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Methodology for a rapid and simultaneous determination of total cysteine, homocysteine, cysteinylglycine and glutathione in plasma by isocratic RP-HPLC

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ABSTRACT

Alterations in the plasma aminothiols levels can be considered as important biomarkers for the diagnosis and screening of several human disorders, namely cardiovascular diseases. We aimed to optimize a rapid, sensitive and accurate RP-HPLC methodology with fluorescence detection, for the simultaneous determination of the total concentrations of cysteine, homocysteine, cysteinylglycine and glutathione in blood plasma, as well as its application to the evaluation of those thiols levels in plasma of a group of Azorean subjects. Aminothiols were reduced with tri-*n*-butylphosphine and derivatized with a thiol-specific fluorogenic reagent ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate. The thiols adducts were separated by an isocratic elution on a Platinum EPS C18 analytical column (53 mm × 7 mm I.D., 3 μm) using a phosphate buffer containing 4% of acetonitrile as a mobile phase. Results indicated an excellent linearity for all the analytes over their respective concentration ranges with correlation coefficients (r^2) ≥0.99. The LOD for the four plasma thiols was lower than 0.10 μmol/L, while LOQ varied from 0.5 to 15 μmol/L. For both intra- and inter-day precision, the RSD (%) values were lower than 1.9%, and the CV (%) values were found under 0.5%. The recovery ranged from 92% to 100% indicating a high degree of the method's accuracy. This method allows a simultaneous, complete analysis of the four plasma aminothiols and the internal standard in 6 min. By reducing the total run time, a larger number of analysis can be performed daily.

1. Introduction

The simultaneous determination of the plasma low molecular-mass weight thiols concentrations, such as homocysteine (Hcy), cysteine (Cys), cysteinyl–glycine (Cys–Gly), and glutathione (GSH), is of potential interest for the understanding the dynamic relationship among them, as well as for clinical diagnosis and screening of various human disorders [1], including diabetes, cancer, neurodegenerative and cardiovascular diseases (CVD) [2,3]. These aminothiols (Fig. 1) are involved in cellular protection against oxygen and nitrogen reactive species, in heavy metal and xenobiotics detoxification, in control of gene expression and in cell signaling [4–6]. In fact, thiol groups (—SH) are critical intra and extracellular redox buffers which promptly undergo oxidative coupling reactions to form disulfides (—S—S—). Therefore, plasma aminothiols interact via redox exchange reactions and oxidized, reduced and protein-bound forms of these species comprise a dynamic system designed as the redox thiol status. Usually, more than 90% of them are protein-bound, and the remainder are mostly in the form of disulfides [7,8].

The metabolic pathways of aminothiols are strongly linked. GSH is the major non-protein thiol inside cells, while extracellular GSH is present at much lower concentrations [2,3]. The action of γ-glutamyltranspeptidase on extracellular GSH removes the γ-glutamyl moiety of the peptide and the remaining Cys–Gly is usually taken within intracellular milieu by membrane dipeptidases to form Cys and Gly as precursors of GSH resynthesis [3,9]. Homocysteine is a critical regulatory intermediate of methionine (Met) cycle, acting as a precursor for Cys in the transsulfuration pathway, as well as for Met synthesis via remethylation, by using betaine as a methyl donor. Cysteine represents the most abundant thiol in plasma followed by Cys–Gly [10].

Increased levels of total plasma Hcy and Cys have been associated with the development of CVD [11,12]. It was found that these thiols can promote atherogenesis on account of their effect on endothelial function, vascular smooth muscle cell activation, and hemostatic activation [13–15]. According to Dhawan and collaborators [16] lower plasma GSH level has been considered as an independent predictor of impaired coronary microvascular function and plaque necrotic core in patients with coronary

