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# An improved method for simultaneous analysis of aminothiols in human plasma by high-performance liquid chromatography with fluorescence detection

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ABSTRACT

Altered levels of aminothiols in biological fluids are thought to be an important risk indicator for several diseases, and reliable methods for the accurate determination of aminothiols concentrations in plasma are thus required. In this paper ammonium 5-bromo-7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-BF) is proposed as a convenient fluorogenic derivatizating reagent for the determination of aminothiols (cysteine, cysteinylglycine, homocysteine and glutathione) by HPLC with fluorescence detection. The reactions of SBD-BF with aminothiols at room temperature are about three-times faster than those of ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (the most frequently employed reagent) at 60 °C. The derivatives of SBD-BF with cysteine, cysteinylglycine, homocysteine and glutathione are easily separated by HPLC and their calibration curves show excellent linearity over the range 0.05–20 μmol/L with excellent $r^2$ values for all analytes. SBD-BF reacts with thiols under mild conditions, *i.e.* at 25 °C over about 30 min, and is proposed as a suitable fluorogenic reagent for thiol derivatization to be introduced in analytical clinical chemistry. The detection limits of Cys, Cys-Gly, Hcy and GSH at a signal-to-noise ratio of 5 were 0.1 μM for Cys, 0.01 μM for Cys-Gly and Hcy, and 0.02 μM for GSH. Furthermore, validation parameters of the proposed method are quite satisfactory. As an application of this method the determination of thiol derivatives in human plasma was carried out on a number of samples.



## 1. Introduction

Biological thiols such as cysteine (Cys), cysteinylglycine (Cys-Gly), homocysteine (Hcy) and glutathione (GSH) widely occur in biological tissues and fluids and are known to play important roles in living systems [1].

The determination of aminothiols, as well as the corresponding disulfides, has gained high interest within the biomedical community over recent years as such molecules are important biomarkers for a wide range of diseases; in particular, it is now widely accepted that increased total plasma homocysteine (tHcy) is a risk factor for cardiovascular diseases [2], as well as other degenerative ones. Numerous HPLC methods with various detection techniques such as UV [3], fluorescence (FL) after labeling and mass spectrometry (MS) or MS/MS [4] have been developed for determination of thiol derivatives in biological samples. The method with MS/MS detection is highly sensitive and selective but it suffers from limitations in terms of equipment and maintenance costs, instead, HPLC with FL detection is most commonly used due to its sensitivity and reasonable simplicity. This method requires pre-column derivatization and different types of fluorogenic reagents for thiols have been reported, *e.g.* monobromobimane [5], *o*-phthaldialdehyde [6], ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F) [7–10] and 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F) [11], and novel labeling reagent such as *N*-(2-acridonyl)maleimide are still currently proposed [12].

Commercially available SBD-F, a popular way of derivatizing the thiol group of sulfur compounds [10], is highly specific to SH groups and does not react competitively with alcohol, phenol or amino functions; in addition, the derivatives of biological thiols and SBD-F are stable, easily separated by HPLC and strongly fluorescent. This reagent, widely employed in clinical chemistry, has recently found a novel application in the determination of Hcy in food [13].

However, the completion of reaction require an exceedingly long time and the conditions are quite drastic, *i.e.* derivatization has to be carried out at 60 °C over 1 h period [14]; moreover, fast oxidation or degradation of thiols, as well as of other species participating into reaction, may easily take place under these conditions.

As we have previously reported in a preliminary communication [15] we have been searching for new fluorogenic reagents,

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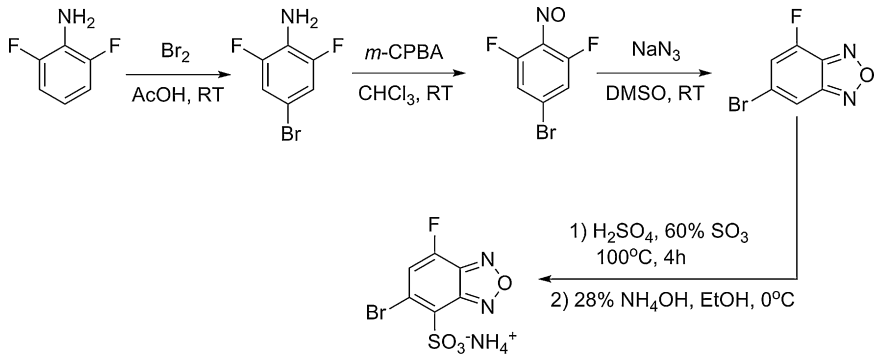


