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Determination of the *in Vivo* Redox Status of Cysteine, Cysteinylglycine, Homocysteine, and Glutathione in Human Plasma

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An assay that measures the reduced, oxidized, and protein-bound forms of cysteine, cysteinylglycine, homocysteine, and glutathione in human plasma is described. Oxidized and protein-bound thiols are converted to their reduced counterparts by the use of $NaBH_4$, and, following derivatization with monobromobimane (mBrB), the thiol–bimane adducts are quantified by reversed-phase ion-pair liquid chromatography and fluorescence detection. The presence of 50 μM dithioerythritol provides linearity of the standard curves at very low thiol concentrations. Selective determination of the oxidized forms was accomplished by blocking free sulfhydryl groups with *N*-ethylmaleimide (NEM) and excess NEM is inactivated by the subsequent addition of $NaBH_4$. The reduced forms of the thiols in plasma were trapped with minimal oxidation by derivatizing blood samples at the time of collection. This was attained by drawing blood directly into tubes containing isotonic solutions of mBrB or NEM. The assay is sufficiently sensitive (<2 pmol) to detect the various forms of the four thiol compounds in human plasma. The analytical recovery of cysteine, cysteinylglycine, homocysteine, and glutathione was close to 100%, and the within-day precision corresponded to a coefficient of variation of 7, 8, 6, and 7%, respectively. The assay has been used to determine the various forms of the four thiol compounds in human plasma. © 1992 Academic Press, Inc.

A growing interest in the biological role of sulfhydryls and disulfides has spurred the development of techniques for measuring these compounds. Our interest has been heightened by a possible role for the sulfur-containing amino acid homocysteine in the development of premature cardiovascular disease and by the confirmation that plasma homocysteine levels are a useful indicator of homocysteinuria, vitamin B_{12}, and folate deficiency (1).

Homocysteine is formed from the endogenous transmethylase inhibitor *S*-adenosylhomocysteine (1,2) and exported to the extracellular space when intracellular production exceeds the metabolic capacity (3). Intracellularly, homocysteine is metabolized either to cystathionine or to methionine (1,4). Cystathionine is further metabolized to cysteine, which is a precursor of glutathione (5). The breakdown of glutathione is catalyzed by γ-glutamyl transpeptidase, which in humans is localized primarily to the external surface of the cell membranes, but is also found in plasma (5,6). This results in the formation of cysteinylglycine, which in addition to cysteine, homocysteine, and glutathione also occurs in plasma (6–8). These thiols are thus metabolically related.

The facile oxidation of sulfhydryl compounds results in a variety of disulfide forms *in vivo* (9). These include the low-molecular-weight compounds cystine, cystinyl-*bis*glycine, homocystine and glutathione disulfide, and their corresponding mixed disulfides. Sulfhydryl compounds also form mixed disulfides with proteins, and in plasma, a major fraction of these compounds is associated with albumin (8,10,11).

To elucidate the function of various sulfhydryl-containing moieties in biochemical processes and their possible interrelationship with respect to redox status, several analytical approaches have been employed. Glutathione and other low-molecular-weight thiols have been analyzed by a variety of spectrophotometric, chromatographic, and enzymatic procedures (12–24). Disulfides of low-molecular-weight thiols have been analyzed by chromatography following precolumn derivatization (12), directly by enzymic methods (25), or by using HPLC with electrochemical detection (26–28). Some assays for disulfides rely on their conversion

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to the corresponding thiols using reductants such as dithiothreitol (20,29), tri-*n*-butylphosphine (30), or $NaBH_4$ (24,31–33) and subsequent derivatization of the sulfhydryl to form a fluorescent chromophore.

A common feature of existing methods is their inability to simultaneously quantify the oxidized and reduced forms of several thiols in biological systems. The determination of thiol status in plasma is particularly challenging since redox conditions change rapidly (within seconds) after blood collection (5,24).

We have developed a panel of closely related techniques for the analysis of the major thiol-containing components in plasma. These techniques are a further development of a previously published method for the measurement of various forms of glutathione in plasma, are based on the derivatization of free thiol with monobromobimane (mBrB),[1] and exploit the ability of $NaBH_4$ to reduce disulfides (both free and protein-bound) and of *N*-ethylmaleimide (NEM) to block free thiols. A novel approach is the derivatization of free thiols at the time of blood collection. This is achieved by injecting isotonic mixtures containing either mBrB or NEM into blood collection tubes prior to sampling. The performance of this analytical method was assessed in human plasma.

MATERIALS AND METHODS

Materials

N-Ethylmaleimide, *N*-ethylmorpholine, dithioerythritol, GSH, GSSG, homocysteine, cysteinylglycine, and cysteine were obtained from Sigma Chemical Co. (St. Louis, MO) and cystinyl-*bis*glycine was from Serva Chemicals (Heidelberg, Germany). $NaBH_4$ was from Fluka Chemie AG (Switzerland). Dimethyl sulfoxide (DMSO), hydrogen bromide (HBr), 5-sulfosalicylic acid (dihydrate), perchloric acid, acetic acid, phosphoric acid, and methanol (for chromatography) were purchased from Merck AG (Darmstadt, Germany), and mBrB was from Calbiochem, Behring Diagnostics (La Jolla, CA) or Molecular Probes (Eugene, OR). Tetrabutylammonium hydroxide was obtained from Aldrich-Chemie (Steinheim, Germany). ODS Hypersil (3 μm) was obtained from Shandon Southern, Ltd. (Chesire, UK). Columns for reversed-phase liquid chromatography (3-μm Hypersil, 150 × 4.6) were slurry packed at 9000 psi using a Shandon column packer.

