PLASMA THIOLS CONCENTRATION LEVELS AND THEIR CORRELATION WITH HEALTH PATIENTS CONDITION

Florentina GÂTEA[1], Cătălina PETRĂREANU[2], Elena LUPEANU[3], Gabriel Lucian RADU[4]

The purpose of this study was to determine the concentrations of cysteine, homocysteine and glutathione in human plasma and to correlate these results with the health condition of human volunteers. The study was performed on 27 volunteers being under surveillance at the „Ana Aslan" Institute Bucharest. A RP-HPLC method with fluorescence detection was used for quantifications of interested thiols. The results revealed that large values of cysteine and homocystein in plasma are related with the presence of cardiovascular disease and the age of patients.

Keywords: Plasma thiols, RP-HPLC-FD, cardiovascular disease.

1. Introduction

Thiols are organic compounds that exist *in vivo* in three forms which include the free thiol (e.g., cysteine), homodisulfides formed between two identical thiols (e.g., glutathione disulfide) and heterodisulfides formed between different thiols (e.g., protein bound thiols). Thiols constitute the major category of the total body antioxidants and they play a significant role in defense against reactive oxygen species [5].

Intracellular homocysteine is a byproduct of methionine metabolism and is either re-methylated to methionine, or catabolized by vitamin B6-dependent transsulfuration to cysteine. Plasma homocysteine concentrations may be determined by genetic and nutritional factors. The supplementation with folic acid alone or in combinations with B6 and/or B12 decreased the homocysteine concentrations in plasma [2, 3].

[1] PhD student, Faculty of Applied Chemistry and Materials Sciences, University POLITEHNICA of Bucharest, Romania

[2] PhD student, Faculty of Applied Chemistry and Materials Sciences, University POLITEHNICA of Bucharest, Romania

[3] Senior researcher, National Institute of Gerontology and Geriatrics "Ana Aslan", Caldarusani, 307, Bucharest, Romania

[4] Prof., Faculty of Applied Chemistry and Materials Sciences, University POLITEHNICA of Bucharest, Romania, gl_radu@chim.upb.ro

[5] PhD student, Faculty of Applied Chemistry and Materials Sciences, University POLITEHNICA of Bucharest, Romania

Cysteine has been identified as the rate-limiting substrate for GSH biosynthesis in the brain, liver, pancreas and lens [4,5]. It appears that cysteine is a rate-limiting amino acid for GSH synthesis in humans [6]. In addition to conversion from homocysteine, intracellular cysteine also results from conversion from other amino acids as well as from the gamma glutamyl transferase dependent recycling of the amino acids from extracellular glutathione [7]. The quantification of the major aminothiol compounds can be easy performed in plasma and they provide a measure of *in vivo* oxidative stress [8].

Maintenance of the thiol redox status is important for normal physiological function. Changes of the redox thiol status have been linked whith coronary artery disease [9], pregnancy [10], early atherosclerosis [11], renal and liver failure [12], rheumatoid arthritis [13], dementia and Alzheimer's disease [14]. These studies have shown that the measurement of plasma aminothiol concentrations has emerged as a useful tool for diagnosing and monitoring the presence of human diseases and metabolic disorders. Simultaneously also appeared the need for validated, simple and accurate methods able to quantify plasma thiols that can be use in clinical laboratories.

Quantification of plasma thiols must take onto account their physical and chemical properties. Thiols are highly polar and water soluble, which makes almost impossible their extraction from biological matrices without derivatization [15]. The redox properties of thiols is mainly due by the sulfhydryl group (–SH) that in the presence of an electron acceptor form the disulfides. Because of the lack of chromophore in their structure, thiols can not be detected with common chromatographic detectors such as UV–vis absorbance and fluorescence.

A variety of analytical methods including chromatographic, capillary electrophoresis (CE), enzymatic, immuno and electrochemical methods are available for the determination of thiols in clinical and environmental samples [16-19]. All methods, except those based on electrochemical and tandem-mass spectrometry detection, depend on pre- or post-column derivatization of thiols.

