0 [116]. The investigated thiol reacts with 4,4′DTP to form a mixed disulfide and 4-thiopyridone (4TP). Pre-column derivatization techniques rely on detection of the aromatic moieties of the mixed disulfide, whereas in post-column techniques the thione anion is detected [117].

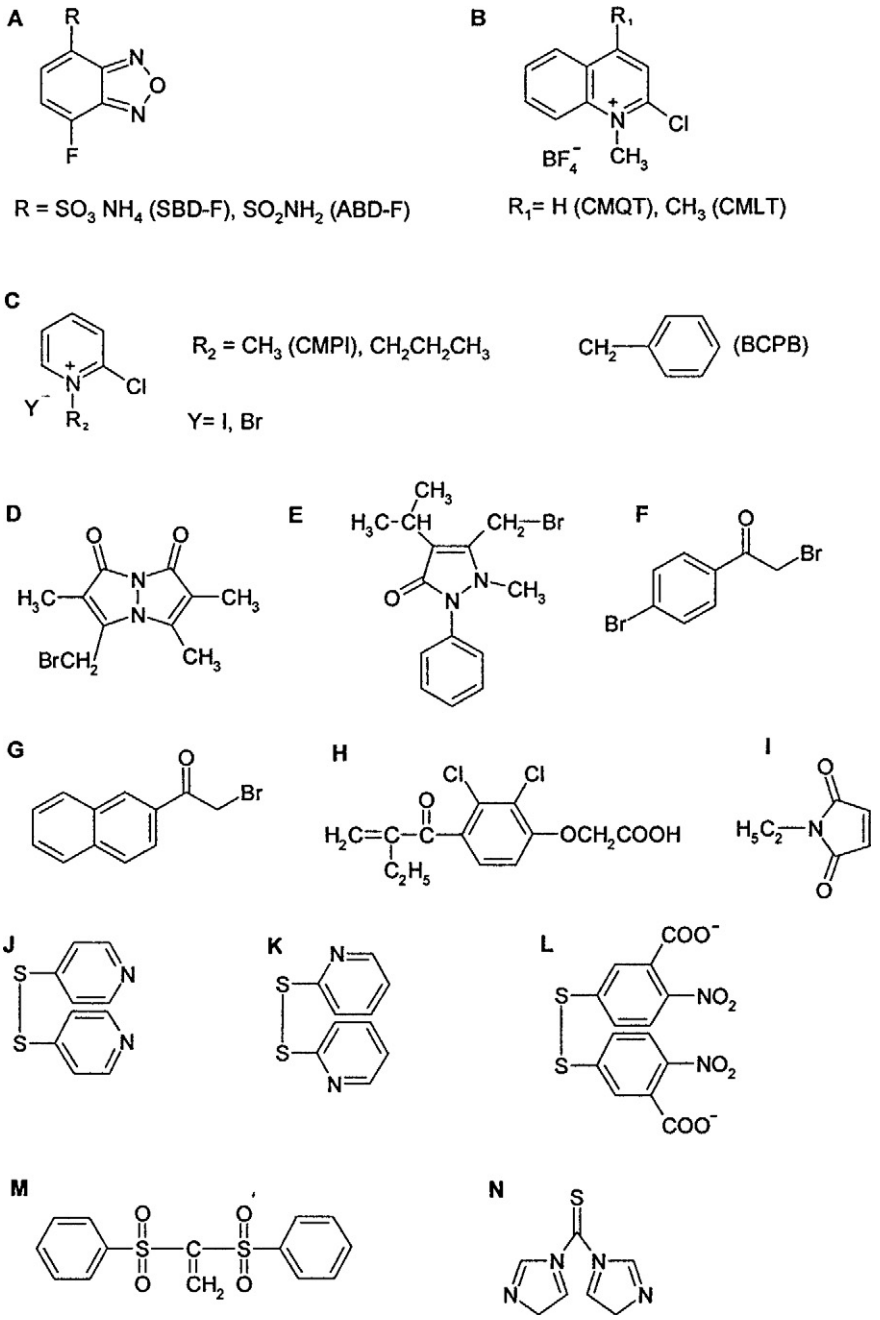

Fig. 4. Structures of UV-derivatization reagents. A, benzofurazans: aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (SBD-F), 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F); B, 2-haloquinolinium salts: 2-chloro-1-methyllepidinium tetrafluoroborate (CMLT), 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT); C, 2-halopyridinium salts: 1-benzyl-2-chloropyridinium bromide (BCBP), 2-chloro-1-methylpyridinium iodide (CMPI), 2-chloro-1-propylpyridinium iodide (CPPI); D, monobromobimane (mBrB); E, 3-bromomethyl-4-isopropylphenazone (BMP); F, p-bromophenacyl bromide (pBPB); G, 2-bromo-2′-acetonaphthone (BAN); H, Ethacrynic acid (EAA); I, N-ethylmaleimide (NEM); J, 4,4′-dithiopyridine (4,4′DTP); K, 2,2′-ditiopyridine (2,2′DTP); L, 5,5-dithio-bis 2-nitrobenzoic acid (DTNB); M, 1,1′-[ethenylidenebis(sulfonyl)]bis-benzene (ESB); N, 1,1-thiocarbonyldiimidazole (TCDI).

Amarnath et al. [118,119] proposed method of derivatization for aminothiols that modifies both amino and thiol groups. Treatment with 1,1′-thiocarbonyldiimidazole (TCDI, Fig. 4N) in slightly basic solutions converts thiols rapidly and quantitatively to a very stable derivatives. 2-Thioxothiazolidine-4-carboxylic acid, 2-thioxothiazolidine-4-carbonylglycine, 2-thioxotetrahydro-1,3-thiazine-4-carboxylic acid and 5,5-dimethyl-2-thioxothiazolidine-4-carboxylic acid were generated in the presence of TCDI from cysteine, cysteinylglycine, homocysteine and penicillamine, respectively. The resulting cyclic derivatives exhibit intense UV absorption with a maximum at 272 nm. Excess TCDI is hydrolyzed completely to imidazole that did not have significant UV absorption above 240 nm.

3. Pre-separation considerations

The analysis of biological samples present a variety problems: (1) large number of individual compounds in the sample, leading to difficulty in resolving the analytes of interest, (2) the presence of components, such as proteins, that can modify the chromatographic or electrophoretic column, (3) low concentrations of exogenous or endogenous compounds of interest, leading to detec-

