
HPLC Analysis of Homocysteine and Related Compounds

Mitsuhiro Wada, Shinichi Nakamura and
Kenichiro Nakashima

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/intechopen.75030>

Abstract

Homocysteine (Hcy), a sulfur-containing amino acid, is a representative intermediate metabolite of methionine (Met) to cysteine (Cys) via several intermediates. An elevated level of Hcy in plasma plays an important role in diseases such as neural tube defects and Down syndrome. Homocystinuria is the most common inborn error of sulfur metabolism and is caused by mutations in the metabolic enzymes of Hcy. These errors can be caused by abnormal levels of Met metabolites and classified on the basis of plasma Met levels. Additionally, Hcy and related compounds such as glutathione play an important role in maintaining homeostasis. Therefore, the simultaneous determination of Hcy and/or related compounds is required for appropriate clinical management of several diseases. The sulfur-containing amino acids and their derivatives in biological samples are quantified sensitively using high-performance liquid chromatography methods coupled with various detection methods such as UV/Vis, fluorescence, chemiluminescence, electrochemical, mass spectrometry, and tandem mass spectrometry. In this chapter, we review recent advances in these analytical methods and their applications.

Keywords: homocysteine, homocysteine-related compounds, sulfur-containing amino acids, HPLC, determination, derivatization

1. Introduction

Homocysteine (Hcy), one of the sulfur-containing amino acids, is a representative intermediate metabolite of methionine (Met) in the cysteine (Cys) biosynthetic pathway, as shown in **Figure 1**. Hcy is remethylated to Met by Met synthase or betaine-Hcy methyltransferase (transmethylation), and Met is transmethylated to Hcy via several steps. The first step in the transmethylation

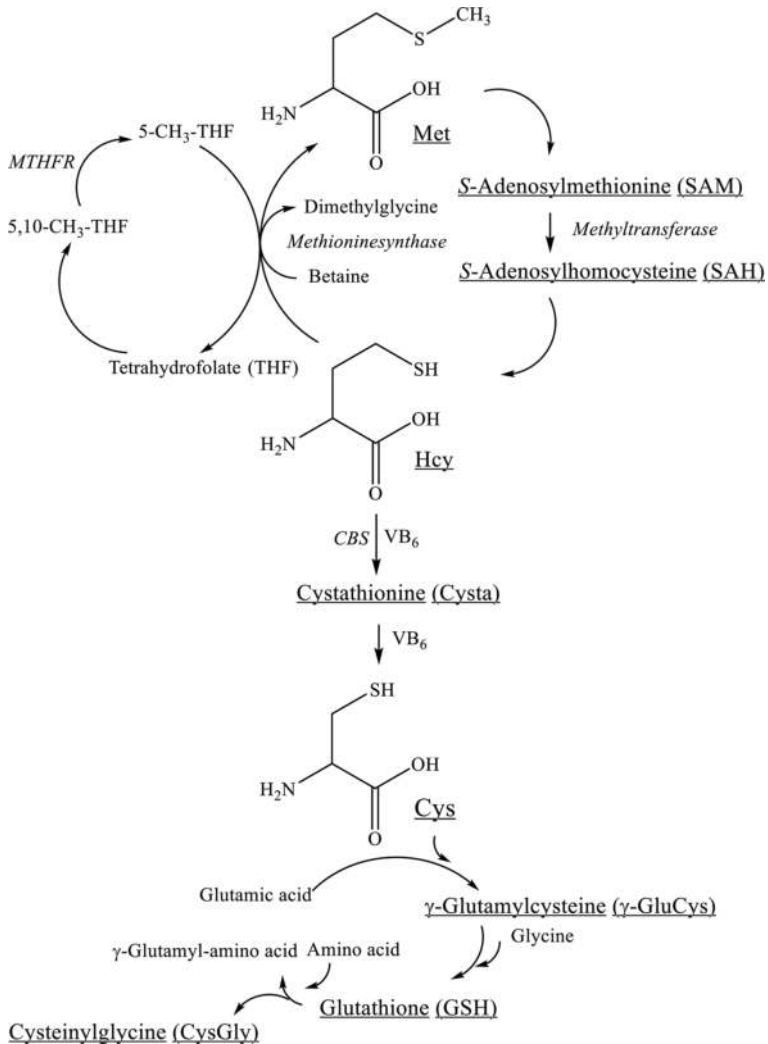


Figure 1. Relationship of Hcy and its related compounds in Met metabolism. The compounds underlined are target compounds in the references.

of Met to Hcy is the activation of Met to *S*-adenosylmethionine (SAM) by Met adenosyltransferase. SAM is converted to *S*-adenosylhomocysteine (SAH); then, SAH is hydrolyzed to Hcy by SAH hydrolase. Hcy is converted to cystathionine (Cysta) by cystathionine β-synthase (CBS) (transsulfuration); then, Cysta is hydrolyzed to Cys by cystathionine-γ-liase [1]. Cys is a fundamental substrate for glutathione (GSH) biosynthesis. In the first step of this biosynthesis, Cys and glutamate generate the dipeptide γ-glutamylcysteine (GluCys). This step is believed to be rate limiting, and enzyme activity is regulated by feedback inhibition with GSH. Next, the addition of glycine to GluCys results in the formation of GSH, catalyzed by glutathione synthase, and finally, the degradation of GSH generates cysteinylglycine (CysGly) [2, 3].

Most Hcy in human plasma is present as the bound form of Hcy, in which Hcy binds with plasma proteins through an —S—S— bond, while free Hcy is present in the oxidized or reduced form. Oxidized forms of Hcy include homocysteine (HcyHcy) and Hcy-Cys disulfide. The bound form of Hcy and the oxidized form are called *S*-linked Hcy. The bound form of Hcy constitutes 70–90% of the total Hcy (~10–15 $\mu\text{mol/L}$) in the body, 10–30% is present as oxidized Hcy, and less than 1% is present as reduced Hcy [4]. The ratio of Cys to Hcy in human plasma may be similar.

Hcy is metabolized to Hcy-thiolactone by methionyl-*t*RNA synthetase in an error-editing reaction during protein biosynthesis when Hcy is mistakenly replaced with Met. An increase in the Hcy level leads to elevated thiolactone levels in human cells and serum. Hcy-thiolactone reacts with proteins by *N*-linking to the ϵ -amino group of protein lysine residues (homocysteinylated), resulting in protein damage [5]. The measurement of *N* ϵ -Hcy-Lys generated by the proteolytic degradation of *N*-Hcy-protein provides an indicator of homocysteinylated [6].

Hcy is important for the clinical diagnosis of a variety of metabolic disorders related to human diseases. For example, an elevated Hcy plasma concentration is believed to be related to cardiovascular disease [7]. Consequently, the determination of Hcy in plasma has been used to diagnose this disease and to evaluate new diagnostic tools for atherosclerosis [8, 9]. Additionally, high plasma levels of Hcy play an important role in neural tube defects [10] and Down syndrome [11]. Homocystinuria is the most common inborn error of sulfur metabolism and is caused by homozygous mutations in the methylenetetrahydrofolate reductase (MTHFR) gene and heterozygous mutations in CBS [12]. These errors can be classified on the basis of plasma Met levels, which tend to be elevated in the case of CBS deficiency and lowered in the case of MTHFR deficiency.

