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Determination of different species of homocysteine in human plasma by high-performance liquid chromatography with ultraviolet detection

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Abstract

This assay measures reduced, free oxidized, protein-bound, and total homocysteine in human plasma. Oxidized species of homocysteine are converted to reduced form by sodium borohydride, and, after precolumn derivatization with 2-chloro-1-methylquinolinium tetrafluoroborate, homocysteine 2-*S*-quinolinium derivative is separated from those of other plasma thiol derivatives, and quantitated by ion-paired reversed-phase high-performance liquid chromatography with ultraviolet detection. The reduced homocysteine sulfhydryl groups are trapped with minimal oxidation by derivatizing blood samples at the time of collection. With the use of this precise and sensitive HPLC method utilizing popular ultraviolet detection, homocysteine in plasma can be detected and quantitated at the level of 0.1 and 0.2 for reduced fraction, and 0.3 and 0.5 nmol/ml for total homocysteine, respectively. The method is applied for determination of different fractions of homocysteine in plasma of apparently healthy men and women. © 2002 Published by Elsevier Science B.V.

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1. Introduction

Homocysteine is situated at a critical regulatory branch point in sulfur metabolism. This thiol containing amino acid is produced by cellular demethylation of dietary methionine. It can be remethylated to methionine, an important amino acid in protein synthesis, or converted to cysteine in the transsulfuration pathway. The release of homocysteine into the extracellular medium represents the third route of cellular homocysteine disposal and is particularly important. Homozygous homocystinuria, a rare genetic disorder usually caused by cystathionine β-synthase deficiency, leads to severe increase of plasma and urine homocysteine to concentrations higher than 100 μmol/l [1] and is associated with venous thrombosis and premature atherosclerosis. Values of total homocysteine, understood as the sum of reduced, free oxidized (homocystine and homocysteine mixed disulphides) and protein-bound homocysteine, between 5 and 15 μmol/l plasma in fasting subjects are considered normal. The two major acquired causes of increased homocysteine levels are chronic renal failure and deficiencies of folate, vitamin B_{12} or vitamin B_6, three vitamins involved in the normal metabolism of

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methionine [2]. Mildly elevated plasma homocysteine levels (16–20 μmol/l) are associated with an increased risk factor for coronary heart disease, peripheral vascular disease, and acute coronary and cerebrovascular events [3–6] in many, but not all [7] prospective and retrospective studies. Some of these inconsistencies may be attributed to differences in analytical methodologies. A variety of methods have been utilized to measure the fasting or post methionine-loading concentrations of some or all potentially atherogenic homocysteine fractions. To date, three separation techniques, gas chromatography–mass spectrometry (GC–MS) [8,9], high-performance liquid chromatography (HPLC) [10–21] and high-performance capillary electrophoresis (HPCE) [22,23], and two immunological methods, the fluorescence polarization immunoassay (FPIA) [24] and the enzyme immunoassay (EIA) [25], were exploited for the determination of total homocysteine in plasma. However, there is a recognized need to measure different species of homocysteine in order to fully understand the dynamic relationship between homocysteine and other biologically important thiols and disulphides in plasma [26,27]. Alteration in the redox status of homocysteine rapidly affects and is related to the total thiol redox status in plasma. This observations may be explained by continuous redox and disulphide exchange reactions in plasma. The comprehensive measurement of homocysteine and its disulphides in human plasma has proven to be problematic because of low concentration and susceptibility to oxidation. The concentration of reduced homocysteine in plasma ex vivo decreases rapidly.

The purpose of this study was to develop an HPLC assay method for the rapid and specific determination of different species of homocysteine in human plasma. The method relies on transformation of homocysteine, in the reaction with ultraviolet thiol specific tagging reagent, to stable derivative, and separation and quantitation by ion-pairing reversed-phase HPLC. Oxidized species are converted by reduction to the thiol form before the derivatization step. In order to circumvent the loss of homocysteine due to oxidation during sample processing, the derivatization reagent, 2-chloro-1-methylquinolinium tetrafluoroborate, is added to whole blood immediately after collection and prior to separation from erythrocytes.

2. Experimental

2.1. Instrumentation

HPLC analyses were performed with a Hewlett-Packard (HP) 1100 Series system equipped with quaternary pump, an autosampler, thermostated column compartment, vacuum degasser and diode-array detector. For instrument control, data acquisition and data analysis, an HP ChemStation for LC 3D system including single instrument HP ChemStation software and Vectra color computer, was used. Water was purified using a Millipore Milli-QRG system.