HPLC with fluorescence detection (RP-HPLC–FD) is the most commonly used method for determination of aminothiols due to its high sensitivity and relative simplicity. In addition, RP-HPLC–FD allows the simultaneous determination of the primary biological aminothiols cysteine, homocysteine, cysteinylglycine, and glutathione [20]. Different types of labeling reagents have been used in the RP-HPLC-FD methods, including monobromobimane [21,22], ortho phthaldialdehyde [23], N-(1-pyrenil)maleimide [24], 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) [25], N-(2-acridonyl)maleimide [26], ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F) and 7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide (ABD-F) [27-29].

The first objective of this study was to quantify three important biological thiols in plasma samples using a chromatographic method with fluorescence detection. The second objective was to identify the correlation between the levels of cysteine, homocysteine and glutathione in plasma with the presence/absence of cardiovascular disease.

2. Experimental

2.1. Materials and apparatus

Plasma samples. The study was performed on 27 volunteers being under surveillance at the „Ana Aslan" Institute Bucharest divided in two groups: control group consisted of 11 volunteers without cardiovascular disease (C-G) and the study group composed of volunteers diagnosed with cardiovascular disease (CVD-G). Each participant gave his/her informed consent prior to inclusion in the study. Peripheral human blood samples were collected into evacuated tubes containing EDTA as anticoagulant. Plasma was separated within 30 min in a refrigerated centrifuge (Hettich 320r) at 4 °C and stored at -20 °C until analysis.

Standard solutions. DL-homocysteine (HCys), L-cysteine (Cys), and L-glutathione (GSH) reference standards, L-penicillamine (internal standard -IS) were purchased from Sigma-Aldrich and most of them were > 99% certified purity. All other chemicals were of analytical purity. Stock solutions of 25 mM HCys, Cys, GSH and IS needed in the method procedure were prepared by dissolving appropriate amount of the compounds in 2mL of 0.1M hydrochloric acid and diluting to the volume of 10 mL. These solutions were kept at 4 °C for several days without noticeable change of the thiol content. A series of working standard solutions of Cys, HCys, GSH at concentrations of 5 µM, 12.5 µM, 25 µM, 50 µM, 500 µM, 1mM, 2mM, and 3mM and an IS solution at 2.5 mM were prepared by appropriately diluting the stock solutions with distilled water.

HPLC analyses were performed with a Shimadzu Chromatography System (Kyoto, Japan) that consisted of two LC-20AD pumps, a SIL-HTC autosampler, an online DGU-20A5 vacuum degasser, a column thermo controller CTO-20AC, and a fluorescence detector RF 100AXL. For instrument control, data acquisition and data analysis, LC Solution software was used. Chromatographic separation was achieved on the Fortis C18 column (250 mm ×4.6 mm i.d., 5µm).

2.2. Methods

Derivatization method
Samples derivatization was with monobrombiman (MBB) as described previously [21] with some minor modifications: 60µL of sample was mixed with,

10 µL of internal standard (2.5 mM), 60 µL of 4 M $NaBH_4$ in 66 mM NaOH and 333 mL/L DMSO (dimethylsulfoxide), 20 µL of 2 mM EDTA (ethylenediaminetatraacetic) acid in 1.65 mM DTE (dithioerythritol), 20 µL of 1-octanol and 40 µL 1.8 M HCl. After 5 minutes (to allow reduction of disulfides) 200 µL 0.5M carbonate buffer pH =8.2, 200 µL of distilled water and 40 µL of 25 mM MBB were added. After stirring the reagent mixture, derivatization of the thiol functions by MBB required 20 min incubation in the dark at room temperature. The derivatization was finished by adding 80 µL of glacial acetic acid.