DNA methylation is regulated by the Met cycle using SAM as a methyl group donor in the presence of methyltransferase. Under normal physiological conditions, SAM is hydrolyzed to adenosine and Hcy by SAH hydrolase. However, this reaction is readily reversible due to equilibrium dynamics that strongly favors SAH synthesis over hydrolysis. SAM and SAH therefore regulate the normal level of methylation in DNA, and deregulation of the methionine cycle has serious cellular consequences, resulting in disease. The ratio of SAM/SAH, called the “methylation index,” may be a useful indicator of the methylation capacity of the cell [13].

Hcy toxicity appears to be the auto-oxidation of Hcy, which reduces the disulfide to a free thiol, followed by metal-independent oxidation of the free thiol to generate reactive oxygen species such as superoxide and hydrogen peroxide [14]. Therewith, other thiol compounds like Cys having a chemical structure similar to Hcy are also recognized to be risk factors of cardiovascular disease [15, 16]. In contrast, GSH is a major antioxidant and detoxifier and has many essential metabolic functions in human. GSH exists in both reduced and oxidized (GSSG) forms. CysGly is a prooxidant that reduces ferric iron to ferrous iron [17]. *N*-Acetylcysteine (NAC) is an endogenous product of Cys metabolism [18], and cysteamine (CA) augments intracellular Cys levels via a disulfide interchange reaction in which CA converts Cys to CA-Cys to generate Cys [19]. Homocysteine is involved in current topics, and the determination methods are useful for the researchers.

High-performance liquid chromatography (HPLC) is the most commonly used chromatographic technique for quantifying Hcy and related compounds. In contrast to gas chromatography, HPLC enables the analysis of polar and thermally labile compounds. Furthermore, HPLC can be coupled with a variety of detection methods, including ultraviolet/visible (UV/Vis), fluorescence (FL), chemiluminescence (CL), mass (or tandem mass/mass) spectrometry (MS or MS/MS), and electrochemical detection (ECD). The researcher selects the most suitable detection tools according to the aims of the analysis [20].

Several eminent reviews have been published over the past decade regarding these detection tools, e.g., HPLC-UV/Vis detection [21], HPLC-FL detection [22], HPLC-luminescence detection [23], HPLC and/or capillary electrophoresis [2, 3], electrochemical assays [24], and chromatographic methods in the study of autism [20], together with their application to the quantification of Hcy and/or related compounds and descriptions of the importance of measuring these compounds. In this chapter, we review HPLC methods and their applications in recent publications (from 2008 to 2017).

2. HPLC analysis of homocysteine and related compounds

As described above, Hcy and related compounds are found in the form of free thiols, disulfides, and protein-bound complexes and participate in metabolism, antioxidant defense, and drug detoxification. Alterations in the concentrations and ratios of free thiols and disulfides provide biomarkers of metabolism and of the redox status in biochemical, physiological, pharmacological, and toxicological studies. Therefore, analytical methods for the determination of Hcy and/or these other compounds in biological samples are extremely important. In this section we describe HPLC methods combined with various detection methods.

Pretreatment of the sample is often required for successful chromatographic analysis of Hcy and related compounds. Biological fluids such as blood (plasma or serum) and urine must be processed prior to HPLC analysis in order to (1) liberate Hcy (including the reduced disulfide), (2) provide desirable characteristics for detection, and (3) remove any interfering compounds. Sample pretreatment consists of reduction, derivatization, and/or cleanup steps. (1) The total Hcy concentration in a biological sample is important in clinical practice. Hcy can be in the reduced and in S-linked forms, such as a disulfide and Hcy bound with plasma proteins. These S-linked forms are converted to the reduced form and analyzed as the total Hcy. The disulfide can be reduced using sodium borohydride [25, 26], tributylphosphine (TBP) [27, 28], tris(2-carboxyethyl)phosphine (TCEP) [6, 29–40], dithiothreitol (DTT) [41–44], 1,4-dithioerythritol (DTE) [45–47], and mercaptoethanol [48]. Bai et al. developed a unique online reduction quartz column packed with a Zn(II)-TCEP complex [33, 49]. The column efficiently converted disulfides (except GSSH) to the reduced form as effectively as a TCEP solution. N-linked Hcy can be liberated from protein and converted to Hcy-thiolactone using harsh conditions (6 mol/L HCl at 120°C for 1 h) after removing Hcy and S-linked Hcy by reduction with DTT [36, 44]. (2) The structures of Hcy and related compounds have low absorbance and are non-fluorescent. Derivatization is therefore essential for the UV/Vis and fluorescence detection of small amounts of these compounds in biological samples. Many derivatization reagents have been developed and applied to various biological samples. Furthermore, derivatization reagents have been recently developed allowing

sensitive MS or MS/MS detection for Hcy and related compounds, as described in detail below. (3) Following their reduction, Hcy and related compounds are deproteinized, then derivatized, and chromatographically separated. Cleanup is achieved using ultrafiltration [31], acid precipitation with trichloroacetic acid [28, 37, 41, 50] or perchloric acid [29, 34, 45], and organic solvent precipitation with methanol [48, 51, 52] or acetonitrile [31, 53]. Further cleanup following the derivatization of Hcy and related compounds may be required, such as a liquid-liquid extraction [45] or a solid-phase extraction [54].

2.1. UV/Vis detection

HPLC-UV/Vis is the most commonly used detection method due to the simple and relatively inexpensive instrumentation required. However, Hcy and related compounds have low absorbance and are present in biological samples in low amounts, which precludes their direct analysis by HPLC-UV/Vis. As mentioned above, this is addressed by derivatization using reagents such as those shown in **Figure 2**. The halopyridine-type derivatization reagents 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT) [27, 39, 40], 2-chloro-1-methyllepidinium tetrafluoroborate (CMLT) [6, 30], and 1-benzyl-2-chloropyridinium bromide (BCPB) [26] react with Hcy and related compounds to form stable *S*-quinolinium or *S*-pyridinium derivatives with intense UV

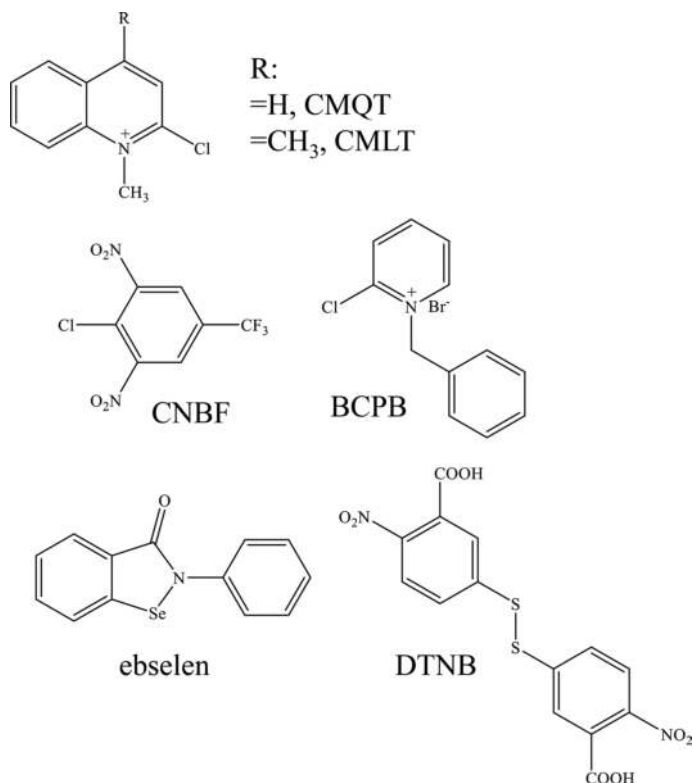


Figure 2. Chemical structures of derivatizing reagents for UV/Vis detection.

absorption. Stachniuk et al. developed an HPLC-UV/Vis method using CMLT derivatization for the determination of Hcy, Cys, GSH, GluCys, CysGly, and NAC in human saliva, plasma, and urine [30]. The analytes were separated within 7 min and monitored by absorbance at 355 nm. The limits of detection (LODs) at a signal-to-noise (S/N) ratio of 3 ranged from 0.05 to 0.12 $\mu\text{mol/L}$. The authors showed a good positive correlation between the concentrations of the analytes in plasma and saliva and suggested that saliva is an alternative to plasma for the quantification of Hcy and related compounds.