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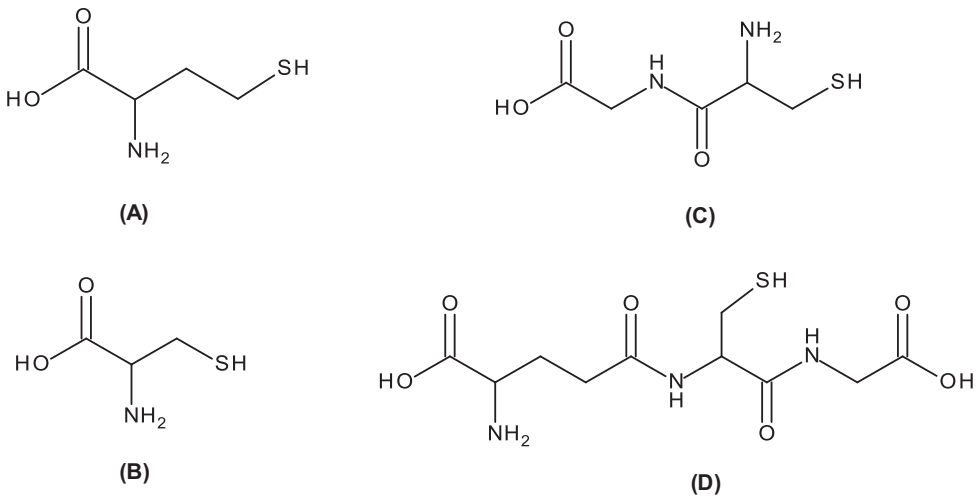

Fig. 1. The structures of the aminothiols (A) Hcy, (B) Cys, (C) Cys–Gly, and (D) GSH.

atherosclerotic heart disease. There is no published work regarding alterations in total Cys–Gly levels in cardiovascular disease patients, albeit high levels of this thiol have been reported in patients with breast cancer or rheumatoid arthritis [17,18]. However, the pathophysiological role of Cys–Gly has not been yet clearly understood.

For the detection and quantification of thiols it is necessary to consider their unfavorable physicochemical properties. In fact, they lack of a strong absorption in UV–vis regions or native fluorescence, are easily oxidized to disulfides, and are generally highly polar and water soluble, which makes their extraction from biological matrices very difficult [19]. Over the years, several analytical methods have been developed for thiols determination such as liquid chromatography (LC) [20,21], gas chromatography [22,23], ion-exchange chromatography [24,25] and capillary electrophoresis [26,27]. High performance liquid chromatography (HPLC) with several detection techniques, such as ultraviolet [4,28,29], fluorescence (FL) [30–32], electrochemical [5,6,33] and mass spectrometry [34–36], is the most reported methodology. All the referred methods have basic limitations in terms of equipment, reagent costs, complexity, sample preparation, run time, number of thiols simultaneously quantified, and/or validation assessment, which delay their use for high-throughput routine clinical or research purposes [8]. In order to enhance the thiol detection, chemical derivatization procedures are required and may be performed either pre-column or post-column.

Different types of labeling reagents have been used, including monobromobimane [30,37], ortho-phthaldialdehyde [38,39], *N*-(1-pyrenil)maleimide [40,41], 9-acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)phenyl)-3-oxo-3H-naphtho[2,1-*b*]pyran (ThioGlo 3) [42], *N*-(2-acridonyl)maleimide [1], ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F) [20,43–45], and 7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide (ABD-F) [46,47]. The halogenobenzofurazans reagents (SBD-F and ABD-F) are the most often used, since they react selectively with thiol compounds (Fig. 2) and do not fluoresce themselves. This avoids potential interference from the excess reagent used [47]. The death rate from coronary artery disease in Azores Archipelago is about twice than in the Portuguese Mainland [48]. In order to understand this fact, our research group has been screening several biomarkers for atherosclerosis in the Azorean populations, particularly the plasma aminothiols status. Therefore, the purpose of the present work was to optimize a rapid, sensitive and accurate RP-HPLC methodology with fluorescence detection for the simultaneous determination of total Hcy, Cys, Cys–Gly and GSH concentrations in plasma, taking into account the reduction of their retention times, in order to increase significantly the number of the daily analyzed samples. This method was applied to blood samples donated by apparently healthy Azorean volunteers.

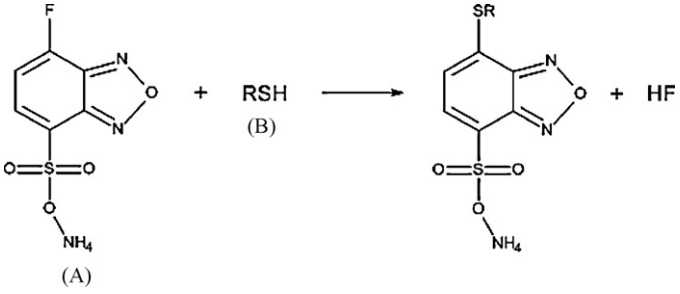

Fig. 2. Derivatization reaction equation of thiols (B) with SBD-F (A).

