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Review

# Simultaneous analysis of multiple aminothiols in human plasma by high performance liquid chromatography with fluorescence detection☆

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ABSTRACT

Aminothiols serve numerous vital functions in biochemistry, including detoxification and regulation of cellular metabolism, enzymatic activity, and protein trafficking and degradation. Plasma aminothiol concentrations are frequently measured for clinical and translational research investigating oxidative stress, and for routine clinical diagnosis and monitoring of vascular injury. Although a variety of techniques are available to measure aminothiol concentrations in plasma, high performance liquid chromatography with fluorescence detection (HPLC–FD) is the most widely used. This review summarizes HPLC–FD methods, including pre-analytical considerations, procedures for sample reduction, derivatization, and chromatographic separation of the primary biological aminothiols cysteine, homocysteine, cysteinylglycine, and glutathione in human plasma.



**Contents**



## 1. Introduction

Aminothiols are low molecular weight thiol-containing amino acids that play important roles in cell signaling, metabolism and detoxification. Several studies have demonstrated that aminothiols are involved in the pathogenesis of human diseases [1,2]. In particular, the association between thiol concentrations, oxidative stress and cardiovascular disease has received much attention [2–5]. As a result, measurement of plasma aminothiol concentrations has emerged as a useful tool for diagnosing and monitoring the presence of human diseases and metabolic disorders including oxidative stress [5–15]. This has generated a need for simple, sensitive, precise, and fully validated analytical methods capable of determining aminothiols for research purposes and for routine

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clinical use. Assays capable of determining multiple analytes are desirable because of extensive interconversion of aminothiols and the need to understand the dynamic relationship between all thiol and disulfide components [16,17], as well as to facilitate assessment of the relationship between thiols and disease status.

A variety of methods are available for determining concentrations of two or more aminothiols, including capillary electrophoresis [18,19], gas or liquid chromatography with mass spectrometric detection [20–22], and HPLC with ultraviolet [23–25], fluorescence [15,26–40], or electrochemical detection [41–44]. Each of these methods has basic limitations in terms of performance, equipment cost, complexity, sample processing and run times, and/or validation parameters assessed, which create challenges or render them impractical for high-throughput routine clinical or research purposes. For instance, methods based on mass spectrometric detection provide highly specific and sensitive quantification of compounds of interest often with less sample manipulation compared to other chromatographic methods. Unfortunately, mass spectrometric detection is cumbersome, and requires expensive instrumentation that may be out of reach for many laboratories [45–47]. Ultraviolet detection suffers from poor sensitivity and specificity [45]. Electrochemical detection represents an important tool for the analysis of redox-reactive compounds such as thiols and disulfides, but it exhibits high oxidation potential that can reduce its performance and possibly contributes to the poorer precision reported than for ultraviolet or fluorimetric detection methods [45,46].

HPLC with fluorescence detection (HPLC–FD) is the most commonly used method for determination of aminothiols due to its high sensitivity, relative simplicity, ease of automation, and high-throughput capability [45,46]. Sample preparation usually involves disulfide reduction, protein precipitation, and derivatization prior to chromatographic separation. HPLC–FD allows the simultaneous determination of the primary biological aminothiols cysteine (CYS), homocysteine (Hcys), cysteinylglycine (CysGly), and glutathione (GSH) (Fig. 1). Simultaneous measurement of multiple aminothiols is desirable since many of them are metabolically related. For example, it is well established that homocysteine and cysteine are key components of the transsulfuration pathway; cysteine is a precursor of glutathione; and cysteinylglycine is a breakdown product of glutathione. Generally speaking, excellent linearity is observed over a long range, extending well above and below the concentrations found in health and disease conditions [31,35]. Reagent costs are comparatively low and the equipment required is available in most research laboratories. Aminothiol concentrations previously reported in healthy subjects and other patient populations using selected HPLC–FD methods are presented in Table 1.