Several unique types of derivatization reagents have been reported. 4-Chloro-3,5-dinitrobenzotrifluoride (CNBF) has an activated halide leaving group that can be easily replaced by a thiol group, leading to the formation of a stable thioether with increased absorbance at 230 nm [32]. Using this approach allowed the quantification of Hcy, Cys, CysGly, and GSH in human plasma, urine, and saliva, with LODs of 0.04–0.08 $\mu\text{mol/L}$. 5,5'-Dithiobis-2-nitrobenzoic acid (DNTP), which utilizes the sulfhydryl-disulfide exchange reaction, has been used for quantifying Hcy, Cys, CysGly, and GSH [43, 47]. Ebselen, a Se-containing derivatization reagent that reacts with the sulfhydryl group, was used for the determination of Hcy and Cys in human serum [55]. The absorbance at 254 nm of the derivatives was monitored, and separation was complete within 11 min. The derivatives could also be determined sensitively by inductively coupled plasma mass spectrometry, with an LOD of 9.6 nmol/L.

2.2. Fluorescence detection

Derivatization allows the sensitive determination of Hcy and related compounds in biological samples by FL detection. Many appropriate derivatization reagents have been developed, and representative compounds cited in this section are shown in **Figure 3**.

Halogenobenzofurazans are often used for the determination of Hcy and related compounds, with ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F) being most commonly used. SBD derivatives are detected by FL using 385 and 515 nm for λ_{ex} and λ_{em} , respectively. Hcy, Cys, CysGly, and GSH are isocratically separated within 6 min [28]. The use of HPLC-FL under hydrophilic interaction chromatography (HILIC) conditions allows the separation of Hcy, NAC, CA, Cys, CysGly, GSH, and GluCys within 10 min. The LODs were 0.02–3.4 nmol/L at an S/N ratio of 3 [56]. The SBD-F is the standard against which newly developed methods are compared. The HPLC-FL method with SBD-F showed a good correlation with the results obtained using a bodipy-based fluorescence sensor for the determination of Hcy, Cys, and GSH in human serum [53]. In a clinical study, Hcy levels in patients with pulmonary hypertension [57], type 2 diabetes [58], and ulcerative colitis [59] were determined by HPLC-FL with SBD-F. A validated HPLC method for the routine determination of Hcy, Cys, and CA was developed [25] and applied to several hundred plasma samples. The results were used to examine the utility of carotid intima-media thickness [9] and cardio-ankle vascular index [8] as screening tools for atherosclerosis in the Japanese population. Recently, Cevasco et al. developed ammonium 5-bromo-7-fluorobenzo-2-oxa-1,3-diazol-4-sulphonate (SBD-BF) as a reagent with improved reactivity to Hcy and related compounds [37]. The reaction of SBD-BF with these substrates at room temperature is about three times faster than with SBD-F at 60°C, and Hcy, Cys, GSH, and CysGly in plasma were determined, with LODs of 0.05–20 $\mu\text{mol/L}$. 4-Fluoro-7-aminosulfonylbenzofurazan (ABD-F), another halogenobenzofurazan, was used for the determination of Hcy, Cys, GSH, and CysGly in cell culture medium [35] and plasma, urine, saliva,

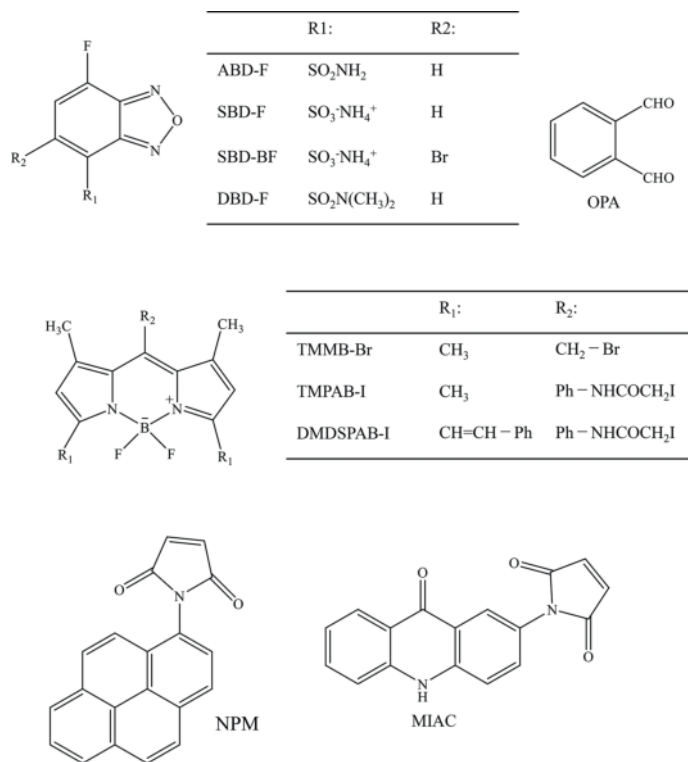


Figure 3. Chemical structures of derivatizing reagents for FL detection.

and cerebrospinal fluid [60]. The derivatization of these substrates was complete after 10 min at 35 or 50°C under alkaline conditions. FL of the ABD derivatives at around 390 and 510 nm for λ_{ex} and λ_{em} allowed sensitive determination, with LOQs of 0.1–0.5 $\mu\text{mol/L}$. Another halogeno-benzofurazan, 4-(*N,N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F), reacts with the thiol and amino groups in Hcy and related compounds, allowing the simultaneous determination of Hcy, Cys, and Met by HPLC-FL detection and thus may be suitable for screening for homocystinuria, an inborn error of sulfur metabolism. The DBD-derivatives were separated within 15 min and quantified sensitively (0.04–0.14 $\mu\text{mol/L}$). The method was applied to maternal plasma after delivery [46] and dried blood spots from newborns [45].

o-Phthalaldehyde (OPA) is another representative derivatization reagent for Hcy and related compounds and can be used for pre- or post-column derivatization methods. Recently, Jakubowski and coresearchers developed several HPLC-FL detection methods combined with on-column derivatization for Hcy in urine [38], Hcy and Met in plasma and urine [29], and Hcy-thiolactone, *S*-linked Hcy, and *N*-linked Hcy in urine [61], plasma [36], and milk [44]. Hcy and related compounds spiked with NAC were injected and separated on a reversed-phase column, using OPA in a NaOH aqueous solution/CH₃CN mixture as the mobile phase. The fluorescence of the OPA-Hcy or OPA-Hcy-thiolactone derivatives generated during separation was monitored using 370 and 480 nm for λ_{ex} and λ_{em} , respectively. The LOQs for Hcy and Hcy-thiolactone were 25 [38] and 20 nmol/L [36], respectively.