2.2. Chemicals

2-Chloro-1-methylquinolinium tetrafluoroborate (CMQT) was prepared in this laboratory according to the procedure described earlier [28]. For thiols derivatization prior to HPLC analysis, a 0.01 or 0.1 *M* water solution was used. Ethylenediaminetetraacetic acid disodium salt (EDTA), tris(hydroxymethyl)aminomethane (Tris), perchloric acid (PCA), and HPLC-grade acetonitrile and methanol were from J.T. Baker (Deventer, Netherlands). Trichloroacetic acid (TCA) 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. L-Cysteine hydrochloride (CSH), DL-cystine (CSSC) and glutathione (GSH) were received from Reanal (Budapest, Hungary). DL-Homocysteine (HCSH) and cysteinylglycine (CGSH) were from Sigma (St. Louis, MO, USA), homocystine (HCSSCH) from Serva (Heidelberg, Germany), and sodium borohydride ($NaBH_4$) from Merck (Darmstadt, Germany). All other reagents were HPLC or analytical reagent grade purchased from commercial sources. Purified water from Millipore Milli-QRG system (Vienna, Austria) was used throughout the experiments. pH of the buffers used 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.

2.3. Blood collection

Specimens were taken from fasting apparently

healthy volunteers of different ages. Blood (3 ml) was drawn, with a tourniquet applied, into an EDTA tube. One portion (1 ml) was within a few seconds mixed with 40 μl of 50 nmol/ml 2MPA (internal standard) and 100 μl of 0.01 *M* CMQT in phosphate-buffered isotonic solution (PBS), in order to block labile homocysteine sulfhydryl groups, followed by plasma preparation (without delay) according to standard procedure. Remaining blood was centrifuged under standard conditions (10 min, 1000 *g*, within <20 min after collection) and plasma was used for determination of total, total free and protein-bound homocysteine. Plasma obtained from CMQT-treated blood as well as normal plasma can be kept at −20°C if analysis is not performed the same day.

2.4. Analytical procedures for determination of homocysteine

2.4.1. Determination of total homocysteine

To 150 μl of plasma, 50 μl of 0.1 *M* EDTA, 10 μl of 0.15 μmol/ml 2,2′MPA (internal standard), 50 μl of *n*-octanol, 100 μl of 6 *M* $NaBH_4$ in 0.1 *M* NaOH and 50 μl of 3 *M* HCl was 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 1 *M* pH 7.6 Tris buffer and 30 μl of 0.1 *M* CMQT was 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 aliquot of solution from above the protein precipitate was transferred into HPLC system.

2.4.2. Determination of protein-bound homocysteine

Plasma (150 μl) was mixed with 3 *M* 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 containing 15 nmol of 2,2′MPA, reduced with $NaBH_4$ and further analysed as described in Section 2.4.1.

2.4.3. Determination of total free homocysteine

The supernatant and washings (acid soluble fraction) from the procedure described in Section 2.4.2 were collected in a test tube and internal standard (2,2′MPA, 15 nmol), 50 μl of *n*-octanol and 100 μl of 6 *M* $NaBH_4$ were added. Further analysis followed the procedure described in Section 2.4.1.

2.4.4. Determination of reduced homocysteine

To 150 μl plasma CMQT-treated according to the procedure described in Section 2.3, 50 μl of 3 *M* PCA was added, the mixture was vortex-mixed and precipitated protein separated by centrifugation for 10 min at 12 000 *g*. The supernatant was transferred to a vial, followed by injection (20 μl) into the chromatographic system.

2.5. Chromatographic conditions

Plasma final analytical solutions (supernatants, 20 μl) were injected using an autosampler into a Hewlett-Packard SB-C_{18} (150×4.6 mm) column packed with 5-μm particles. Separation of homocysteine–CMQT derivative was isocratic using a mobile phase (flow-rate, 1 ml/min; temperature, 25°C) consisting of 0.05 *M* TCA buffer, adjusted to pH 3.2 with lithium hydroxide solution of the same concentration, and acetonitrile in the ratio of 88:12 (v/v). In order to remove late-eluting UV-absorbing sample components, e.g. cysteine and cysteinylglycine–CMQT derivatives as well as CMQT excess, acetonitrile content was increased to 40% in 3 min started at 5 min followed by decrease to 12% during the following 3 min. 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 authentic compounds.