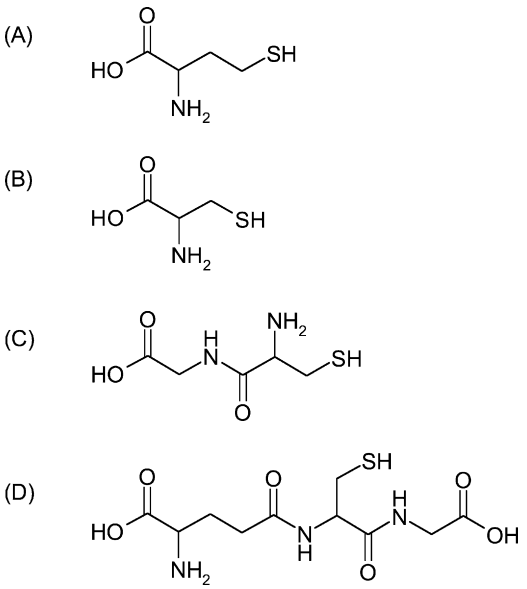


**Fig. 1.** Structures of (A) Hcys, (B) CYS, (C) CysGly, and (D) GSH.

**Table 1**
Aminothiol concentrations reported in healthy subjects and other patient populations.

| Author | Population | Concentration[a,b] (μM) | | | | |
|---|---|---|---|---|---|---|
| | | *N* | Cys | Hcys | GSH | CysGly |
| Ivanov et al. [39] | H, M | 17 | 219.3 ± 31.1 | 11.7 ± 3.7 | 7.1 ± 2.6 | NR |
| | H, F | 16 | 215.3 ± 34.6 | 9.8 ± 2.9 | 6.6 ± 2.5 | NR |
| Pastore et al. [34] | H, M | 20 | 212 ± 23 | 9.2 ± 2.0 | 12.8 ± 4.6 | 65.7 ± 4.6 |
| | H, F | 24 | 206 ± 14 | 6.7 ± 0.5 | 12.8 ± 3.6 | 53.2 ± 7.7 |
| Williams et al. [35] | H | 20 | 120.5 ± 9.8[c] | 6.5 ± 0.3[c] | 5.7 ± 0.3[c] | 25.8 ± 1.4[c] |
| | S | 20 | 164.2 ± 15.2[c] | 9.9 ± 0.6[c] | 4.6 ± 0.2[c] | 21.8 ± 1.2[c] |
| Jacobsen et al. [33] | H, M | 36 | 209.6 ± 28.4 | 9.3 ± 1.9 | NR | 30.8 ± 4.5 |
| | H, F | 35 | 190.7 ± 27.5 | 7.9 ± 2.3 | NR | 26.5 ± 3.7 |
| Mansoor et al. [32] | H, M | 8 | 264.3 ± 33.3 | 11.9 ± 1.5 | 7.6 ± 1.3 | 31.8 ± 5.4 |
| | H, F | 10 | 236.0 ± 26.2 | 10.9 ± 2.1 | 6.2 ± 1.4 | 27.6 ± 3.4 |
| Rizzo et al. [58] | H | 36 | 118.6 ± 31.2 | 7.5 ± 2.8 | NR | 17.8 ± 7.4 |
| Pfeiffer et al. [37] | H, M | 27 | 298 ± 29 | 9.1 ± 1.8 | NR | NR |
| | H, F | 43 | 280 ± 32 | 7.8 ± 1.7 | NR | NR |
| Durand et al. [38] | H, M | 15 | 210.1 ± 27.3 | 9.2 ± 1.8 | 4.5 ± 0.9 | 30.3 ± 7.3 |
| | H, F | 15 | 191.5 ± 30.3 | 7.3 ± 1.6 | 4.3 ± 1.7 | 24.7 ± 3.8 |
| Sjoberg et al. [15] | H | 15 | 270 ± 40 | 11 ± 3 | 4.1 ± 1.4 | 26 ± 7 |
| | CKD | 47 | 329 ± 62 | 20 ± 7 | 4.9 ± 3.4 | 29 ± 10 |
| Carducci et al. [27] | H | 46 | 168 ± 28 | 7.7 ± 1.9 | NR | NR |
| Tcherkas et al. [28] | H | 19 | 260 ± 11 | 8.3 ± 0.5 | NR | NR |
| | CVD | 34 | 255 ± 12 | 11.7 ± 1.1 | NR | NR |

[a] Concentrations μM unless otherwise specified.
[b] Concentration data presented as mean ± SD unless otherwise specified.
[c] Mean ± SE.
*N*, number of subjects; H, healthy subjects; M, males only; F, females only; S, stroke patients; CKD, chronic kidney disease patients; CVD, cardiovascular disease patients; NR, not reported.

This review summarizes HPLC–FD methods, including pre-analytical considerations, procedures for sample reduction, derivatization, and simultaneous chromatographic separation of multiple aminothiols (e.g., cysteine, homocysteine, cysteinylglycine, and glutathione) in human plasma.

## 2. Pre-analytical considerations

### 2.1. Total aminothiols and aminothiol redox species

Thiol groups (–SH) are critical intracellular and extracellular redox buffers which readily undergo oxidative coupling reactions to form disulfides (–S–S–). Aminothiols are present *in vivo* in their reduced form and a variety of disulfide derivatives. Disulfide moieties may be bound to protein, or non-protein bound as homocystine or cystine or as mixed disulfides (e.g., CYS–Hcys)[48]. All redox forms are measured together in assays that determine total aminothiol concentrations. However, an increase or decrease in the total concentration of an aminothiol does not necessarily indicate a proportional change in concentration for each redox form [15]. Also, the activity of one redox form may contribute more to the pathogenesis of a disease. For example, reduced Hcys is more likely to be atherogenic than oxidized or protein-bound Hcys [49]. Therefore, it may be informative to examine the individual redox forms of aminothiols. Altered redox status of aminothiols has been observed in a number of diseases including vascular and renal disease [7,14,15,50].