Difluoroboraindacene (BODIPY) is an intense fluorogenic and stable compound, and several derivatives were recently synthesized as useful fluorescence derivatizing reagents for Hcy and related compounds. 1,3,5,7-Tetramethyl-8-bromomethyl-difluoroboradiaza-*s*-indacene (TMMB-Br) was used for the determination of Hcy, Cys, NAC, and GSH in human plasma [41]. The LODs ranged from 0.2 to 0.8 nmol/L by monitoring FL using 505 and 525 nm for λ_{ex} and λ_{em} . Furthermore, 1,3,5,7-tetramethyl-8-phenyl-(4-iodoacetamido)difluoroboradiaza-*s*-indacene (TMPAB-I) was developed for quantifying Hcy, Cys, NAC, GSH, coenzyme A [62], and 6-mercaptopurine, and 1,7-dimethyl-3,5-distyryl-8-phenyl-(4'-iodoacetamido)difluoroboradiaza-*s*-indacene (DMDSPAB-I) was developed for quantifying Hcy, Cys, NAC, GSH, CysGly, and penicillamine [63]. The excitation and emission wavelengths of DMDSPAB-I are very long (620 and 630 nm, respectively), which is useful for FL detection, allowing a high quantum yield of 0.557 and LODs ranging from 0.24 to 0.72 nmol/L for the substrates.

The *N*-substituted maleimide-type FL derivatization reagents *N*-(1-pyrenyl)maleimide (NPM) [31] and *N*-(2-acridonyl)-maleimide (MIAC) [50] were used. Among them, NPM has been applied to quantifying Hcy, Cys, CysGly, and GSH in plasma from healthy controls and uremic patients. The concentrations of the total, free, and reduced forms of Hcy, Cys, and CysGly in patients were higher than those in healthy controls, while the concentrations of the three forms of GSH were lower in the healthy controls.

A novel post-column resonance light scattering (RLS) detection method combined with HPLC was developed for quantifying Hcy and Cys in human urine [34]. Fluorosurfactant-capped gold nanoparticles (AuNPs) were used as a post-column RLS reagent. The detection principle was based on the enhanced RLS intensity of AuNPs upon the addition of Hcy or Cys (at $\lambda_{\text{ex}} = \lambda_{\text{em}} = 560 \text{ nm}$).

2.3. Chemiluminescence detection

Recently, McDermott et al. reported an HPLC-CL detection method using manganese (IV) as a post-column reagent [64]. Thiols or disulfides reacted with manganese (IV) emit red light with a maximum of 735 nm. Hcy-related compounds, including Cys, NAC, GSH, GSSG, CysCys, and HcyHcy, were determined with a single chromatographic separation. The LODs for the compounds ranged from $5 \times 10^{-8} \text{ M}$ to $1 \times 10^{-7} \text{ M}$. This method, with simple sample pretreatment involving deproteinization, was applied to the determination of GSH and GSSG in the whole blood.

An HPLC-CL detection method using fluorosurfactant-prepared triangular gold nanoparticles (AuNPs) as a post-column CL reagent was developed for the determination of amino-thiols [65]. The triangular AuNPs were generated by trisodium citrate reduction of HAuCl_4 in the presence of nonionic fluorosurfactant (such as zonyl FSN-100) and act as a catalyst for the luminol- H_2O_2 CL system. The reduced aminothiols decrease the CL intensity of the triangular AuNPs-luminol- H_2O_2 system. After the reduction of thiols by TCEP and deproteinization with HClO_4 , Hcy, Cys, GSH, CysGly, and GluCys in human plasma and urine were separated by HPLC and then mixed with the AuNPs-luminol- H_2O_2 system. The LODs ranged from 0.016 to 0.1 pmol at $S/N = 3$. Furthermore, an automated system involving online reduction using a quartz column packed with the Zn(II)-TCEP complex was developed for quantifying

Hcy, HcyHcy, Cys, CysCys, CysGly, GSH, and GluCys [49]. The seven compounds in human plasma and urine could be determined, with LODs in the range of 8.3–25.4 nmol/L.

2.4. Electrochemical detection

ECD methods are suitable for the detection of Hcy and related compounds due to the electrochemical activity of these compounds, allowing thiols to be directly detected without derivatization. This detection method is frequently used because of the simplicity, inexpensiveness, and sensitivity of the instruments and the approach. Furthermore, the disulfide compound can be detected without cleavage of the disulfide bond.

Khan et al. reported an HPLC-ECD method for the simultaneous determination of Hcy, Met, Cys, CysCys, GSH, GSSH, NAC, and ascorbic acid (ASA) in human plasma and erythrocytes using dopamine as an internal standard (IS) [66]. The analytes were extracted from the biological fluids by simple liquid-liquid extraction. The nine compounds and the IS were separated within 25 min, and their LODs ranged from 0.6 to 25 ng/mL at an S/N ratio of 3. Furthermore, an ion-pairing reversed-phase-HPLC-ECD method was reported [67] in which total Cys, Hcy, and GSH were reduced by TCEP, and Met and ASA in human plasma and blood cell were determined with LODs of 60–80 pg/mL in a total analysis time of 20 min. More recently, Hannan et al. reported an HPLC-ECD method for nine compounds and malondialdehyde in rabbit serum [68], allowing the endogenous antioxidant capacity and lipid peroxidation level to be monitored simultaneously.

Lehotay's research group reported a two-dimensional HPLC-ECD method for determination of the enantiomers of Hcy, Cys, and Met using a combination of ODS and teicoplanin aglycone columns [69, 70]. The chiral separation of Hcy, Cys, and Met enantiomers was realized in a single 130 min analytical run. The D-enantiomers were more strongly retained by the chiral selector than the L-enantiomers, and the LODs of the method ranged from 0.05 to 0.5 µg/mL. As a clinical application, the amino acid enantiomers in the serum of healthy volunteers and multiple sclerosis patients were determined. The D-enantiomers of the amino acids were not detected in all samples, but the total L-Met levels in the patients were significantly higher than those in the healthy subjects. An improved method using a teicoplanin aglycone column separated the enantiomers using an ion-pairing reversed-phase mode and a low column temperature [71].

2.5. Mass spectrometry or tandem mass spectrometry detection

HPLC-MS and HPLC-MS/MS are characterized by high sensitivity and rapid separation which enables the efficient and accurate analysis of biological samples. Recently, several methods for the direct or indirect (with derivatization) determination of Hcy and related compounds have been developed.