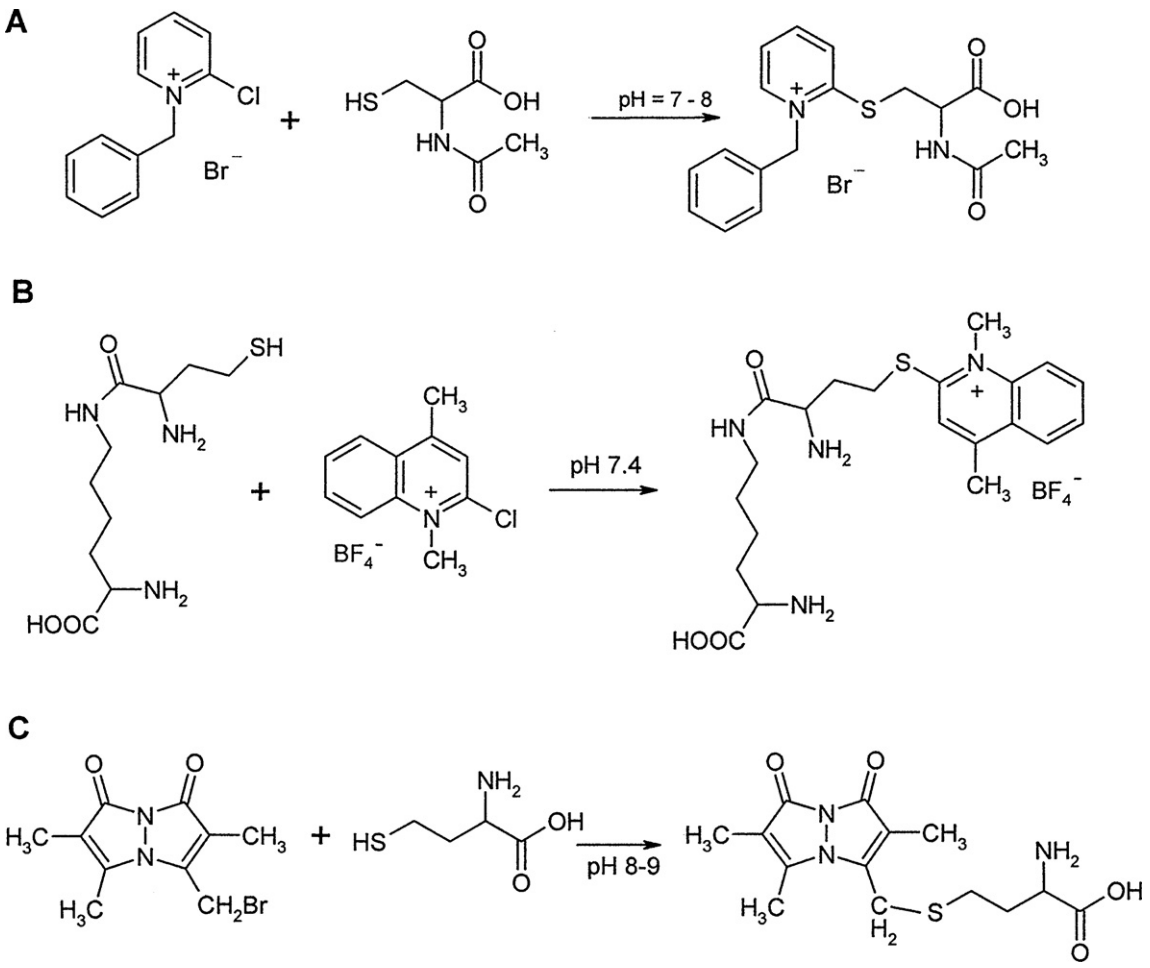

Fig. 5. Derivatization reaction equations of: A, NAcCys with BBCP; B, Nε-HcyLys with CMLT; C, homocysteine with mBrB.

tion difficulties, and (4) conjugation of analytes to protein and/or low-molecular-mass components of the analyzed mixture. Moreover, 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.

3.1. Most frequently analyzed samples

The biological fluids that are commonly analyzed are plasma and urine. Whole blood is less often analyzed, with exception in the case of small animals and forensic toxicology where this may be necessary. Bile, sweat, tears, milk and salvia can also be analyzed. The ease with which samples can be analyzed increases with the degree of fluidity, bone being the most difficult to handle, and cerebrospinal fluid the easiest. The biological fluid most commonly analyzed is plasma. Urine is usually free from proteins and lipids but contains many other components, the concentrations of which depend largely on diet and lifestyle. Detection and determination of urinary components, including thiols, is therefore very difficult. In analysis of urinary thiols it is often difficult to obtain 24-h urine collections. Therefore, random urine samples frequently are collected for analysis of thiols, and to compensate for variations in

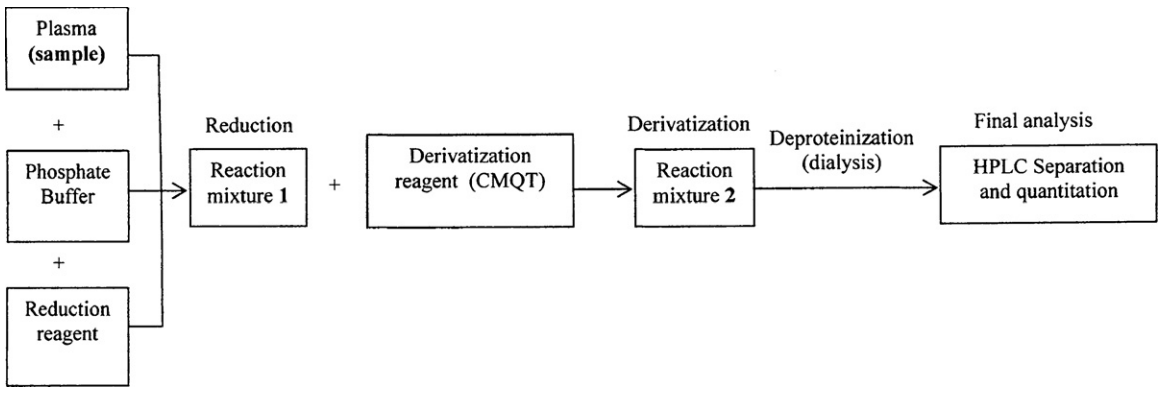

Fig. 6. The schematic of the automatic analytical system for determination of total main plasma thiols. Reproduced from Fig. 1 in Ref. [75].

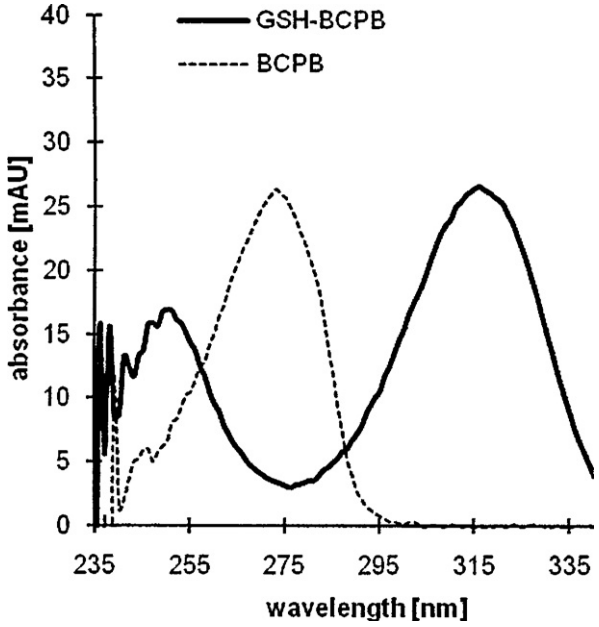

Fig. 7. Comparison of the absorption spectra of BCPB derivatization reagent (dotted line) and BCPB glutathione derivative (continuous line).

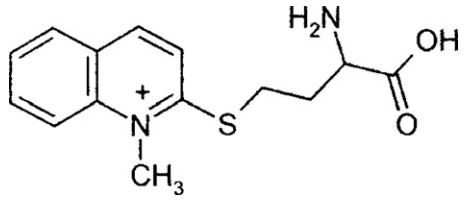

Fig. 8. Chemical structure of Hcy-CMQT derivative.

urinary output, the urinary concentrations of the thiols are corrected against creatinine values [91]. Urinary thiol concentration is usually expressed in mmol per mol of creatinine.

