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Analysis of plasma thiols by high-performance liquid chromatography with ultraviolet detection

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Abstract

The procedure for measurement of different forms of four plasma thiols cysteine, cysteinylglycine, glutathione and homocysteine is proposed. The analytes are derivatized with thiol-specific ultraviolet labeling reagent, 2-chloro-1-methylquinolinium tetrafluoroborate, and separated from each other, reagent excess and plasma matrix constituents by reversed-phase high performance liquid chromatography with detection at 355 nm. Oxidized forms are converted to their thiol counterparts by reductive cleavage with sodium borohydride prior to derivatization step. In order to circumvent the loss of reduced fraction of thiols due to oxidation during sample preparation, the derivatization reagent is added to whole blood immediately after collection and before separation of plasma from erythrocytes. The method measures total thiols, total free thiols, protein-bound thiols and reduced thiols. The method is linear within the physiological and pathological ranges of thiols and is applied for plasma samples donated by apparently healthy volunteers.

Keywords: Derivatization, LC; Thiols; Organosulfur compounds; Chloromethylquinolinium tetrafluoroborate

1. Introduction

The biological thiols can by classified as high-molecular-mass protein thiols and low-molecular mass free thiols. Disulfide linkages, –S–S– between two cysteinyl residues are important determinants of protein structures. Low-molecular-mass thiols and their disulfides, such as cysteine, glutathione and homocysteine, are critical cellular components that play numerous important roles in metabolism and homeostasis. They play a central role in the antioxidant defense network, including radical quenching. 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, such as Vitamins E and C [1]. Cysteine is a critical substrate for protein synthesis, and rate-limiting precursor of glutathione and taurine synthesis. Homocysteine is a critical regulatory intermediate of the methionine cycle that serves as a precursor for cysteine on the transsulfuration pathway as well as for methionine via remethylation. Altered levels of thiols in plasma have been linked with specific pathological conditions. Mildly elevated plasma homocysteine levels have been associated with an increased risk for cardiovascular and cerebrovascular diseases in men [2], and highly elevated plasma and urine levels are a clinically relevant indicator of well known, but fortunately rare, group of inborn errors of metabolism called homocystinuria [3]. Cysteinylglycine, a product of enzymatic degradation of glutathione, is a second to cysteine most abundant thiol in plasma.

The most significant of the multiple roles of thiol compounds in vivo may be their function as redox buffers, regulating protein thiol–disulfide composition in cellular and extracellular compartments. Most thiols are ready to react with reactive oxygen species that is to neutralize them to relatively less toxic products at the expanse of reducing power of thiol, which itself gets oxidized to a disulfide. The facile oxidation of sulfhydryl compounds results in a variety of disulfides forms in vivo. These include low-molecular-mass symmetrical and unsymmetrical disulfides and disulfides with proteins, in human plasma mainly with albumin. Reduced, free oxidized and protein-bound forms of cysteine, cysteinylglycine, glutathione and homocysteine comprise the plasma redox thiol status [4]. Because of the importance of biological thiols and disulfides and the possible use of plasma levels as biomarkers of health status, there is a need to fully understand the dynamic relationship between all of the thiols and disulfide components in human plasma. The measurement of thiols and disulfides in plasma has proven to be difficult. Most thiols are present in low concentration

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(0.1–10 nmol/ml) and are very unstable in the isolated plasma [5,6].

Because of this problem, few methods for the accurate measurement of thiols and disulfides in plasma have been described. Mansoor et al. [7], in order to prevent the loss of thiols due to oxidation, added *N*-ethylmaleimide or monobromobimane directly to blood collection tubes prior to venipuncture. Thus existing thiols in plasma would be blocked with these reagents. Followed by HPLC analysis with fluorescence detection. Analysis of the plasma from the blood collected in monobromobimane solution yielded the amounts of reduced thiols; analysis the sample from tube with *N*-ethylmaleimide gave the amounts of oxidized forms. In another approach Andersson et al. [8,9] chilled the blood immediately after collection for several minutes. During the cooling period, sulfosalicilic acid solution was added in order to protect the sulfhydryl species against oxidation. Using different procedures for sample preparation it was possible to determine by HPLC reduced, free and protein-bound fractions of main plasma thiols. The HPLC system utilized isocratic reversed-phase separation and postcolumn derivatization with 4,4′-ditiodipyridine and ultraviolet detection.