[1] Abbreviations used: CV, coefficient of variation; DMSO, dimethyl sulfoxide; DTE, dithioerythritol; GSH, reduced glutathione; GSSG, glutathione disulfide; XSH, reduced thiol component; XSSX, soluble disulfide of a thiol component; XSSR, soluble mixed disulfide of a thiol component; ProSSX, mixed disulfide between a thiol component and protein; mBrB, monobromobimane; NEM, *N*-ethylmaleimide; ODS, octadecylsilyl; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline.

Solution B was 65% DMSO and 35% water (v/v) containing 51 mM NaCl and 140 mM HBr.

Methods

Standards. Standard solutions of 800 μM cysteine, 100 μM (reduced equivalents) cystinyl-*bis*glycine, 40 μM homocysteine, and 40 μM glutathione were separately prepared in 5% 5-sulfosalicylic acid containing 50 μM DTE. Equal volumes of each standard were mixed, giving a final standard containing 200 μM cysteine, 25 μM (reduced equivalents) cystinyl-*bis*glycine, 10 μM homocysteine, and 10 μM glutathione.

Sample collection and processing. Blood was routinely collected into evacuated tubes containing heparin as an anticoagulant and either mBrB or NEM as thiol-derivatizing reagent or no additions. The mBrB-containing tubes were prepared as follows: mBrB was dissolved in 100% acetonitrile at a concentration of 180 mM, and 75 μl of this solution was diluted to 500 μl with PBS and injected into 5-ml evacuated blood collection tubes. The NEM-containing tubes were prepared as follows: NEM was dissolved in PBS at a concentration of 120 mM, and 500 μl of this solution was injected into 5-ml evacuated blood collection tubes. The blood was immediately centrifuged at 10,000*g* for 1 min at room temperature to remove cells and platelets. From the different plasma preparations thus obtained, aliquots were drawn and further treated as described below.

Principles for the determination of the various thiol components. The principles of the different procedures described below involve reduction of the various oxidized forms to thiols by $NaBH_4$ and subsequent derivatization of the free sulfhydryl groups with mBrB. It is important that the various components be added in the order quoted.

Total plasma thiol components (XSH + XSSX + XSSR + ProSSX, procedure 1). To 30 μl of the untreated plasma was added 30 μl of 2.0 M $NaBH_4$ in 0.05 M NaOH. Plasma proteins were then precipitated by adding 60 μl of a 20% solution of sulfosalicylic acid containing 100 μM DTE (final concentration, 50 μM). The samples were kept on ice for about 30 min for release of gas. Precipitated protein was removed by centrifugation. To 60 μl of the supernatant were added 30 μl of 1.4 M $NaBH_4$ in 0.05 M NaOH, 130 μl of solution B, 50 μl of 1.0 M *N*-ethylmorpholine (final pH, 9.0), and 10 μl of 20 mM mBrB in 100% acetonitrile. After a 10-min incubation at room temperature (20°C) in the dark, 20 μl of 1.06 M perchloric acid was added. This method is referred to as procedure 1.

Free oxidized plasma thiol components (XSSX + XSSR, procedure 2). To 500 μl of NEM-treated plasma was added 55 μl of a 50% solution of sulfosalicylic acid (final concentration, 5%) containing 500 μM DTE (final

concentration, 50 μM). Precipitated protein was removed by centrifugation. To 30 μl of the supernatant were added 30 μl of 1.4 M $NaBH_4$ in 0.05 M NaOH, 160 μl of solution B, 50 μl of 1.0 M *N*-ethylmorpholine (final pH, 9.0), and 10 μl of 20 mM mBrB in 100% acetonitrile. After a 10-min incubation at room temperature (20°C) in the dark, 20 μl of 1.06 M perchloric acid was added. This method is referred to as procedure 2.

Protein-bound thiol components in plasma (ProSSX, procedure 3). To 30 μl of the untreated plasma was added 150 μl of 5% sulfosalicylic acid/50 μM DTE. Following precipitation of the protein and centrifugation, 30 μl of 2.0 M $NaBH_4$ in 0.05 M NaOH was added to the pellet. To the dissolved pellet were added 30 μl of a 40% solution of sulfosalicylic acid containing 100 μM DTE, 30 μl of 1.4 M $NaBH_4$ in 0.05 M NaOH, 130 μl of solution B, 50 μl of 1.0 M *N*-ethylmorpholine (final pH, 9.0), and 10 μl of 20 mM mBrB in 100% acetonitrile. After a 10-min incubation at room temperature (20°C) in the dark, 20 μl of 1.06 M perchloric acid was added. This method is referred to as procedure 3.

Reduced plasma thiol components (XSH, procedure 4). To 500 μl of mBrB-treated plasma was added 55 μl of a 50% solution of sulfosalicylic acid (final concentration, 5%) containing 500 μM DTE (final concentration, 50 μM). Precipitated protein was removed by centrifugation. To 30 μl of the supernatant were added 30 μl of 5% sulfosalicylic acid/50 μM DTE, 160 μl of distilled water, 50 μl of 1.0 M *N*-ethylmorpholine (final pH, 9.0), and 10 μl of acetonitrile. After a 10-min incubation at room temperature (20°C) in the dark, 20 μl of 1.06 M perchloric acid was added. This method is referred to as procedure 4.

Chromatography. Samples of 25 μl were injected into a 150 × 4.6-mm column packed with 3-μm particles of ODS-Hypersil (C18), equipped with a guard column packed with pelliguard L-18. The temperature was 25°C and the flow rate 1.5 ml/min. Elution solvent A was (1 liter) 10 mmol tetrabutylammonium phosphate, 2.5 ml glacial acetic acid diluted with distilled water, pH adjusted to 3.4 with 2.0 M NaOH. Solvent B was (1 liter) 200 ml acetonitrile, 10 mmol tetrabutylammonium phosphate, 2.5 ml glacial acetic acid diluted with distilled water, pH adjusted to 3.4 with 2.0 M NaOH. Solvent C was 75% acetonitrile. The elution profile was as follows: 0–13 min, 6–22% B; 13.1–25 min, 22–40% B; 25.1–30 min, 40–75% B. After each run, the column was washed for 5 min with solvent C to remove late-eluting fluorescent material. The retention times for the bimane derivatives of cysteine, cysteinylglycine, homocysteine, and glutathione were 7.0, 8.0, 12.5, and 31.2 min, respectively.