Accurately measuring individual redox components for an aminothiol is technically challenging because interconversion between redox forms (e.g., cysteine oxidized to cystine) continues *ex vivo*. Reduced aminothiols are oxidized within minutes if whole blood or plasma is left at room temperature [51]. Methods require complex sample processing immediately following blood collection and prior to storage. Moreover, reduced aminothiols will be oxidized during storage at −70 °C [15]. Acidic sodium citrate has been used as an anticoagulant to stabilize the reduced form of aminothiols for two weeks [35]. The mechanism for this is unknown, but it has been hypothesized that the acidic environment stabilizes the sulfhydryl group [35]. Most methods require derivatization and analysis of the reduced form immediately or within 24 h of sample collection [15,32,51]. Currently, measurement of reduced and oxidized forms of aminothiols is best suited for smaller studies due to the complex, time intensive nature of sample processing. For clinical studies with large numbers of subjects, it is far more practical to measure total aminothiol concentrations. The thiol forms (e.g., total, reduced) measured by selected HPLC–FD methods are presented in Table 2.

### 2.2. Sample collection and handling

Blood collection and processing prior to sample storage is a critical step in the accurate determination of aminothiol concentrations. Inadequate or inconsistent processing may lead to artificially high or low concentrations of aminothiols.

Sampling conditions for homocysteine measurement have been well investigated and reviewed previously [52–56]. Several seemingly benign variables, such as a subject's food intake and posture during sample collection, have been shown to influence homocysteine concentrations [54–56]. Other more obvious variables are also a concern. For example, due to the high concentrations of glutathione found in erythrocytes (∼1 mM), even slight hemolysis may result in elevated concentrations of plasma glutathione. Recommendations based on this information support the establishment of standardized protocols [56]. All aspects of sample collection should be uniform because minor but sys-

**Table 2**
Operating conditions of selected methods for simultaneous determination of aminothiols.

| Method | Thiols measured | Thiol form measured | Anti-coagulant | Sample volume (μL) | Reducing agent | Prot precip | Derivatizing agent | Thiol standard form | Calibrator matrix | IS (conc) |
|---|---|---|---|---|---|---|---|---|---|---|
| Ivanov et al. [39] | Cys, Hcys, GSH | Total | Sodium citrate | 62 | TPP | SSA | mBrB | Disulfide (Hcys, GSH) | Plasma | None |
| Fiskerstrand et al. [36] | Cys, Hcys, CysGly | Total; free | EDTA | 30 | $NaBH_4$ | SSA | mBrB | Disulfide (Cys, Hcys) | 0.1 M HCl | None |
| Pastore et al. [34] | Cys, Hcys, GSH, CysGly | Total | EDTA | 10 | $NaBH_4$ | HCl | mBrB | Disulfide (Cys, Hcys) | 0.1 M HCl | None |
| Williams et al. [35] | Cys, Hcys, GSH, CysGly | Total; free; reduced | Sodium citrate | 30 | $NaBH_4$ | SSA | mBrB | Reduced | 0.1 M HCl | None |
| Jacobsen et al. [33] | Cys, Hcys, CysGly | Total | EDTA | 100 | $NaBH_4$ | PCA | mBrB | Disulfide (Hcys only) | Serum | None |
| Mansoor et al. [32] | Cys, Hcys, GSH, CysGly | Total; free; PB; reduced | Heparin | 30 | $NaBH_4$ | SSA | mBrB | Reduced | 5% SSA + 50 μM DTE | None |
| Krijt et al. [40] | Cys, Hcys, GSH, CysGly | Total | EDTA | 100 | TCEP | TCA | SBD-F | Reduced | Plasma; PBS | MPG (12.3 μM) |
| Rizzo et al. [58] | Cys, Hcys, CysGly | Total | EDTA | 200 | DTT | TCA | SBD-F | Reduced | 0.12 M PCA | NAC (612 μM) |
| Pfeiffer et al. [37] | Cys, Hcys | Total | EDTA | 50 | TCEP | TCA | SBD-F | Disulfide (Hcys only) | Plasma; PBS | cysteamine (10 μM) |
| Durand et al. [38] | Cys, Hcys, GSH, CysGly | Total | EDTA | 240 | TBP | PCA | SBD-F | Reduced | Plasma | NAC (500 μM) |
| Sjoberg et al. [15] | Cys, Hcys, GSH, CysGly | Total; free; reduced | EDTA | 100 | TBP | TCA | SBD-F | NR | NR | None |
| Nolin et al. [31] | Cys, Hcys, GSH, CysGly | Total | EDTA | 50 | TCEP | TCA | SBD-F | Reduced | PBS | MPG (25 μM) |
| Carducci et al. [27] | Cys, Hcys | Total; free | Heparin | 50 | 2-ME | UF | OPA | Disulfide | $H_2O$ | None |
| Tcherkas et al. [28] | Cys, Hcys | Total | EDTA | 100 | 2-ME | Methanol | OPA | Reduced | 0.05 M PCA | None |

Prot Precip, protein precipitation reagent; IS, internal standard; Cys, cysteine; Hcys, homocysteine; CysGly, cysteinylglycine; GSH, glutathione; PB, protein-bound; EDTA, ethylenediaminetetraacetic acid; TPP, triphenylphosphine; $NaBH_4$, sodium borohydride; TCEP, trix-(2-carboxyethyl) phosphine; DTT, dithiothreitol; TBP, tributylphosphine; 2-ME, 2-mercaptoethanol; SSA, sulfosalicylic acid; HCl, hydrochloric acid; PCA, perchloric acid; TCA, trichloroacetic acid; UF, ultrafiltration; mBrB, monobrombimane; SBD-F, ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid; OPA, *o*-phthaldialdehyde.