In this paper, we describe an HPLC method for determination of different fractions of main plasma thiols. The method is based on derivatization of thiols with 2-chloro-1-methylquinolinium tetrafluoroborate to stable 2-*S*-quinolinium derivatives, and separation and quantitation by ion-pairing reversed-phase liquid chromatography. Disulfides are converted to their thiol counterparts by reductive cleavage. In order to avoid the oxidation of thiols ex vivo, the derivatization reagent is added to blood immediately after collection.

2. Experimental

2.1. Chemicals and reagents

L-Cysteine hydrochloride (CSH), DL-cystine (CSSC), glutathione (GSH), and oxidized glutathione (GSSG) were from Reanal (Budapest, Hungary). DL-Homocysteine (HCSH), DL-homocystine (HCSSCH), and cysteinylglycine (CGSH) were received from Sigma (St. Louis, MO, USA). 2-Chloro-1-methylquinolinium tetrafluoroborate (CMQT) was prepared in this laboratory according to the procedure described earlier [10]. Perchloric acid (PCA), ethylenediaminetetraacetic acid disodium salt (EDTA), 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). 3-Mercaptopropionic acid (3MPA), 3,3′-dithiodipropionic acid (3,3′MPA), and 2-mercaptopropionic acid (2MPA) were purchased from Fluka (Buchs, Switzerland). 2,2′-Dithiodipropionic acid (2,2′MPA) was prepared in this laboratory by oxidation of 2MPA. Trichloroacetic acid (TCA) and sodium borohydride ($NaBH_4$) were from Merck (Darmstadt, Germany). All other reagents were HPLC or analytical reagent grade purchased from commercial sources. The pH of the buffers was adjusted by potentiometric titration. The titration system was calibrated with standard pH solutions. All reagents were tested and found to be stable for unattended analysis.

2.2. Apparatus

HPLC analyses were performed with a Hewlett-Packard (Waldbronn, Germany) HP 1100 Series system equipped with quaternary pump, an autosampler, thermostated column compartment, vacuum degasser and diode-array detector and controlled by HP ChemStation software. UV spectra were recorded on a Hewlett-Packard HP 8453 (Waldbronn, Germany) diode array UV-Vis spectrophotometer. Water was purified using a Millipore Milli-QRG system (Vien, Austria). For pH measurement, a Hach One (Loveland, USA) pH meter was used.

2.3. Sample collection and storage

Blood (3 ml) was collected by venipuncture from fasting apparently healthy volunteers of different age into evacuated tubes containing EDTA. For determination of reduced thiols one portion (1 ml) was within few seconds mixed with 100 μl of 0.01 M CMQT in phosphate-buffered isotonic solution (PBS) in order to block labile sulfhydryl groups, followed by plasma preparation (without delay) according to the standard procedure (centrifugation for 10 min, $1000 \times g$, within <20 min after collection). Received plasma was immediately processed according to the procedure described in Section 2.4.1. Remaining blood was centrifuged under standard conditions and plasma was used for determination of total, total free and protein-bound thiols. Plasma can be kept at −20 °C if analysis is not performed the same day.

2.4. Analytical procedures for determination of thiols

2.4.1. Determination of reduced thiols, procedure 1

One hundred and fifty microliters of CMQT treated, according to the procedure described in Section 2.3, plasma was mixed with 10 μl internal standards (50 nmol/ml with respect to both 2MPA and 3MPA) according to the procedure described in Section 2.3. After 1 min 50 μl of 3M PCA were added, the mixture was vortex-mixed and precipitated protein separated by centrifugation for 10 min at $12{,}000 \times g$. The supernatant was transferred to a vial, followed by injection (20 μl) into the chromatographic system.

2.4.2. Determination of total thiols, procedure 2

To 150 μl of plasma 50 μl of 0.1 M EDTA, 10 μl of internal standards solution (0.15 μmol/ml with respect to 2,2′MPA and 1.5 μmol/ml with respect to 3,3′MPA), 50 μl of *n*-octanol, 100 μl of 6 M $NaBH_4$ in 0.1 M NaOH and 50 μl of 3 M HCl were added. The mixture was vortex-mixed

followed by addition, after 1.5 min, of 50 μl 3 M HCl in order to decompose excess of $NaBH_4$. Next, 100 μl of 0.2 M pH 7.6 phosphoric buffer and 20 μl of 0.1 M CMQT were added. The mixture was vortex-mixed, put aside for 1 min and acidified with 100 μl of 3 M PCA followed by centrifugation (12,000 × *g*, 10 min). A 20 μl of solution from above the protein precipitate was transferred into the HPLC system.