**Fig. 1.** Synthetic route to SBD-BF.

with benzofurazan structure, suitably more reactive than those previously proposed and we reasoned that the presence of an additional, reactivity enhancing substituent could solve the problem. Accordingly, the ammonium 5-bromo-7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-BF) was synthesized in satisfactory yield and characterized by means of spectroscopic and analytical data. In this work we disclose our preliminary results in full details together with the synthesis and characterization of SBD-BF as well as additional, new data regarding precision and recovery which support nicely the validity of the proposed protocol. Furthermore, examples of the application of this method to thiol determination in human plasma are reported.

## 2. Experimental

### 2.1. Chemicals and reagents

*L*-Homocysteine, *L*-cysteine, glutathione (reduced form), sodium azide and tris (2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Fluka (Germany); cysteinylglycine was obtained from Bachem (Switzerland); 2,6-difluoroaniline was purchased from Alfa Aesar (Germany); trichloroacetic acid (TCA) was purchased from Merck (Germany); 3-chloroperoxybenzoic acid and EDTA were obtained from Sigma. All other chemicals employed were of the highest purity available.

### 2.2. Synthesis of SBD-BF

SBD-BF was synthesized as depicted in Fig. 1: bromination of 2,6-difluoroaniline was carried out with bromine in acetic acid as described in the literature [16] and the resulting 4-bromo-2,6-difluoroaniline was oxidized with 3-chloroperoxybenzoic acid to the corresponding nitrosoderivative which was then reacted with sodium azide [17] to afford 6-bromo-4-fluoro-2,1,3-benzoxadiazole. The desired product was finally obtained after sulfonation [18] and subsequent neutralization with ammonium hydroxide; the crude salt was purified by recrystallization from ethanol, affording pure SBD-BF in an overall 48% yield. $^1$H NMR spectrum was recorded at 300 MHz in DMSO-$d_6$: $\delta$ 7.74 (d, 1, $J_{H\text{-}F}$ = 10 Hz), 7.08 (bs, 4). Anal. Calcd for $C_6H_5N_3O_4SBrF$: C, 22.9; H, 1.6; N, 13.3. Found: C, 23.0; H, 1.8; N, 13.2. MS (ESI, *neg*): *m*/*z* 295.0 ($M^-$, 97%), 297.0 ($M^-$+2, 100%); isotopic pattern and relative peak intensities gave additional evidence of the presence of one bromine and one sulfur atom in the anion.

### 2.3. Plasma samples

The plasma samples were obtained by a standardized procedure: human whole blood samples were collected into tubes containing EDTA as an anticoagulant and immediately centrifugated at 2000 × *g* for 15 min; samples were stored at −80 °C until analysis.

### 2.4. Sample preparation

One hundred microliters of samples (human plasma or calibration solutions) was mixed with 10 μL of a solution of TCEP (75 mmol/L in borate buffer, pH 7.4) and allowed to react at room temperature for 15 min. 90 μL of a solution of TCA (100 mL/L in water with EDTA 1 mmol/L) was then added and the sample was centrifuged for 6 min at 14,500 × *g*. An aliquot of the resulting solution (100 μL) was mixed with 380 μL of a solution of SBD-BF (25 mmol/L in 0.125 mol/L borate buffer with EDTA 5 mmol/L, pH 9.5) and 20 μL of NaOH 1 mol/L, incubated at room temperature for 30 min, and then acidified with 50 μL of HCl 1 mol/L. In a final step, after derivatization the sample was diluted five times with water and then 20 μL of the aliquot was injected into a HPLC instrument.

### 2.5. Apparatus and chromatographic conditions

HPLC analysis was performed on a Agilent 1100 system (Agilent Technologies Inc.) equipped with an online vacuum degasser, a high-pressure gradient quaternary pump, a manual sample injector (loop 20 μL), a column oven and a fluorescence detector. Data analysis was done using Agilent ChemStation software (Agilent Technologies).

The HPLC separation of the four aminothiols was performed on a reversed-phase C18 column Synergy Hydro (Phenomenex, Torrance, CA, USA) 150 mm × 3 mm (i.d.) with 4 μm particle size, protected by a Phenomenex C18 security guard column (4.0 mm × 3.0 mm). Mobile phases were 0.1% aqueous trifluoroacetic acid (Solvent A) and 5% tetrahydrofuran in methanol (Solvent B).