**Table 3**
Effect of freeze-thaw on aminothiol concentrations in human plasma from healthy subjects.

| Analyte (μM) | Control | FT1 | %Δ | FT2 | %Δ | 3 h ice | %Δ |
|---|---|---|---|---|---|---|---|
| CYS | 281.3 ± 34.4 | 281.2 ± 35.3 | 0.0 ± 2.2 | 283.0 ± 35.5 | 0.6 ± 2.6 | 279.6 ± 33.5 | −0.5 ± 2.1 |
| Hcys | 8.6 ± 2.6 | 8.5 ± 2.5 | 0.0 ± 2.3 | 8.6 ± 2.6 | 0.7 ± 2.7 | 8.5 ± 2.5 | −0.3 ± 2.3 |
| GSH | 6.6 ± 1.5 | 6.1 ± 1.5[a] | −7.0 ± 4.9 | 5.8 ± 1.5[a] | −11.3 ± 4.1 | 6.5 ± 1.6 | −0.3 ± 12.1 |
| CysGly | 31.7 ± 5.6 | 32.2 ± 5.7[a] | 1.7 ± 2.2 | 32.8 ± 5.8[a] | 3.6 ± 2.8 | 31.8 ± 5.6 | 0.3 ± 2.7 |

CYS, cysteine; Hcys, homocysteine; GSH, glutathione; CysGly, cysteinylglycine; FT1, one freeze-thaw cycle; FT2, two freeze-thaw cycles; %Δ, percentage change versus control. Note that the headings represent conditions prior to sample preparation/derivatization. Control samples were centrifuged and frozen at −70 °C within 1 h of blood draw, then thawed on a later date for experimentation. 3 h ice samples were stored as whole blood for 3 h on ice water prior to centrifugation and freezing, and then thawed on a later date for experimentation. FT1 and FT2 samples were centrifuged and frozen within 1 h of blood draw, then subjected to 1 or 2 freeze-thaw cycles, each of which consisted of freezing at −70 °C for 3 h following by thawing at room temperature for 30 min prior to experimentation. Concentrations determined via method of Nolin et al. [31]. Comparisons made by the two-sided paired Student's *t*-test. Data expressed as mean ± SD.

[a] $P < 0.01$ vs. control.

tematic variation in procedure may influence the results and, consequently, study outcome. Although these recommendations pertain to total homocysteine measurement, the benefits of a standardized protocol apply to measurement of all aminothiols.

Following sample collection, whole blood tubes must be placed immediately in ice water and centrifuged as soon as possible. Cooling whole blood slows down or inhibits homocysteine export by red blood cells and enzyme activity that may cause interconversion of aminothiols (i.e., break down of GSH to CysGly through the activity of gamma-glutamyl transferase). Total concentrations of homocysteine, cysteine, CysGly and glutathione are constant in whole blood kept on ice water for up to 3 h [46,52]. Plasma obtained from centrifugation should remain cooled during aliquotting then frozen immediately.

### 2.3. Sample stability

Once frozen at −70 °C, total aminothiol concentrations are constant for several years until sample preparation and analysis can be performed [46,52]. Total plasma homocysteine and cysteine concentrations are constant during repeated freeze-thaw cycles [52]. However, significant changes in total plasma CysGly and glutathione concentrations have been observed following freeze-thaw. In a recent comparison of paired samples ($N = 19$) undergoing one versus two freeze thaw cycles, we found that total CysGly concentrations increased 1.7% ($P < 0.01$) and 3.6% ($P < 0.01$), respectively, and total GSH concentrations decreased 7.0% ($P < 0.01$) and 11.3% ($P < 0.01$), respectively (previously unpublished, Table 3).

### 2.4. Plasma or serum and choice of anticoagulant

Significant differences in homocysteine concentrations between plasma and serum have been reported [52,57]. We found significant differences in measured concentrations of CYS, Hcys, CysGly, and GSH in serum versus EDTA-plasma samples obtained from healthy subjects and from subjects with end stage renal disease (previously unpublished, Table 4). Whole blood must be placed on ice water immediately following sample collection to minimize interconversion of aminothiols and thus preserve individual aminothiol concentrations. This makes it logistically difficult to measure aminothiols in serum since it must remain at room temperature to allow clotting. Therefore, it is recommended that aminothiols be measured in plasma [54]. As depicted in Table 2, although heparin- and citrate-plasma have been utilized [35,36], EDTA-plasma is more commonly used and is the preferred anticoagulant to prevent metal catalyzed reactions and cell activation in studies investigating oxidative stress.