2.4.3. Determination of protein-bound thiols, procedure 3

Plasma (150 μl) was mixed with 3M PCA (70 μl) and precipitated protein was separated by centrifugation during 10 min at 10,000 × *g*. The supernatant was decanted and protein was washed with two 50 μl portions of water. Next, the protein pellet was resuspended with 150 μl of water solution containing 15 nmol of 2,2′MPA and 150 nmol of 3,3′MPA, reduced with $NaBH_4$ and analyzed as described in procedure 2.

2.4.4. Determination of total free thiols, procedure 4

The supernatant and washings (acid soluble fraction) from the procedure 3 were collected in a test tube and internal standards (2,2′MPA, 15 nmol and 3,3′MPA, 150 nmol in the volume of 150 μl), 50 μl of *n*-octanol and 100 μl of 6 M $NaBH_4$ were added. Further analysis followed procedure 2.

2.5. Chromatography

Samples (20 μl) were injected using an autosampler into a Zorbax SB-C_{18} (150 mm × 4.6 mm), column (Agilent Technologies, Waldbronn, Germany) packed with 5 μm particles. The temperature was 25 °C, the flow-rate 1 ml/min and the detector wavelength 355 nm. For separation of the 2-*S*-quinolinium derivatives of thiols from each other and from reagent excess gradient elution was used. The elution profile was as follows: 0–4 min 12% B; 4–7 min 12–40% B; 7–8 min 40% B; 8–10 min 40–12% B. Elution solvent (A) was 0.05 M trichloroacetic acid buffer (pH 3.2 prepared from 0.05 M trichloroacetic acid and 0.05 M lithium hydroxide) and (B) acetonitrile. 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 for authentic compounds.

2.6. Calibration

2.6.1. Preparation of calibration standards

Stock solutions of 10 μmol/ml cysteine, cysteinylglycine, glutathione and homocysteine and their symmetrical disulfides needed in the method development procedure were prepared by dissolving appropriate amount of the compound in 2 ml of 0.1 M hydrochloric acid and diluting to the volume of 10 ml. The thiol solutions were standardized by titration with *o*-hydroxymercurybenzoate [11]. 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. For preparation of calibration standards for determination of disulfide forms of CSH, CGSH, GSH, HCSH in human plasma (total, total free and protein-bound), portions of 150 μl of plasma from apparently healthy donors were placed each in a sample tube and spiked with the growing amount of working standard solution of disulfides to provide, assuming 100% of the future reduction of the disulfide bonds, concentration of exogenous CSH of 7, 10, 20, 40, 70, 100, 150, 200, 300, CGSH of 1, 3, 5, 10, 15, 20, 40, 50, GSH of 0.5, 1.0, 3.0, 5.0, 10.0, 15.0, 20.0, 30.0, 40.0, and HCSH of 0.5, 1.0, 3.0, 5.0, 10.0, 20.0, 30.0, 40.0, 50.0 nmol/ml plasma. For reduced thiols, a 15 ml of fresh blood was mixed with 1.5 ml of 0.01 M CMQT in PBS and plasma was prepared under standard conditions. Portions (150 μl) of this CMQT-treated plasma were placed each in a sample tube with internal standards (2MPA and 3MPA) and spiked with growing amounts of 10 μl (50 nmol/ml) working standard solution of appropriate thiol to provide concentration of exogenous cysteine of 0.5, 1.0, 2.0, 4.0, 5.0, 7.0, 10.0 nmol/ml plasma, cysteinylglycine of 0.5, 1.0, 1.5, 2.0, 3.0, 5.0, 7.0, 10.0 nmol/ml plasma, glutathione of 0.3, 0.5, 0.7, 1.0, 1.5, 2.0, 5.0 nmol/ml plasma, and homocysteine of 0.2, 0.3, 0.5, 0.7, 1.0, 1.5 and 2.0 nmol/ml plasma.