2. Materials and methods

2.1. Chemicals

L-Cysteine (≥99.5%), cysteamine hydrochloride (IS, ≥98%), DL-homocysteine (≥95%), L-glutathione (≥99%), ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (≥99.5%), tri-*n*-butylphosphine (TBP, ≥97%), trichloroacetic acid (TCA, ≥98%), hydrochloric acid (HCl, 37%), potassium tetraborate tetrahydrate were obtained from Fluka (Sigma–Aldrich chemie, Steinheim, Germany). Cysteinylglycine (Cys–Gly, 85%), N,N-dimethylformamide (≥99.9%) and acetonitrile (ACN) were purchased from Sigma–Aldrich (Sigma–Aldrich chemie, Steinheim, Germany). Sodium hydroxide (≥99%) and ethylenediaminetetraacetic acid disodium (EDTA, ≥99%) were obtained from Riedel-de Haën (Sigma–Aldrich chemie, Steinheim, Germany). Potassium dihydrogenophosphate (KH_2PO_4) and ortho-phosphoric acid were provided by Merck (Darmstadt, Germany). The deionized water used for the preparation of the mobile phase and buffers solutions was produced by Milli-Q water purification system (Millipore, Bedford, MA, USA). All solvents were HPLC grade and reagents of the highest purity available.

2.2. Optimization of the analytical conditions

The composition of the mobile phase significantly influences the retention time (RT) and the peak response of the analytes. The

most common used organic solvents for the aminothiols separation using RP-HPLC include methanol, ACN and tetrahydrofuran (until 0.2%). Various buffers including sodium phosphate buffer (NaH_2PO_4) 50 mmol/L and potassium phosphate buffer (KH_2PO_4) in the range of 0.010–0.2 mol/L in different compositions were experimented both in gradient and isocratic elution modes. The peak response and the analytes resolution of the referred aminothiols were recorded against all different types of the mobile phase, having in consideration the shorter run time, superior sensitivity and great peak resolution of the target aminothiol peaks.

Flow rate of the mobile phase is another parameter that influences the resolution of the analytes and the respective RT. Aminothiols were analyzed using different flow rates in the range of 0.40–1.20 mL/min and its effect in the separation and peak shape was investigated. The flow rate that revealed superior sensitivity and good resolution of all analytes was selected. Furthermore the flow rate was optimized under different column temperatures. The effect of column temperature in the range of 25–40 °C on peak resolution, sensitivity and RT of all analytes were also investigated and the changes observed in the chromatograms were recorded.

2.3. Chromatographic conditions and apparatus

The study was carried out using an Agilent Technologies (Avondale, PA, USA) HPLC system model 1200 coupled with a fluorescence detector (FD). The system was controlled by ChemStation software from Agilent Technologies. The separation was performed using a Platinum EPS C18 analytical column (53 mm × 7 mm I.D., 3 μm particle size; Alltech Associates, Deerfield, IL, USA) fitted to a C18 (30 mm × 2 mm, I.D.) pre-column guard cartridge.

Chromatographic analysis of all aminothiols was performed by using 0.1 mol/L KH_2PO_4 (pH 2.0) containing 40 mL/L of ACN as the mobile phase, filtered through a 0.45 μm cellulose acetate membrane (Whatman, Dassel, Germany), which allowed symmetrical and well resolved peak of the individual compounds. The temperature of the column oven was maintained at 35 °C that shows the best resolution and shorter run time and the flow rate of 1 mL/min was adopted in our study that allows increased heights of the peaks and decreased their respective width and RT. An aliquot of 20 μL was injected through a Rheodyne 71251 injection valve fitted with a 20 μL sample loop (Rheodyne, Cotati, CA, USA) after filtration through a 0.45 μm PVDF filter (Alltech Associates, Inc. Deerfield, IL, USA). The fluorescence signals were measured with excitation at 385 nm and emission at 515 nm using a Hewlet Packard-FD (Avondale, PA, USA) model 1100. The detector signal was recorded and processed (peak area determination) by Agilent technologies ChemStation software, using an internal standard methodology.