2.6. Calibration

2.6.1. Preparation of calibration standards

Stock solutions of 10 μmol/ml homocysteine, homocystine and related thiols and disulphides needed in the method development procedure were prepared by dissolving an 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 [29]. 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 disulphide forms of homocysteine in human plasma (total free and protein-bound), portions of 150 μl of plasma from apparently healthy donors were each placed in a sample tube and spiked with increasing amounts of working standard solution of homocystine to provide, assuming 100% of the future reduction of the disulphide bonds, concentration of exogenous homocysteine of 0.5, 1.0, 3.0, 5.0, 10.0, 20.0, 30.0, 40.0 and 50.0 nmol/ml plasma. For reduced homocysteine 15 ml of fresh blood was mixed with 50 μl of 0.6 μmol/ml 2MPA (internal standard) and 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 and spiked with increasing amounts of working standard solution of homocysteine to provide concentrations of exogenous homocysteine of 0.20, 0.30, 0.50, 0.70, 1.00 and 2.00 nmol/ml plasma.

2.6.2. Calibrations curves

The calibration standards for total and reduced homocysteine were processed according to procedures described in Sections 2.4.1 and 2.4.4, respectively. The calibration ranges of homocysteine were from 0.5 to 50 and from 0.2 to 2 nmol/ml plasma, respectively. The peak height ratios of homocysteine–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 standard

Several thiols and their disulphides were tested as candidate internal standards for the analytical procedures leading to determination of reduced and oxidized forms of homocysteine, respectively. Each of these compounds was added to homocysteine or homocystine water standard solution to achieve a final concentration of 10 nmol/ml and the resultant mixture was subjected to all steps of the appropriate analytical procedure. The performance of the chosen compound was checked with human plasma as well.

2.8. Loss of homocysteine in plasma

Known amounts of homocysteine standard were added to fresh plasma, plasma that had been stored at −20°C, and to water. At different times thereafter, samples were analyzed by the CMQT-HPLC method. To 1.5 ml of plasma or water, 15 μl of 1-μmol/ml standard homocysteine 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 each containing 100 μl of 1 *M* pH 7.6. Tris 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 3 *M* PCA, vortex-mixed and centrifuged during 10 min at 10 000 *g*. A 20-μl aliquot of supernatant was chromatographed under conditions described in Section 2.5.

3. Results and discussion

Recently we have described [28] a sensitive and specific liquid chromatographic method for the determination of hydrophilic thiols. The method is based on conversion of the thiols to highly UV-absorbing derivatives by reaction with 2-chloro-1-methylquinolinium tetrafluoroborate and separation and quantitation by ion-paired reversed-phase high-performance liquid chromatography. This analytical scheme has been applied to biological samples, and procedures for determination of mesna [30] and cysteine [31] in plasma and urine, respectively, have been reported. The objective of this work was to extend the above derivatization scheme for determination of different forms of homocysteine in human plasma. This was accomplished by elaboration of optimal conditions for each step of the analytical procedure.

3.1. Derivatization of homocysteine

Homocysteine reacts rapidly with 2-chloro-1-methylquinolinium tetrafluoroborate (Fig. 1A) according to the nucleophilic displacement scheme of chlorine atom by sulfur from homocysteine sulfhydryl group leading to formation of stable thioether linkage, 2-*S*-quinolinium derivative. This derivative

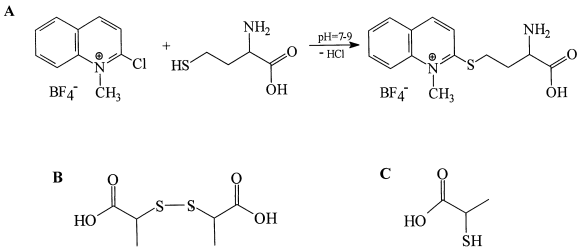

Fig. 1. Chemical derivatization reaction equation of homocysteine with 2-chloro-1-methylquinolinium tetrafluoroborate (A) and chemical structure of the internal standards for determination of oxidized forms of homocysteine (B) (2,2′-dithiodipropionic acid) and reduced homocysteine (C) (2-mercaptopropionic acid).

possesses a well defined absorption maximum in the upper ultraviolet region (350 nm) with a high molar absorptivity coefficient. The reaction occurs in a fully water environment and under mild conditions. Bathochromic shift accompanying the reaction, shown in Fig. 2, from the reagent maximum at 328 nm to that of the derivative at 350 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 authentic sample) and avoid a huge peak of unreacted derivatization reagent on the chromatogram.