3.2. Sample collection and handling

Sampling is a critical step in the procedure of accurate determination of aminothiols. Non-standard processing leads to under- or overestimation of concentration of aminothiols. Food intake, diurnal variations, posture during blood collection, sample centrifugation, hemolysis, storage conditions, may change the plasma total homocysteine concentration [120]. For example, blood samples collected in the supine position have ~10% lower mean total homocysteine concentrations than those collected in the sitting position [120,121]. After blood collection, but before removal of the blood cells, many authors observe time- and temperature-dependent increase in total homocysteine concentration [120–123]. The increase is prevented by immediate centrifugation and removal of blood cells or by keeping samples cooled on ice until centrifugation [120,121]. Hemolysis may interfere with some assays but will usually not change the plasma total homocysteine concentrations [120]. However, hemolysis can either cause overestimation of glutathione in the plasma because the GSH level in erythrocytes is 500-fold higher than that in plasma, or, without refrigeration, leads to underestimation of GSH and overestimation of GSSG in whole blood and plasma when autoxidation and proteolysis of GSH in the plasma are not repressed [8,124,125]. Sampling conditions for homocysteine measurement have been well investigated and reviewed in details elsewhere [120,121,126]. All aspects of sample collection should be uniform because minor but systematic variation in procedure may influence the results and, consequently, study outcome. Therefore, Refsum et al. [120] recommend procedures for sample collection and handling. Time of day, fasting state, and posture during sample collection should be uniform. One type of collection tube should be used. Blood samples should be centrifuged within 1 h or kept cold until centrifugation (<8 h). Plasma/serum samples can be mailed to the laboratory at room temperature. Centrifugation and removal of the blood cells,

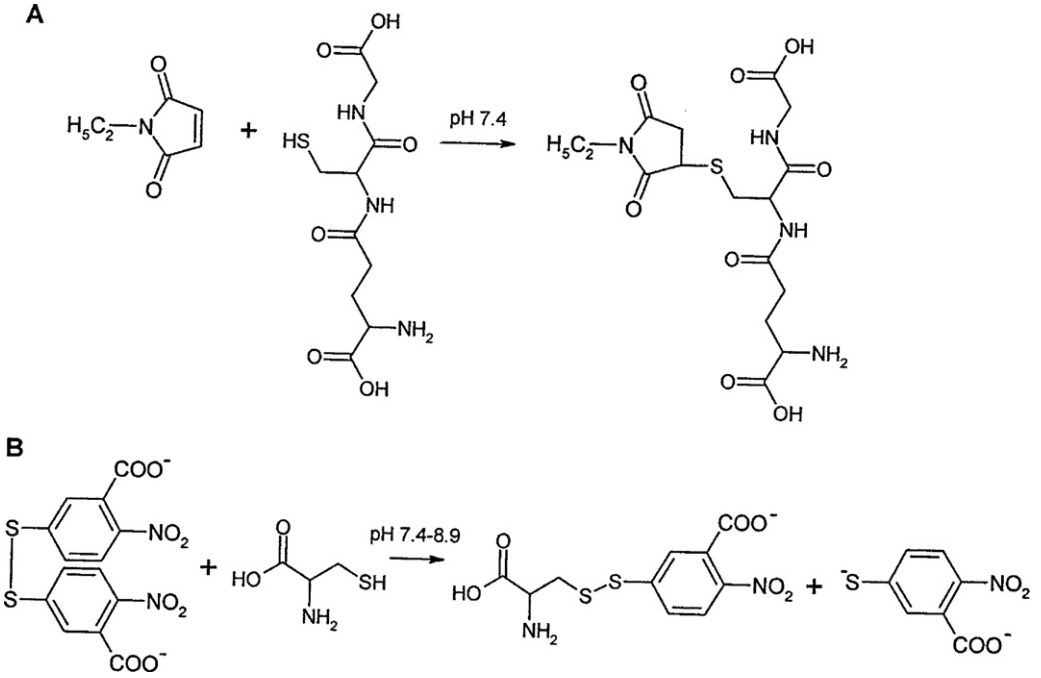

Fig. 9. Equations for the derivatization reaction of: A, GSH with NEM; B, Cys with DTNB.

or cooling of the sample, should take place as soon as possible [120]. Homocysteine and other total aminothiols in plasma are stable in the frozen state (−80 °C) for years; unfrozen at low temperatures, for several weeks; and at room temperature for several days, making feasible the transport of unfrozen samples to the laboratory [127].

Collection and storage of urine samples is less difficult. Experiments [69] on stability of the endogenous thiols in urine ex vivo have proved that both the thiols cysteine and cysteinylglycine are fairly stable during the early hours after urine collection. This means that urine, kept at room temperature, can be safely analyzed for the thiol redox status within 4–5 h. Total loss of reduced cysteine in urine was observed after 14 days at room temperature and after 21 days in the refrigerator (4 °C). A decrease of 96% was observed within 3 months in urine stored at −20 °C [69].

Concentration of aminothiols in serum is at average about 10% higher than in plasma. Since to receive serum, blood must remain at room temperature to allow clotting it is recommended to determine aminothiols in plasma. Although several anticoagulants including heparin and citrate are available, EDTA is most commonly used. EDTA-plasma is much more safe from metal catalyzed reactions.

Most of the aminothiols in human biofluids are present in the disulfide forms rendering them inaccessible to derivatization reagent. In order to determine their total contents disulfide bonds must be cleaved to liberate a free thiol. For this purpose dithiothreitol, dithioerythritol, 2-mercaptoethanol, sodium or potassium borohydride and trialkylphosphines were used. Sulfhydryl reagents dithiothreitol, dithioerythritol or 2-mercaptoethanol can form derivatives with derivatization reagents, finally interfering with peaks of analytes. Poorly soluble in water tributylphosphine (TBP) does not react with thiol specific reagents but is an irritant with an unpleasant odor. Introduced in 1997 tris(2-carboxyethyl)phosphine (TCEP) for determination of homocysteine in plasma [128] is non volatile, soluble and stable in slightly acidic and basic water solutions, and odorless. There are reports showing unequal efficiency of phosphines as –S–S– linkage reducing reagents. Krij et al. [129] compared TCEP to TBP and concluded that there was low agreement between the two reducing reagents in the determination of total Hcy, Cys, CysGly and GSH. This finding is supported by the authors' unpublished results. In general, TCEP is preferred for its user friendliness and better repeatability. Different reducing reagents including sulfhydryl compounds and potassium or sodium borohydride should not be used interchangeably.

