Journal of Chromatography B, 877 (2009) 3400–3404

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Fully automated method for simultaneous determination of total cysteine, cysteinylglycine, glutathione and homocysteine in plasma by HPLC with UV absorbance detection☆

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ARTICLE INFO

Article history:
Received 13 January 2009
Accepted 8 June 2009
Available online 13 June 2009

Keywords:
Cysteine
Cysteinylglycine
Glutathione
Homocysteine
HPLC
On-line sample preparation
Chemical derivatization

ABSTRACT

A fully automated HPLC method for the simultaneous determination of total thiols in plasma samples has been developed. The method involves reductive conversion of disulfides to their reduced counterparts with the use of tris(2-carboxyethyl)phosphine. After reduction the newly formed sulfhydryl groups are reacted with 2-chloro-1-methylquinolinium tetrafluoroborate to form 2-S-quinolinium derivatives followed by deproteinization by dialysis. The reaction products are separated by reversed-phase HPLC, detected and quantified by UV absorbance detection at 355 nm. The recommended HPLC procedure enables measurement of four main plasma aminothiols cysteine, cysteinylglycine, glutathione, and homocysteine with low imprecision (mean relative standard deviations within calibration range, 3.47%, 5.34%, 4.25% and 3.26%, respectively) and good sensitivity. Accuracy, expressed as the mean measured amount as percentage of added amount, was within 97.5–103.0%, 98.3–102.5%, 96.3–99.5% and 97.1–99.1%, respectively. The lower limit of quantification for all thiols was 0.5 μM. The whole unattended instrument acquisition time amounts 13 min.

1. Introduction

Plasma sulfur amino acids, including cysteine, cysteinylglycine, glutathione and homocysteine are being investigated as potential indicators of health status and disease risk [1]. Especially increased plasma homocysteine, closely related with methionine metabolism, is considered an independent risk factor for cardiovascular disease [2,3]. Glutathione is recognized as a key physiological antioxidant that not only detoxifies reactive oxygen species directly, but also enhances the functional ability of other crucial antioxidants vitamin E and C [4]. In biological membranes, vitamin E is present in a low molar ratio in contrast with phospholipids, which are abundant and highly susceptible to oxidative damage. Thiols such as reduced glutathione and dihydrolipoate support the recycling of vitamins C and E.

Thus determination of total homocysteine and related thiols plays an important role in diagnosis and therapy of folate and cobalamin deficiencies. Since the response of a cell to oxidative stress typically involves alterations in thiol content [5], plasma aminothiol concentrations are increasingly utilized for clinical and translational research involving oxidative stress [6], and for routine clinical diagnosis and monitoring of various human diseases and metabolic disorders [7,8]. Therefore, there is a need for fully validated, simple, precise and sensitive assays capable of simultaneous determining aminothiols in human plasma.

Chromatographic methods have been developed extensively for total homocysteine determinations despite the recent introduction of immunoassays. Among chromatographic methods, gas chromatography coupled to mass spectrometry [9], high performance liquid chromatography [10–13] and capillary electrophoresis [14] has been developed. To our knowledge only few fully automated procedures for determining total homocysteine and other metabolically related thiols has been described [15–17]. Aside from the great susceptibility to oxidation, which can occur before or during analytical process, most thiols lack the structural properties necessary for the production of signals compatible with common HPLC detectors such as UV absorbance and fluorescence. Therefore, the analyst must resort to derivatization for signal enhancement and labile sulfhydryl group blocking if fluorescence or UV–vis detection methods are employed.

In this paper a new method that allows analyzing about 80 samples within 24 h by successful implementation in a fully automated procedure: the (1) reduction of disulfide bonds with tris(2-carboxyethyl)phosphine, (2) derivatization of thiols with 2-chloro-1-methylquinolinium tetrafluoroborate, (3) deproteinization by dialysis and (4) separation and quantifi-

☆ This paper is part of the special issue "Analysis of Thiols", I. Dalle-Donne and R. Rossi (Guest Editors).