The pH effect on the derivatization yield was studied in a range between 7 and 9. When pH was

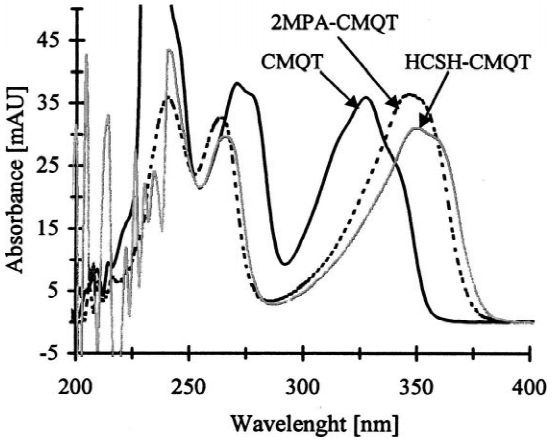

Fig. 2. Comparison of the absorption spectra of derivatization reagent (CMQT) with homocysteine (HCSH–CMQT) and internal standard (2MPA–CMQT) derivatives.

increased above 8.2, yield of homocysteine–CMQT derivatives started to decrease (data not shown), most likely because of hydrolysis of the thioether bond. For routine derivatization pH 7.6, 7-fold CMQT excess and room temperature were chosen as optimum conditions for both homocysteine and internal standard (2MPA).

3.2. Cleavage of the disulphide bonds

The bulk of plasma homocysteine occurs in the disulphide form rendering it inaccessible to derivatization reagent, and in order to determine its total content disulphide bonds must be cleaved with a reducing agent to liberate a free thiol. For this purpose sodium borohydride ($NaBH_4$) was used. We examined the time and temperature–response curves for $NaBH_4$ solution added in excess in order to determine conditions required to fully reduce disulphide bonds of homocysteine as well as of 2,2′-dithiodipropionic acid (internal standard, Fig. 1B). The results indicated that maximum reduction was reached (data not shown here) after 1.5 min of incubation at room temperature. These condition of reduction were adopted for analysis of plasma for total and total free and protein-bound homocysteine.

3.3. Optimum chromatographic conditions

Preliminary experiments within method development for plasma homocysteine determination were carried out on standard water solutions containing homocysteine and three other plasma thiol pool components, cysteine, cysteinylglycine and glutathione. Cysteine and cysteinylglycine–CMQT derivatives show much longer retention times than that of homocysteine (CSH–CMQT, 8.2 and CGSH–CMQT, 8.6 min; peaks not shown), but glutathione derivative elutes in close proximity (Fig. 6D), and in order to avoid coelution we had to know their retention behavior under various conditions. Accordingly we have investigated the influence of TCA buffer concentration, acetonitrile content, pH, temperature and flow-rate of the mobile phase used on the retention factors and resolution. As can be seen from Fig. 3, variations of the retention factors of GSH–CMQT and HCSH–CMQT derivatives are similar with increase of acetonitrile content and TCA