Direct injection of biological samples into the HPLC or CE system is not recommended. Proteins are the most abundant components in biological samples and their presence in injected samples may attenuate the performance of the analytical procedure and shorten the lifetime of the instrumentation. The removal of any protein species is usually carried out by protein precipitating agents. Conventionally, acidification, addition of an organic solvent (acetonitrile, methanol, acetone), ultrafiltration and dialysis are commonly employed as means of protein elimination. Sulfosalicylic acid, trichloroacetic acid, trifluoroacetic acid, perchloric acid and meta-phosphoric acid are the acidic agents used for deproteinization [130]. This type of deproteinization is satisfactory for total thiol determination in biological samples. In the case of GSH and GSSG, and other thiol redox couples as biomarkers of exercise-induced oxidative stress, acidification of samples can induce partial oxidation of thiol group upon denaturation of oxyhemoglobin (for example in whole blood) with concomitant generation of hydrogen peroxide that results in overestimation of the disulfide forms. Addition of NEM [131] or CMQT [72], ultrafiltration [132], or application of a new strategy for assessment of the redox status via differential rates of GSH oxidation by CE-ESI-MS [46] has been proposed to prevent oxidation artifacts.

3.3. Total aminothiols and their redox species

The facile oxidation of sulfhydryl compounds results in a variety of disulfide forms present in living creatures. Reduced, free oxidized and protein-bound forms of cysteine, cysteinylglycine, glutathione and homocysteine comprise the plasma redox thiol status. All redox forms are measured together in assays that determine total aminothiol concentrations (Fig. 10). In healthy subjects, cysteine is the most abundant plasma aminothiol (total concentration ~250 μM). About 65% is protein bound, 30% is free oxidized, and 3–4% is reduced [72,133]. The total plasma concentration of cysteinylglycine is less (~30 μM), about 60% is protein-bound, 30% is free oxidized, and 10% is reduced [72,133]. The intracellular glutathione is found in milimolar concentrations. Plasma contain lower GSH levels (total concentration, ~6 μM), 65% of plasma glutathione is in the reduced form [3,72,133]. Normal human plasma contains total concentration of homocysteine close to 10 μM [72,120,133–135]. Of this total, only 1–2% occurs as the thiol, homocysteine. The remaining 98% is in the form of disulfides. About 75% of the total is bound to protein, whereas the remainder occurs in non-protein-bound forms: homocystine, homocysteine-cysteine disulfide, and more minor amounts of other mixed disulfides, e.g. homocysteine-cysteinylglycine disulfide [135]. The term total thiol, as applied to urine samples, refers to the sum of concentrations of reduced and oxidized (symmetrical and unsymmetrical disulfides) forms. Normal urine is generally free of proteins and accordingly it does not contain protein-bound thiols. Total cysteine in adult human urine consists of 6% in the reduced form and 94% in the oxidized form, and approximately 14% of total urinary cysteinylglycine is reduced and 86% oxidized [69].

Since altered redox status of aminothiols has been observed in a number of diseases [136], knowledge of concentration of particular redox forms is desirable. However, for the reason of interconversion between thiol and disulfide forms after sample collection, autooxidation of thiols, lack of adequately sensitive methods and technical challenges in sampling, measurement of the individual redox components is difficult. Reduced forms are oxidized ex

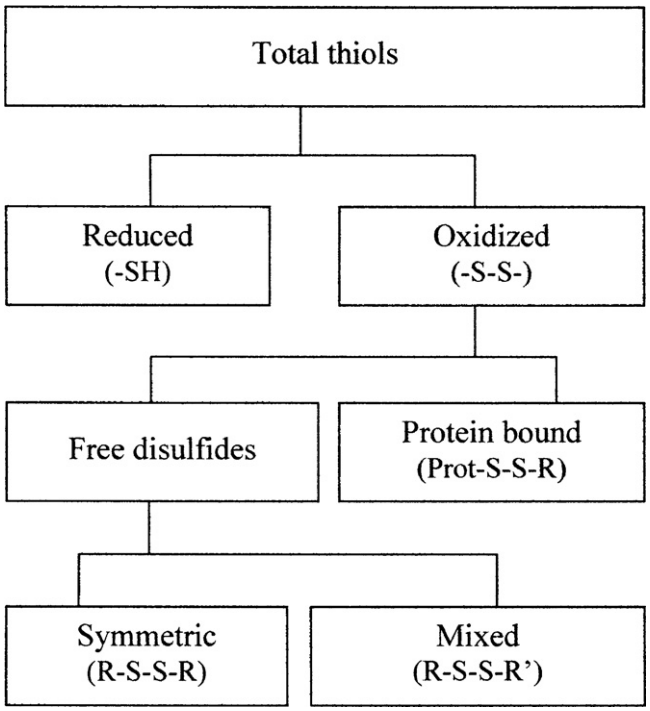

Fig. 10. Red-ox forms of thiols in human plasma.

vivo in minutes [72,137]. Rossi et al. [125] have found that oxidation of amino thiol occurs not only after restoration of neutral – slightly alkaline pH following deproteinization, but even during deproteinization with acid itself. Consequently, in most clinical studies the total thiols are determined. A rapid reaction with NEM [125,131] or CMQT [72] protected these oxygen-sensitive analytes, by irreversible thioether linkage formation, in the early stages of plasma sample preparation. Described in these reports procedures enable determination of all thiol redox forms reduced, free oxidized and protein-bound. A shift in the thiol/disulfide equilibrium after irreversible blocking reduced forms is possible as well. According to Rosenfeld [138] such problems can be expected during analysis of plasma for redox species of all thiols.

4. Separation of ultraviolet-derivatized thiols

Ultraviolet-absorbing derivatives of aminothiols are usually separated by HPLC or CE. These techniques applied to bioanalysis of thiols do not use just one simple separation step, but rather consist of several sample pretreatment steps which simplify the matrix, often preconcentrate and chemically modify the analytes, but at the same time are sources of assay variability. In order to minimize the contribution of sample preparation, injection variation and column/capillary deterioration to the final results, the internal standard mode of quantification can be applied. Internal standard is a stable labeled, structurally similar analog added to both calibration standards and samples at known and constant concentration to facilitate quantification of the target analytes. With an internal standard method, compound of known purity that does not cause interference in the analysis is added to the sample mixture. If a known quantity of standard is added to the unknown prior to any manipulations, the ratio of standard to analyte remains constant because the same fraction of each is lost in any operation. An ideal internal standard for thiol measurements should possess similar physicochemical properties, go through all steps of the procedure, and elute close to the analyte. In theory, almost every exogenous thiol may be used as internal standard. N-acetylcysteine [139,140], cysteamine [141,142], and 2-mercaptopropionylglycine [142,143] have been frequently used as internal standards. Since the selection of an internal standard concentration that yield precise and accurate quantification for multiple thiols with different concentration ranges can constitute a challenge, some procedures apply two compounds [70–72]. 2-Mercaptopropionic acid and 2,2′-dithiodipropionic acid were used for reduced and oxidized forms of glutathione and homocysteine, and 3-mercaptopropionic acid and 3,3′-dithiodipropionic acid for reduced and oxidized forms of cysteine and cysteinylglycine in human plasma or urine [70–72]. Such approach, at the same time, fulfils the requirements for proximity of the elution time (Fig. 11) and to be subject to all steps of the analytical procedure including reduction of disulfide bounds [72]. Endogenous compounds can be used as internal standards provided their native concentrations are at least one order of magnitude lower than analytes.

4.1. High performance liquid chromatography separation

Ultraviolet-absorbing derivatives of aminothiols are usually separated by reversed-phase chromatography with ion-pairing using either isocratic or gradient elution.