2.6.2. Calibrations curves

The calibration standards for total and reduced thiols were processed without delay according to procedures described in Sections 2.4.2 and 2.4.1, respectively. The calibration ranges for cysteine were 7.0–300.0 and 0.5–10.0 nmol/ml plasma, for cysteinylglycine 1–50 and 0.5–10.0 nmol/ml plasma, for glutathione 0.5–40.0 and 0.3–5.0 nmol/ml plasma and homocysteine 0.5–50 and 0.2–2.0 nmol/ml plasma, respectively. The peak height ratios of respective analyte-CMQT derivative to that of internal standard were plotted versus analyte concentration and the curves were fitted by least-square linear regression analysis.

2.7. Search for internal standards

Several thiols and disulfides were added first to water standard solutions of thiol and disulfide analytes and next to plasma, and the resulted mixtures were subjected to analysis according to the procedures 1 and 2 (Sections 2.4.1 and 2.4.2), respectively.

2.8. Decay of thiols in plasma

Known amounts of cysteine, cysteinylglycine, glutathione and homocysteine standards were added to fresh plasma, plasma that had been stored at −20 °C, and to water. At different times thereafter, samples were analyzed as follows. To 1.5 ml of plasma or water 15 μl of 1 μmol/ml of each standard thiol solution was added and the mixture was vortex-mixed. After 1, 5, 10, 15, 20, 25, 30 and 45 min 150 μl portions of the mixture were transferred

into a sample tube containing each 100 μl of 0.2 M pH 7.6 phosphoric buffer, 50 μl of 0.1 M EDTA, and 20 μl of 0.1 M CMQT solution. After 1.5 min reaction mixtures were acidified with 100 μl of 3M PCA, vortex-mixed and centrifuged during 10 min at 10,000 × *g*. A 20 μl of supernatant was chromatographed under conditions described in Section 2.5.

3. Results and discussion

Recently, we have published [12] an HPLC method for determination of different forms of homocysteine existing in human plasma. The method measures reduced homocysteine, total free, protein-bound and total homocysteine, and is based on conversion of the thiol to highly UV-absorbing derivative by reaction with 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT) followed by separation and quantitation by ion-pairing reversed-phase high-performance liquid chromatography. Because of importance of biological thiols and their disulfides as potential indicators of disease risk and health status, there is a need to fully understand the dynamic relationship between all of the thiol and disulfide components in human plasma. To this end, we extended our CMQT derivatization HPLC-UV method for homocysteine to metabolically related plasma thiols including cysteine, cysteinylglycine and glutathione and their disulfides. This method is used based upon its ability to detect and quantitate relevant thiols and disulfides with the use of standard HPLC system equipped with by far the most popular UV-Vis detector.

3.1. Chromatography

In order to establish optimum RP-HPLC conditions for separation of CMQT derivatives of analytes from each other end those of internal standards, prepared according to the procedure described previously [10,12], regent excess and other unidentified matrix components several mobile phase compositions as well as pH, temperature and flow-rates were tested in order to find out their influence on the peak heights, retention factors and resolutions. Preliminary experiments within method development for plasma thiols determination were carried out on standard solution. As can be seen from the chromatogram shown in Fig. 1 the four CMQT derivatives elute by pairs; homocysteine in close proximity to glutathione and cysteinylglycine in close proximity to cysteine. By this reason we have investigated the influence of several parameters, among others mobile phase acetonitrile content, and TCA buffer pH and concentration on resolution, and results are shown in Fig. 2. Increase in TCA buffer concentration caused increase in resolution for both pairs of peaks. Resolution between homocysteine and glutathione decreased when pH was increased approaching zero at pH 2.7. In the same range of pH resolution between cysteine and cysteinylglycine showed opposite