The separation was carried out at a flow rate of 600 μL/min equilibrating the column at 100% A; the gradient increased linearly up to 26% of B in 13 min, then it reached in 1 min 100% of B and maintained for 10 min for eluting the excess reagent and other hydrophobic plasma components. Re-equilibration was carried out over 15 min; total analysis time was 40 min.

The column heater was set at 30 °C. Typically, 20 μL of sample were injected onto the column and derivatized aminothiols were detected by fluorescence with excitation at 385 nm and emission at 515 nm.

### 2.6. Calibration and linearity

Four standard concentrations of the aminothiols covering a range of 0.2–20 μmol/L for Cys, and 0.05–20 μmol/L for Cys-Gly, Hcy and GSH were employed as calibration samples. These were analyzed in triplicate and calibration curves for each aminoth-

iol were obtained by linear regression analysis of the aminothiol peak area plotted against the nominal aminothiol concentration (μmol/L).

### 2.7. Recovery and precision

Recovery of aminothiols from plasma was assessed by analyzing human plasma obtained from normal healthy subjects after addition of samples of aminothiols at various concentrations. The concentrations in spiked biological samples were determined in triplicate.

The intra-day reproducibility of the method was established by replicate analyses ($n = 3$) of samples at three different concentrations whereas inter-day reproducibility was established by replicate analyses of the same samples on 6 days.

## 3. Results and discussion

### 3.1. Derivatization of thiols with SBD-BF

This new reagent, which possesses a bromine atom in the position 5, was subjected, together with SBD-F, to a preliminary study in order to compare their reactivities towards thiols. The results of this kinetic study, carried out with Cys and Hcy nucleophiles by means of the UV–Vis technique, clearly indicate that in the reactions of SBD-BF with Hcy and Cys (kinetic runs carried out under *pseudo-first order* conditions in borate buffer 0.1 mol/L, ionic strength 0.05, pH = 9.55, following the absorbance changes at 380 nm) the second order rate constants at 25 °C (8.73 L mol$^{-1}$ s$^{-1}$ and 3.78 L mol$^{-1}$ s$^{-1}$, respectively) are about three-times higher than those of SBD-F (3.49 L mol$^{-1}$ s$^{-1}$ and 1.34 L mol$^{-1}$ s$^{-1}$, respectively) at 60 °C (other conditions being equal). Our results show that SBD-BF is conveniently more reactive than SBD-F, as it reacts with thiols under milder conditions and in shorter times, *i.e.* at 25 °C over about 30 min, although a reducing agent is still required.

The reactions were monitored also by means of $^1$H NMR spectroscopy and, since only fluorine (and not bromine) gives spin–spin coupling with $^1$H, we obtained unequivocal evidence that fluorine (rather than bromine) undergoes substitution by thiols.

In the course of the kinetic study we observed that, as expected, Cys and, even more easily, Hcy undergo oxidation to disulfide during the single kinetic run. Consequently, we have carried out additional experiments under nitrogen atmosphere and in the presence of a reducing agent. Several reductants have been previously proposed for the reduction of disulfide bonds before derivatization for total plasma thiol measurements: the most used tri-*n*-butylphosphine (TBP) suffers from severe drawbacks, *e.g.* it is corrosive, has an unpleasant odor and requires toxic *N*,*N*-dimethylformamide (DMF) as a solvent; dithiothreitol (DTT) [19] and TCEP [20,21] seem to offer the most reliable and reproducible results. The above reported rate constants were determined in the presence of TCEP since in the derivatization step excess DTT (which is a thiol) will compete with the aminothiols under determination in the reaction with the fluorogenic agent, giving rise to fluorescent products which could interfere in the quantitative assessment.

The emission spectra of the derivatives of Cys with SBD-F and SBD-BF, recorded with a spectrofluorimeter PerkinElmer MPF 44 A, are practically superimposable (with $\lambda_{ex} = 380$ nm and $\lambda_{em} = 510$ nm), thus suggesting that our new reagent SBD-BF could be a suitable pre-column derivatization reagent for reversed-phase HPLC fluorimetric determination of thiols.