Most commonly used reversed-phase HPLC separation is based upon the non-polar, or hydrophobic, interaction between non-polar sample molecules and the non-polar stationary phase. Aminothiols or their derivatization products, often containing ionizable or ionic moieties, can be too polar to be retained by reversed-phase mode. In instances such like this, and when mobile

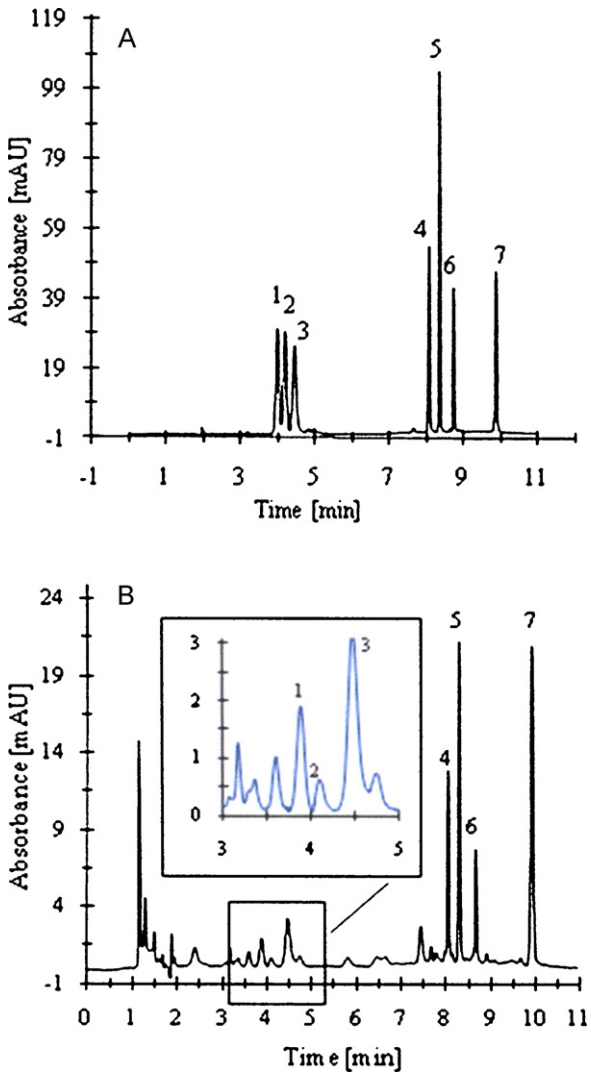

Fig. 11. Typical chromatograms of main plasma thiols: (A) water standard solution; (B) reduced thiols in plasma. Peaks: 1, GSH; 2, Hcy; 3, 2-mercaptopropionic acid; 4, 3-mercaptopropionic acid; 5, Cys; 6, CysGly; 7, CMQT. Reproduced from Fig. 1 in Ref. [72].

phase pH manipulation does not help, addition of ion-pairing reagent can prove to be useful, and allow the development of a good separation. The reagent is added to the mobile phase and allowed to equilibrate with the column. The nonpolar end of the reagent is held on the nonpolar stationary phase, be it C_8 or C_{18}, leaving the charged functional group in the mobile phase. Ionic analytes of the opposite charge are attracted to the column causing better retention. Sulfonic acids (alkyl, C_4–C_8) are used to enhance retention of cationic analytes. For anionic species tetraalkylamonium halides can be applied. Alkyl sulfonic acids (sodium salts) are expensive, strongly bind to the stationary phase and require long column equilibration time [144]. According to Dolan [145] ion-pairing reagents can never be washed fully from the column, even with extensive column flushing, and is advised to dedicate a particular column to ion-pairing application only. A satisfactory improvement of retention of the cationic thiols analytes can be achieved by chromatographing them with the mobile phase containing trichloroacetic acid [61–67,69–85,89,91]. Trichloroacetic acid acts as pairing reagent and at the same time constitute the main component of the mobile phase buffer.

HPLC separation can be performed using either isocratic or gradient elution. For multiple aminothiols with different concen-

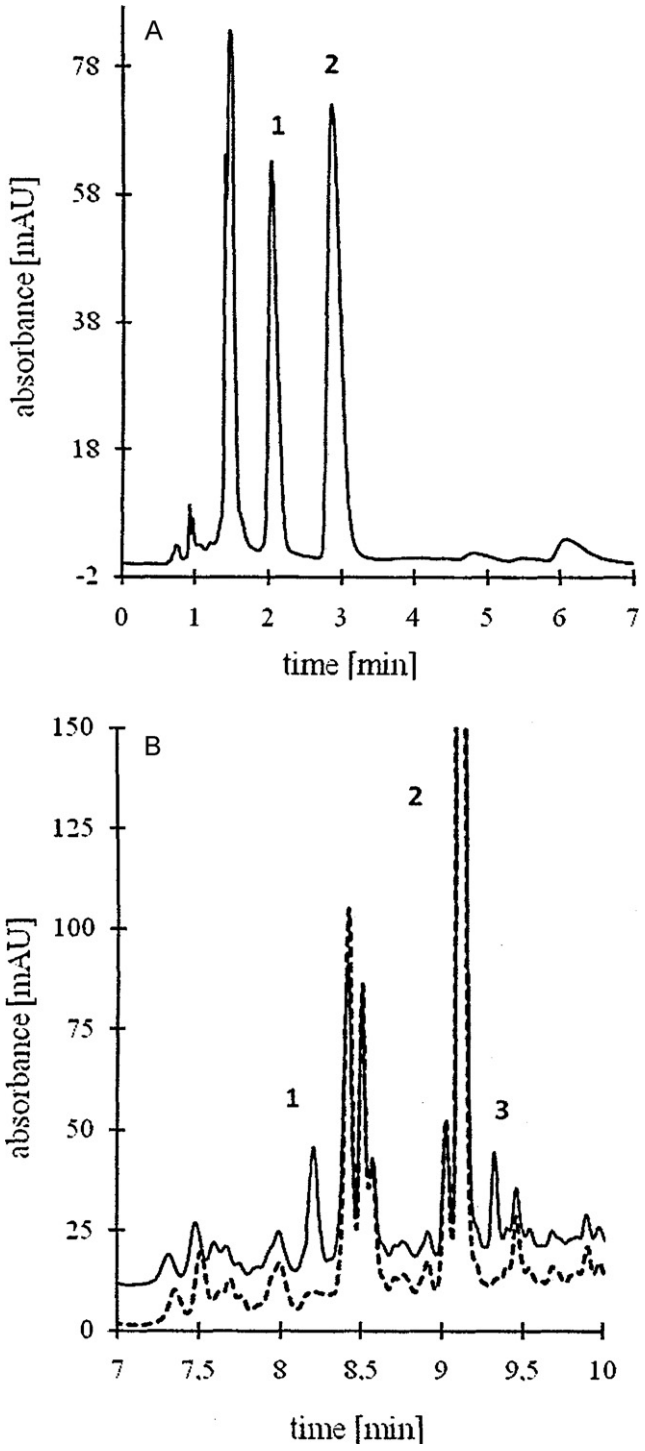

Fig. 12. Chromatograms of urine sample after reduction and derivatization with CMQT. A, isocratic elution [70]; B, gradient elution [83]. Peaks: 1, 2-mercaptopropionylglycine; 2, cysteine; 3, D-penicillamine. Reproduced from Fig. 7 in Ref. [70] and from Fig. 3 in Ref. [83].

tration ranges in plasma, and particularly in urine, gradient elution is preferred [75–85], as is the case for cysteamine and endogenous thiols in plasma [80] or cysteine [70] and drugs tiopronin and D-penicillamine in urine [83] shown in Fig. 12. When endogenous thiols in plasma are not of interest isocratic elution for separation of cysteamine can be applied in order to shorten analysis time [80].