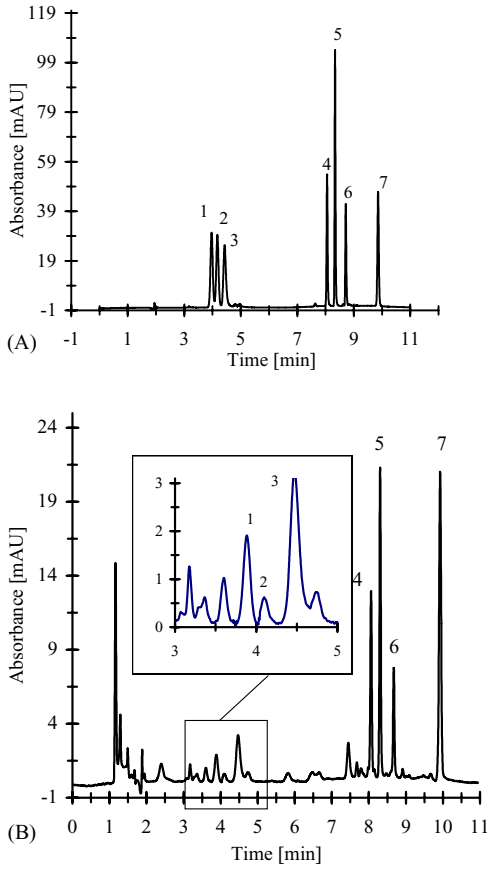

Fig. 1. Typical chromatograms of main plasma thiols. (A) water standard solution, concentration of analytes and internal standards in final analytical solution 20 nmol/ml in respect to each; (B) reduced thiols in plasma. Peaks: 1, GSH 2.51 nmol/ml; 2, HCSH 0.31 nmol/ml; 3, 2MPA 3.33 nmol/ml; 4, 3MPA 3.33 nmol/ml; 5, CSH 12.1 nmol/ml; 6, CGSH 2.36 nmol/ml; 7, CMQT.

behavior. The retention factors for all four analytes showed droping trend with pH increase, and for glutathione derivative it decreased more rapidly than that for homocysteine causing alteration of elution sequence of the two peaks starting from pH 2.7 (data not shown here). This was a result of growing negative charge on glutathione moiety of the GSH-CMQT derivative, resulted from progressive deprotonation of its carboxylic groups when pH increases, which decreased the net positive charge of the derivative as a whole. Smaller positive charge caused weaker, as compared with HCSH-CMQT derivative possessing only one carboxylic group, interaction with the trichloroacetate pairing agent and resulted in poorer retention.

Above briefly mentioned experiments enabled us to establish optimum chromatographic conditions for routine analysis of plasma for cysteine and metabolically related thiols, specified in Section 2.5. Under these conditions, CMQT derivatives of cysteine, cysteinylglycine, glutathione and homocysteine eluted (Fig. 1) after 8.36 (R.S.D., 0.02%), 8.71 (R.S.D., 0.11%), 3.92 (R.S.D., 0.32%) and 4.32 (R.S.D., 0.31%) min, respectively.

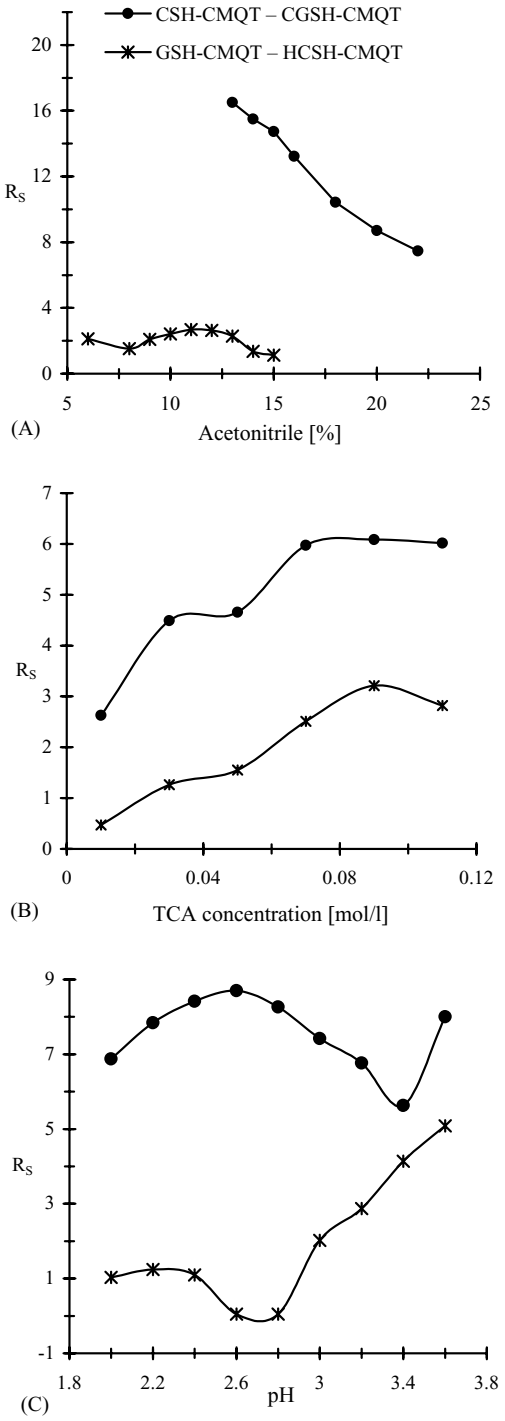

Fig. 2. Resolution of two pairs of peaks of CMQT derivatives: cysteine–cysteinylglycine and glutathione–homocysteine as a function of mobile phase acetonitrile content (A), TCA buffer concentration (B), and mobile phase pH (C).