2.4. Standards preparation

Each aminothiol standard and IS stock solutions (1 mmol/L) were prepared by dissolving them in 5 mmol/L HCl, and further stored in a dark flask at +4 °C. On a daily basis, they were diluted with 5 mmol/L HCl to obtain the working standard solutions of Cys (15–500 μmol/L), Cys–Gly (5–80 μmol/L), Hcy (2–50 μmol/L), GSH (0.5–7.5 μmol/L) and keeping the IS concentration constant (5 μmol/L). Calibration curves for Cys, CysGly, Hcy, and GSH were generated by linear regression analysis.

2.5. Plasma samples

Subjects in this study were apparently healthy (with no declared chronic diseases), aged 20–60 years, all born and living in the Azores Archipelago (Portugal), and gave informed consent to participate. Those that had been taking vitamin supplements were excluded. Blood samples were collected by venipuncture under fasten conditions into heparinised tubes, and immediately centrifuged at 2500 × *g* for 15 min at 4 °C to separate erythrocytes from plasma, which was stored at −80 °C until further analysis.

2.6. Sample preparation

After thawed at room temperature, 100 μL of plasma (or calibration solutions) samples were transferred to plastic eppendorf tubes (*ca.* 1.5 mL) containing 50 μL of IS solution (5 μmol/L). 15 μL of TBP in dimethylformamide (100 mL/L) were carefully added and the mixture was incubated for 30 min at +4 °C. Deproteinization was achieved by adding 150 μL of cold TCA (10%), containing 1 mmol/L EDTA, and followed by centrifugation at 15,500 × *g* for 10 min. An aliquot of 60 μL, taken from the middle of the clear supernatant, was mixed with 120 μL of potassium borate (125 mmol/L, pH 10.5), containing 4 mmol/L EDTA and 60 μL of SBD-F (1.0 g/L dissolved in a solution of 125 mmol/L potassium borate, pH 9.5). This mixture was incubated for 60 min at 60 °C and the samples were cooled and kept at +4 °C until further analysis.

2.7. Validation of the methodology

The precision, accuracy, recovery, sensitivity, linearity, limit of detection (LOD), and limit of quantification (LOQ) were evaluated in order to validate de present methodology.

The precision of the chromatographic methodology was evaluated by performing the repeatability of the HPLC analysis of the four plasma thiols spiked with IS in intra-day and inter-day measurements of the RT by repeated plasma samples injections (two determinations from two different plasma extraction from the same individual). The intra- and inter-day results were expressed as mean, standard deviation (±SD), coefficient of variation (%CV) and relative standard deviation (%RSD). The intra-day variations were studied by analyzing plasma samples spiked with IS for 10 days.

The accuracy of this method was evaluated by determining the recovery of Cys, Cys–Gly, Hcy and GSH in samples with known concentrations of aminothiols. Three different standard amounts of each of the thiols were added to the plasma sample which was subjected to the HPLC chromatographic analysis. The recovery was calculated based on the difference between the total concentration determined in the spiked samples and the concentration in the non-spiked samples.

The sensitivity of the method was determined by quantifying the LOD and LOQ for each aminothiol. LOD was evaluated by considering the analyte concentration that yield a signal-to-noise ratio (S/N) of 3. LOQ is the minimum concentration of the analyte that can be determined at an acceptable precision and accuracy under the analytical conditions used (corresponding to the lowest acceptable point in the calibration curve). For LOD and LOQ determinations, dilutions of each analyte were performed. The linearity of the method was evaluated from de calibration curves constructed at eight concentration points of each aminothiol in the spiked plasma samples. For each calibration curve, the absolute peak-area ratios of the aminothiol to the IS area were calculated and plotted against the ratio of the nominal aminothiol to the IS concentrations.

2.8. Statistical analysis

Linear regression was applied to develop an equation to predict the plasma aminothiols concentrations. Differences in mean values (Mean ± SD) between gender were tested by Student's *t*-test or Mann–Whitney *U*-test for unpaired observations. Statistical analysis was performed by using SPSS 15.0 for software for windows (SPSS Institute, Chicago, IL). A *P*-value of <0.05 was considered to be statistically significant.