In general, liquid chromatographic methods for determination of thiols and their disulfides in biological samples involve three cardinal steps reduction of disulfide bonds, derivatization of free thiols with a proper UV-introducing reagent, and separation by HPLC. Detailed information on the experimental procedures of these steps, as well as some minor operations, utilized by numerous methods are available in Tables 1 and 2 .

4.2. Capillary electrophoresis separation

Several modes of operation are available within capillary electrophoresis technique including capillary zone electrophoresis (CZE), electrokinetic chromatography, isotachophoresis, capillary gel electrophoresis, capillary electrofocusing and capillary electrochromatography. For determination of UV-derivatized hydrophilic thiols almost exclusively CZE was used. In majority of cases derivatization for CE is detection oriented, associated with the introduction of UV-absorbing or fluorescent group and incorporation of a charge in order to give a more appropriate charge-to-mass ratio. In this review only UV-derivatization of thiols are considered. The main drawback of CE is a low concentration sensitivity, which is due to the restricted range of analyte loading, arising from the relatively small volumes that can be injected in the capillary (10–100 nL). Besides derivatization several solutions were proposed to circumvent this problem, e.g. use of capillaries with extended path length, more selective detectors, two-dimensional CE and on-capillary stacking. Different kinds of stacking have been proposed [146]. Shihabi invented acetonitrile-salts stacking and applied it, among others, to determination of glutathione [147]. This technique of on-capillary concentration was more recently incorporated into procedures for determination of cysteamine [87] and homocysteine [86] in plasma, and cysteine and glutathione in orange juice [88]. The procedures consisted of four essential steps: reduction of disulfide forms of the analytes to their thiol counterparts with tris(2-carboxyethyl)phosphine [86,88] or tri-*n*-butylphosphine [87], derivatization of thiols to their S-quinolinium derivatives with CMQT, separation of so-formed derivatives by capillary zone electrophoresis with acetonitrile stacking, and detection and quantification with the use of ultraviolet detector at 355 nm. The probable mechanism of the stacking is proposed to be a transient pseudo-isotachophoresis.

Russel and Rabenstein reported [113] possibility for simultaneous CZE determination of endogenous thiols and main thiol drugs in standard water solution and erythrocytes by means of separation of mixed disulfides after reaction with Ellman's reagent. Reduced glutathione derivatized with Ellman's reagent and oxidized glutathione without derivatization were determined in water standard solution and human lymphocytes by Raggi et al. [114].

Solid phase extracted thiolic peptides from marine microalga, derivatized with monobromobimane (mBrB) were separated and determined by CZE [97]. The proposed procedure has proven its capability to determine peptides including γ-GluCys, GSH, phytochelatins and desglycyl-phytochelatins. Another mBrB based CZE method for human blood total thiols was proposed by Ivanov et al. [95].

ABD-F derivatized plasma thiols were separated by CZE [93]. The method is not enough sensitive to determine total homocysteine in plasma donated by apparently healthy volunteer. Racemic homocysteine derivatized with ABD-F interacted with γ-cyclodextrin was successfully separated into L- and D-ABD-homocysteine enantiomers [92].

Piccoli et al. described CZE method which allows a simultaneous and quantitative determination of reduced (NEM-derivatized) and oxidized glutathione (without derivatization) in mammalian red blood cells. The procedure does not require removal of the excess of N-ethylmaleimide [148].

Table 2
Performance characteristics of UV-derivatization HPLC methods for hydrophilic thiols.

Analyte	Derivatization reagent[a]	Sample	Chromatographic conditions	Reduction	Overall analytical time [min]	Linear range	LOD	Ref.
Hcy GSH Cys	mBr B	Human plasma	Gradient elution Mobile phase: pH 3.6 MeCN/ammonium nitrate Flow rate 1.5 ml/min *T* 25 °C Isocratic elution pH 2.2 MeCN/0.1 M TFA in water Flow rate 0.75 ml/min *T* 25 °C, 250 nm	TPP	42	(Hcy, GSH) 0.5–500 nmol/ml plasma (Cys) 0.5–750 nmol/ml plasma	2 pmol in peak	[94]
Hcy GSH Cys	mBrB	Human plasma	Gradient elution Mobile phase: pH 2.2 MeCN/0.1 M TFA in water Flow rate 0.075 (0.080) μl/min *T* 35 °C, 210, 250 nm	TPP	91	(Hcy, GSH) 1–1000 nmol/ml plasma (Cys) 1–1100 nmol/ml plasma	2 pmol in peak	[96]
NAcCys Captopril	BCPB	Pharmaceutical formulations	Isocratic elution Mobile phase: pH 2.5 citrate buffer/sodium octane sulfonate/acetone Flow rate 0.7 ml/min *T* 45 °C, 314 nm	NA	38	2–20 μg/ml	0.2 ng in peak	[62]
Hcy Cys	CMPI	Human urine	Gradient elution Mobile phase: pH 2.5 TCA/LiOH/MeOH Flow rate 0.5 ml/min *T* 40 °C, 312 nm	TBP	84	5–150 nmol/ml urine 20–200 nmol/ml urine	NR	[64]
Cys	CMPI	Human plasma	Isocratic elution Mobile phase: pH 2.2 TCA/LiOH/MeCN Flow rate 1 ml/min *T* 40 °C, 312 nm	2-mercapto-ethanol	75	20–300 nmol/ml plasma	10 pmol in peak	[65]
Cys Hcy CysGly GSH	BCPB	Human plasma	Gradient elution Mobile phase: pH 2.6 TCA/LiOH/MeCN Flow rate 1 ml/min *T* 25 °C, 315 nm	TCEP	45	(Cys) 0–300 nmol/ml plasma (Hcy, GSH) 0–40 nmol/ml plasma (CysGly) 0–50 nmol/ml plasma	(0.2 nmol/ml) plasma 4 pmol in peak	[66]
Cys CysGly	BCPB	Human urine	Gradient elution Mobile phase: pH 2.6 TCA/LiOH/MeCN Flow rate 1 ml/min *T* 25 °C, 315 nm	TCEP	30	(Cys) 50–300 nmol/ml urine (CysGly) 5–50 nmol/ml urine	4 pmol in peak	[67]

Table 2 (*Continued*)