Instrumentation. A Spectra-Physics SP 8700 solvent delivery system coupled to a Perkin–Elmer ISS 100 autosampler was used. The detector was a Shimadzu RF-535 fluorometer equipped with concave diffraction grating excitation and emission monochromators operating at excitation wavelength 400/13-nm bandpass and emission wavelength 475/15-nm bandpass. Plotting and integration of peaks were performed by a Model 4290 computing integrator from Spectra-Physics.

Recovery and precision studies. Cysteine, cysteinylglycine, homocysteine, and glutathione or their corresponding disulfides dissolved in water containing DTE were added to plasma that had been frozen and thawed once or to an equal volume of water to give a final concentration of the exogenously added thiol components of 100, 20, 15, and 5 μM (reduced equivalents), respectively. The final concentration of DTE was 50 μM. From the plasma values obtained (procedure 1) was subtracted the amount of the respective endogenous thiol components calculated by analyzing parallel samples of plasma. Net values of exogenously added thiol components were thus achieved. The recoveries of thiol components were calculated as the percentage recovered from plasma relative to the amount detected in the water matrix. To determine the within-run precision (CV) of the assay, we assayed 10 replicates of plasma supplemented with cysteine, cysteinylglycine, homocysteine, and glutathione or their corresponding disulfides at a concentration of 100, 20, 15, and 5 μM (reduced equivalents), respectively.

Standard curves and detection limits. We prepared standard curves by adding known concentrations of cysteine, cystinyl-*bis*glycine, homocysteine, and glutathione to 5% sulfosalicylic acid containing 50 μM DTE ranging from 6.25 to 200 μM for cysteine, 0.78 to 25 μM for cystinyl-*bis*glycine (reduced equivalents), 0.31 to 10 μM for homocysteine, and 0.31 to 10 μM for glutathione.

RESULTS

Reduction of Disulfides with $NaBH_4$ and Formation of Bimane Adducts

The initial objective was to extend the previously published method for the determination of the various forms of glutathione (24) to include three other important thiols in plasma: cysteine, cysteinylglycine, and homocysteine. This was accomplished by modifying the derivatization conditions. Most important was the decrease in the mBrB concentration to 20% of that used previously. This reduced some interfering reagent peaks to acceptable levels. Furthermore, it was necessary to include 10 mM of the ion-pair reagent tetrabutylammonium phosphate in the mobile phase to obtain satisfactory separation.

Figure 1A shows a typical HPLC chromatogram obtained from a standard sample containing 200 μM cysteine, 25 μM cysteinylglycine, 10 μM homocysteine, and 10 μM glutathione, which had been treated with $NaBH_4$

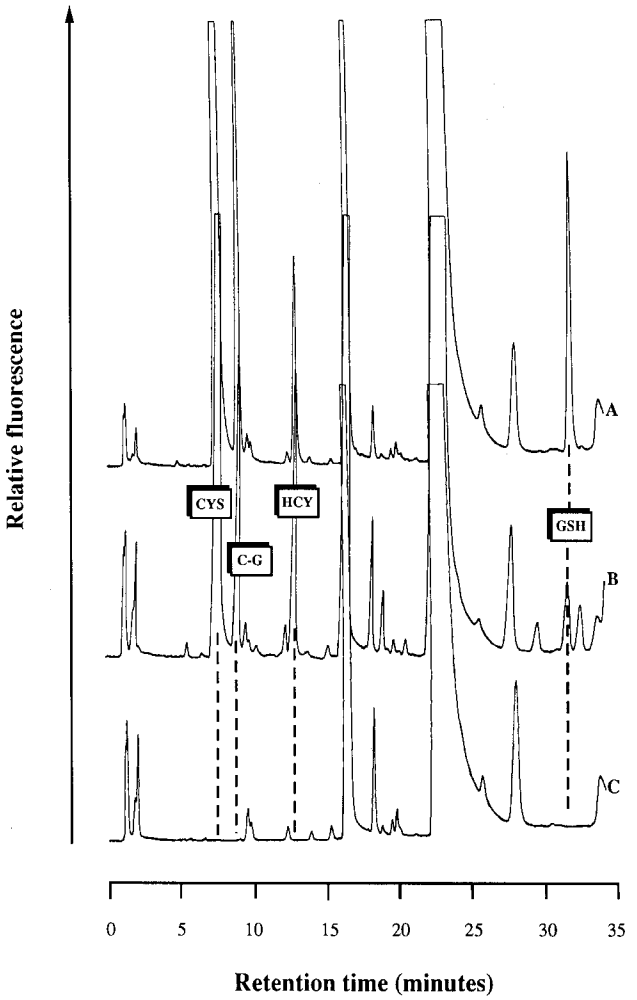

FIG. 1. Reversed-phase high-performance liquid chromatogram of a mBrB-derivatized sample of (A) standard solution composed of 200 μM cysteine (CYS), 25 μM cysteinylglycine (C-G), 10 μM homocysteine (HCY), and 10 μM glutathione (GSH) in 5% sulfosalicylic acid/50 μM DTE; (B) whole plasma (total thiol components); and (C) 5% sulfosalicylic acid/50 μM DTE (blank). Procedure 1 was used as described under Materials and Methods.

before derivatization with mBrB. This shows baseline separation of the thiol–bimane adducts, which in turn are well separated from the reagent peaks (Fig. 1C). Figure 1B shows a chromatogram obtained from $NaBH_4$-treated human plasma derivatized with mBrB.