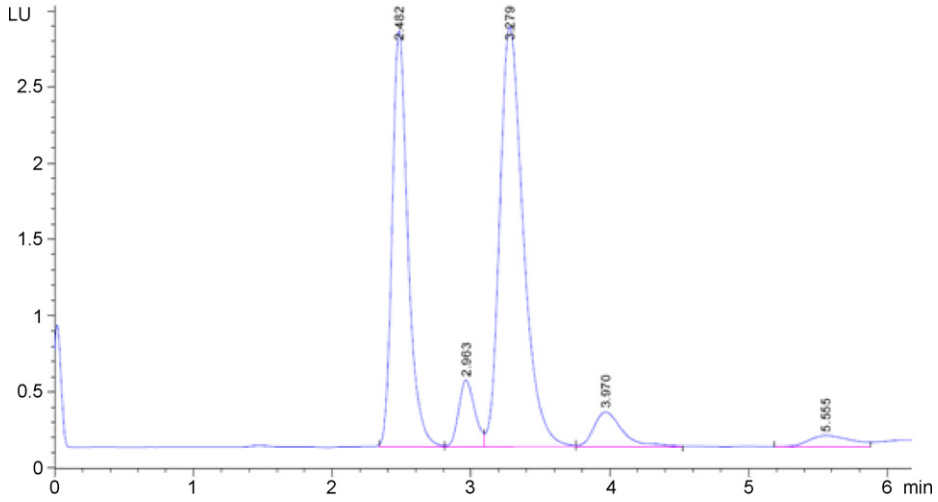

Fig. 3. HPLC chromatogram obtained from the analysis of plasma sample with FLD detection. Chromatographic conditions: Platinum EPS C18 analytical column (53 mm × 7 mm I.D.); mobile phase: 0.1 mol/L KH2PO4 buffer pH 2.0 containing 40 mL/L of ACN and the flow rate was 1 mL/min; temperature 35 °C; FLD detection (excitation at 385 nm and emission at 515 nm). Legend: Cys (2.482 min), IS (2.963 min), Cys–Gly (3.279 min), Hcy (3.970 min) and GSH (5.555 min).

Table 1
The regression equations, the linear range and the limits of detection (LOD) and quantification (LOQ) of Cys, Cys–Gly, Hcy and GSH.

Thiols	Linear range (μmol/L)	Linear equation	r^2 [a]	LOD (μmol/L)	LOQ (μmol/L)
Cys	15–500	$y = 0.1376x - 0.0047$	0.996	0.09	15
Cys–Gly	5–80	$y = 1.0571x + 0.211$	0.998	0.01	5
Hcy	2–50	$y = 0.4283x + 0.0389$	0.995	0.05	2
GSH	0.5–7.5	$y = 0.895x - 0.0272$	0.995	0.03	0.5

[a] r^2 – correlation coefficient.

3. Results and discussion

The simultaneous determination of multiple aminothiols levels in the plasma has emerged as an useful tool not only for clinical and translational research involving oxidative stress, metabolic and redox regulation, but also used for monitoring the disease status. The method described in this paper represents an optimization of the methodologies reported by Araki and Sako [20] and Minniti et al. [32]. The main attention of those authors was the detection and quantification of total plasma homocysteine, whereas our study focused in the determination of the four aminothiols and IS in the same HPLC run.

3.1. Chromatographic separation

Representative chromatogram of the human plasma sample from apparently healthy volunteers with the addition of IS is illustrated in Fig. 3. Retention times for Cys, IS, Cys–Gly, Hcy, and GSH were 2.48, 2.96, 3.27, 3.97, and 5.55 min, respectively. The identification of five peaks was done by comparison of the retention times with those corresponding to the pure standards run separately in the same analytical conditions and confirmed by spike of the authentic standards to the plasma sample. The isocratic elution of the five analytes was completed within 6 min (Fig. 3), and because it was not necessary to regenerate the column, a new run was performed every 8 min which allows a high-throughput capability. Other previous reported methods showed a total analysis time ranging from 8 to 40 min, and gradient elution was also used [20,31,32,43]. Our method has the advantage of saving time and volume of mobile phase that are of crucial importance for routine analysis and also for the environment by reducing the solvent waste. Under these elution conditions, all the peaks of interest were well separated and there were no others interfering peaks from endogenous compounds. Therefore, we can support the previously methodology reported by Minniti *et al.* [32], that SBD-F is a suitable labeling reagent for the sensitive determination of the plasma aminothiols by HPLC-FLD. SBD-F adducts presents a high fluorescence and exhibit an excellent stability which is suitable for pre-column derivatization. It is important to store the SBD-F adducts samples in a cool and dark place until use, since they are light sensitive [32]. In this assay we preferred to employ cysteamine hydrochloride as an IS than 2-mercaptopropionylglycine or N-acetylcysteine, because this allowed us to shorten the total run time of the complete aminothiols separation. Our results also showed that the presence of an IS did not affect the precision of the described method (Table 2) in opposition to the previously reported by Accinni et al. [49], and also the IS used allows the correction of possible systematic errors due to sample handling.