Analyte	Derivatization reagent[a]	Sample	Chromatographic conditions	Reduction	Overall analytical time [min]	Linear range	LOD	Ref.
Cys	CMQT	Chemically defined media	Gradient elution Mobile phase: pH 5 ammonium acetate/MeCN Flow rate 1.5 ml/min *T* 25 °C, 355 nm	TCEP	Cys, 9 $(Cys)_2$, 12	0.25–500 nmol/ml	36 nM	[68]
CoA 4-Phosphopantetheine Pantetheine cysteamine	CMQT	Biological media (mixture of enzymes and peptides)	Gradient elution Mobile phase: pH 5 ammonium acetate/MeCN Flow rate 3 ml/min *T* 25 °C, 355 nm	NR	60	NR	NR	[90]
Hcy GSH CysGly Cys	CMQT	Human plasma	Gradient elution Mobile phase: pH 3.2 TCA/LiOH/MeCN Flow rate 1 ml/min *T* 25 °C, 355 nm	$NaBH_4$	24	(Hcy) 0.5–50 nmol/ml plasma (GSH) 0.5–40 nmol/ml plasma (CysGly) 1–50 nmol/ml plasma (Cys) 7–300 nmol/ml plasma	(Hcy, GSH) 0.1 nmol/ml plasma (2 pmol in peak) (Cys) 2 nmol/ml plasma (40 pmol in peak) (Cysgly) 0.5 nmol/ml plasma (10 pmol in peak)	[72]
GSH Cys Hcy CysGly	CMQT	Human saliva	Gradient elution Mobile phase: pH 1.65 TCA/LiOH/MeCN Flow rate 1.2 ml/min *T* 25 °C, 355 nm	TBP	52	(GSH) 0.5–30 nmol/ml plasma (Hcy) 0.5–30 nmol/ml plasma (Cys) 0.5–100 nmol/ml plasma (CysGly) 0.5–30 nmol/ml plasma	0.5 nmol/ml saliva (LLQ)	[76]
Cys	CMQT	Human urine	Gradient elution Mobile phase: pH 3.25 TCA/LiOH/MeCN Flow rate 2 ml/min *T* 40 °C, 355 nm	TBP	(total Cys) 49 (reduced Cys) 19	20–400 nmol ml urine	0.23 nmol ml urine (4.6 pmol in peak)	[70]
Hcy	CMQT	Human plasma	Gradient elution Mobile phase: pH 3.2 TCA/LiOH/MeCN Flow rate 1 ml/min *T* 25 °C, 355 nm	$NaBH_4$	20.5	0.5–50 nmol/ml plasma	0.3 nmol/ml (6 pmol in peak)	[71]
Cys	CMQT	Human urine	Gradient elution	TCEP	27	(Cys) 50–300 nmol/ml urine	NR	[69]

Table 2 (*Continued*)

Analyte	Derivatization reagent[a]	Sample	Chromatographic conditions	Reduction	Overall analytical time [min]	Linear range	LOD	Ref.
CysGly			Mobile phase: pH 3.2 TCA/LiOH/MeCN Flow rate 1.5 ml/min *T* 25 °C, 355 nm			(CysGly) 10–50 nmol/ml urine		
Tiopronin D-penicillamine	CMQT	Human urine	Gradient elution Mobile phase: pH 2 TCA/LiOH/MeCN Flow rate 1 ml/min *T* 25 °C, 355 nm	$NaBH_4$	20	1–200 mmol/ml urine	0.5 nmol/ml (10 pmol in peak)	[83]
Hcy Cys GSH CysGly	CMQT	Human plasma	Gradient elution Mobile phase: pH 1.65 TCA/LiOH/MeCN Flow rate 1.2 ml/min *T* 25 °C, 355 nm	TCEP	25	(Hcy, GSH) 1–30 nmol/ml (CysGly) 1–60 nmol/ml (Cys) 10–300 nmol/ml	(LOQ) 0.5 nmol/ml (10 pmol in peak)	[75]
mesna	CMQT	Human urine	Isocratic elution Mobile phase: pH 2.3 TCA/LiOH/MeCN Flow rate 1.2 ml/min *T* 25 °C, 350 nm	TBP	45	0.2–800 nmol/ml urine	0.1 nmol/ml urine (2 pmol in peak)	[79]
Nε-HcyLys γ-GluCys	CMLT	Human plasma	Gradient elution Mobile phase: pH 2.3 TCA/NaOH/MeCN Flow rate 1. ml/min *T* 25 °C, 355 nm	TCEP	35	(*Nε*-Hcy-Lys) 0.1–6.0 nmol/ml (γ-Glu-Cys) 0.5–30 nmol/ml plasma	nmol/ml plasma (4 pmol in peak)	[89]
Tiopronin	pBPB	Human plasma	Gradient elution Mobile phase: pH 1.8 trifluoroacetic acid/MeCN Flow rate 1.2 ml/min *T* 30 °C, 263 nm	NA	85	20–4000 ng/ml urine	(captopril) 10 ng/ml plasma (hydrochlorothiazide) 3.3 ng/ml plasma	[100]
Captopril	pBPB	Human serum	Isocratic elution Mobile phase: acetic acid/MeCN/water Flow rate 1 ml/min *T* 25 °C, 263 nm	NA	30	50–1000 ng/ml serum	15 ng/ml (0.15 ng in peak)	[99]
Captopril	BAN	Human plasma	Isocratic elution Mobile phase: *n*-hexane/2-propanol/MeOH/acetic acid Flow rate 2 ml/min *T* 25 °C, 246 nm	NA	60	12.5–500 ng/ml	10 ng/ml (0.25 ng in peak)	[102]
Captopril	BMP	Human urine	Isocratic elution Mobile phase: pH 6.5 Sodium acetale/MeCN/MeOH Flow rate 1 ml/min *T* 35 °C, 254 nm	NA	60	800–16000 ng/ml	3 ng (in peak)	[103]

Table 2 (*Continued*)

Analyte	Derivatization reagent[a]	Sample	Chromatographic conditions	Reduction	Overall analytical time [min]	Linear range	LOD	Ref.
Cys	NEM	Syntetic peptides	Gradient elution Mobile phase: TFA/water/MeCN Flow rate 1 ml/min *T* 25 °C, 214 nm	NR	45	NR	0.1 nmol/ml	[105]
GSH NAcCys Captopril MPG	ESB	Thiol drugs	Isocratic elution Mobile phase: pH 4 Triethylamine phosphate/methanol/MeCN Flow rate 1 ml/min *T* 25 °C, 254 nm	NA	25	(GSH) 0.03–0.18 mg/ml (NAcCys, Thiola) 0.05–0.20 mg/ml (Captopril) 0.03–0.15 mg/ml	0.1 nmol (in peak)	[107]
GSH	DTNB	Erythrocytes	Gradient elution Mobile phase: pH 3.83 KH_2PO_4/MeOH Flow rate 1.2 ml/min *T* 39 °C, 330 nm	NR	52	0.5–3 nmol/ml	0.041 μmol/ml (0.82 nmol in peak)	[111]
GSH	DTNB	Cell homogenate	Gradient elution Mobile phase: formic acid/MeCN Flow rate 0.8 ml/min *T* 25 °C, 326 nm	$NaBH_4$	71	NR	7.5 pmol (in peak)	[112]
GSH Hcy Cys CysGly	DTNB	Human plasma and cell extracts	Isocratic elution Mobile phase: pH 3.8 KH_2PO_4/MeOH Flow rate 1.2 ml/min *T* 37 °C, 330 nm	DTT	20	(GSH) 0.5–30 nmol/ml (Hcy) 0.5–45 nmol/ml (Cys) 0.2–750 nmol/ml (CysGly) 0.5–80 nmol/ml plasma	NR	[110]
Cys Hcy CysGly	TCDI	Human plasma, urine and cerebrospinal fluid	Isocratic elution Mobile phase: acetic acid/acetonitrile/MeOH Flow rate 1 ml/min *T* 25 °C, 250–300 nm	TCEP	80	(Cys) 80–230 nmol/ml (Hcy) 2.5–10 nmol/ml (CysGly) 12–50 nmol/ml	2 pmol (in peak)	[119]
Cys GSH γ-GluCys CysGly Hcy	4,4′DTP	Human plasma and urine	Isocratic elution Mobile phase: MeOH/phosphate buffer/octyl sulfate Flow rate 0.8 ml/min *T* 25 °C, 324 nm	DTT	76	NR	50 nmol/l (1.5 fmol in peak)	[117]

NR, not reported.
NA, not applied.
[a] Derivatization reaction conditions described in Table 1.