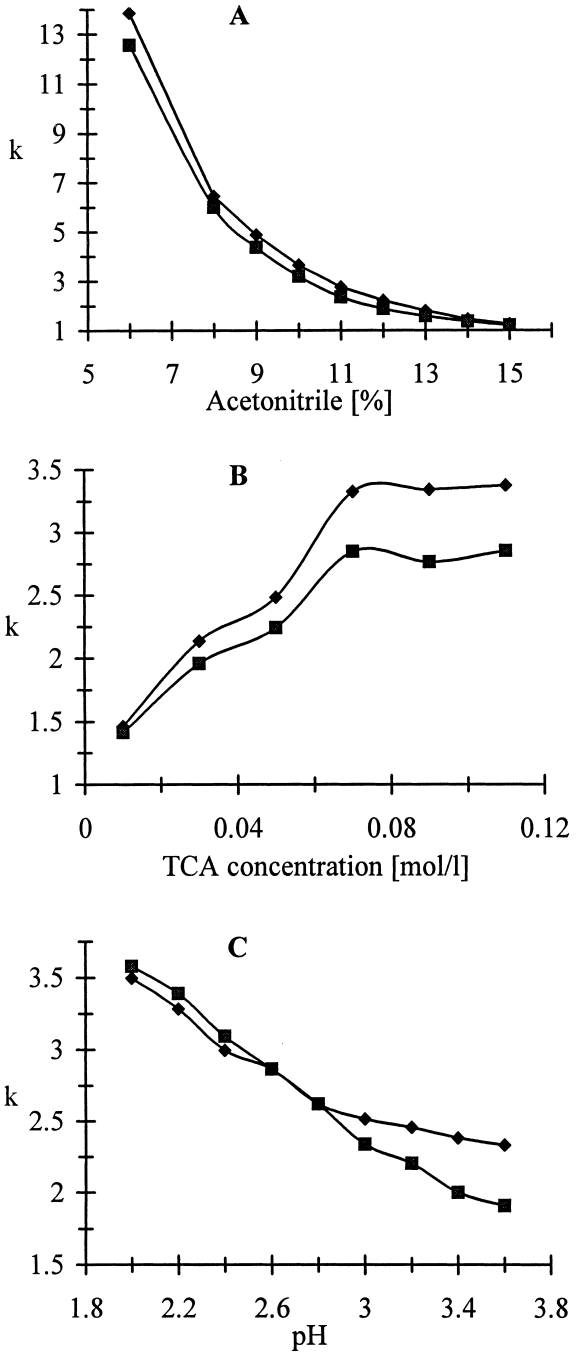

Fig. 3. Effect of mobile phase acetonitrile content (A), TCA, buffer concentration (B), and pH (C) on the retention factors of the CMQT derivatives of glutathione and homocysteine. Symbols: ♦, homocysteine derivative; ■, glutathione derivative.

buffer concentration within the ranges investigated. The retention factor of GSH–CMQT derivative decreased more rapidly than that of HCSH–CMQT when pH was increased causing alteration of elution sequence of the two peaks starting from pH between 2.6 and 2.8. Growing negative charge on glutathione moiety of the derivative, resulted from progressive deprotonation of its carboxylic groups when pH increases, decreased the net positive charge of the GSH–CMQT derivative as a whole. Smaller positive charge caused weaker, as compared with HCSH–CMQT possessing only one carboxylic group, interaction with the trichloroacetate pairing agent and resulted in poorer retention. Resolution between these two peaks decreased when pH was increased, approaching zero at pH 2.7, and started to grow with further pH increase (Fig. 4). Increase in TCA buffer concentration caused increase in resolution within the concentration range of 0.01–0.09 mol/l. Temperature and flow-rate of the mobile phase had minor influence on retention parameters (data not shown).

Based on the above, briefly mentioned, experimental results we have chosen optimum chromatographic conditions, specified in Section 2.5, under which homocysteine–CMQT derivative eluted after 4.28±0.02 min (RSD, 0.47%, $n=10$). Typical chromatograms for various forms of homocysteine in plasma donated by apparently healthy volunteers are shown in Fig. 6. The peak of homocysteine–CMQT derivative is well separated from internal standard derivative (4.51±0.03 min, RSD, 0.76%), derivatiza-

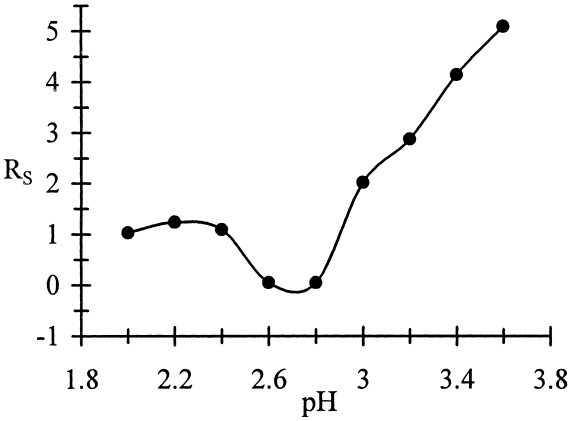

Fig. 4. Resolution between closely eluted CMQT derivatives of glutathione and homocysteine as a function of mobile phase pH.

tion reagent excess and other endogenous plasma thiols, known to react with CMQT [28,31] including closely eluting glutathione–CMQT derivative. Commonly used thiol drugs such as mesna or captopril do not disturb analysis (data not shown).

3.4. Poor stability of homocysteine in plasma ex vivo

We observed very low levels of reduced homocysteine in fresh human plasma and somewhat higher in plasma that had been stored at −20°C. These low levels appear to be due to the poor stability of homocysteine in plasma resulted from great susceptibility to the oxidative reactions. Indeed, rapid losses for reduced homocysteine were observed by us when added to fresh or stored plasma to provide initial concentration of 10 nmol/ml plasma. A decrease of 65% was observed within 5 min, and 100 and 90% in fresh and plasma stored at −20°C, respectively, after 45 min of incubation at room temperature (Fig. 5). Within the same time, and under the same conditions, concentration of homocysteine in water remained constant. While similar problems regarding instability have been revealed previously for thiols in plasma [26,32], some authors continue to report data