3.2. Method validation

Linear regression analysis for the plasma aminothiols was performed by internal standard method. Each calibration curve and respective correlation coefficient are showed in Table 1, as well as the linear range for all molecules. The LOD of the assay for the four plasma thiols was lower than 0.10 μmol/L (Table 1), while the LOQ varied from 0.5 to 15 μmol/L (Table 1). These results are in accordance to the literature described by Cevasco et al. [31]. The majority of the published methodologies only reported the fully validation parameters for Hcy, which left us without information regarding the others three aminothiols [20,44,50]. The results presented in Table 2 show that for both intra- and inter-day precision the RSD (%) values were lower than 1.9%, and the CV (%) values were found under 0.5%. We observed that no similar results were achieved by others researchers [20,43,50]. The recovery was calculated based on the difference between the total concentration determined in the spiked plasma samples and the concentration in the non-spiked samples (Table 3). Three different amounts of

Table 2
Intra- and inter-day precision data for retention time (RT), standard deviation (SD), relative standard deviation (RSD), and coefficient of variation (CV) of the four aminothiols and internal standard (IS).

Thiols	Intra-day precision ($n = 10$)[a]				Inter-day precision ($n = 10$)[a]			
	Mean RT (min)	SD (min)	RSD (%)[b]	CV (%)[c]	Mean RT (min)	SD (min)	RSD (%)[b]	CV (%)[c]
Cys	2.4681	0.0078	0.541	0.320	2.4771	0.0044	0.316	0.179
IS	2.9634	0.0057	0.329	0.194	2.9552	0.0099	0.766	0.339
Cys–Gly	3.2808	0.0099	0.673	0.304	3.2755	0.0138	0.911	0.424
Hcy	3.9431	0.0174	1.276	0.442	3.9702	0.0075	0.516	0.190
GSH	5.5418	0.0213	1.840	0.386	5.5620	0.0211	1.710	0.379

[a] Two determinations from two different plasma extraction from the same individual.
[b] RSD (%) = (Mean deviation)^2/SD × 100.
[c] CV (%) = (SD/Mean) × 100.

Table 3
Recovery of Cys, Cys–Gly, Hcy and GSH from human plasma sample.

Plasma thiols	Concentration (μmol/L)			Recovery (%)
	Plasma	Spiked	Found	
Cys	300	50	342.90	97.98
		100	370.36	92.59
		200	476.85	95.37
Cys–Gly	33	10	41.91	97.47
		20	50.80	95.86
		40	69.97	95.86
Hcy	12.5	5	17.49	99.99
		10	21.74	96.66
		20	29.99	92.30
GSH	1.65	1	2.48	93.82
		2.5	3.82	92.28
		5	6.12	92.09

each aminothiol standard were added to the sample which was subjected to the derivatization procedure and chromatographic analysis. Excellent recovery (92–100%; Table 3) of the four plasma thiols was observed for all concentrations, consistent with previous findings [31,43].

3.3. Application of the method

The proposed validated method was successfully applied to the determination of total plasma aminothiols levels in 326 plasma samples from apparently healthy subjects. The results illustrated in Table 4 show that total Hcy, Cys–Gly and GSH levels were significantly higher in men than in women ($P < 0.001$). The average concentrations of all aminothiols are similar to those reported in literature [3,30,51].

The method can be applied in clinical analysis for the assessment of oxidative stress through plasma aminothiols status. It can also be easily automated by using an autosampler with controlled temperature (+4 °C).

Table 4
Total plasma aminothiols levels in the apparently healthy subjects, according to gender.

Plasma thiols (μmol/L)	All[a] (326)	Women[a] (189)	Men[a] (137)
Cys	199 ± 39	196 ± 39	203 ± 39
Cys–Gly	32 ± 6	29 ± 5	35 ± 6*
Hcy	10 ± 3	9 ± 3	11 ± 4*
GSH	1.5 ± 0.5	1.4 ± 0.5	1.6 ± 0.6*

[a] Values are mean ± SD for (n) subjects
* Significant differences between genders $P < 0.001$.