We examined the concentration–response curve for $NaBH_4$ in order to determine the concentration required to fully reduce the different disulfides, but which was below the level interfering with the formation and stability of the thiol–bimane adducts. Figure 2 shows that the formation of fluorescent material from each of the four disulfides was dependent on the concentration of $NaBH_4$ and increased to an optimum at 1.2–1.4 M. The optimal $NaBH_4$ concentration was the same for all of the thiol components, and we therefore routinely used a $NaBH_4$ concentration of 1.4 M (refers to the standard solution) to reduce free oxidized thiol compounds.

Reduction of Total Oxidized Thiol Components in Plasma

We determined the formation of the thiol–bimane adducts in whole plasma as a function of increasing concentration of $NaBH_4$ (Fig. 3). The optimal concentration of $NaBH_4$ required to obtain maximal yield of the four thiol components from plasma was slightly above that observed for thiol components dissolved in 5% sulfosalicylic acid/50 μM DTE (standard solution) (Fig. 2). Again the optimal $NaBH_4$ concentration was the same for all of the thiols. On the basis of these results we used 2.0 M $NaBH_4$ (final concentration, 0.21 M) when total plasma thiol components were measured (procedure 1).

Linearity of the Assay for Cysteine, Cysteinylglycine, Homocysteine, and Glutathione

It was necessary to add 50 μM DTE (5.4 μM final in the assay mixture) to the samples in order to obtain a linear standard curve for glutathione at low concentrations (24). This was also required for cysteine, cysteinylglycine, and homocysteine (results not shown). Figure 4 shows typical standard curves obtained for the analysis of the four thiol components in plasma over the concentration range corresponding to their plasma levels. The curves demonstrate linearity over this range and all of them pass through the origin.

Stability of the Thiol–Bimane Adducts

The stability of the thiol–bimane adducts was tested at various temperatures for 14 days. The recovery of

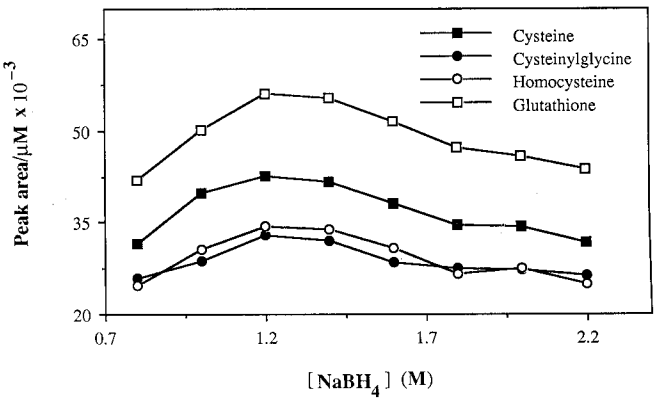

FIG. 2. The effect of the concentration of $NaBH_4$ on the reduction of a standard solution containing the disulfide forms of cysteine (100 μM), cysteinylglycine (12.5 μM), homocysteine (5 μM), and glutathione (5 μM). The thiol components were prepared in 5% sulfosalicylic acid/50 μM DTE and pretreated with $NaBH_4$ at concentrations indicated and then derivatized with mBrB using procedure 1 (as described under Materials and Method). The concentrations of $NaBH_4$ refer to the standard solution.

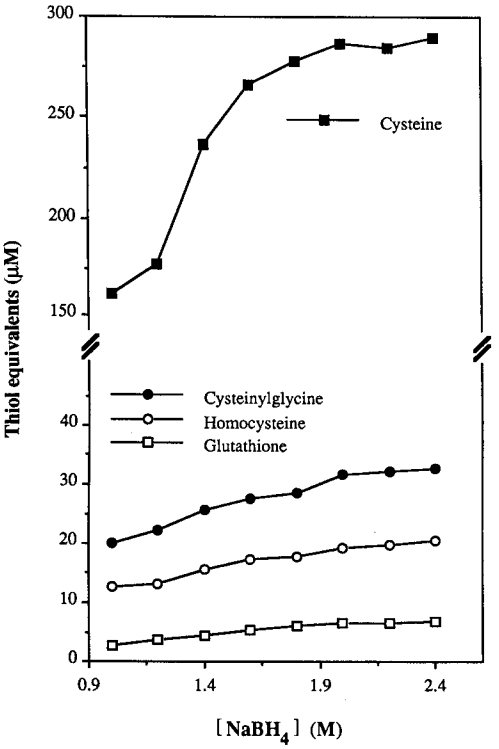

FIG. 3. The effect of the concentration of $NaBH_4$ on the yield of cysteine, cysteinylglycine, homocysteine, and glutathione from plasma. Plasma was pretreated with $NaBH_4$ at the concentrations indicated (refers to the standard solution) and then derivatized with mBrB using procedure 1.

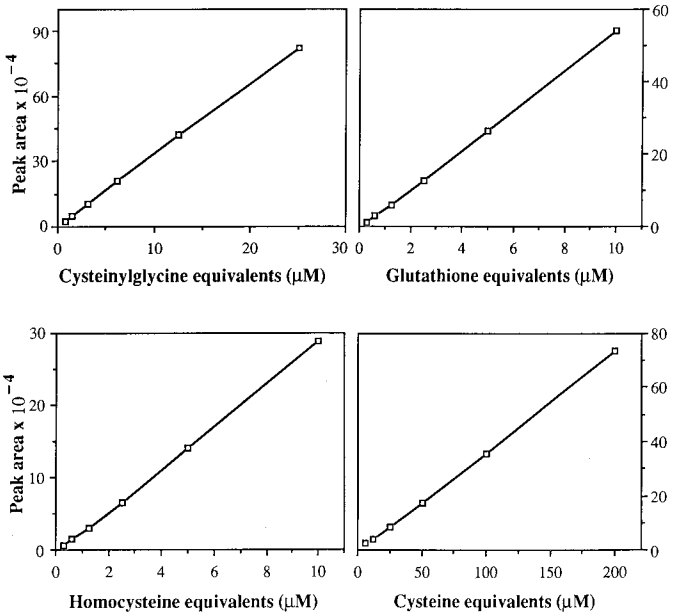

FIG. 4. Standard curves for cysteine, cysteinylglycine, homocysteine, and glutathione. The standards were dissolved in 5% sulfosalicylic acid containing 50 μM DTE. Samples were analyzed by procedure 1.

total thiols at −80, −20, and 4°C was nearly unchanged, although a very slight decline could be demonstrated at −20 and 4°C. At room temperature the decline was more pronounced (Fig. 5). Freezing and thawing the samples resulted in a minor decline in the recovery.