Time course studies on the derivatization reactions of Cys, Cys-Gly, Hcy and GSH with SBD-BF were carried out at first separately and subsequently with a mixture of the thiols as follows. 100 μL aliquots of the reaction mixture were withdrawn at various time intervals, acidified with 50 μL of HCl 1 mol/L and injected (20 μL) into a HPLC instrument equipped with a fluorescence detector. As shown in Fig. 2 the peak area *vs.* time indicated that a plateau was always reached within 10–15 min which remained unchanged over the entire incubation time (90 min), thus suggesting that the derivatization reactions proceeded to completion in a conveniently short time.

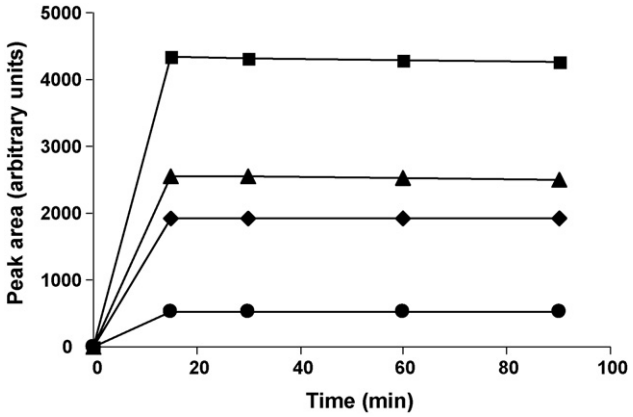


**Fig. 2.** Time courses of derivatization reaction of thiols with SBD-BF. Symbols are Cys (●), Cys-Gly (■), Hcy (▲) and GSH (♦).

### 3.2. Chromatographic separation

Chromatograms of standard solutions of the derivatized aminothiols in borate buffer and human plasma spiked with standard thiol derivatives are shown in Fig. 3. The eluted peaks of the four analytes were distinctly separated: Cys, Cys-gly, Hcy and GSH were eluted in this order with the following retention times (min): 7.4, 9.2, 9.7 and 11.1.

### 3.3. Linearity, limit of quantification (LOQ) and limit of detection (LOD)

Linear calibration curves were obtained for each analyte in the concentration ranges of 0.2–20 μmol/L for Cys, 0.05–20 μmol/L for Cys-Gly, Hcy and GSH. The regression equations and the correlation coefficients ($r^2$) were calculated as follows: $y = 77.6070x - 8.2661$ ($r^2 = 1.0000$) for Cys, $y = 368.2561x - 14.4403$ ($r^2 = 1.0000$) for Cys-Gly, $y = 228.1895x - 10.7662$ ($r^2 = 1.0000$) for Hcy, and $y = 181.2304x - 7.2888$ ($r^2 = 1.0000$) for GSH.

The calculations of LOD and LOQ were carried out at a S/N values of 5:1 and 10:1 for LOD and LOQ, respectively; the LOD (LOQ) values were 0.1 μM (0.2 μM) for Cys, 0.01 μM (0.05 μM) for Cys-Gly and Hcy, and 0.02 μM (0.05 μM) for GSH.

The sensitivity of the proposed method to Cys and Hcy was significantly higher than that employing SBD-F (detection limits: Cys, 0.47 μM; Hcy, 0.16 μM [22]).

### 3.4. Recovery and precision

The mean recoveries of all analytes from plasma at different concentrations ranged from 76% and 97% (Table 1).

Intra-day and inter-day precisions are reported in Table 2. Intra-day values are below 1% and inter-day values are lower than 3.7% (significantly better than those recently reported for SBD-F [22]), thus giving us confidence about the reproducibility of the method.