**Table 4**
Comparison of aminothiol concentrations in human plasma versus serum.

| Analyte (μM) | Healthy subjects | | ESRD subjects | |
|---|---|---|---|---|
| | Plasma | Serum | Plasma | Serum |
| CYS | 217 ± 41 | 229 ± 44[a] | 369 ± 58 | 384 ± 60[a] |
| Hcys | 6.8 ± 2.4 | 7.4 ± 2.5[a] | 19.0 ± 3.1 | 20.4 ± 3.2[a] |
| GSH | 5.4 ± 1.0 | 5.8 ± 1.0[b] | 4.9 ± 0.8 | 5.3 ± 1.4 |
| CysGly | 26.7 ± 4.4 | 28.3 ± 4.0[a] | 41.0 ± 13.3 | 44.8 ± 14.1[a] |

CYS, cysteine; Hcys, homocysteine; GSH, glutathione; CysGly, cysteinylglycine; ESRD, end-stage renal disease. Concentrations determined via method of Nolin et al. [31]. Comparisons made by the two-sided paired Student's *t*-test. Data expressed as mean ± SD.

[a] $P < 0.001$ vs. plasma.

[b] $P < 0.02$ vs. plasma.

## 3. HPLC analysis

### 3.1. Reduction

Sample preparation involves reduction of disulfides, precipitation of proteins and derivatization of available thiols. Complete reduction of disulfide bonds prior to derivatization is critical to accurate quantification of total aminothiols. Adding a reducing agent to plasma samples releases protein-bound aminothiols and reduces free disulfides. Several reducing agents have been used (Table 2), including sodium borohydride [33,34,36], dithiothreitol (DTT) [58], tributylphosphine (TBP) [15,26,38,57], and tris-(2-carboxyethyl) phosphine (TCEP) [31,40,59].

DTT and sodium borohydride are problematic because they are reactive with thiol-specific fluorogenic reagents. During derivatization, the labeling reagent is consumed and a background fluorescent product is generated. Upon chromatographic separation, this background peak can obscure the detection of aminothiol adducts. Although steps may be taken to remove excess reducing agent prior to derivatization (e.g., solid phase extraction), there is a risk of reoxidation of aminothiols, likely creating a large source of error.

Trialkylphosphines TBP and TCEP are powerful reductants, which stoichiometrically and irreversibly reduce disulfides and need to be present in only slight molar excess for complete reduction of disulfides. They are non-reactive toward many other functional groups and do not interfere with subsequent derivatization. TBP has a highly disagreeable odor and is poorly soluble in water, so it must be dissolved in dimethylformamide for use. TCEP is non-volatile, stable and water-soluble, making it easy to use and equally efficient in plasma or aqueous solutions, so it has emerged as a preferred reductant [40,59]. In a comparison of methods using TCEP versus TBP as a reducing agent, Krijt et al showed TCEP is less sensitive to temperature and calibrator matrix [40]. Therefore, when using TCEP as the reducing agent, calibration standards may be prepared in either plasma or PBS. EDTA is added to all solutions used in analytical sample preparation to prevent re-oxidation.

Immediately following reduction, proteins are removed by ultrafiltration [27] or acid precipitation with trichloroacetic acid, perchloric acid, or sulfosalicylic acid. Rizzo et al tested these deproteinizing agents and found that trichloroacetic acid improved assay sensitivity and precision [58]. Perchloric acid and sulfosalicylic acid were not appropriate for detecting low concentrations of aminothiols [58]. Alternatively, Durand et al described compounds of interest eluting as doublet peaks as a result of using trichloroacetic acid and avoided this problem by using perchloric acid [38]. Samples are then centrifuged to obtain protein-free supernatant ready for derivatization.

### 3.2. Derivatization reagents

Fluorogenic reagents used to detect aminothiols in plasma include monobromobimane (mBrB) [32–36,39], *o*-phthaldialdehyde (OPA) [27,28], dansyl chloride [29,30], and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) [15,26,31,37,38,40]. Monobromobimane reacts rapidly, but not specifically, with thiols at pH 8.0 at room temperature. Derivatization and chromatographic conditions have been repeatedly improved to obtain satisfactory separation between aminothiols of interest and interfering peaks [60].

OPA also has the advantage of a short derivatization time at room temperature. OPA reacts with all primary amino groups in the presence of a thiol to produce a fluorescent isoindole. However, current methods for simultaneous measurement of multiple aminothiols with OPA involve alkylation of free thiols with iodoacetate and addition of 2-mercaptoethanol as a reducing agent and source of thiols for derivatization. Although this method allows detection of other amino acids in addition to thiol-containing amino acids, complex chromatography is required to separate aminothiols from other derivatized amino acids. Dansyl chloride may be used to determine redox forms of aminothiols in one sample, rather than depending on two or more analyses [29,30]; however, this method is not useful for measuring total aminothiol concentrations.