Analysis of Redox Status in Human Plasma

Thiols are rapidly oxidized (9). Marked alterations in the redox status of the plasma thiols have been found within 10 to 20 s after blood sampling. This cannot be prevented by adding EDTA or antioxidants to the vacutainers (data not shown). Therefore, we attempted to immediately trap thiols by drawing blood directly into isotonic solutions containing the thiol-specific reagents. These solutions were injected into the sampling tubes in a manner that maintained a vacuum sufficient to collect 5 ml of blood. In order to measure both the free thiols and the free oxidized forms we developed two such mixtures. Free thiols were measured by collecting the blood directly into tubes containing mBrB (procedure 4) and the free oxidized forms by collecting the blood into tubes containing NEM and subsequently treating the plasma with $NaBH_4$ to reduce the oxidized forms and simultaneously destroy the excess NEM (procedure 2).

Figure 6B shows a typical HPLC chromatogram of plasma that has been rapidly derivatized with mBrB. It contained no reagent hydrolysis peaks. This is in contrast to the chromatogram of the protein-bound thiols (Fig. 6A, procedure 3), although none of these peaks interfere with the determination of cysteine, cysteinylglycine, homocysteine, and glutathione.

We determined the mBrB concentration giving the highest yield of the different thiols in plasma. Figure 7 shows that a final concentration of 2.7 mM mBrB was

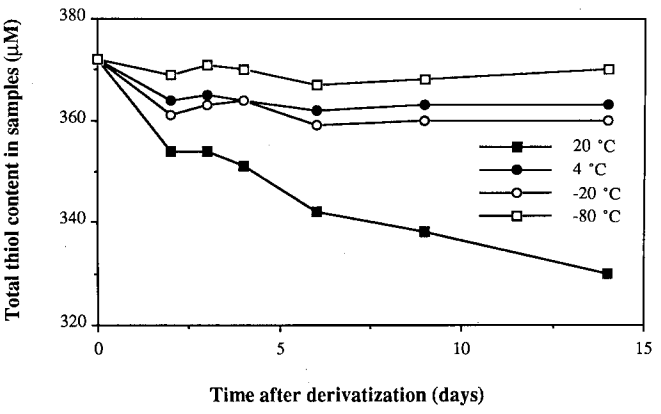

FIG. 5. Stability of thiol–bimane adducts. Plasma was derivatized with mBrB using procedure 1 and divided into four fractions. The fractions were stored at 20, 4, −20, or −80°C for the time indicated and then analyzed by HPLC.

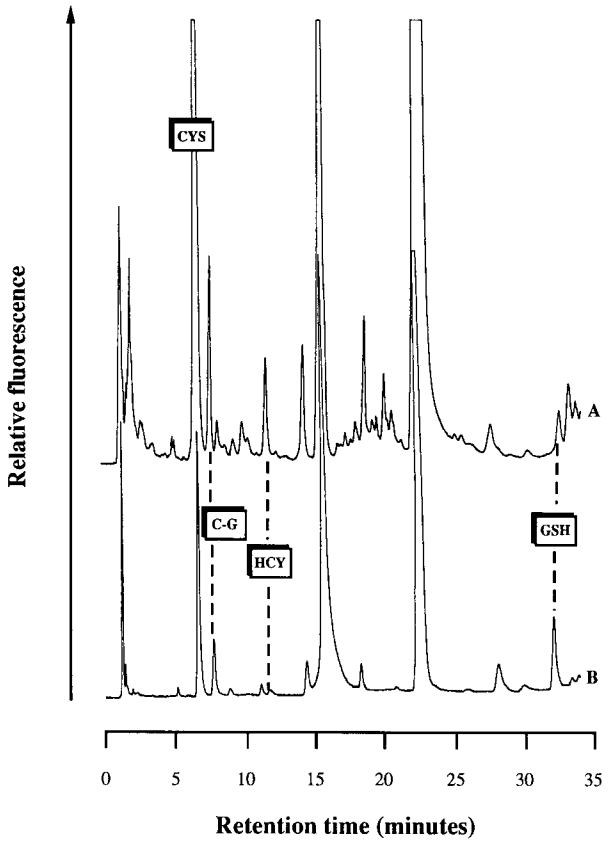

FIG. 6. Reversed-phase high-performance liquid chromatogram of a mBrB-derivatized sample of (A) $NaBH_4$-treated plasma protein precipitate (protein-bound form; procedure 3 was used) and (B) mBrB-treated plasma (reduced form; procedure 4 was used). See Materials and Methods for details.

sufficient for this purpose, and this concentration was therefore used throughout the study.

Figure 8B shows a HPLC of plasma immediately derivatized with NEM, where the reduction step with $NaBH_4$ was omitted before derivatization with mBrB. The chromatogram clearly demonstrates that NEM efficiently blocks all free sulfhydryl groups on the thiols studied. However, $NaBH_4$ treatment reduces the oxidized thiol components, which then react with mBrB. This is demonstrated in Fig. 8A.