3.2. Internal standard approach

In order to minimize the contributions of sample preparation, injection variation and column deterioration to the final results, the internal standard mode of quantitation was applied. From amongst the thiol and disulfide

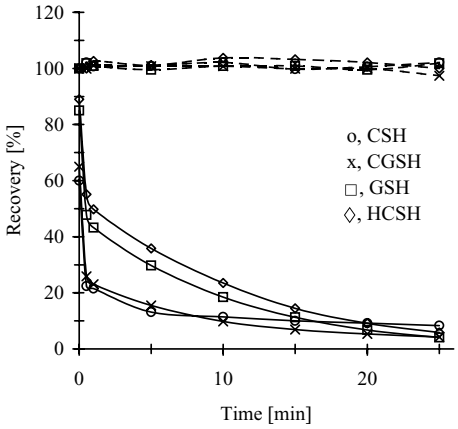

Fig. 3. Loss of thiols added to human plasma (—) and water (- - -). Details are provided in the text.

candidate internal standard tested, 2MPA and 3MPA and their disulfides 2,2′MPA and 3,3′MPA were chosen. 2MPA and 2,2′MPA serve for reduced and oxidized forms of glutathione and homocysteine, and 3MPA and 3,3′MPA for reduced and oxidized forms of cysteine and cysteinylglycine. This distribution fulfills the requirements for a good internal standard; it should possess similar physicochemical properties, go through all steps of the procedure, and elute close to the analyte. *N*-acetylcysteine, *N*-(2-mercaptopropionyl)glycine and penicillamine were not taken into consideration because they can be present in plasma as drugs.

3.3. Loss of thiols in plasma

Rapid losses of reduced thiols were observed by us when added to fresh or stored plasma to provide initial concentration of 10 nmol/ml plasma. After 2 min from addition the recovery for CSH, CGSH, GSH and HCSH was 21, 21, 43 and 50%, respectively. Under the same circumstances, concentration of thiols in water remained constant (Fig. 3). The similar decay of thiols was reported by others [5,6] and this has to be taken under consideration in the case of any attempt to measure reduced plasma thiols.

3.4. Linearity, imprecision, detection and quantitation limits

The linearity between the concentration of thiols and the peak height ratios of respective analyte-CMQT derivative to that of internal standard was determined by analyzing normal plasma spiked with the standard solution of thiols prepared as described in Section 2.6. The linearity was demonstrated using seven- to nine-point calibration curves, and at each concentration four replicates were assayed, independently for reduced and total thiols as described in Sections 2.4.2 and 2.4.1. Detailed data for method calibration as well as imprecision and limits of detection and quantitation for total and reduced thiols are inserted in Table 1.

Table 1
Validation data

Thiol	Retention time		Regression equation	R^2	Linear range (nmol/ml)	R.S.D. (%)[a]		LLQ[b] (nmol/ml)	LLD (nmol/ml)[c]
	(Min)	R.S.D. (%)[a]				Maximum	Minimum		
Reduced thiols									
CSH	8.34	0.14	$y = 0.1885x + 0.4776$	0.9989	0.5–10	5.5	1.9	0.5	0.3
CGSH	8.71	0.14	$y = 0.2038x + 0.1054$	0.9986	0.5–10	5.7	0.2	0.5	0.3
GSH	4.03	0.46	$y = 0.5595x + 0.6988$	0.9972	0.3–5	7.8	2.1	0.3	0.1
HCSH	4.23	0.46	$y = 0.3572x + 0.1116$	0.9985	0.2–2	8.6	1.9	0.2	0.1
Total thiols									
CSH	8.36	0.02	$y = 0.0356x + 6.588$	0.9973	7–300	11.2	2.2	7	2
CGSH	8.71	0.01	$y = 0.0292x + 0.7121$	0.9984	1–50	10.5	4.1	1	0.5
GSH	3.92	0.32	$y = 0.1727x + 0.8803$	0.9963	0.5–40	10.7	3.4	0.5	0.1
HCSH	4.23	0.31	$y = 0.1272x + 1.4398$	0.9963	0.5–50	7.3	2.0	0.5	0.1

[a] Relative standard deviation.
[b] Lower limit of quantitation.
[c] Lower limit of detection.