4. Conclusion

The described validated HPLC-FLD method for the simultaneous determination of total plasma aminothiols levels was highly sensitive, precise, accurate, selective, reproducible and rapid. We achieved a complete elution within 6 min with well resolved peaks of all analytes which can be used for high-throughput routine clinical analysis allowing the screening of populations that are at risk of development of CVD, in comparison with other reported methods where fewer compounds have been determined in a single run with longer analysis time using complex and expensive methodologies.

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References

[1] B. Benkova, V. Lozanov, I.P. Ivanov, A. Todorova, I. Milanov, V. Mitev, J. Chromatogr. B 870 (2008) 103–108.
[2] E. Camera, M. Picardo, J. Chromatogr. B 781 (2002) 181–206.
[3] A. Pastore, G. Federici, E. Bertini, F. Piemonte, Clin. Chim. Acta 333 (2003) 19–39.
[4] E. Bald, G. Chwatko, R. Glowacki, K. Kusmierek, J. Chromatogr. A 1032 (2004) 109–115.
[5] B. Bailey, J. Waraska, I. Acworth, Methods Mol. Biol. 594 (2010) 327.
[6] A. Khan, M.I. Khan, Z. Iqbal, Y. Shah, L. Ahmad, S. Nazir, D.G. Watson, J.A. Khan, F. Nasir, A. Khan, Ismail, Talanta 84 (2011) 789–801.
[7] D. Di Giuseppe, S. Frosali, R. Priora, F.C. Di Simplicio, G. Buonocore, C. Cellesi, P.L. Capecchi, F.L. Pasini, P.E. Lazzerini, H. Jakubowski, J. Lab. Clin. Med. 144 (2004) 235–245.
[8] M.E. McMenamin, J. Himmelfarb, T.D. Nolin, J. Chromatogr. B 877 (2009) 3274–3281.
[9] A. Meister, J. Biol. Chem. 263 (1988) 17205–17208.
[10] D.M. Townsend, K.D. Tew, H. Tapiero, Biomed. Pharmacother. 58 (2004) 47–55.
[11] L. El-Khairy, P.M. Ueland, H. Refsum, I.M. Graham, S.E. Vollset, Circulation 103 (2001) 2544–2549.
[12] C. Antoniades, A.S. Antonopoulos, D. Tousoulis, K. Marinou, C. Stefanadis, Eur. Heart J. 30 (2009) 6–15.
[13] S. Yardim-Akaydin, Y. Özkan, E. Özkan, M. Torun, B. Simsek, Clin. Chim. Acta 338 (2003) 99–105.
[14] J. Loscalzo, J. Clin. Invest. 98 (1996) 5.
[15] S. Lentz, J. Thromb. Haemost. 3 (2005) 1646–1654.
[16] S.S. Dhawan, P. Eshtehardi, M.C. McDaniel, L.V. Fike, D.P. Jones, A.A. Quyyumi, H. Samady, Atherosclerosis 219 (2011) 266–272.
[17] J. Lin, J.A.E. Manson, J. Selhub, J.E. Buring, S.M. Zhang, Cancer Res. 67 (2007) 11123.
[18] A. Hernanz, A. Plaza, E. Martín-Mola, E. De Miguel, Clin. Biochem. 32 (1999) 65–70.
[19] T. Toyo'oka, J. Chromatogr. B 877 (2009) 3318–3330.
[20] A. Araki, Y. Sako, J. Chromatogr. B: Biomed. Appl. 422 (1987) 43–52.
[21] K.J. Huang, C.H. Han, J.Y. Sun, D.J. Niu, Z.W. Wu, Y.P. Xue, L.J. Zhang, X.Q. Xiong, Chromatographia (2010) 1–6.
[22] H. Kataoka, K. Takagi, M. Makita, J. Chromatogr. B: Biomed. Appl. 664 (1995) 421–425.
[23] J.I. Sigit, M. Hages, K.A. Brensing, U. Frotscher, K. Pietrzik, K. Bergmann, D. Lutjohann, Clin. Chem. Lab. Med. 39 (2001) 681–690.