SBD-F exhibits excellent sensitivity and specificity towards sulfhydryl groups and is the most popular derivatizing agent. SBD-F and its hydrolysis products are non-fluorescent, resulting in chromatograms with well-resolved aminothiol peaks and no interfering reagent peaks. The drawbacks of using SBD-F are a long derivatization time and elevated temperature requirements (1 h at 60 °C). Thiol–SBD-adducts are stable for at least 8 h when protected from light [61]. With the use of an internal standard, we have shown that loss of fluorescence does not affect calculated results for up to 18 h [31].

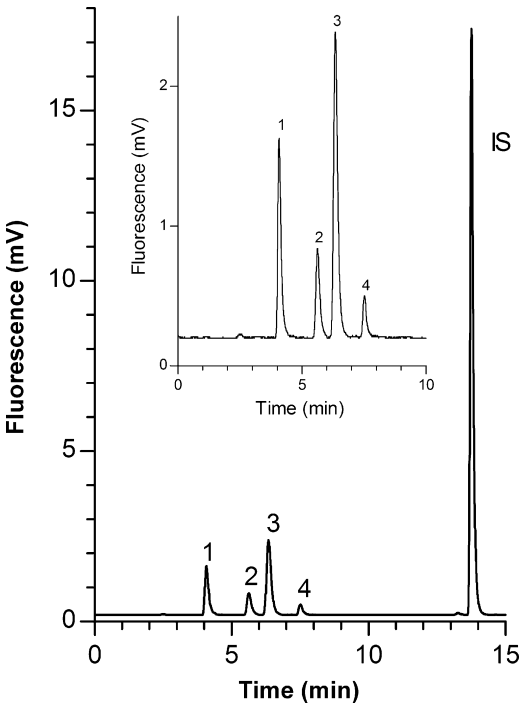


**Fig. 2.** Representative chromatogram of aminothiol standard solution containing 25.0 μM CYS, 3.13 μM Hcys, 6.25 μM CysGly, and 1.56 μM GSH. Each thiol compound was derivatized with SBD-F (1 h at 60 °C as reported previously [31]) and separated on a Waters Symmetry $C_{18}$ column (4.6 mm × 150 mm; 5 μm particles) at 29 °C with two mobile phases, A: 0.1 M acetate buffer (pH 4.5)–methanol [97:3] and B: methanol. SBD-adducts were separated over 8 min with 100% A at a flow rate of 0.8 mL/min, followed by 80%:20% A:B (changed linearly over 1 min) at a flow rate of 1.0 mL/min for 6 min. Peaks: 1, CYS; 2, Hcys; 3, CysGly; 4, GSH; IS, 2-mercaptopropionylglycine.

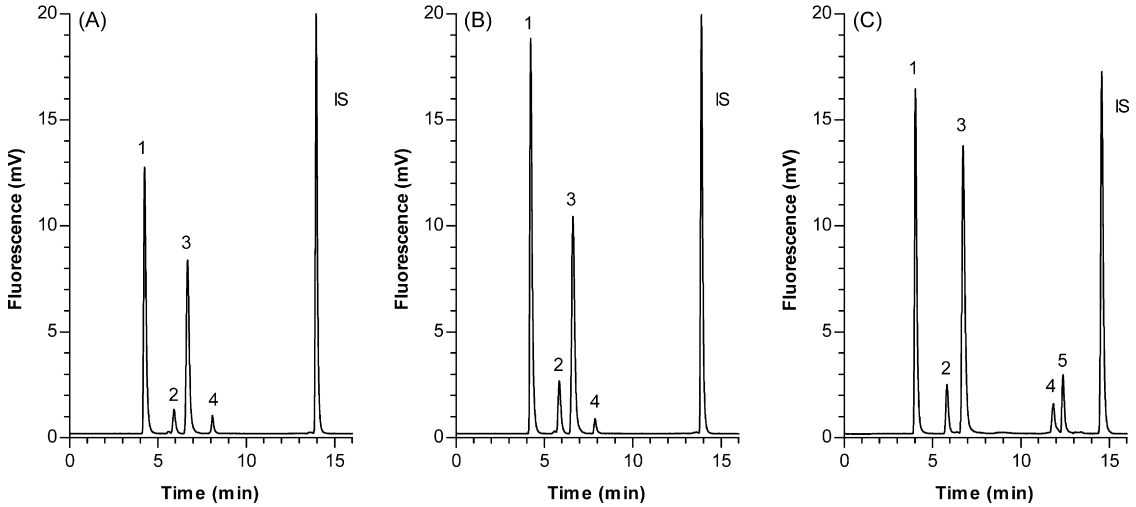


**Fig. 3.** Representative chromatograms of total aminothiols in plasma samples obtained from (A) a healthy subject, (B) a patient with chronic kidney disease, and (C) a healthy subject immediately prior to administration of the last dose of *N*-acetylcysteine 600 mg administered orally twice daily for 14 days. All samples were prepared and derivatized according to Nolin et al. [31]. In (A) and (B) SBD-adducts were separated as described in Fig. 2. For (C), chromatographic conditions were optimized to measure NAC on a Waters Symmetry $C_{18}$ column (4.6 mm × 150 mm; 5 μm particles) at 29 °C with two mobile phases, A: 0.1 M acetate buffer (pH 4.0)–methanol [98:2] and B: methanol. SBD-adducts were separated over 7 min with 100% A at a flow rate of 1.0 mL/min, followed by 80%:20% A:B (changed linearly over 1 min) for 7 min. Peaks: 1, CYS; 2, Hcys; 3, CysGly; 4, GSH; 5, NAC; IS, 2-mercaptopropionylglycine.