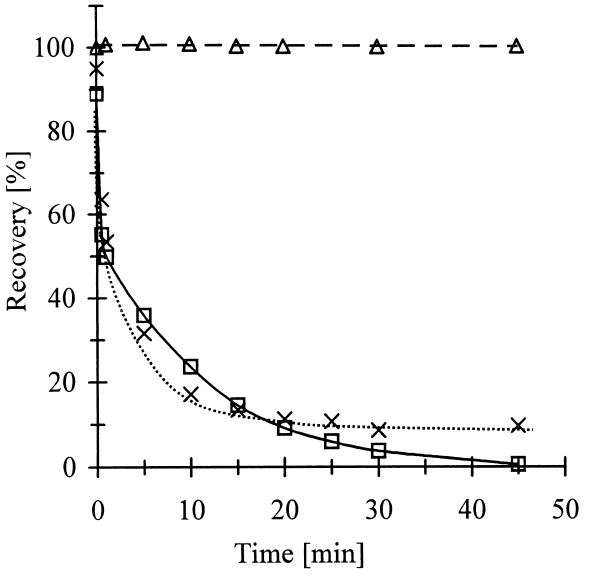

Fig. 5. Decay of homocysteine added to plasma and water. Symbols: △, water; □, fresh plasma; ×, stored plasma. Experimental conditions described in Sections 2.4.4 and 2.8.

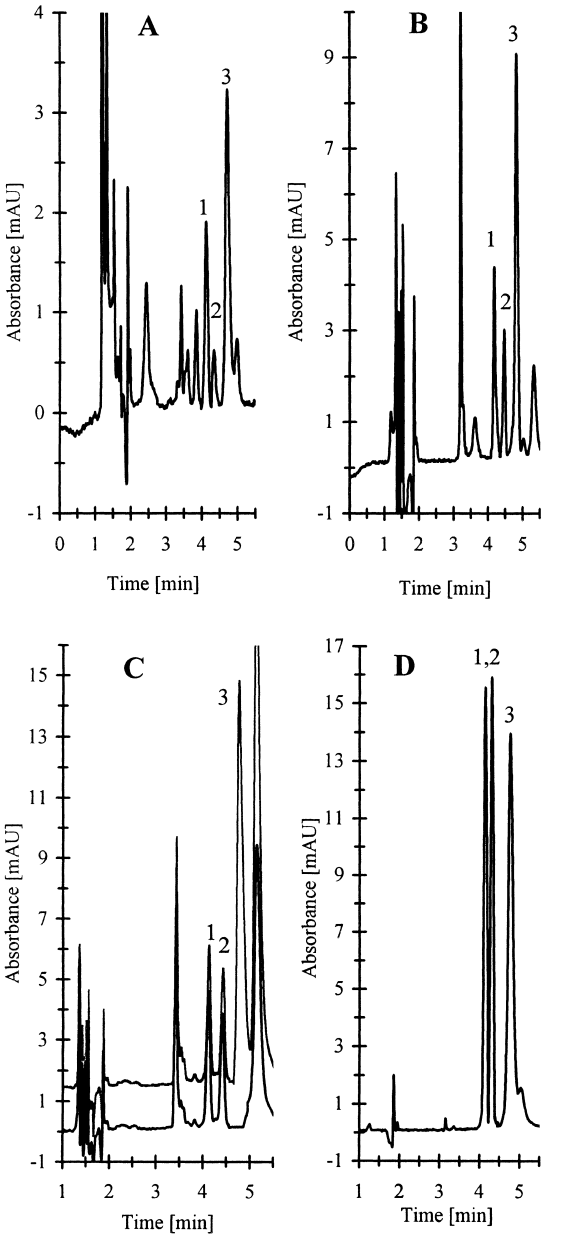

Fig. 6. Typical HPLC chromatograms of plasma samples and water standard solution: (A) reduced homocysteine (concentration 0.49 nmol/ml plasma, 7 pmol on column); (B) protein-bound homocysteine (concentration 8.7 nmol/ml plasma, 42.4 pmol on column); (C) total homocysteine (concentration 12.1 nmol/ml plasma, 43 pmol on column); thick line, plasma blank; thin line, internal standard added to plasma; (D) standard water solution of GSH, HCSH and 2MPA (concentration 10 nmol/ml of each, 200 pmol on column). Peaks: 1, GSH–CMQT; 2, HCSH–CMQT; 3, 2MPA–CMQT.

on thiol content without taking this lack of stability into consideration.