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doi:10.1016/j.jchromb.2009.06.012

cation by reversed-phase HPLC with UV absorbance detection, is described.

2. Experimental

2.1. Chemicals and reagents

2-Chloro-1-methylquinolinium tetrafluoroborate (CMQT) was prepared in this laboratory according to the procedure described earlier [18]. For thiols derivatization prior to HPLC analysis, a 0.01 or 0.1 M water solution of CMQT was used. Perchloric acid (PCA), sodium hydrogen phosphate heptahydrate ($Na_2HPO_4{\cdot}7H_2O$), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$), and HPLC-grade acetonitrile were from J.T. Baker (Deventer, The Netherlands). DL-Homocysteine (HCSH), cysteinylglycine (CGSH), glutathione (GSH) and cysteine (CSH) and their disulfides were purchased from Sigma (St. Louis, MO, USA). Tris(2-Carboxyethyl)phosphine (TCEP), trichloroacetic acid (TCA) and lithium hydroxide (LiOH) were from Merck (Darmstadt, Germany). Purified water from Millipore Milli-QRG system (Vienna, Austria) was used throughout the experiments. All reagents were tested and found to be stable for unattended analysis.

2.2. Calibration standards preparation and storage

2.2.1. Standard solutions

Stock solutions of 10 mM HCSH, CSH, CGSH, GSH and their symmetrical disulfides needed in the method development procedure were prepared by dissolving appropriate amount of the compounds in 2 mL of 0.1 M 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. The working solutions were prepared by appropriate dilutions with water as needed, and processed without delay.

2.2.2. Plasma preparation

Blood was collected into evacuated tubes containing EDTA, immediately placed on ice, and centrifuged at 800 × *g* for 15 min at room temperature. Plasma was then used for the determination of total thiols without delay or stored at −20 °C. Majority of blood donors were young school boys and girls (8–15 years old). For preparation of calibration standards, plasma samples from an apparently healthy donor were placed each in polypropylene tube and spiked with the appropriate amount of working standard solution of disulfides.

The sample processor collects the sample and the reagents and dispenses them into a 700 μL derivatization vial, where reduction and derivatization are carried out at room temperature.

2.3. Apparatus

A programmable sample procesor, ASTED XL (Gilson, Viliers-le-Bel, France) consisted of dialyser, injection sample loop (100 μL), model 402 dilutor fitted with a two 1 mL volume syringes, compatible with chromatographic system was used for unattended determination of plasma thiols. The processor automatically and on-line prepares the plasma sample for final chromatographic analysis. It operates in a concurrent sequencial mode with the HPLC system; one sample undergoes the chromatographic analysis while the next one is being prepared. The dialyser block with cellulose acetate dialysis membrane (EZEE-MOUNT, 15 kDA molecular mass cut-off) was used. The volumes of the donor and recipient channels were 100 and 175 μL, respectively. Sample was aspirated at a 3 mL/min flow rate into the donor channel of the dialysis block. Recipient solution (175 μL) was aspirated (1.5 mL/min) by 402 syringe pump to the sample loop. After each dialysis the sample line and the donor channel were flushed with 5 mL of deionized water. Final HPLC analysis was performed on a Hewlett-Packard HP 1100 liquid chromatograph (Waldbronn, Germany) encompassing a quaternary pump, autosampler, thermostated column compartment, vacuum degasser and diode-array detector. The schematic of the automatic analytical system is shown in Fig. 1.

2.4. Analytical procedure for determination of thiols

The plasma samples were pipetted into the 0.7 mL vials placed in the rack on the tray of the sample processor (80 samples for 24 h analysis time). The vials in the second rack of the processor's tray (defined as "Reagent" in the manufacturer manual) were filled with phosphate buffer (0.2 M, pH 7.4), TCEP phosphate buffer solution (0.25 M), CMQT water solution (100 mM), and 3 M perchloric acid.