The concentration of NEM required to mask free thiols in plasma was determined. Figure 9 demonstrates that NEM reduced the amount of acid-soluble free thiols plus disulfides in a dose-dependent manner, until a plateau was obtained. A maximal effect was seen at about 12 mM NEM (Fig. 9). The fraction not trapped by NEM corresponded to the amount existing as disulfides.

Recovery and Precision of the Assay

The recoveries of the different thiol components (added to plasma) as their respective bimane adducts were close to 100% (Table 1). The within-day precision (CV) of the assay of cysteine, cysteinylglycine, homocysteine, and glutathione was 7.2, 7.8, 6.6, and 7.1%, respectively. The CV of the corresponding disulfides was 7.0, 8.4, 6.8, and 7.5, respectively.

Quantification of the Reduced, Oxidized, and Protein-Bound Forms of Cysteine, Cysteinylglycine, Homocysteine, and Glutathione in Plasma from Healthy Subjects

Table 2 shows results obtained from the analysis of the reduced forms of the four thiols in plasma obtained from blood collected directly into tubes containing mBrB. The reduced forms of cysteine, cysteinylglycine, and homocysteine account for 2–12% of these thiol components in plasma; the highest reduced fraction was observed for cysteinylglycine and the lowest for homocysteine. In contrast, most glutathione in plasma occurs in the reduced form.

Table 3 shows results obtained from the analysis of the free oxidized forms of the four thiol components in plasma obtained from blood collected directly into tubes containing NEM. About one-third of the amount of cysteine and cysteinylglycine and about 16% of homocysteine exist in the free oxidized form. The remaining and predominating form is the protein-bound fraction (Table 4). In contrast, glutathione was equally distributed between the free oxidized and the protein-bound form.

Directly measured thiol components by procedure 1 (Table 5) equaled the sum of the reduced, oxidized, and

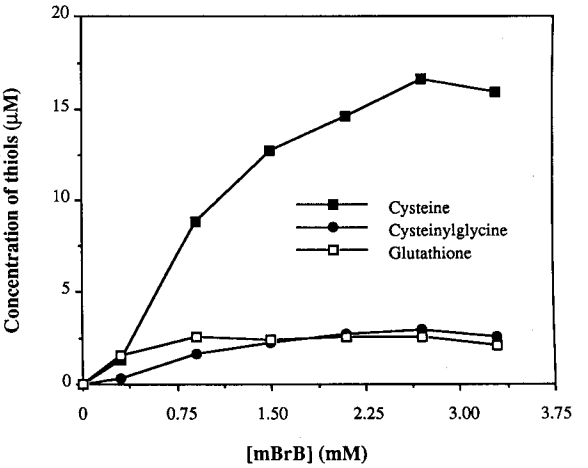

FIG. 7. Optimization of the mBrB concentration required for maximum yield of reduced thiol components in human plasma. mBrB was dissolved in 100% acetonitrile at concentrations of 20, 60, 100, 140, 180, and 220 mM. Seventy-five microliters of each solution, diluted to 500 μl with PBS, was injected into 5-ml evacuated blood collection tubes containing heparin. Blood from healthy subjects was drawn and the isolated plasma further treated as described in procedure 4. Final mBrB concentrations in the tubes are indicated.

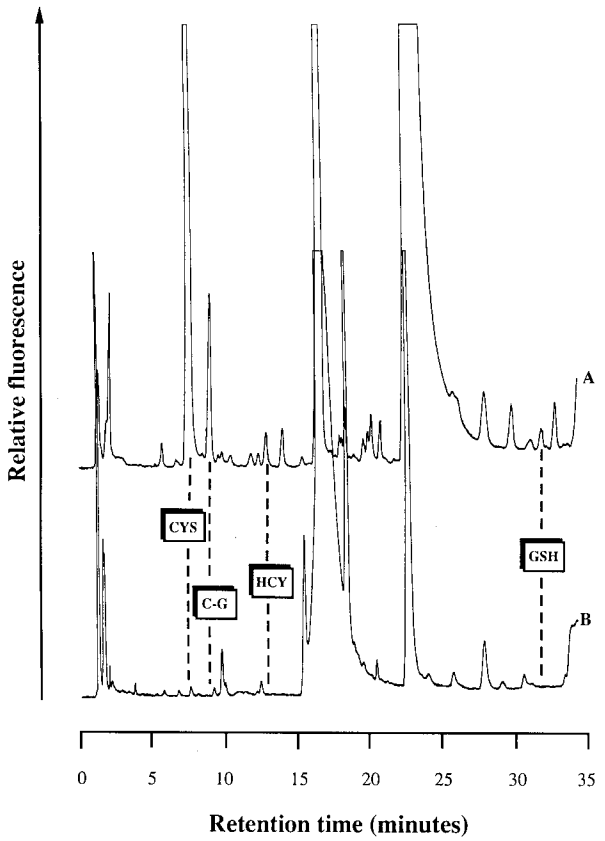

FIG. 8. Reversed-phase high-performance liquid chromatogram of a mBrB-derivatized sample of (A) NEM-treated plasma that subsequently has been reduced with $NaBH_4$ (oxidized form) and (B) as A but without $NaBH_4$. Procedure 2 was used as described under Materials and Methods.

protein-bound forms (Tables 2, 3, and 4). Notably, the plasma level of all these sulfur compounds were somewhat higher in males than in females (Table 5). Table 6 summarizes the results presented in Tables 2, 3, and 4 and shows the percentile distribution of the different thiol forms in human plasma. It clearly demonstrates that the predominating form of cysteine, cysteinylglycine, and homocysteine is protein-bound, followed by the oxidized form. Notably, the level of the reduced form of the cysteine and homocysteine was extremely low. In contrast, glutathione is unique among the thiol components in plasma as it predominantly occurs in the reduced form.