3.5. Validation

3.5.1. Linearity

The relationship between detector response and homocysteine concentration was continuous and repeatable and was demonstrated using a mine-point calibration curve. At each concentration, four replicates were assayed as described in Section 2.4. The calibration curves were linear in the range from 0.2 to 2, and from 0.5 to 50 nmol/ml plasma, for reduced and oxidized forms of homocysteine, respectively. These calibration ranges can be easily extended up if required. The equations for the linear regression lines and coefficients of correlation were $y = 0.1272x + 1.4398$ and $r^2 = 0.9963$ for oxidized forms, and $y = 0.3572x + 0.11$ and $r^2 = 0.9985$ for reduced homocysteine, respectively.

3.5.2. Dilution parallelism

A plasma sample with elevated level of total homocysteine was diluted with phosphate buffered isotonic solution from 0 to 32-fold and the resulting solutions were analyzed according to procedure for total homocysteine described in Section 2.4.1. The results shown in Table 1 show good linearity of the assays.

3.5.3. Recovery and imprecision

The recoveries were measured by the addition of the homocysteine or homocystine standards to plasma samples of known concentration of endogenous reduced or total homocysteine concentration from an equivalent plasma matrix. After addition of appropriate internal standard solution, the samples were processed according to the procedures for determination of reduced (Section 2.4.4) and total homocysteine (Section 2.4.1). Three concentration of exogenous homocysteine were studied: one near the lower limit of quantitation, one near the center, and one near the upper boundary of the standard curve. Recoveries and within and between analysis imprecision, expressed as relative standard deviation values, were calculated and are shown in Table 2.

Table 2
Recoveries, within-analysis and between-analyses imprecision of homocysteine added to plasma

Homocysteine added (nmol/ml)	Recovery (%)	RSD (%, $n=4$)	
		Within analysis	Between analyses
Total			
0.5	99.6	4.9	5.2
20.0	96.1	5.0	4.8
50.0	102.8	3.1	3.0
Reduced			
0.2	105.3	4.5	4.7
1.0	100.2	3.2	3.1
2.0	99.4	1.8	2.2

3.5.4. Internal standard

In order to minimize the contributions of sample preparation, injection variations and column deterio-

Table 1
Dilution parallelism

Plasma:PBS ratio (v/v)	Total homocysteine (nmol/ml)			
	Observed ($n=3$)		Calculated	$\frac{\text{Observed}}{\text{Calculated}} \cdot 100$
	Mean (SD)	RSD (%)		
1:0	19.6 (0.94)	4.8	19.6	100.0
1:1	10.1 (0.67)	6.7	9.81	103.1
1:2	6.59 (0.26)	3.9	6.54	100.8
1:4	3.76 (0.08)	2.1	3.92	95.7
1:8	2.23 (0.08)	3.5	2.18	102.5
1:16	1.11 (0.05)	4.3	1.15	96.4
1:32	0.61 (0.02)	3.7	0.59	102.0
			Mean (SD)	100.1 (2.9)

ration to the final results, the internal standard approach was used. 2-Mercaptopropionic acid, 3-mercaptopropionic acid, thiomalic acid and thioglycolic acid were tested as candidate internal standard. *S*-Quinolinium derivatives of all of these compounds, with the exception of that of 2-mercaptopropionic acid, eluted too far from homocysteine derivative or coeluted with matrix components. Therefore, 2-mercaptopropionic acid and its oxidized form (2,2′MPA) were used as internal standards for determination of reduced and oxidized form of homocysteine, respectively.

3.5.5. Detection and quantitation limits

The lower limits of detection and quantitation for reduced homocysteine were estimated by analysis of plasma samples containing derivatization reagent (Section 2.4.4) spiked with decreasing amounts of homocysteine. They were established to be 0.1 and 0.2 nmol/ml plasma. At these concentrations the signal-to-noise ratios were 3 and 6, respectively. The corresponding set of data for total homocysteine acquired by progressive dilution of plasma with buffered isotonic solution, followed by analysis as for total homocysteine (Section 2.4.1), was 0.3 and 0.5 nmol/ml plasma.