A 50 μL of plasma sample was automatically transported into the vial of the Results Area (manufacturer's manual) in the tray of ASTED sample processor and mixed with 50 μL of phosphate buffer followed by addition of 5 μL of TCEP solution. After 8 min a 5 μL of CMQT derivatizing reagent was added. Just after mixing of the reagents a 50 μL of 3 M PCA was added. A 100 μL of the

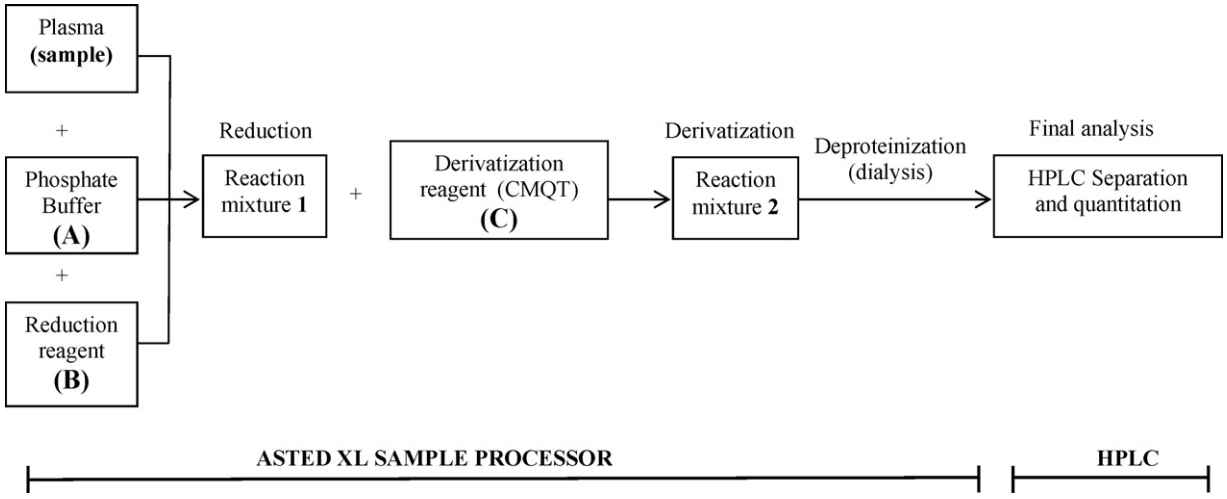

Fig. 1. The schematic of the automatic analytical system; (A) phosphate buffer solution (0.2 M), (B) tris(2-carboxyethyl)phosphine phosphate buffer solution (0.25 M), and (C) 2-chloro-1-methylquinolinium tetrafluoroborate water solution (0.1 M).

Table 1
Sample preparation programme including reduction, derivatization and deproteinization steps.

Gilson sample processor program steps	Reagent	Volume (μL)	Concentration, pH
1. Begin cycle			
2. Add reagent A and B to sample	Phosphate buffer—reagent A	50	0.2 M, pH 7.4
	Tris(2-carboxyethyl)phosphine—reagent B	5	0.25 M
	Plasma—sample	50	
3. Mix result method Asp/Disp		75	
4. Rinse needle (inside/outside)	Deionized water	250/500	
5. Wait delay 8 min			
6. Add reagent C to result	2-chloro-1-methylqunolinium tetrafluoroborate—reagent C	5	0.1 mM
7. Mix result method Asp/Disp		75	
8. Rinse needle	Deionized water	250/500	
9. Wait delay 1 min			
10. Add reagent D to result	Perchloric acid—reagent D	50	3 M
11. Mix result method Asp/Disp		100	
12. Dialyse result (static mode)		100	
13. Inject trace coordination YES			
14. Wash dialyser reservoir	Donor channel—deionized water	3 × 175	
	Recipient channel—trichloroacetic acid solution/acetonitrile (65:35, v/v)	3 × 100	0.07 M, pH 4
15. Rinse needle	Deionized water	250/500	
16. End cycle			

reaction mixture was drawn to a donor channel of the dialyser and held static for 3 min. After that the dialysate from recipient channel was transferred into the 100 μL volume injection loop. Consecutive steps of the sample preparation and injection programme are given in Table 1. From the loop the sample was transferred to the analytical column using the HPLC mobile phase. As the separation was proceeding on the analytical column, the dialyser donor and recipient channels were flushed with 5 mL of water and 5 mL recipient channel solution, respectively. In this way the system was ready for the next sample processing.