2.5.1. Direct analysis

Several HPLC-ESI-MS or HPLC-MS/MS methods for determination of the total Hcy concentration in a blood sample (plasma or serum) have been developed. Hcy was reduced with

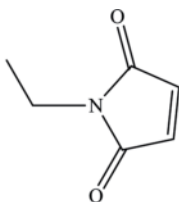
suitable reagents; then sample preparation was completed with a simple deproteinization step [48, 52]. Wang et al. reported a method for the sensitive determination of compounds related to Hcy metabolism, such as Hcy, Met, SAM, SAH, Cysta, FA, THF, 5-MT, 5-FT, serine, and histidine in human serum [72], with LODs of 0.05–1 ng/mL. Using this method, 96 serum samples comprising 46 neural tube defect cases and 50 controls were analyzed. The results showed that SAH is a risk factor for neural tube defects. Another HPLC-ESI-MS/MS method for quantifying Hcy, Cys, SAM, SAH, Cysta, Met, GSH, and CysGly in plasma using *N*-(2-mercaptopropionyl)-glycine as an IS was used to identify biomarkers for diabetic nephropathy [51].

An HPLC-ESI-MS/MS method for the determination of SAM and SAH in cultures of ovarian cancer cells was developed [13]. LODs of approximately 0.5 ng/mL for both targeted analytes allowed the designed strategy to evaluate the effect of cisplatin on changes in the methylation index between epithelial ovarian cell lines sensitive to (A2780) and resistant to (A2780CIS) to this drug after exposure to cisplatin. In addition, a stable-isotope dilution UPLC-MS/MS method for both compounds has been reported [54]. This method showed high sensitivity (0.5 for SAM and 0.7 nmol/L for SAH) and selectivity, low RSD (less than 3.3 RSD% for intra-assays and less than 10.1 RSD% for inter-assays), fast sample preparation (40 samples in 60 min), and a short analysis run time (3 min).

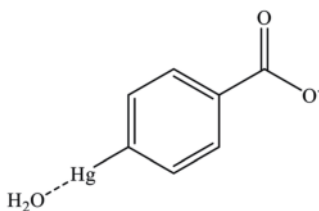
Urinary Hcy sulfonic acid is a biomarker candidate for diseases used in metabolomics approaches and was quantified by HPLC coupled with time-of-flight mass spectrometric detection (-Q-TOF/MS). An increase in Hcy sulfonic acid concentration in the urine of patients with nephrolithiasis was caused by melamine [73], and a decrease was observed in pregnant patients with intrahepatic cholestasis [74] compared with healthy volunteers.

2.5.2. Derivatization methods

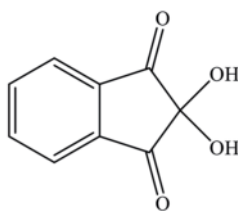
HPLC-MS (or HPLC-MS/MS) detection is often used for the determination of compounds in biological samples, but the sensitivity of this method can be inadequate due to low ionization efficiency of the analyzed specimen. Hcy and related compounds ionize poorly in comparison with other amino acids, and thus the introduction of nucleophilic groups and/or hydrophobic residues into these compounds might be useful. Derivatization reagents used to make the thiol group resistant to oxidation are shown in **Figure 4**. *N*-Ethylmaleimide (NEM) was used for the determination of Hcy in human plasma using an HPLC-MS system. The total and reduced (using DTT) concentrations of Hcy were determined with an LOD of 10 nM. Furthermore, HPLC-Orbitrap MS methods combined with *p*-(hydroxymercuri)benzoate (PHMB) as an organic mercury derivatization reagent were developed. The levels in yeast of Hcy, Cys, GSH, CysGly, GluCys, and SAH, reduced using TCEP, were determined [75]. The derivatives could be detected by HPLC-ICP-MS, but the LODs (12–128 fmol/injection) and precision for the Orbitrap MS method are higher than those of the HPLC-ICP-MS method (440–1100 fmol/injection) [17]. Thirty-six amino acids, including Hcy, Met, Hcy-Cys disulfide, Cys, Met-sulfone, Met-sulfoxide, HcyHcy, and CysCys, were determined after post-column derivatization with ninhydrin [76]. The derivatized amino acids were separated on a hydrophilic interaction liquid chromatography column with an analysis time of 18 min and LODs of 0.1 μ mol/L. As a clinical application, 97 plasma samples were analyzed for inborn errors of amino acid metabolism. An



N-ethylmaleimide (NEM)



p-hydroxymercuribenzoate (PHMB)



ninhydrin

Figure 4. Chemical structures of derivatizing reagents for MS or MS/MS detection.

increase or decrease in metabolites was identified in 95 of the samples, providing a clinical sensitivity of 97.9%.

3. Conclusions

HPLC methods coupled with various detection methods reported over the past decade were reviewed, focusing on their application for the determination of Hcy and related compounds. FL detection methods combined with novel or traditional derivatization reagents remain important for clinical studies. Also, UV/Vis and ECD are powerful, highly sensitive methods for analyzing biological samples. MS and MS/MS detection are powerful tools for identifying biomarkers of disease using a metabolomics approach. Further development of methods in the next decade by analytical researchers is anticipated.

Conflict of interest

The authors have declared no conflict of interest.

Author details

Mitsuhiro Wada^{1*}, Shinichi Nakamura¹ and Kenichiro Nakashima²

*Address all correspondence to: m-wada@phoenix.ac.jp

1 School of Pharmaceutical Sciences, Kyushu University of Health and Welfare, Nobeoka, Miyazaki, Japan

2 Faculty of Pharmaceutical Sciences, Nagasaki International University, Sasebo, Nagasaki, Japan

References

- [1] Perla-Kaján J, Twardowski T, Jakubowski H. Mechanisms of homocysteine toxicity in humans. *Amino Acids*. 2007;**32**:561-572. DOI: 10.1007/s00726-006-0432-9
- [2] Kuśmierk K, Chwatko G, Głowacki R, Kubalczyk P, Bald E. Ultraviolet derivatization of low-molecular-mass thiols for high performance liquid chromatography and capillary electrophoresis analysis. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. 2011;**879**:1290-1307. DOI: 10.1016/j.jchromb.2010.10.035
- [3] Isokawa M, Kanamori T, Funatsu T, Tsunoda M. Analytical methods involving separation techniques for determination of low-molecular-weight biothiols in human plasma and blood. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. 2014;**964**:103-115. DOI: 10.1016/j.jchromb.2013.12.041
- [4] Refsum H, Ueland PM, Nygård O, Vollset SE. Homocysteine and cardiovascular disease. *Annual Review of Medicine*. 1998;**49**:31-62. DOI: 10.1146/annurev.med.49.1.31
- [5] Jakubowski H. Homocysteine thiolactone: Metabolic origin and protein homocysteinylation in humans. *Journal of Nutrition*. 2000;**130**:377S-381S
- [6] Głowacki R, Borowczyk K, Bald E. Determination of *Nε*-homocysteinyll-lysine and γ -glutamylcysteine in plasma by liquid chromatography with UV-detection. *Journal of Analytical Chemistry*. 2014;**69**:583-589. DOI: 10.1134/S1061934814060082
- [7] Boushey CJ, Beresford SA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. *Journal of the American Medical Association*. 1995;**274**:1049-1057
- [8] Kadota K, Takamura N, Aoyagi K, Yamasaki H, Usa T, Nakazato M, Maeda T, Wada M, Nakashima K, Abe K, Takeshima F, Ozono Y. Availability of cardio-ankle vascular index