**Table 5**
Performance characteristics of selected methods for simultaneous determination of aminothiols.

| Author | Thiol | Linear range[a] (μM) | Intra-assay CV (%) | Inter-assay CV (%) | LOD |
|---|---|---|---|---|---|
| Ivanov et al. [39] | Cys | 0.5–750 | 4.73 | <4.91 | >2 pmol |
| | Hcys | 0.5–500 | 4.59 | <4.91 | >2 pmol |
| | GSH | 0.5–500 | 4.81 | <4.91 | >2 pmol |
| Fiskerstrand et al. [36] | Cys | 0.5–1000 | 2.2 | 2.3 | NR |
| | Hcys | 0.5–1000 | 2.6 | 2.2 | NR |
| | CysGly | 0.5–1000 | 1.9 | 1.8 | NR |
| Pastore et al. [34] | Cys | 1.5–300 | 3.4 | 4 | 50 nM |
| | Hcys | 0.625–100 | 2.4 | 4.9 | 50 nM |
| | GSH | 0.625–100 | 2.8 | 6.1 | 50 nM |
| | CysGly | 0.626–100 | 1.9 | 3.8 | 50 nM |
| Williams et al. [35] | Cys | 1–1000 | NR | NR | NR |
| | Hcys | 0.5–250 | NR | NR | NR |
| | GSH | 0.5–200 | NR | NR | NR |
| | CysGly | 1–200 | NR | NR | NR |
| Jacobsen et al. [33] | Cys | 0–492 | 2.35 | 3 | 10 pmol |
| | Hcys | 0–200 | 3.31 | 4.85 | 4 pmol |
| | CysGly | 0–123 | 3.96 | 7.46 | 10 pmol |
| Mansoor et al. [32] | Cys | 6.25–200 | 7.2 | NR | >2 pmol |
| | Hcys | 0.31–10 | 6.6 | NR | >2 pmol |
| | GSH | 0.31–10 | 7.1 | NR | >2 pmol |
| | CysGly | 0.78–25 | 7.8 | NR | >2 pmol |
| Krijt et al. [40] | Cys | 0–400 | 2.3 | 3.9 | NR |
| | Hcys | 0–150 | 1.9 | 3.8 | NR |
| | GSH | 0–50 | 1.9 | 13 | NR |
| | CysGly | 0–150 | 1.2 | 3.2 | NR |
| Rizzo et al. [58] | Cys | 0.01–300 | 0.27 | 6.25 | 77.6 pmol |
| | Hcys | 0.01–300 | 0.98 | 3.2 | 16.8 pmol |
| | CysGly | 0.01–300 | 0.41 | 8 | 13.3 pmol |
| Pfeiffer et al. [37] | Cys | NR | 1.1–1.8 | 2.4 | NR |
| | Hcys | 0–200 | 1.1–1.8 | 5.6 | 0.16 μM |
| Durand et al. [38] | Cys | 100–400 | 1.8 | 4 | NR |
| | Hcys | 10–40 | 2.31 | 2.81 | NR |
| | GSH | 10–40 | NR | 5.65 | NR |
| | CysGly | 20–80 | 1.88 | 3.22 | NR |
| Nolin et al. [31] | Cys | 25–800 | 2.5 | 5.1 | NR |
| | Hcys | 3.13–100 | 2 | 5.5 | NR |
| | GSH | 1.56–50 | 2.6 | 3.9 | NR |
| | CysGly | 6.25–200 | 1.9 | 4 | NR |
| Carducci et al. [27] | Cys | 4–1000 | 3.6 | 3.8 | 2.4 pmol |
| | Hcys | 0.4–150 | 3.8 | 4.2 | 0.8 pmol |
| Tcherkas et al. [28] | Cys | 3–250 pmol | NR | NR | 0.5 pmol |
| | Hcys | 3–250 pmol | NR | 6.1 | 0.8 pmol |

[a] Concentrations μM unless otherwise specified. CV, coefficient of variation; LOD, limit of detection; NR, not reported.