2.5. Chromatography

For the separation of 2-S-quinolinium derivatives of homocysteine and metabolically related thiols from each other and derivatization reagent excess an Agilent Technologies Zorbax SB-C18 (150 mm × 4.6 mm i.d.) column, packed with 5 μm particles, and a gradient elution were used. The elution profile was as follows: 0–4 min, 11% B; 4–8 min, 11–35% B; 8–12 min, 35–11% B (A:B, v/v). Elution solvent B was acetonitrile and solvent A was 0.07 M TCA water solution, adjusted to pH 1.65 with LiOH of the same concentration. The flow rate was 1.2 mL/min, the temperature 25 °C and analytical wavelength 355 nm.

2.6. Calibration

Calibration curves for plasma total homocysteine and metabolically related thiols were constructed by processing 50 μL of calibration standard samples of plasma, spiked with appropriate disulfides and next processed according to the procedure described in Section 2.4. The concentration ranges of GSH and HCSH were from 1.0 to 30 μM, for CSH from 10 to 300 μM, and for CGSH from 1.0 to 60 μM of plasma. The calibration ranges can be easily extended up if needed.

3. Results

3.1. Reduction and derivatization

Because of high susceptibility to oxidation more than 90% of plasma thiols stores in the disulfide forms including 64% bound to proteins. Thus, determination of total thiol content must comprise disulfide bond reduction step. Moreover, reducing agent is necessary both for the reduction of the disulfide bonds and to keep the thiol in the reduced form until derivatization. Reducing agent must be compatible with the thiol-specific derivatization agent. Thiols such as 2-mercaptoethanol and dithiothreitol are not suitable as reductants in our assay because they consume CMQT and may produce additional derivatives finally interfering with chromatogram. The same was observed by other researchers [15,19] when monobromobimane was used as derivatization reagent. Use of sodium borohydride is very inconvenient and may lead to problems with derivatization reaction because of pH control difficulties. Additionally, sodium borohydride decomposition reaction during sample preparation is concomitant with significant sample foaming. Thus, for simultaneous determination of total thiols in plasma samples TCEP, which react with disulfides at room temperature, was used. TCEP reduces all dimer and mixed disulfides completely but not protein bound cysteine. The latter is reduced in only 80% however with good repeatability. The detailed study on TCEP capacity to reduce protein bound cysteine are now in progress in our laboratory and will be published soon.

In this assay we exploited CMQT a highly reactive and thiol specific UV derivatization reagent [18]. The 2-S-quinolinium derivatives resulted from the reaction are stable thioethers exhibiting well defined absorption maximum at 348 nm and molar absorptivity coefficient about $2 \times 10^4\ M^{-1} \times cm^{-1}$. Bathochromic shift from reagent maximum (328 nm) to the maximum of the derivative (348 nm) is analytically advantageous. It was thanks to this phenomenon that we could recommend the use of large excess of CMQT in order to drive the reaction to completion (in real world sample) and avoid a huge peak of unreacted derivatization reagent on the chromatogram.

3.2. Dialysis

In order to optimize a dialysis, pH and concentration of the recipient dialyser channel solution, and dialysis time were optimized. Finally the dialysis was performed with recipient dialyser channel solution consisted of 0.07 M trichloroacetic acid solution adjusted to pH 4 with 0.07 M LiOH and acetonitrile (65:35, v/v). The dialysis was performed in static mode during 8 min. In order to check between run dialysis repeatability a series of sequential analyses ($n = 6$) were performed with water standards and normal plasma samples spiked with different amounts, representing calibration ranges, of CSH, CGSH, GSH and HCSH (as disulfides) and the standard deviation and the imprecision for the peak height was calculated (Table 2). In all cases imprecision was less than 7%. The

Table 2
The typical results for the between run dialysis imprecision.