- (CAVI) as a screening tool for atherosclerosis. *Circulation Journal*. 2008;**72**:304-308. DOI: 10.1253/circj.72.304
- [9] Takamura N, Abe Y, Nakazato M, Maeda T, Wada M, Nakashima K, Kusano Y, Aoyagi K. Determinants of plasma homocysteine levels and carotid intima-media thickness in Japanese. *Asian Pacific Journal of Clinical Nutrition*. 2007;**16**:698-703
- [10] Mills JL, Scott JM, Kirke PN, McPartlin JM, Conley MR, Weir DG, Molloy AM, Lee YJ. Homocysteine and neural tube defects. *Journal of Nutrition*. 1996;**126**:756S-760S
- [11] Coppedè F. The complex relationship between folate/homocysteine metabolism and risk of Down syndrome. *Mutation Research*. 2009;**682**:54-70. DOI: 10.1016/j.mrrev.2009.06.001
- [12] Melnyk S, Pogribna M, Pogribny I, Hine RJ, James SJ. A new HPLC method for the simultaneous determination of oxidized and reduced plasma aminosulfhydryls using coulometric electrochemical detection. *Journal of Nutritional Biochemistry*. 1999;**10**:490-497
- [13] Iglesias González T, Cinti M, Montes-Bayón M, Fernández de la Campa MR, Blanco-González E. Reversed phase and cation exchange liquid chromatography with spectrophotometric and elemental/molecular mass spectrometric detection for *S*-adenosyl methionine/*S*-adenosyl homocysteine ratios as methylation index in cell cultures of ovarian cancer. *Journal of Chromatography A*. 2015;**1393**:89-95. DOI: 10.1016/j.chroma.2015.03.028
- [14] Hogg N. The effect of cyst(e)ine on the auto-oxidation of homocysteine. *Free Radical Biology and Medicine*. 1999;**27**:28-33
- [15] Kumar A, John L, Alam MM, Gupta A, Sharma G, Pillai B, Sengupta S. Homocysteine- and cysteine-mediated growth defect is not associated with induction of oxidative stress response genes in yeast. *Biochemical Journal*. 2006;**396**:61-69. DOI: 10.1042/BJ20051411
- [16] Wronska-Nofer T, Nofer JR, Stetkiewicz J, Wierzbicka M, Bolinska H, Fobker M, Schulte H, Assmann G, von Eckardstein A. Evidence for oxidative stress at elevated plasma thiol levels in chronic exposure to carbon disulfide (CS₂) and coronary heart disease. *Nutrition, Metabolism and Cardiovascular Diseases*. 2007;**17**:546-553. DOI: 10.1016/j.numecd.2006.03.002
- [17] Bakirdere S, Bramanti E, D'ulivo A, Ataman OY, Mester Z. Speciation and determination of thiols in biological samples using high performance liquid chromatography-inductively coupled plasma-mass spectrometry and high performance liquid chromatography-Orbitrap MS. *Analytica Chimica Acta*. 2010;**680**:41-47. DOI: 10.1016/j.aca.2010.09.023
- [18] Schmidt LE, Knudsen TT, Dalhoff K, Bendtsen F. Effect of acetylcysteine on prothrombin index in paracetamol poisoning without hepatocellular injury. *Lancet*. 2002;**360**:1151-1152. DOI: 10.1016/S0140-6736(02)11194-9
- [19] Wood PL, Khan MA, Moskal JR. Cellular thiol pools are responsible for sequestration of cytotoxic reactive aldehydes: central role of free cysteine and cysteamine. *Brain Research*. 2007;**1158**:158-163. DOI: 10.1016/j.brainres.2007.05.007
- [20] Zurawicz E, Kaluzna-Czaplińska J, Rynkowski J. Chromatographic methods in the study of autism. *Biomedical Chromatography*. 2013;**27**:1273-1279. DOI: 10.1002/bmc.2911

- [21] Kuśmierek K, Chwatko G, Głowacki R, Bald E. Determination of endogenous thiols and thiol drugs in urine by HPLC with ultraviolet detection. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. 2009;**877**:3300-3308. DOI: 10.1016/j.jchromb.2009.03.038
- [22] Guo XF, Wang H, Guo YH, Zhang ZX, Zhang HS. Simultaneous analysis of plasma thiols by high-performance liquid chromatography with fluorescence detection using a new probe, 1,3,5,7-tetramethyl-8-phenyl-(4-iodoacetamido)difluoroboradiaza-*s*-indacene. *Journal of Chromatography A*. 2009;**1216**:3874-3880. DOI: 10.1016/j.chroma.2009.02.083
- [23] Nakashima K. Development and application of sensitive methods with luminescence detections for determination of biologically active compounds. *Journal of Health Science*. 2011;**57**:10-21. DOI: 10.1248/jhs.57.10
- [24] Baron M, Sochor J. Estimation of thiol compounds cysteine and homocysteine in sources of protein by means of electrochemical techniques. *International Journal of Electrochemical Science*. 2013;**8**:11072-11086
- [25] Ichinose S, Nakamura M, Maeda M, Ikeda R, Wada M, Nakazato M, Ohba Y, Takamura N, Maeda T, Aoyagi K, Nakashima K. A validated HPLC-fluorescence method with a semi-micro column for routine determination of homocysteine, cysteine and cysteamine, and the relation between the thiol derivatives in normal human plasma. *Biomedical Chromatography*. 2009;**23**:935-939. DOI: 10.1002/bmc.1205
- [26] Kuśmierek K, Bald E. Reversed-phase liquid chromatography method for the determination of total plasma thiols after derivatization with 1-benzyl-2-chloropyridinium bromide. *Biomedical Chromatography*. 2009;**23**:770-775. DOI: 10.1002/bmc.1183
- [27] Khalighi HR, Mortazavi H, Alipour S, Nadizadeh K. Relationship between salivary and plasma level of homocysteine in coronary artery disease. *Dental and Medical Problems*. 2015;**52**:22-25
- [28] Ferin R, Pavão ML, Baptista J. Methodology for a rapid and simultaneous determination of total cysteine, homocysteine, cysteinylglycine and glutathione in plasma by isocratic RP-HPLC. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. 2012;**911**:15-20. DOI: 10.1016/j.jchromb.2012.10.022
- [29] Borowczyk K, Chwatko G, Kubalczyk P, Jukubowski H, Kubalska J, Głowacki R. Simultaneous determination of methionine and homocysteine by on-column derivatization with *o*-phthalaldehyde. *Talanta*. 2016;**161**:917-924. DOI: 10.1016/j.talanta.2016.09.039
- [30] Stachniuk J, Kubalczyk P, Furmaniak P, Głowacki RA. Versatile method for analysis of saliva, plasma and urine for total thiols using HPLC with UV detection. *Talanta*. 2016;**155**:70-77. DOI: 10.1016/j.talanta.2016.04.031
- [31] Ma L, He J, Zhang X, Cui Y, Gao J, Tang X, Ding M. Determination of total, free, and reduced homocysteine and related aminothiols in uremic patients undergoing hemodialysis by precolumn derivatization HPLC with fluorescence detection. *RSC Advances*. 2014;**4**:58412-58416. DOI: 10.1039/c4ra10138c