DISCUSSION

The procedures described herein represent a significant advance in the methodology for the analysis of various thiol components in plasma. First of all, the procedures allow the analysis of thiol status in plasma before significant oxidation or redistribution occurs. This is obtained by immediate derivatization of the sulfhydryl groups by mBrB or NEM in an isotonic medium.

This method is based on a previously published assay designed for the determination of the reduced, oxidized, and protein-bound forms of glutathione in human plasma (24). In the previous article (24), the principles and motives for the choice of reagents were carefully evaluated and are not described here. However, the chromatographic procedure had to be substantially altered since four bimane derivatives were measured simultaneously. We found that acetonitrile gave better resolution of the peaks than did methanol, and that the interfering peaks were greatly reduced by lowering the mBrB concentration. Separation was further improved by adding 10 mM tetrabutylammonium phosphate to the mobile phase. As demonstrated in Figs. 1, 6, and 8, these modifications resulted in a acceptable separation of all four thiol–bimane adducts.

A $NaBH_4$ concentration of 1.4 M (final concentration at the time of derivatization, 0.15 M) was optimal for the reduction of each of the free disulfides, i.e., disulfides present in the deproteinized plasma (Fig. 2). However, a

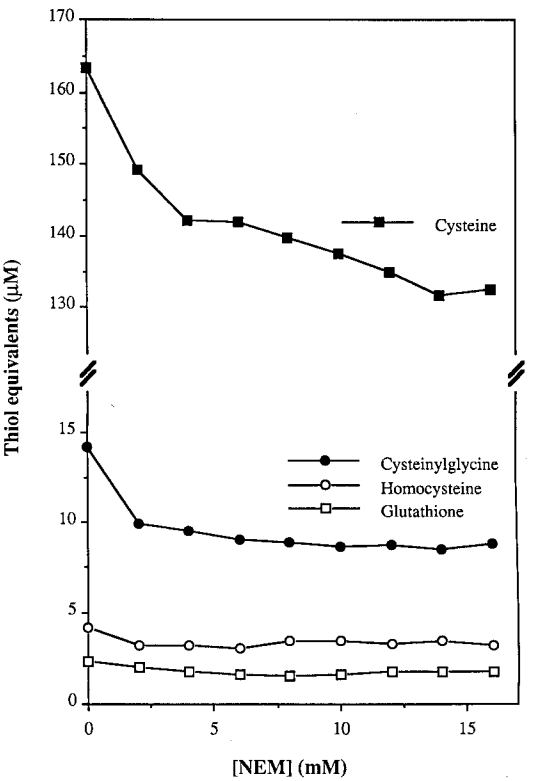

FIG. 9. Determination of the optimal NEM concentration needed to block sulfhydryl groups in human plasma. NEM solutions of varying concentrations were prepared in PBS and 500 μl of each solution was injected into 5-ml evacuated blood collection tubes containing heparin. Blood from healthy subjects was drawn and the isolated plasma further treated as described in procedure 2. Final NEM concentrations in the tubes are indicated.

TABLE 1

Analytical Recovery of Cysteine, Cysteinglycine, Homocysteine, Glutathione, and Their Corresponding Disulfides in Plasma

	Mean	CV (%)
Cysteine	108	7.2
Cysteinylglycine	95	7.8
Homocysteine	102	6.6
GSH	112	7.1
Cystine	101	7.0
Cystinyl-*bis*glycine	112	8.4
Homocystine	90	6.8
GSSG	115	7.5

Note. Cysteine, cysteinylglycine, homocysteine, and glutathione were added to plasma at concentrations of 100, 20, 15, and 5 μM, respectively. The corresponding disulfides were added in equivalent amounts. $n = 10$ each. Values are percentages of added thiol compound that were measured.

somewhat higher concentration of $NaBH_4$ (2.0 M) was needed to obtain maximum yield of the total thiol components (Fig. 3) and ProSSX (results not shown). This difference may be related to the high protein content inhibiting $NaBH_4$, or to the reduction or displacement of protein-bound thiol components being dependent on a higher concentration of reductant. Notably, the optimal $NaBH_4$ concentration required to obtain maximal yield of the four thiols in plasma was the same for all species.

The necessity of adding DTE (50 μM) to the solvent in which GSH or GSSG is dissolved to obtain linear standard curves was stressed in the previous paper (24). This proved to be critical for the linearity of the four standard curves demonstrated (Fig. 4). Separate standard curves for the reduced and oxidized forms of the four thiol components were not required since these curves were identical.

The sensitivity of the method allows the detection of quantities > 2 pmol. Reagent impurities resulting in small interfering peaks determine the detection limit. However, high chromatographic resolution has minimized such interference and this, together with the linearity of the standard curve at low concentrations, is the major reason for the low detection limit compared to those of previously published methods (>10 pmol) (20,34).

Satisfactory analytical precision was obtained (Table 1) without inclusion of an internal standard. There are objections to the inclusion of thiols as internal standards to compensate for oxidation or other chemical reactions (35). Differing rates of reaction for different thiols may be a source of erratic results.