Concentration (μM)	Imprecision (RSD, %)							
	Water standard sample				Plasma sample			
	GSH	HCSH	CSH	CGSH	GSH	HCSH	CSH[a]	CGSH
1	4.8	6.3	3.8	2.5	5.6	6.5	2.1	3.9
15	5.1	4.9	4.2	3.1	4.9	5.2	1.8	4.3
30	2.3	1.8	2.0	1.5	3.7	3.7	2.0	2.8

[a] Spiked concentrations of cysteine (as cystine) were 10, 150 and 300 μM.

mean recovery of the dialysis for CSH, CGSH, GSH and HCSH was 48.2%, 45.3%, 44.6% and 42.5%, respectively.

3.3. Chromatography

For optimum HPLC conditions for separation of CMQT derivatives of analytes from each other, reagent excess and other unidentified matrix components, several mobile phase compositions and concentration, as well as pH, temperature and flow rate were tested. Preliminary experiments within method development were carried out on standard solutions. After thorough study of the above-mentioned chromatographic variables, the separation conditions chosen constitute a necessary compromise between maximum detectibility, mobile phase pH stability and chromatographic resolution. Finally the mobile phase described in Section 2 was used. Under these conditions derivatives of GSH, HCSH, CSH and CGSH were eluted after 8.1, 8.4, 9.7, and 10.1 min, respectively. Typical chromatograms, obtained during standard thiol solution and plasma sample analyses are presented in Fig. 2. Identification of peaks was based on comparison of retention times and diode array spectra, taken at real time of analysis, with corresponding set of data obtained by analyzing sample containing authentic compounds.

3.4. Linearity, precision and accuracy

The peak heights of thiol derivatives were plotted versus analyte concentration and the curves were fitted by least-square linear regression analysis. Calibration data including regression equations are shown in Table 3. Standard errors for slope for CSH, CGSH, GSH and HCSH, were 0.00671, 0.001458, 0.00172, 0.00246, mAU, and for intercept 0.86077, 0.03578, 0.022101 and 0.03156 mAU/mol × L, respectively. The lower limit of quantification value (LOQ) for all thiols was 0.5 μM. The LOQ values were assessed in buffered saline (a proxy matrix, 0.9% NaCl in pH 7.4 10 mM phosphate buffer) samples as the minimum quantity of thiol that could be measured without interference from the baseline noise (signal-to-noise ratio of 6:1, imprecision within 15% and accuracy 85–115%).

The developed method was applied to the analysis of plasma samples donated by 50 apparently healthy volunteers. Average total plasma CSH, CGSH, GSH and HCSH content was 281.8, 37.0, 6.6 and 6.1 μM of plasma, respectively. The maximum imprecision values (RSD%, $n = 3$) for determination of CSH, CGSH, GSH and HCSH, in plasma were 7.1%, 6.2%, 6.5% and 4.8%, respectively. Notably, such good precision was obtained with no outliners excluded and without including an internal standard. Accuracy determination was accomplished by analysis of plasma spiked with known amounts of thiols in their oxidized forms. Three concentrations representing the entire range of the calibration curve were studied: one near the lower end, one near the center and one near the upper boundary of the standard curve (10, 150 and 300 μM for CSH; 1, 30 and 60 μM for CGSH and 1, 15 and 30 μM for GSH and HCSH). The spiked plasma samples were processed according to the recommended analytical procedure for total thiols determination. Measured concentrations were assessed by application of calibration curve obtained on that occasion. Accuracy, expressed as the mean measured amount as percentage of added amount, was for CSH, CGSH, GSH and HCSH within 97.5–103.0%, 98.3–102.5%, 96.3–99.5% and 97.1–99.1%, respectively.