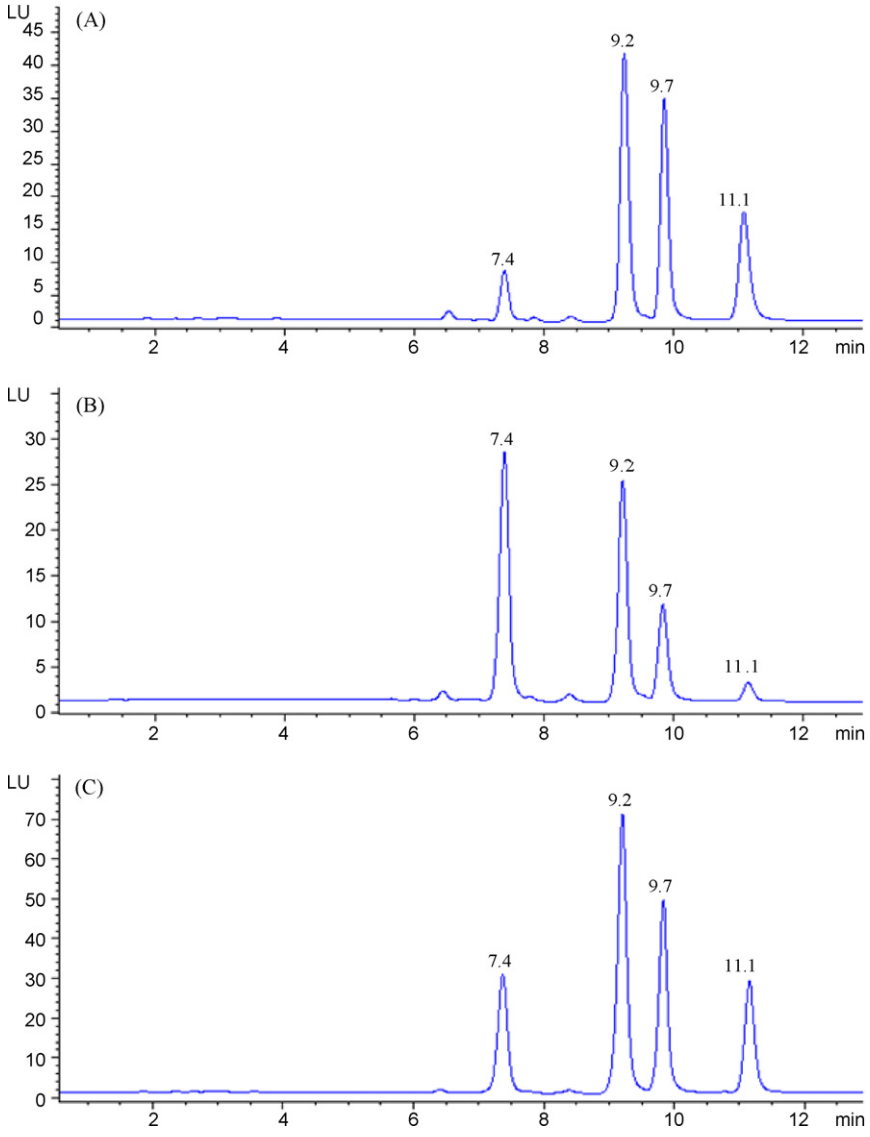


**Fig. 3.** Chromatograms of derivatized Cys, Cys-Gly, Hcy, and GSH. (A) In borate buffer, 1 μM each; (B) in human plasma after dilution (see Section 2.4) and (C) the same as B, spiked with 1 μM each.

**Table 1**
Recovery of Cys, Cys-Gly, Hcy and GSH in human plasma.

| | Concentration (μM)[a] | | | |
|---|---|---|---|---|
| | Plasma | Spiked | Measured | % Rec |
| Cys | 6.12 | 2.00 | 6.18 | 76.11 |
| | | 5.00 | 8.97 | 80.66 |
| | | 15.00 | 18.49 | 87.55 |
| Cys-Gly | 0.49 | 2.00 | 2.30 | 92.19 |
| | | 5.00 | 5.10 | 92.98 |
| | | 15.00 | 14.00 | 90.36 |
| Hcy | 0.17 | 2.00 | 2.10 | 96.66 |
| | | 5.00 | 4.93 | 95.37 |
| | | 15.00 | 14.14 | 93.21 |
| GSH | 0.05 | 2.00 | 1.87 | 93.30 |
| | | 5.00 | 4.62 | 91.49 |
| | | 15.00 | 13.21 | 87.81 |

[a] Concentration of injected sample. Actual concentration in plasma = concentration of injected sample ×55 (dilution factor, see Section 2.4).

**Table 2**
Intra- and inter-day reproducibility for Cys, Cys-Gly, Hcy and GSH in spiked human plasma.

| | Concentration (μM)[a] | Intra-day[b] RDS(%) | Inter-day[c] RDS(%) |
|---|---|---|---|
| Cys | 7.96 | 0.18 | 2.74 |
| | 11.04 | 0.41 | 0.89 |
| | 19.76 | 0.46 | 0.84 |
| Cys-Gly | 3.27 | 0.35 | 3.26 |
| | 6.79 | 0.36 | 1.00 |
| | 17.89 | 0.19 | 0.70 |
| Hcy | 3.13 | 0.69 | 3.69 |
| | 6.72 | 1.03 | 1.26 |
| | 18.03 | 0.64 | 0.77 |
| GSH | 2.88 | 0.70 | 3.48 |
| | 6.14 | 0.31 | 2.05 |
| | 17.12 | 0.17 | 1.87 |

[a] Concentration of injected sample. Actual concentration in plasma = concentration of injected sample ×55 (dilution factor, see Section 2.4).
[b] Calculated from three replicates for each concentration.
[c] Calculated from three replicates for each concentration over 6 days.