Table 2
Different forms of thiols (nmol/ml) in plasma from volunteers

Sex	Age (year)	CSH		CGSH		GSH		HCSH	
		Mean (S.D.)	R.S.D. (%)	Mean (S.D.)	R.S.D. (%)	Mean (S.D.)	R.S.D. (%)	Mean (S.D.)	R.S.D. (%)
Total thiols									
Female	31	334.2 (25.9)	7.5	23.1 (1.8)	7.8	12.1 (0.8)	6.6	4.7 (0.4)	9.0
Female	25	194.5 (9.8)	5.0	15.6 (1.5)	10.0	6.1 (0.5)	7.5	10.1 (0.5)	5.1
Female	49	244.5 (19.4)	8.1	17.0 (1.3)	7.6	5.4 (0.5)	9.3	12.1 (0.7)	5.7
Female	51	258.8 (25.8)	10.0	22.4 (2.2)	9.8	12.1 (1.1)	9.1	15.7 (0.5)	3.2
Male	29	210.8 (13.2)	6.3	27.8 (0.9)	3.4	15.3 (1.0)	9.2	12.2 (0.6)	4.8
Male	30	263.8 (16.8)	6.4	27.3 (1.8)	6.5	18.4 (0.8)	10.0	11.7 (0.9)	7.6
Male	24	202.0 (5.6)	2.8	26.7 (2.7)	10.0	8.4 (0.8)	8.8	10.4 (0.8)	7.4
Male	59	494.2 (51.0)	10.3	36.1 (5.2)	14.4	17.1 (1.7)	10.0	13.8 (0.3)	2.2
Reduced thiols									
Female	31	11.7 (0.2)	2.5	2.0 (0.1)	4.5	2.3 (0.04)	3.5	0.29 (0.01)	2.8
Female	25	12.6 (0.2)	1.9	3.7 (0.2)	6.7	3.8 (0.1)	3.6	0.30 (0.02)	6.3
Female	49	12.5 (0.5)	4.2	3.0 (0.1)	2.4	1.4 (0.05)	3.6	0.18 (0.02)	8.3
Female	51	7.1 (0.4)	5.3	2.7 (0.1)	4.1	5.4 (0.2)	4.1	0.36 (0.02)	5.1
Male	29	5.9 (0.2)	3.6	2.6 (0.1)	5.2	3.6 (0.2)	6.1	0.24 (0.02)	7.9
Male	30	7.2 (0.1)	2.0	2.8 (0.1)	5.0	4.3 (0.3)	7.2	0.49 (0.03)	6.7
Male	24	5.6 (0.2)	3.4	4.0 (0.1)	3.2	4.5 (0.2)	5.3	0.27 (0.02)	6.2
Male	59	7.7 (0.4)	5.3	3.0 (0.1)	2.2	6.0 (0.4)	7.3	0.13 (0.01)	7.5
Total free thiols									
Female	31	100.2 (6.9)	6.9	10.3 (0.3)	3.3	6.6 (0.2)	3.0	1.7 (0.1)	5.9
Female	25	35.1 (1.5)	4.2	5.1 (0.5)	10.5	4.3 (0.3)	7.0	1.3 (0.1)	7.5
Female	49	52.2 (4.3)	8.2	5.7 (0.2)	4.2	3.3 (0.1)	4.2	5.3 (0.4)	7.5
Female	51	35.3 (0.8)	2.2	6.1 (0.2)	3.0	5.8 (0.3)	6.8	3.4 (0.2)	5.8
Male	29	31.2 (2.9)	9.2	5.2 (0.5)	9.9	4.9 (0.2)	4.3	1.0 (0.1)	9.6
Male	30	36.4 (1.1)	3.1	8.0 (0.2)	3.0	4.5 (0.2)	6.0	2.1 (0.2)	9.1
Male	24	41.9 (1.6)	3.8	11.5 (0.6)	5.2	6.4 (0.5)	7.2	1.3 (0.1)	10.6
Male	59	43.6 (1.8)	4.1	6.8 (0.4)	5.5	6.4 (0.1)	2.2	5.1 (0.4)	7.6
Protein-bound thiols									
Female	31	234.5 (9.4)	4.1	12.8 (0.8)	6.3	5.3 (0.2)	3.9	3.1 (0.07)	2.2
Female	25	160.1 (10.1)	6.4	10.4 (0.4)	4.0	1.9 (0.1)	5.1	8.4 (0.3)	3.6
Female	49	193.3 (12.8)	6.7	11.3 (0.6)	5.0	2.2 (0.07)	3.4	6.9 (0.3)	3.7
Female	51	222.3 (18.7)	8.4	16.9 (1.1)	6.7	6.9 (0.5)	7.8	12.2 (0.8)	6.8
Male	29	177.9 (5.7)	3.3	22.8 (9.3)	4.1	10.7 (1.0)	9.1	11.0 (0.8)	7.5
Male	30	227.4 (15.2)	6.7	19.1 (1.5)	8.1	13.9 (1.4)	10.4	9.4 (0.6)	5.9
Male	24	161.5 (4.6)	2.8	15.1 (0.6)	3.9	1.7 (0.07)	4.1	9.5 (0.3)	3.4
Male	59	454.5 (24.1)	5.3	30.0 (2.9)	9.7	11.0 (0.7)	6.7	8.9 (0.6)	6.2