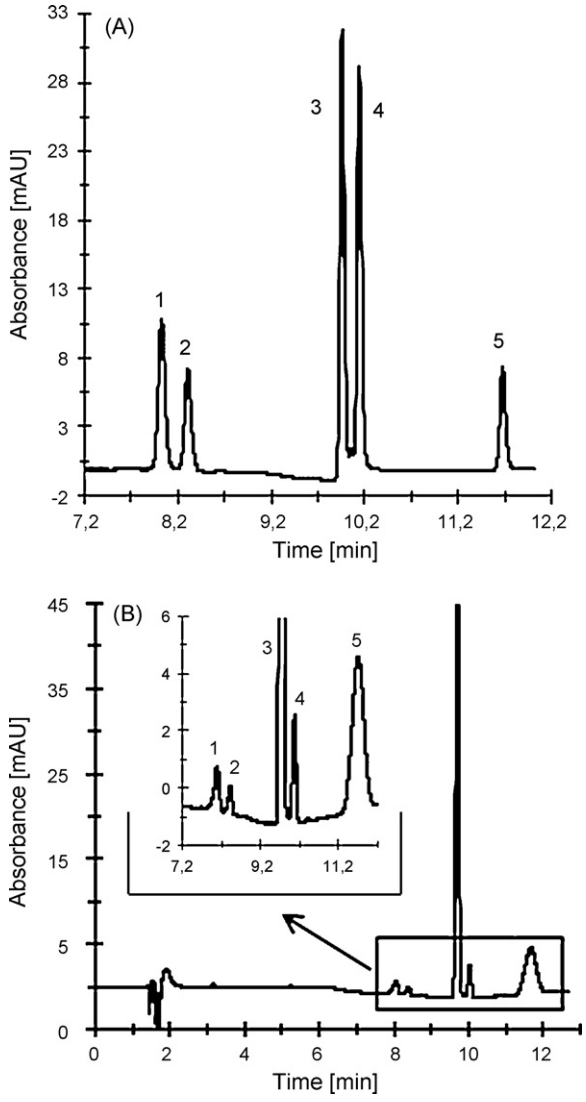

Fig. 2. Typical chromatogram of main plasma thiols. (A) Water standard solution (concentration of each thiol—5 μM); (B) chromatogram of the representative plasma sample analyzed according to fully automated procedure. Peaks: 1; glutathione (10.7 μM), 2; homocysteine (6.2 μM), 3; cysteine (142 μM), 4; cysteinylglycine (30.2 μM), 5; excess of 2-chloro-1-methylquinolinium tetrafluoroborate.

4. Discussion

Recently, we have reported an HPLC method for determination of different forms of main plasma thiols based on manual sample

Table 3
Plasma calibration curves parameters for cysteine, cysteinylglycine, glutathione and homocysteine.

Thiol	Regression equation	R^2	Range (μM)	Imprecision (RSD, %)	
				Max	Min
CSH	$y = 0.2789x + 5.8385$	0.9954	10–300	5.8	1.3
CGSH	$y = 0.,1404x + 1.6389$	0.9991	1–60	3.9	2.1
GSH	$y = 0.1437x + 0.2035$	0.9989	1–30	7.2	0.2
HCSH	$y = 0.1595x + 0.181$	0.9981	1–30	5.1	0.0