**Table 3**
Aminothiol concentrations in plasma of healthy subjects.

| | Population | Concentration (μM) | | | |
|---|---|---|---|---|---|
| | | Hcy | Cys | Cys-Gly | GSH |
| 1 | F | 4.9 | 148.2 | 19.3 | 8.0 |
| 2 | F | 11.4 | 252.8 | 32.5 | 10.3 |
| 3 | F | 10.2 | 184.9 | 37.4 | 12.4 |
| 4 | F | 10.4 | 280.5 | 40.5 | 9.8 |
| 5 | M | 10.9 | 214.0 | 43.7 | 13.6 |
| 6 | M | 8.6 | 215.8 | 27.9 | 13.2 |
| 7 | M | 9.0 | 219.6 | 38.3 | 17.8 |
| 8 | F | 6.4 | 146.9 | 27.6 | 9.8 |
| 9 | M | 13.1 | 203.9 | 30.5 | 12.5 |
| 10 | M | 7.7 | 198.8 | 31.6 | 12.5 |
| 11 | F | 8.5 | 219.0 | 34.3 | 12.6 |
| 12 | M | 7.2 | 154.4 | 36.2 | 6.8 |
| 13 | F | 7.6 | 127.4 | 40.3 | 7.3 |
| 14 | M | 7.5 | 208.1 | 45.2 | 7.4 |
| 15 | M | 7.0 | 168.8 | 32.9 | 6.0 |
| 16 | F | 7.4 | 195.5 | 30.5 | 15.9 |
| 17 | F | 9.9 | 240.2 | 42.5 | 10.1 |
| 18 | F | 7.0 | 148.3 | 38.3 | 6.6 |
| 19 | F | 6.1 | 202.3 | 28.2 | 9.4 |
| 20 | M | 7.6 | 225.6 | 33.6 | 8.4 |
| 21 | F | 12.0 | 266.0 | 56.6 | 16.0 |
| 22 | M | 7.5 | 166.6 | 29.1 | 12.9 |
| 23 | F | 10.9 | 312.7 | 40.0 | 7.6 |
| 24 | M | 8.2 | 229.6 | 51.7 | 7.0 |
| 25 | M | 11.5 | 224.6 | 43.9 | 11.3 |
| 26 | F | 9.2 | 205.6 | 39.6 | 11.2 |
| 27 | F | 9.7 | 248.4 | 42.3 | 13.4 |
| 28 | M | 10.6 | 245.8 | 52.2 | 14.5 |
| 29 | F | 8.8 | 227.6 | 39.1 | 11.8 |

### 3.5. Determination of thiol derivatives in human plasma

Thiol levels in blood plasma from 29 healthy subjects were measured to assess the applicability of the proposed method to such biological fluid: results are reported in Table 3 and the measured concentrations of thiol compounds are in good agreement with those reported in the literatures [23–25].

## 4. Conclusion

The properties of SBD-BF mark it as a very promising fluorogenic reagent for thiol derivatization to be introduced in analytical clinical chemistry, not only for its reactivity, which is conveniently high, but also for other properties, such as hydrophilicity and selectivity toward the thiol function and for the excellent stability of fluorescent products, having a long-wavelength fluorescence similar to that of thiol—SBD-F adducts, obtained under the new, milder reaction conditions now attainable.

The present method offers a simple, sensitive, and reproducible HPLC method that has been fully validated for simultaneously determining total concentrations of the aminothiols Cys, Hcy, Cys-Gly, and GSH in human plasma. Although for analysis of plasma aminothiols the sensitivity of the used method is not a critical point, the sensitivity for Cys, Hcy, Cys-Gly and GSH of the proposed method was higher than those of previous method with SBD-F [9].

Control experiments carried out on plasma samples demonstrated value and straightforward applicability of this protocol to the practice, therefore this method might be promising for the routine use.

Future work has been planned to develop a protocol employing SBD-BF as derivatization reagent for aminothiols in other biological samples (*e.g.* cerebrospinal fluid, urine).

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