Table 3
Optimization conditions for determination of hydrophilic thiols by capillary electrophoresis in the form of their UV-derivatives.

Reagent[a]	Analyte	Sample	Electrophoretic conditions	Overall time of analysis	Linear range	Limit of detection	Ref.
Ellman	GSH	Human lymphocytes	Fused silica capillary with polyacrylamide coating (27 cm total length, 75 or 50 μm i.d.), 0.05 M pH 7.0 phosphate buffer, 25 °C, 8 kV, injection 3.45×10^4 Pa for 5 s, detection 200 nm	≈8 min (electrophoresis)	NR	0.5 μM	[114]
	GSSG						
	GSH	Erythrocytes	Fused silica capillary (24 cm total length, 25 μm i.d.), 0.01 M sodium phosphate (pH 7.4), −12 kV, injection 5 psi for 3 s, detection 357 nm	≈30 min	5 μM^{-1} mM	5 μM	[113]
	Captopril	Standards			16 μM^{-1} mM	16 μM	
	Cys				28 μM^{-1} mM	28 μM	
	Hcy (neg. polarity)				12 μM^{-1} mM	12 μM	
	D-penicillamine				59 μM^{-1} mM	59 μM	
mBrB	GSH	Marine microalga	Fused silica capillary (98 cm total length, 50 μm i.d.), 0.1 M sodium borate (pH 7.6), 25 °C, 15 kV, injection 50 mbar for 20 s, detection 390 nm	≈2 h	7.5–100 μM	1.41 μM	[97]
	Cys						
	γ-GluCys						
	Hcy	Human blood	Fused silica capillary (65 cm total length, 50 μm i.d.), 0.08 M pH 9 sodium phosphate, 30 °C, 250 V/cm, injection 50 mbar for 7 s, detection 234 nm	10 min (electrophoresis)	0.5–500 μM	5 μM	[95]
	Cys				0.5–750 μM		
	GSH				0.5–500 μM		
ABD-F	Hcy (chiral separation)	Standards	Fused silica capillary (57 cm total length, 50 μm i.d.), 0.05 M pH 2.25 sodium phosphate with 0.02 or 0.05 M γ-CD, 15 °C, 20 or 25 kV, injection 3.5 kPa for 10 s, detection 220 nm	≈2 h (with 1 h homocystine reduction)	NR	NR	[93]
	Hcy	Human plasma	Bare fused silica capillary (27 cm total length, 50 μm i.d.), 0.05 M sodium phosphate (pH 2.1), 25 °C, 15 kV, injection 0.5 psi for 5 s, detection 220 nm	≈1 h	1–200 μM	0.2 μM	[92]
	Cys					2 μM	
	GSH					1 μM	
2,2′-DTP	GSH	Human plasma and human erythrocytes	(MEKC method) Bare fused silica capillary (64.5 cm total length, 50 μm i.d.), 0.05 M SDS in 0.05 M phosphate buffer (pH 7.5), 20 or 25 °C, 28 kV, multistep injection, detection 343 nm	≈20 min	0.05–2 mM	5 μM	[149]
	Hcy	Human plasma		≈40 min	0.03–3 μM	6 μM	[150]
	Cys	Human urine		≈40 min	0.05–5 mM	2.5 μM	[151]
CMQT	Hcy	Human plasma	Fused silica capillary (52 cm total length, 50 μm i.d.), 0.2 M Tris/HCl (pH 2.1), 25 °C, 25 kV, injection 50 nL, detection 355 nm	≈50 min	5–80 μM	1 μM	[86]
	Cysteamine	Human plasma	Fused silica capillary (64.5 cm total length, 50 μm i.d.), 0.2 M Tris/HCl (pH 2.1), 25 °C, 30 kV, injection 50 mbar for 51 s (60 nL), detection 355 nm	≈50 min	2.5–20 μM	0.8 μM	[87]
	Cys	Orange juice		≈40 min	2.5–30 μM	1 μM	[88]
	GSH						

NR, not reported.
[a] Derivatization reaction conditions described in Table 1.

The only micellar electrokinetic chromatography procedure for quantitation of thiols reported by Glatz et al. is based on an on-capillary detection reaction with 2,2′-dipyridyl disulfide. The thiols, after pre-separation, are quantitatively transformed to mixed disulfides concomitantly with formation of an equimolar amount of 2-thiopyridone that is further separated and detected. The concentration of the thiol is thus estimated indirectly. Initially this method was applied to reduced GSH in human plasma and erythrocytes [149] and next to total Hcy in plasma [150] and total Cys in human urine [151].

Experimental details of the above mentioned CE procedures are placed in Tables 1 and 3.

5. Conclusion

Derivatization is an indispensable step in most bioanalytical methods for thiols. The derivatization utilizing a suitable labeling reagent followed by chromatographic or electrophoretic separation and detection is the reliable means for sensitive and selective assays. Although a variety of detection methods are available to measure thiol concentrations, ultraviolet detection is widely used. Ultraviolet detection is less specific and less sensitive but simpler as compared with fluorescence or electrochemical detection, nevertheless, its sensitivity and reliability, achieved in the manual or fully automated mode, is sufficient for detection and quantitation of

majority of aminothiols and thiol-drugs in human biofluids in physiological and pathological conditions. Moreover, the UV detector is known for its stability and low demand in terms of maintenance, and belongs to the standard instrumentation in hospital laboratories. This review summarizes UV-derivatization HPLC and CE methods, including pre-analytical considerations, procedures for sample reduction, derivatization, and liquid phase separation of the primary biological aminothiols cysteine, homocysteine, cysteinylglycine, and glutathione. Some experimental details of analytical procedures for determination of thiol containing drugs – cysteamine, 2-mercaptopropionylglycine, D-penicillamine, captopril, mesna and methimazole are also reviewed. After conventional sample preparation (e.g. liquid–liquid or solid-phase extraction), where a relatively large biofluid sample is needed, the microanalytical abilities of CE are not utilized. In such a case more sensitive and reproducible HPLC technique is recommended. The justification for using CE as the final analytical step is its excellent resolving capabilities for analytes which can be difficult to separate by HPLC. Injection of body fluids without or with little sample preparation (derivatization) is more often possible in CE because the bare capillary is less susceptible to irreversible modification by sample components than HPLC column. Even if adsorption occurs capillary can be cleaned with aggressive agents. Another very important stimulus to the application of CE methodology is the strong charge on derivative, as is the case of 2-S-pyridinium and 2-S-quinolinium derivatives. Green chemist would emphasize minimization of solvents and reagent usage in CE. The authors' intention was to provide satisfactory information on the UV-derivatization HPLC and CE methods currently available for hydrophilic thiols, and to sensitize the reader for particular problems in the analysis including artifacts related to sample preparation.

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