- [32] Zhang W, Li P, Geng Q, Duan Y, Guo M, Cao Y. Simultaneous determination of glutathione, cysteine, homocysteine, and cysteinylglycine in biological fluids by ion-pairing high-performance liquid chromatography coupled with precolumn derivatization. *Journal of Agricultural and Food Chemistry*. 2014;**62**:5845-5852. DOI: 10.1021/jf5014007
- [33] Zhang L, Lu B, Lu C, Lin JM. Determination of cysteine, homocysteine, cystine, and homocystine in biological fluids by HPLC using fluorosurfactant-capped gold nanoparticles as postcolumn colorimetric reagents. *Journal of Separation Science*. 2014;**37**:30-36. DOI: 10.1002/jssc.201300998
- [34] Xiao Q, Gao H, Yuan Q, Lu C, Lin JM. High-performance liquid chromatography assay of cysteine and homocysteine using fluorosurfactant-functionalized gold nanoparticles as postcolumn resonance light scattering reagents. *Journal of Chromatography A*. 2013; **1274**:145-150. DOI: 10.1016/j.chroma.2012.12.016
- [35] Steele ML, Ooi L, Münch G. Development of a high-performance liquid chromatography method for the simultaneous quantitation of glutathione and related thiols. *Analytical Biochemistry*. 2012;**429**:45-52. DOI: 10.1016/j.ab.2012.06.023
- [36] Głowacki R, Bald E, Jakubowski H. An on-column derivatization method for the determination of homocysteine-thiolactone and protein N-linked homocysteine. *Amino Acids*. 2011;**41**:187-194. DOI: 10.1007/s00726-010-0521-7
- [37] Cevasco G, Piatek AM, Scapolla C, Thea S. An improved method for simultaneous analysis of aminothiols in human plasma by high-performance liquid chromatography with fluorescence detection. *Journal of Chromatography A*. 2010;**1217**:2158-2162. DOI: 10.1016/j.chroma.2010.02.012
- [38] Głowacki R, Borowczyk K, Bald E, Jakubowski H. On-column derivatization with *o*-phthaldialdehyde for fast determination of homocysteine in human urine. *Analytical and Bioanalytical Chemistry*. 2010;**396**:2363-2366. DOI: 10.1007/s00216-010-3456-7
- [39] Gowacki R, Bald E. Determination of n-acetylcysteine and main endogenous thiols in human plasma by HPLC with ultraviolet detection in the form of their *s*-quinolinium derivatives. *Journal of Liquid Chromatography and Related Technologies*. 2009;**32**:2530-2544. DOI: 10.1080/10826070903249666
- [40] Głowacki R, Bald E. Fully automated method for simultaneous determination of total cysteine, cysteinylglycine, glutathione and homocysteine in plasma by HPLC with UV absorbance detection. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. 2009;**877**:3400-3404. DOI: 10.1016/j.jchromb.2009.06.012
- [41] Guo XF, Zhu H, Wang H, Zhang HS. Determination of thiol compounds by HPLC and fluorescence detection with 1,3,5,7-tetramethyl-8-bromomethyl-difluoroboradiaza-*s*-indacene. *Journal of Separation Science*. 2013;**36**:658-664. DOI: 10.1002/jssc.201200936
- [42] Ivanov AV, Luzyanin BP, Kubatiev AA. The use of *N*-ethylmaleimide for mass spectrometric detection of homocysteine fractions in blood plasma. *Bulletin of Experimental Biology and Medicine*. 2012;**152**:289-292. DOI: 10.1007/s10517-012-1510-5

- [43] Alkaraki AK, Hunaiti AA, Sadiq MF. Simultaneous aminothiols determination in Down syndrome individuals using a modified HPLC method. *International Journal of Integrative Biology*. 2010;**10**:90-93
- [44] Jakubowski H. New method for the determination of protein N-linked homocysteine. *Analytical Biochemistry*. 2008;**380**:257-261. DOI: 10.1016/j.ab.2008.05.049
- [45] Wada M, Kuroki M, Minami Y, Ikeda R, Sekitani Y, Takamura N, Kawakami S, Kuroda N, Nakashima K. Quantitation of sulfur-containing amino acids, homocysteine, methionine and cysteine in dried blood spot from newborn baby by HPLC-fluorescence detection. *Biomedical Chromatography*. 2014;**28**:810-814. DOI: 10.1002/bmc.3142
- [46] Wada M, Hirose M, Kuroki M, Ikeda R, Sekitani Y, Takamura N, Kuroda N, Nakashima K. Simultaneous determination of homocysteine, methionine and cysteine in maternal plasma after delivery by HPLC-fluorescence detection with DBD-F as a label. *Biomedical Chromatography*. 2013;**27**:708-713. DOI: 10.1002/bmc.2848
- [47] Özyürek M, Baki S, Güngör N, Çelik SE, Güçlü K, Apak R. Determination of biothiols by a novel on-line HPLC-DTNB assay with post-column detection. *Analytica Chimica Acta*. 2012;**750**:173-181. DOI: 10.1016/j.aca.2012.03.056
- [48] Ivanov AV, Luzyanin BP, Moskovtsev AA, Rotkina AS, Kubatiev AA. Determination of total homocysteine in blood plasma by capillary electrophoresis with mass spectrometry detection. *Journal of Analytical Chemistry*. 2011;**66**:317-321. DOI: 10.1134/S1061934811030075
- [49] Bai S, Chen Q, Lu C, Lin JM. Automated high performance liquid chromatography with on-line reduction of disulfides and chemiluminescence detection for determination of thiols and disulfides in biological fluids. *Analytica Chimica Acta*. 2013;**768**:96-101. DOI: 10.1016/j.aca.2013.01.035
- [50] Benkova B, Lozanov V, Ivanov IP, Todorava A, Milanov I, Mitev V. Determination of plasma aminothiols by high performance liquid chromatography after precolumn derivatization with N-(2-acridonyl)maleimide. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. 2008;**870**:103-108. DOI: 10.1016/j.jchromb.2008.06.015
- [51] Jiang Z, Liang Q, Luo G, Hu P, Li P, Wang Y. HPLC-electrospray tandem mass spectrometry for simultaneous quantitation of eight plasma aminothiols: Application to studies of diabetic nephropathy. *Talanta*. 2009;**77**:1279-1284. DOI: 10.1016/j.talanta.2008.08.031
- [52] Persichilli S, Gervasoni J, Iavarone F, Zuppi C, Zappacosta B. A simplified method for the determination of total homocysteine in plasma by electrospray tandem mass spectrometry. *Journal of Separation Science*. 2010;**33**:3119-3124. DOI: 10.1002/jssc.201000399
- [53] Jia M-Y, Niu L-Y, Zhang Y, Yang Q-Z, Tung C-H, Guan Y-F, Feng L. BODIPY-based fluorometric sensor for the simultaneous determination of Cys, Hcy, and GSH in human serum. *ACS Applied Materials and Interfaces*. 2015;**7**:5907-5914. DOI: 10.1021/acsami.5b00122