3.5. Application of the method to authentic plasma samples

The optimized CMQT derivatization HPLC-UV method for determination of different species of thiols was applied to authentic plasma samples. The blood samples prepared according to the procedure outlined in Section 2.3 were donated by eight apparently healthy volunteers (four men and four women, 19–55 years old). Results of analysis for total, reduced, total free and protein-bound cysteine, cysteinylglycine, glutathione and homocysteine are inserted in Table 2.

4. Conclusion

Our CMQT derivatization HPLC-UV method for determination of different forms of main plasma thiols encompasses four procedures which enable assessment of concentration of (1) reduced thiols, (2) total free thiols, (3) protein-bound thiols, and (4) total thiols. The free oxidized thiols can be calculated by subtraction of reduced thiols from the content of total free thiols. Analytical figures of merit demonstrated during the method validation protocol compare well with those of known methods [7–9] for determination of different species of thiols in plasma. We believe that this method fulfills experimental and clinical requirements for determining plasma thiols and can be applied to routine assessment of plasma thiol redox status in physiological and pathological conditions. Practicality and low cost of single analysis are central features of routine laboratory tests, and the herein described CMQT derivatization HPLC-UV method, because it uses cheap reagents and is easily affordable by clinical laboratories equipped with standard HPLC-UV systems can contribute to answer the very important question of whether is a causative relationship between oxidative stress and premature development of certain diseases including atherosclerosis.

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References

[1] C.K. Sen, L. Packer, Am. J. Clin. Nutr. 72 (2000) 653S.
[2] D.W. Jacobsen, Clin. Chem. 44 (1998) 1833.
[3] H. Mudd, H.L. Levy, F. Skorby, in: C.R. Seriver, A.L. Beandet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Basis of Inherited Disease, vol. 1, seventh ed., McGraw-Hill, New York, 1995, p. 1279.
[4] P.M. Ueland, M.A. Mansoor, A.B. Guttormsen, F. Muller, P. Aukrust, H. Refsum, A.M. Svardal, J. Nutr. 126 (1996) 1284S.
[5] A. Andersson, A. Lingren, B. Hultberg, Clin. Chem. 41 (1995) 361.
[6] W.A. Kleinman, J.P. Richie Jr., Biochem. Pharmacol. 60 (2000) 19.
[7] M.A. Mansoor, A.M. Svardal, P.M. Ueland, Anal. Biochem. 200 (1992) 218.
[8] A. Andersson, A. Isaksson, L. Brattstrom, B. Hultberg, Clin. Chem. 39 (1993) 1590.
[9] A. Andersson, A. Lingren, M. Arnadottir, H. Prytz, B. Hultberg, Clin. Chem. 45 (1999) 1084.
[10] E. Bald, R. Głowacki, J. Liq. Chromatogr. 24 (2001) 1323.
[11] M. Wroński, Talanta 24 (1977) 347.
[12] G. Chwatko, E. Bald, J. Chromatogr. A 949 (2002) 141.