[24] A. Andersson, L. Brattstrom, A. Isaksson, B. Israelsson, B. Hultberg, Scand. J. Clin. Lab. Invest. 49 (1989) 445–449.
[25] A. Briddon, Amino Acids 15 (1998) 235–239.
[26] C. Carru, L. Deiana, S. Sotgia, G.M. Pes, A. Zinellu, Electrophoresis 25 (2004) 882–889.
[27] A. Zinellu, A. Lepedda Jr., S. Sotgia, E. Zinellu, B. Scanu, F. Turrini, R. Spirito, L. Deiana, M. Formato, C. Carru, Clin. Biochem. 42 (2009) 796–801.
[28] K. Amarnath, V. Amarnath, H.L. Valentine, W.M. Valentine, Talanta 60 (2003) 1229–1238.
[29] K. Kuśmierek, E. Bald, Biomed. Chromatogr. 23 (2009) 770–775.
[30] M.A. Mansoor, A.M. Svardal, P.M. Ueland, Anal. Biochem. 200 (1992) 218–229.
[31] G. Cevasco, A.M. Piatek, C. Scapolla, S. Thea, J. Chromatogr. A 1217 (2010) 2158–2162.
[32] G. Minniti, A. Piana, U. Armani, R. Cerone, J. Chromatogr. A 828 (1998) 401–405.
[33] S. Melnyk, M. Pogribna, I. Pogribny, R.J. Hine, S.J. James, J. Nutr. Biochem. 10 (1999) 490–497.
[34] J.H. Suh, R. Kim, B. Yavuz, D. Lee, A. Lal, B.N. Ames, M.K. Shigenaga, J. Chromatogr. A 877 (2009) 3418–3427.
[35] M. Rafii, R. Elango, G. Courtney-Martin, J.D. House, L. Fisher, P.B. Pencharz, Anal. Biochem. 371 (2007) 71–81.
[36] X. Guan, B. Hoffman, C. Dwivedi, D.P. Matthees, J. Pharm. Biomed. Anal. 31 (2003) 251–261.
[37] A. Ivanov, I. Nazimov, L. Baratova, J. Chromatogr. A 870 (2000) 433–442.
[38] C. Carducci, M. Birarelli, M. Nola, I. Antonozzi, J. Chromatogr. A 846 (1999) 93–100.
[39] K. Mopper, D. Delmas, Anal. Chem. 56 (1984) 2557–2560.
[40] Y. Higashi, M. Yamashiro, R. Yamamoto, Y. Fujii, J. Liq. Chromatogr. Related Technol. 26 (2003) 3265–3275.
[41] C. Parmentier, P. Leroy, M. Wellman, A. Nicolas, J. Chromatogr, J. Chromatogr. B: Biomed. Appl. 719 (1998) 37–46.
[42] N. Ercal, P. Yang, N. Aykin, J. Chromatogr. B: Biomed. Appl. 753 (2001) 287–292.
[43] T.D. Nolin, M.E. McMenamin, J. Himmelfarb, J. Chromatogr. B 852 (2007) 554–561.
[44] A.J. Garcia, R. Apitz-Castro, J. Chromatogr. B 779 (2002) 359–363.
[45] S. Ichinose, M. Nakamura, M. Maeda, R. Ikeda, M. Wada, M. Nakazato, Y. Ohba, N. Takamura, T. Maeda, K. Aoyagi, Biomed. Chromatogr. 23 (2009) 935–939.
[46] S. Tokgoz, C. Bilgi, E. Cakir, M.K. Erbil, T. Kutluay, Turk. J. Med. Sci. 33 (2003) 71–76.
[47] T. Santa, C. Aoyama, T. Fukushima, K. Imai, T. Funatsu, Biomed. Chromatogr. 20 (2006) 656–661.
[48] Eurostat, Health Statistics: Atlas on Mortality in the European Union, Edition ed., Eurostat, Luxembourg, 2009.
[49] R. Accinni, J. Campolo, S. Bartesaghi, G. De Leo, C. Lucarelli, C.F. Cursano, O. Parodi, J. Chromatogr. A 828 (1998) 397–400.
[50] B. Frick, K. Schröcksnadel, G. Neurauter, B. Wirleitner, E. Artner-Dworzak, D. Fuchs, Clin. Chim. Acta 331 (2003) 19–23.
[51] P.M. Ueland, M.A. Mansoor, A.B. Guttormsen, F. Müller, P. Aukrust, H. Refsum, A.M. Svardal, J. Nutr. 126 (1996) 1281S–1284S.