preparation [13]. In this report we propose a fully automated assay for the title plasma thiols with a short turnaround time. The procedure utilizes the same fast and simple derivatization chemistry scheme with the use of CMQT [18] combined with a short time HPLC separation of the analytes ensuring high sample throughput. Automation forced the change of previously applied [13] reducing agent and HPLC separation conditions. Sodium borohydride [13] was replaced by more user friendly TCEP. During optimization of the method we carried out every experiment in such a way that the steps could be performed by the Gilson ASTED XL automatic sample processor. For simplicity these experiments were performed manually; only the optimal design was programmed into the processor and then verified against manual sample preparation. Despite substantial dilution of sample during dialysis, procedure does not need preconcentration step before the final HPLC analysis. The whole unattended instrument acquisition time amounts 13 min with no need of manual sample preparation, and the method enables analysis of 80 plasma samples for 24 h. Analytical figures of merit, demonstrated during the method validation protocol, are well within the criteria for biological sample analysis [20,21]. This high throughput method is precise, accurate, low in reagents cost and linear. The method has also other advantages: (1) good solubility and stability of the derivatizing reagent in water, (2) high reactivity and selectivity of the reagent toward thiols, (3) low absorption of the reagent at analytical wavelength and high optical yield of the derivatives, (4) low hydrolysis rate of the reagent and derivatives under slightly alkaline conditions of derivatization reaction, (5) short column equilibrium time, (6) simultaneous determination of the four thiols with high precision, (7) high stability of all reagents enabling unattended runs, and (8) the use of UV-detector known for its stability and low demand in terms of maintenance. In order to avoid carry over effect, donor and recipient channels of the dialyzer have to be washed with deionized water and trichloroacetic acid–acetonitrile, respectively.

Plasma aminothiols concentration monitoring has emerged as a useful tool for diagnosing and monitoring the presence of human disease and metabolic disorders including oxidative stress. An assay capable of simultaneously determining multiple aminothiols is desirable because of extensive interconversion between them and the need to understand the dynamic relationship between all thiol and disulfide components [8,7,22] as well as to facilitate assessment of the relationship between thiols and disease status. Few methods for determination of one or more thiols have been reported [15,16,23–29], but only three describe a procedure for the four plasma thiols [13,23,27]. In numerous studies, total HCSH concentrations were found to be increased in patients with arteriosclerosis, renal failure, or other diseases. Determination of CSH and GSH in these conditions was reported rarely. Since HCSH shows prooxidative properties and GSH antioxidative, and because there is extensive interconversion between these metabolites, their simultaneous measurement in plasma can facilitate understanding their role in human health and disease. Automated assays depend on the stability of the reagents, plasma samples and thiol derivatives if derivatization step is employed. For example, monobromobimane is subject to photolysis and is unstable in water and room temperature. Even under optimal conditions (2–3 °C, dark glass vials), there was a time-dependent formation of multiple fluorescent peaks, some of which co-chromatographed with the thiol derivatives [15,16]. *o*-Phthalaldehyde (OPA) is a fluorogenic reagent, non-thiol specific since it reacts with all primary amino groups, so that the determination of aminothiols in plasma samples requires a very good separation [30]. In addition, in some cases derivatization with OPA, requires previous carboxymethylation with iodoacetate in order to provide conditions for satisfactory fluorescence yield of the derivative, rendering the assay more difficult for full automation. Measurement of HCSH in plasma by an immunoassay appeared to have become the preferred analytical approach [17], but despite their low instrumental costs, immunoassays have a high reagent cost compared to chromatographic methods. The advantage of HPLC methods, as compared to immunoassay, is that they allow the joint determination of different plasma thiol compounds.

In conclusion, a fully automated method for determination of total main thiols in human plasma has been developed. It has proved possible to use a sample preparation robot to successfully mimic a common manual process used for the preparation of plasma for assessment of aminothiols content. The CSH, CGSH, GSH and HCSH human plasma concentrations were measured using automated precolumn derivatization, on-line dialysis and reversed phase HPLC with UV absorbance detection and quantification.

Acknowledgement

This work was supported by grant from the University of Lodz.