- [54] Kirsch SH, Knapp JP, Geisel J, Herrmann W, Obeid R. Simultaneous quantification of *S*-adenosyl methionine and *S*-adenosyl homocysteine in human plasma by stable-isotope dilution ultra performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. 2009;**877**:3865-3870. DOI: 10.1016/j.jchromb.2009.09.039
- [55] Espina JG, Montes-Bayón M, Blanco-González E, Sanz-Medel A. Determination of reduced homocysteine in human serum by elemental labelling and liquid chromatography with ICP-MS and ESI-MS detection. *Analytical and Bioanalytical Chemistry*. 2015;**407**:7899-7906. DOI: 10.1007/s00216-015-8956-z
- [56] Isokawa M, Funatsu T, Tsunoda M. Fast and simultaneous analysis of biothiols by high-performance liquid chromatography with fluorescence detection under hydrophilic interaction chromatography conditions. *The Analyst*. 2013;**138**:3802-3808. DOI: 10.1039/c3an00527e
- [57] Costa De Campos Barbosa TM, Das Graças Carvalho M, Nicácio Silveira J, Rios JG, Komatsuzaki F, Carvalho Godói L, Yoshizane Costa GH. Homocysteine: Validation and comparison of two methods using samples from patients with pulmonary hypertension. *Jornal Brasileiro de Patologia e Medicina Laboratorial*. 2014;**50**:402-409. DOI: 10.5935/1676-2444.20140048
- [58] Valente A, Bronze MR, Bicho M, Duarte R, Costa HS. Validation and clinical application of an UHPLC method for simultaneous analysis of total homocysteine and cysteine in human plasma. *Journal of Separation Science*. 2012;**35**:3427-3433. DOI: 10.1002/jssc.201200672
- [59] Chen M, Mei Q, Xu J, Lu C, Fang H, Liu X. Detection of melatonin and homocysteine simultaneously in ulcerative colitis. *Clinica Chimica Acta*. 2012;**413**:30-33. DOI: 10.1016/j.cca.2011.06.036
- [60] Persichilli S, Gervasoni J, Castagnola M, Zuppi C, Zappacosta B. A reversed-phase HPLC fluorimetric method for simultaneous determination of homocysteine-related thiols in different body fluids. *Laboratory Medicine*. 2011;**42**:657-662. DOI: 10.1309/LMOIAH19RG5BKBIQ
- [61] Jakubowski H. Quantification of urinary *S*- and *N*-homocysteinylation protein and homocysteine-thiolactone in mice. *Analytical Biochemistry*. 2016;**508**:118-123. DOI: 10.1016/j.ab.2016.06.002
- [62] McMenamin ME, Himmelfarb J, Nolin TD. Simultaneous analysis of multiple amino-thiols in human plasma by high performance liquid chromatography with fluorescence detection. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. 2009;**877**:3274-3281. DOI: 10.1016/j.jchromb.2009.05.046
- [63] Zhang L-Y, Tu F-Q, Guo X-F, Wang H, Wang P, Zhang H-S. A new BODIPY-based long-wavelength fluorescent probe for chromatographic analysis of low-molecular-weight thiols. *Analytical and Bioanalytical Chemistry*. 2014;**406**:6723-6733. DOI: 10.1007/s00216-014-8013-3

- [64] McDermott GP, Terry JM, Conlan XA, Barnett NW, Francis PS. Direct detection of biologically significant thiols and disulfides with manganese(IV) chemiluminescence. *Analytical Chemistry*. 2011;**83**:6034-6039. DOI: 10.1021/ac2010668
- [65] Li Q, Shang F, Lu C, Zheng Z, Lin JM. Fluorosurfactant-prepared triangular gold nanoparticles as postcolumn chemiluminescence reagents for high-performance liquid chromatography assay of low molecular weight amino thiols in biological fluids. *Journal of Chromatography A*. 2011;**1218**:9064-9070. DOI: 10.1016/j.chroma.2011.10.021
- [66] Khan A, Khan MI, Iqbal Z, Shah Y, Ahmad L, Nazir S, Wason DG, Khan JA, Nasir F, Ismail. A new HPLC method for the simultaneous determination of ascorbic acid and amino thiols in human plasma and erythrocytes using electrochemical detection. *Talanta*. 2011;**84**:789-801. DOI: 10.1016/j.talanta.2011.02.019
- [67] Khan MI, Iqbal Z. Simultaneous determination of ascorbic acid, amino thiols, and methionine in biological matrices using ion-pairing RP-HPLC coupled with electrochemical detector. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. 2011;**879**:2567-2575. DOI: 10.1016/j.jchromb.2011.07.013
- [68] Hannan PA, Khan JA, Iqbal Z, Ullah I, Rehman UW, Rehman M, Nasir F, Khan A, Muhammad S, Hassan M. Simultaneous determination of endogenous antioxidants and malondialdehyde by RP-HPLC coupled with electrochemical detector in serum samples. *Journal of Liquid Chromatography and Related Technologies*. 2015;**38**:1052-1060. DOI: 10.1080/10826076.2015.1012522
- [69] Deáková Z, Duráková Z, Armstrong DW, Lehotay J. Separation of enantiomers of selected sulfur-containing amino acids by using serially coupled achiral-chiral columns. *Journal of Liquid Chromatography and Related Technologies*. 2015;**38**:789-794. DOI: 10.1080/10826076.2014.968666
- [70] Deáková Z, Duráková Z, Armstrong DW, Lehotay J. Two-dimensional high performance liquid chromatography for determination of homocysteine, methionine and cysteine enantiomers in human serum. *Journal of Chromatography A*. 2015;**1408**:118-124. DOI: 10.1016/j.chroma.2015.07.009
- [71] Bystrická Z, Bystrický R, Lehotay J. Thermodynamic study of HPLC enantioseparations of some sulfur-containing amino acids on teicoplanin columns in ion-pairing reversed-phase mode. *Journal of Liquid Chromatography and Related Technologies*. 2016;**39**:775-781. DOI: 10.1080/10826076.2016.1247715
- [72] Wang Y, Zhang HY, Liang QL, Yang HH, Wang YM, Liu QF, Hu P, Zheng XY, Song XM, Chen G, Zhang T, Wu JX, Luo GA. Simultaneous quantification of 11 pivotal metabolites in neural tube defects by HPLC-electrospray tandem mass spectrometry. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. 2008;**863**:94-100. DOI: 10.1016/j.jchromb.2008.01.010
- [73] Duan H, Guan N, Wu Y, Zhang J, Ding J, Shao B. Identification of biomarkers for melamine-induced nephrolithiasis in young children based on ultra high performance

liquid chromatography coupled to time-of-flight mass spectrometry (U-HPLC-Q-TOF/MS). *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. 2011;**879**:3544-3550. DOI: 10.1016/j.jchromb.2011.09.039

- [74] Ma L, Zhang X, Pan F, Cui Y, Yang T, Deng L, Shao Y, Ding M. Urinary metabolomic analysis of intrahepatic cholestasis of pregnancy based on high performance liquid chromatography/mass spectrometry. *Clinica Chimica Acta*. 2017;**471**:292-297. DOI: 10.1016/j.cca.2017.06.021
- [75] Rao Y, Xiang B, Bramanti E, D'Ulivo A, Mester Z. Determination of Thiols in Yeast by HPLC coupled with LTQ-Orbitrap mass spectrometry after derivatization with *p*-(hydroxymercuri)benzoate. *Journal of Agricultural and Food Chemistry*. 2010;**58**:1462-1468. DOI: 10.1021/jf903485k
- [76] Prinsen HCMT, Schiebergen-Bronkhorst BGM, Roeleveld MW, Jan JJM, Sain-van Velden MGM, Visser G, van Hasselt PM, Verhoeven-Duif NM. Rapid quantification of underivatized amino acids in plasma by hydrophilic interaction liquid chromatography (HILIC) coupled with tandem mass-spectrometry. *Journal of Inherited Metabolic Disease*. 2016;**39**:651-660. DOI: 10.1007/s10545-016-9935-z