Chromatographic method

The elution protocol (flow, 1mL/min, total run time of 40 min, temperature $25^{o}C$) employed linear gradients of solvents A (water with 0.1% acetic acid) and B (100 % acetonitrile) as follows: 0 min 1 % B; 20 min, 18,5 % B; 22 min, 100 % B; 30 min, 100 % B; 33 min 1 % B; 36 min 1 % B; reinject. The MBB derivatives of thiols were fluorimetrically detected (exitation 375 nm; emission 475 nm, fig.1.). The injection volume was 10µL.
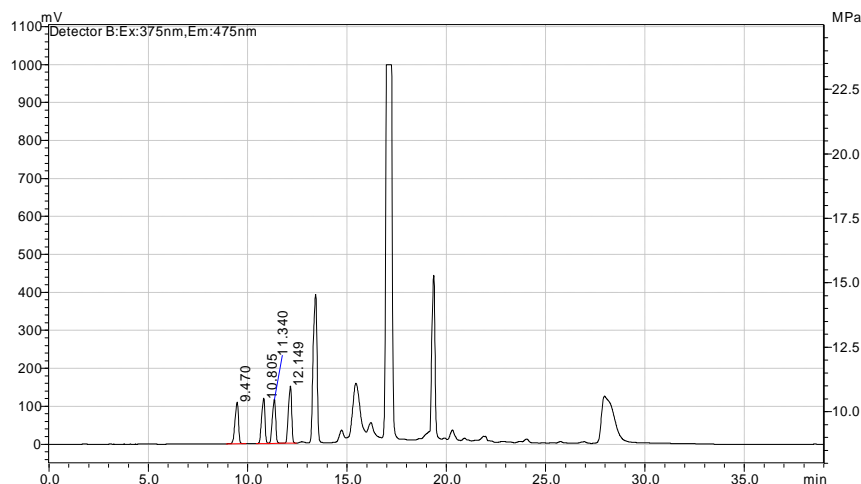

Fig. 1.

Representative chromatogram of cysteine-bimane (t_R=9.470), homocysteine-bimane (t_R=10.805), glutathione-bimane (t_R=11.340) and penicillamine-bimane (t_R=12.149) adducts. The remaining peaks are monobromobimane hydrolysis products.

3. Results and discussion

The RP-HPLC-FD method used for cysteine, homocysteine and glutathione in human plasma was characterized in terms of linearity, selectivity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy.

The calibration curves were prepared from the working standard Cys, HCys and GSH solutions at nine concentrations ranging from 25 nM to 90µM mL^{-1}. The ratio of peak areas of Cys, HCys, GSH to that of the IS was used for the quantification of these thiols in plasma. The selectivity of the method was evaluated by comparing the chromatograms of standards with the corresponding spiked plasma samples. The LOD was determined using a signal to-noise ratio (S/N) of 3. The LOQ was determined as the lowest plasma concentration that produced an S/N of 10 and could be quantified with a relative standard deviation (RSD) lower than 20% and accuracy between 80–120% (Table 1).

Intra-and inter-day precision were evaluated by the analysis of five replicates of each quality control sample at three different concentrations (0.5, 5 and 60 µM mL^{-1}) in the same day and during three days. The coefficient of variation (CV) was taken as a measure of precision and was calculated by the equation: CV (%) = (SD/mean) x 100%. The intra-day precision for low, mid and high concentration of Cys, HCys and GSH was found to be 5.8, 4.1 and 5.6%, respectively. The inter day precision was found to be 6.1, 4.9 and 6.4%, respectively for low, mid and high concentration of Cys, HCys and GSH.

Table 1.

Performance parameters of the RP-HPLC-FD method for thiols quantification

Analite	Cys	HCys	GSH
t$_R$ (min.)	9.476±0.021	10.827±0.024	11.351±0.051
Curve equation	y= 47621.7E-06x-12423.2	y=54713.5E-06x-8579.99	y=54957.1E-06x-2783.49
R^2	0.9996	0.9992	0.9988
Linearity (mol L^{-1})	2.5×10^{-8} -9×10^{-5}	5×10^{-8} -9×10^{-5}	5×10^{-8} -9×10^{-5}
LOD (mol L^{-1})	0.54×10^{-9}	0.27×10^{-9}	0.29×10^{-9}
LOQ (mol L^{-1})	1.66×10^{-9}	0.83×10^{-9}	0.88×10^{-9}

t$_R$ – retention time

Accuracy was determined by recovery test. Recoveries of all of the quantified constituents were determined using a plasma sample for which the

respective chemical contents had been predetermined, spiked at three different concentration levels: recovery (%) = (amount determined – original amount)/amount added) x 100. Triplicate sample analysis was conducted for the determination of recovery at each spiked level. The mean recoveries of Cys, HCys, GSH at each quality control sample level (0.5, 5 and 60 μM mL^{-1}) were $87.4 \pm 7.3\%$, $89.1 \pm 7.5\%$, and $90.8 \pm 7.8\%$, respectively, and the extraction recovery of the IS was $92.1 \pm 7.8\%$ at 5 μM mL^{-1}.