3.5.6. Application to authentic plasma samples

The optimized CMQT-HPLC–UV procedure for determination of different forms of homocysteine was applied to the analysis of plasma samples of apparently healthy volunteers, 19–65 years old (nine men and six women). Table 3 summarizes the results of plasma analysis for particular forms of homocysteine. This distribution clearly demonstrates that the predominating form of homocysteine in both men and women is protein bound, followed by free oxidized form. The level of reduced form is very low. Reduced fraction shows about the same level ($P > 0.05$) in men and in women (0.29 vs. 0.33 nmol/ml), but free oxidized and protein-bound homocysteine levels are significantly higher in men than in women. Consequently, total homocysteine in men is higher than in women (11.96 ± 2.46 and 8.88 ± 2.77 (mean$\pm$SD) nmol/ml, respectively, $P < 0.05$). Similar results (11.85 ± 1.51) for men, but higher (10.91 ± 2.05) for women obtained by HPLC with bimane precolumn derivatization and fluorescent detection were reported earlier [33]. Our results are consistent with a mean value of 10.2 ± 1.9 nmol/ml for total homocysteine (eight men and six women, aged 31–65 years) obtained by HPLC method with postcolumn derivatization with 4,4′-dithiodipyridine and ultraviolet detection [34]. Mean values for total homocysteine in plasma obtained by HPLC method with electrochemical detection [32] (8.30 ± 2.29 for men and 6.59 ± 1.90 for women) and our results obtained by HPLC and UV detection after precolumn derivatization with 2-chloro-1-methylpyridinium iodide [35] (7.42 ± 0.37 for men and 5.39 ± 0.24 for women) are lower than with present method.

The mean value for reduced homocysteine in plasma of apparently healthy volunteers (regardless of sex) in our experiment (0.31 nmol/ml) was higher than previously reported (0.24 nmol/ml) by Mansoor et al. [33], with homocysteine trapped by bimane at the time of blood collection. Hultberg et al. [34] and Andersson et al. [35] reported 0.18 nmol/ml without binding the homocysteine sulfhydryl group at the time of blood collection. This result is consistent with ours obtained without adding the CMQT reagent at the time of blood collection.

The procedure described herein represents a significant advance in the methodology for the determination of homocysteine in plasma over our previously reported method [21]. First of all, thanks to a new derivatization reagent, 2-chloro-1-methylquinolinium tetrafluoroborate, enhanced sensitivity enables reliable determination of the reduced and total free form of homocysteine in addition to protein bound and total content. The improvements also

Table 3
Different forms of homocysteine (nmol/ml) in plasma donated by nine men and six women

Form	Mean (SD)	Range	% of Total (SD)
Men			
Reduced	0.29 (0.10)	0.13–0.49	2.4 (1.1)
Free oxidized[a]	3.08 (1.81)	0.73–5.55	25.8 (12.2)
Protein-bound	8.58 (1.91)	5.18–11.2	71.8 (11.9)
Women			
Reduced	0.33 (0.09)	0.18–0.44	3.7 (2.0)
Free oxidized[a]	2.14 (1.68)	1.03–5.11	24.1 (13.1)
Protein-bound	6.41 (2.22)	3.04–8.74	72.2 (12.6)

[a] Calculated by subtraction of reduced from total free homocysteine (acid soluble fraction).

encompass much better resolution, lower quantitation limits (0.2 or 0.5 vs. 2.5 nmol/ml plasma), better recovery and much shorter analysis time. Besides, the use of CMQT has several advantages: (i) very short derivatization time (1 min) in water under mild conditions and consequent amenability to automation, (ii) good solubility and stability of the reagent and its thiol derivative in water, (iii) high optical yield of the homocysteine derivative (ϵ over 20 000 l mol^{-1} cm^{-1}) at absorption maximum falling in relatively clean UV region, (iv) very good compatibility with reversed-phase HPLC conditions, and (v) easy and cheap synthesis of the reagent.

In terms of sensitivity, simplicity and other analytical parameters, the CMQT-HPLC–UV method can compete with widely recognized HPLC methods using fluorimetric and electrochemical detection [10–19,32] known for their inherent sensitivity as well as for some drawbacks associated with their application to analysis of biological samples. Practicality and low cost of single analysis are central features of routine laboratory tests, and the herein described CMQT-HPLC–UV method, because it is easily affordable by clinical laboratories with HPLC–UV systems, can contribute to answer the very important question of whether there is a causative relationship between slightly elevated homocysteine levels in human blood and premature development of atherosclerosis.

4. Conclusion

The present CMQT-HPLC–UV method includes four procedures elaborated for the determination of (i) free reduced homocysteine, (ii) total free homocysteine, (iii) protein-bound homocysteine, and (iv) total homocysteine. The free oxidized homocysteine is calculated by subtraction of free reduced homocysteine from that of total free. The method does not differentiate between homocystine and homocysteine mixed disulphides. Due to time dependent decay of homocysteine in plasma, its free reduced fraction is determined after derivatization at a blood collection time. All procedures are based on derivatization of the free sulfhydryl group with the UV-tagging agent CMQT, and separation and quantitation of homocysteine 2-*S*-quinolinium derivative by ion-paired reversed-phase HPLC. These assays have proven reliable for the determination of different species of homocysteine in human plasma, and the principles may be adopted for the determination of other thiols in plasma as well as in other biological samples.

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