References

[1] J. Himmelfarb, E. McMenamin, E. McMonagle, Kidney Int. 61 (2002) 705.
[2] M.J. Stampfer, M.R. Malinow, W.C. Willett, L.M. Newcomer, B. Upson, D. Ullmann, P.V. Tishler, C.H. Hennekens, J. Am. Med. Assoc. 268 (1992) 877.
[3] W.A. Kleinman, J.P. Richie, Biochem. Pharmacol. 60 (2000) 19.
[4] C.K. Sen, L. Packer, Am. J. Clin. Nutr. 72 (2000) 653.
[5] D.A. Dickinson, H.J. Forman, Biochem. Pharmacol. 64 (2002) 1019.
[6] J. Himmelfarb, R.M. Hakim, Curr. Opin. Nephrol. Hypertens. 12 (2003) 593.
[7] V. Ducros, K. Demuth, M.P. Sauvant, M. Quillard, E. Causse, M. Candito, M.H. Read, J. Drai, I. Garcia, M.F. Gerhardt, J. Chromatogr. B 781 (2002) 207.
[8] A. Pastore, G. Federici, E. Bertini, F. Piemonte, Clin. Chim. Acta 333 (2003) 19.
[9] A. Windelberg, O. Arseth, G. Kvalheim, P.M. Ueland, Clin. Chem. 51 (2005) 2103.
[10] I.O. Melnikov, I.V. Nazimov, E.A. Stukacheva, Y.M. Glubokov, J. Anal. Chem. 61 (2006) 1093.
[11] K. Gempel, K.D. Gerbitz, B. Casetta, M.F. Bauer, Clin. Chem. 46 (2000) 122.
[12] J.I. Sigit, M. Hages, K.A. Brensing, U. Frotscher, K. Pietrzik, K. von Bergmann, D. Lutjohann, Clin. Chem. Lab. Med. 39 (2001) 681.
[13] E. Bald, G. Chwatko, R. Głowacki, K. Kuśmierek, J. Chromatogr. A 1032 (2004) 109.
[14] C. Chassaing, J. Gonin, C.S. Wilcox, I.W. Wainer, J. Chromatogr. B 735 (1999) 219.
[15] T. Fiskerstrand, H. Refsum, G. Kvalheim, P.M. Ueland, Clin. Chem. 39 (1993) 263.
[16] H. Refsum, P.M. Ueland, A.M. Svardal, Clin. Chem. 35 (1989) 1921.
[17] A. Leino, Clin. Chem. 45 (1999) 569.
[18] E. Bald, R. Głowacki, J. Liquid Chromatogr. Rel. Technol. 24 (2001) 1323.
[19] R.C. Fahey, G.L. Newton, Methods Enzymol. 143 (1987) 85.
[20] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, J. Pharm. Sci. 81 (1992) 309.
[21] S. Braggio, R.J. Barnaby, P. Grossi, M. Cugola, J. Pharm. Biomed. Anal. 14 (1996) 375.
[22] T.D. Nolin, M.E. McMenamin, J. Himmerfalb, J. Chromatogr. B 852 (2007) 554.
[23] A. Pastore, R. Massoud, C. Motti, A.L. Lo Russo, G. Fucci, C. Cortese, G. Federici, Clin. Chem. 44 (1998) 825.
[24] B. Frick, K. Schrocksnadel, G. Neurauter, B. Wirleitner, E. Artner-Dworzak, D. Fuchs, Clin. Chim. Acta 331 (2003) 19.
[25] A. Andersson, A. Isaksson, L. Brattstrom, B. Hultberg, Clin. Chem. 39 (1993) 1590.
[26] A. Andersson, A. Lingren, M. Arnadottir, H. Prytz, B. Hultberg, Clin. Chem. 45 (1999) 1084.
[27] M.A. Mansoor, A.M. Svardal, P.M. Ueland, Anal. Biochem. 200 (1992) 218.
[28] V. Ducros, Ann. Biol. Clin. 66 (2008) 169.
[29] S. Li, J. Jia, G. Liu, W. Wang, Y. Cai, Y. Wang, C. Yu, J. Chromatogr. B 870 (2008) 63.
[30] C. Carducci, M. Birarelli, M. Nola, I. Antonozzi, J. Chromatogr. A 846 (1999) 93.