Mean levels of homocysteine and cysteine measured in plasma volunteers were about 16.92 μmol/L (range 8.87–20.74 μmol/L) and 231.67 μmol/L (range 146.77–266.73 μmol/L), respectively for the control group (with age between 51-68 years, mean=61.63). As might be expected for a healthy population-based sample, 80% of this group had homocysteine levels < 20 μmol/L. For the CVD group (with age between 46-83 years, mean=60) mean levels of homocysteine and cysteine were about 24.63 μmol/L (range 18.22–29.47 μmol/L) and 362.25 μmol/L (range 149.13–501.29 μmol/L), respectively. Mean levels of glutathione were about1.52 μmol/L (range 0.92–2.07 μmol/L) for control group and 1.66 μmol/L (range 0.769–2.836 μmol/L) for the CVD group.

A positive correlation coefficient between cysteine and homocystein levels was calculated both the control group and study group (Fig. 2). Positive values were also obtained for correlations coefficients between the concentrations of cysteine and glutathione and homocysteine and glutathione in plasma of the control group (r= 0.8188, respectively r=0.6931) and study group (r= 0.4161, respectively r=0.3957).
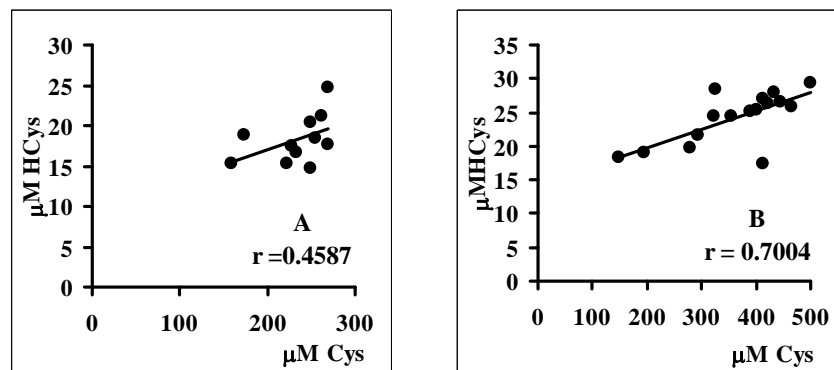

Fig. 2 Correlation between concentration levels of cysteine and homocysteine in plasma, for the control group (A) and study group (B)

The highest values for cysteine and homocysteine concentrations were obtained for volunteers with ages between 68-83 years (430.46± 28.81 μmol/L

respectively, 25.5 ± 1.4 μmol/L), therefore another factor that may influences these values was the age of volunteers.

4. Conclusions

A simple, sensitive and reproducible RP-HPLC-FD method has been validated for simultaneously determining total concentrations of the aminothiols HCys, Cys and GSH in human plasma. The complete separation of thiols was achieved within 12 min. All calibration curves expressed good linearity ($R^2 > 0.998$) within the test range. The recovery of this method was in the range 87.4–92.1%, for the intra-day and inter-day assays, the values of deviation coefficient were less than 6.4%. The assay was successfully applied to the quantification of thiols from 27 samples of human plasma and it is well suited for high-throughput quantitative determination of aminothiols in clinical studies.

The results reveal that the large majority of volunteers with cardiovascular disease showed elevated plasma homocysteine concentrations (>20 μmol/L) in comparison with those without cardiovascular disease. At the same patients there was a high correlation of homocysteine concentration levels with those of cysteine (r=0.7004) and a low correlation with the levels concentration of glutathione (r=0.3957). Plasma concentrations of cysteine and homocysteine increases with age.

Acknowledgements

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