### 3.3. Internal standard

Sample reduction, protein precipitation and derivatization are sources of assay variability. The use of an internal standard, added at the beginning of sample preparation, can greatly improve assay performance. Although methods without an internal standard, and thus poorer precision, can certainly yield relevant results, their use may lead to low statistical power in clinical trials and to a severe underestimation of associations in epidemiological studies [62]. Addition of an internal standard also compensates for the matrix dependency of external calibration. Results calculated from standards prepared in plasma or aqueous media may be variable. Using an internal standard, Kuo et al demonstrated no differences in calibration slopes of aqueous or plasma based standards [26]. *N*-acetylcysteine (NAC) [38,61], cysteamine [26,37], and 2-mercaptopropionylglycine [31,57] have been used as internal standards. These compounds may be present endogenously and/or administered to research subjects as a therapeutic drug. If an endogenous compound is used as an internal standard, it should be added at a final concentration that is at least two orders of magnitude greater than endogenous concentrations to ensure assay precision is not compromised [63]. It can be a challenge to select an internal standard concentration that yields precise and accurate quantitation for multiple aminothiols with different concentration ranges. The method validation process should include tests representing the full range of expected concentrations for each aminothiol of interest.

### 3.4. Chromatography

Fluorescent adducts of aminothiols are usually separated by reversed-phase chromatography with ion-pairing using either isocratic or gradient elution. Retention times of aminothiols are very sensitive to changes in mobile phase pH. Excellent resolution of SBD-adducts of CYS, Hcys, CysGly and GSH using an acetate buffer of pH 4.5 has been achieved (Fig. 2) [31]. Sufficient separation of aminothiols in standard solutions does not guarantee that these compounds can be measured free from interference in biological samples. Moreover, in plasma, interfering

endogenous compounds may differ between patient groups. It is important to test chromatographic conditions with samples from each subject group to be studied to ensure there are no compounds co-eluting with aminothiols of interest. For example, in studies examining oxidative stress where subjects may be receiving thiol–antioxidant therapy like NAC, chromatographic conditions may need to be optimized to prevent NAC interference (Fig. 3). The operating conditions of selected HPLC–FD methods are presented in Table 2.

## 4. Method evaluation

Although much effort is spent in developing methods to measure aminothiol concentrations, very few methods have been fully validated for determination of more than two aminothiols simultaneously [31,34,38]. Optimizing these assays for simultaneous determination of multiple analytes requires significant effort to ensure they are validated. Numerous excellent reviews of assay development and validation procedures have been published previously [64–66]. The performance characteristics of selected HPLC–FD methods capable of simultaneously determining multiple aminothiols in plasma are depicted in Table 5. Briefly, particular attention must be given to assay calibration, linearity, limits of detection and quantitation, accuracy, precision, sample stability, carryover, and recovery. A detailed discussion of these is beyond the scope if this review, but each has been presented in detail previously as they pertain to the validation of a method for the simultaneous determination of total Hcys, CYS, CysGly and GSH via HPLC with fluorescence detection [31]. There is also a need for chemicals of highest purity for use in preparation of calibrators and QC samples for use in method validation. Calibration of aminothiol assays has relied heavily on the use of commercial aminothiols of varying purity as primary calibrators. In the preparation of standards, the disulfide form of aminothiols has been used because it is often available commercially at a high purity, and a large portion of aminothiols are present in plasma as disulfides. However, disulfide aminothiols have low aqueous solubility, making it difficult to prepare concentrated stock solutions, and some aminothiols, including most potential internal standards, are not available commercially in disulfide form. With each laboratory using their own calibration material, comparison between studies can be difficult. Several of the methods discussed and referenced here have been used in interlaboratory studies to compare plasma total homocysteine measurements [67,68]. A common conclusion from these studies is variability could be greatly reduced by the use of standard reference material. In response, Standard Reference Material 1955 for homocysteine and folate was developed and characterized [69]. For the time being however, similar reference material for the analysis of other aminothiols is not available.

## 5. Concluding remarks

The role of homocysteine and other aminothiols in health and disease is widely studied. The need for studies measuring homocysteine in conjunction with other metabolites, including CYS and GSH, has been reinforced by inconsistent results from interventional studies aimed at reducing homocysteine concentrations [16], and illustrates the need for accurate, precise, robust, validated aminothiol assays. Numerous methods for measurement of aminothiols have been developed, most of which are based on HPLC with fluorescence detection. This analytical approach exhibits high sensitivity and precision and is well suited for high-throughput quantitative determination of multiple aminothiols for research purposes and for routine clinical use. The ideal method for measurement of aminothiols would have a fast and simple preparation protocol that could be performed at room temperature, using an efficient reducing agent and a selective fluorescent probe. Fluorescent adducts would be separated by robust chromatography, using isocratic conditions to elute well-separated peaks. This method would include an internal standard to enhance assay performance, and provide quantitative results using calibrators made from certified reference material. For a complete aminothiol profile, this method would measure total concentrations of aminothiols, and with some modification could separately measure redox forms (i.e., oxidized and reduced forms). Currently, no published method meets all of these criteria. Based on Table 2, only methods using SBD-F as a derivatizing agent have incorporated an internal standard; therefore, we prefer these methods. Regardless of the method selected, careful consideration of pre-analytical factors, procedures for sample reduction, derivatization, chromatographic separation, and method validation is essential to ensure accurate and precise results.

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