Many reports indicate that the reduced form of glutathione (GSH) oxidizes very rapidly in human plasma to form GSSG (6,16,21,36). A period of 2.5 min is sufficient for oxidation of a substantial fraction of this thiol (3). Cysteine, cysteinylglycine, and homocysteine may oxidize even more rapidly than glutathione, partly because the steric hindrance is smaller (9). Acidification of the thiol-containing solution with 5-sulfosalicylic acid and the addition of EDTA to remove metal cations are routinely used to minimize oxidation. Addition of EDTA has a minor effect (6,21,37), and both strategies increase the time elapsed between blood collection and derivatization. Due to the high reactivity of thiols, the analysis of reduced, oxidized, and protein-bound forms of these substances in plasma may be unreliable if proper precautions are not taken. Therefore, several investigators have emphasized the need for rapid derivatization of

TABLE 2

Concentration (μM) *in Vivo* of Four Thiols in Plasma Obtained from 10 Healthy Females and 8 Healthy Males

Thiol	Mean ± SD	Range	Reduced/oxidized ±SD	% of reduced thiol components	% of total thiol components
			Females		
Cysteine	10.18 ± 2.88	6.84–16.73	0.126 ± 0.027	57.5	3.6
Cysteinylglycine	3.18 ± 0.43	2.41–3.74	0.412 ± 0.074	18.0	1.1
Homocysteine	0.25 ± 0.09	0.14–0.37	0.132 ± 0.046	1.4	0.1
Glutathione	4.10 ± 1.40	2.14–5.96	3.552 ± 0.937	23.2	1.5
			Males		
Cysteine	8.25 ± 3.74	2.82–11.36	0.097 ± 0.040	49.9	2.6
Cysteinylglycine	2.92 ± 1.04	1.61–4.31	0.311 ± 0.120	17.7	0.9
Homocysteine	0.23 ± 0.16	0.07–0.44	0.144 ± 0.065	1.4	0.1
Glutathione	5.14 ± 0.80	3.92–6.37	3.690 ± 1.140	31.1	1.6

Note. Blood was collected into evacuated tubes containing mBrB and immediately centrifuged at 10,000*g* for 1 min. From the plasma, aliquots were withdrawn and analyzed for the content of thiols as described under Materials and Methods, procedure 4. The content of total thiol components was determined directly by procedure 1.

TABLE 3

Concentration (μM) *in Vivo* of the Free Oxidized Form of Four Thiol Components in Plasma Obtained from 10 Healthy Females and 8 Healthy Males

Thiol	Mean ± SD	Range	Oxidized/reduced ±SD	% of free oxidized thiol components	% of total thiol components
			Females		
Cysteine	80.47 ± 7.78	70.41–92.90	8.243 ± 1.705	88.0	28.7
Cysteinylglycine	7.89 ± 1.61	5.73–10.84	2.496 ± 0.436	8.6	2.8
Homocysteine	1.94 ± 0.44	1.22–2.50	8.547 ± 3.400	2.1	0.7
Glutathione	1.18 ± 0.40	0.75–1.87	0.300 ± 0.076	1.3	0.4
			Males		
Cysteine	85.18 ± 9.58	74.10–98.89	13.534 ± 9.108	86.7	27.0
Cysteinylglycine	9.67 ± 2.27	6.18–12.99	3.605 ± 1.240	9.8	3.1
Homocysteine	1.92 ± 0.38	1.50–2.39	12.151 ± 7.882	2.0	0.6
Glutathione	1.48 ± 0.36	1.02–1.89	0.300 ± 0.093	1.5	0.5

Note. Blood was collected into evacuated tubes containing NEM and immediately centrifuged at 10,000*g* for 1 min. From the plasma, aliquots were withdrawn and analyzed for the content of free oxidized thiol components as described under Materials and Methods, procedure 2. The content of total thiol components was determined directly by procedure 1.

plasma samples (5,16). In the present assay, we minimized the possibility for autoxidation in plasma to occur. It is well established that both mBrB and NEM spontaneously form conjugates with thiols under physiological conditions (13,16,38). In order to minimize the time between blood collection and derivatization we produced isotonic mixtures containing mBrB and NEM and added these mixtures into evacuated tubes. During blood collection, the plasma thiols were immediately derivatized and essentially no oxidation could take place. The amount of thiols could then be determined in the mBrB tubes and the amount of free disulfides in the NEM tubes (after destruction of excess NEM and reduction of the disulfides with $NaBH_4$). Optimal concentrations of mBrB (Fig. 7) and NEM (Fig. 9) were obtained in a medium in which no hemolysis could be demonstrated.

We determined the amount of cysteine, cysteinylglycine, homocysteine, and GSH and the fraction of the different forms present in human plasma. The dominating thiol component is cysteine (236 and 264 μM in females and males, respectively) followed by cysteinylglycine (28 and 32 μM), homocysteine (11 and 12 μM), and GSH (6 and 8 μM) (Table 5). The total values of the

TABLE 4

Concentration (μM) of the Protein-Bound Form of Four Thiol Components in Plasma Obtained from 10 Healthy Females and 8 Healthy Males

Thiol	Mean ± SD	Range	Free/protein bound ±SD	% of protein-bound thiol components	% of total thiol components
			Females		
Cysteine	154.57 ± 17.93	109.50–173.56	0.593 ± 0.078	85.5	55.1
Cysteinylglycine	15.11 ± 3.15	10.59–21.79	0.750 ± 0.136	8.4	5.4
Homocysteine	9.81 ± 2.85	6.30–15.57	0.237 ± 0.062	5.4	3.5
Glutathione	1.32 ± 0.24	0.90–1.63	4.109 ± 1.452	0.7	0.5
			Males		
Cysteine	175.81 ± 21.26	135.00–207.95	0.537 ± 0.073	85.0	55.7
Cysteinylglycine	18.99 ± 2.31	16.30–24.00	0.663 ± 0.110	9.2	6.0
Homocysteine	10.38 ± 2.61	7.25–13.80	0.212 ± 0.039	5.0	3.3
Glutathione	1.64 ± 0.63	0.69–2.47	4.808 ± 2.561	0.8	0.5

Note. Blood was collected into evacuated tubes containing no sulfhydryl-reactive agent and immediately centrifuged at 10,000*g* for 1 min. From the plasma, aliquots were withdrawn and analyzed for the content of protein-bound thiol components as described under Materials and Methods, procedure 3. The content of total thiol components was determined directly by procedure 1.

TABLE 5

Total Concentration (μM) of Four Thiol Components in Plasma Obtained from 10 Healthy Females and 8 Healthy Males

Thiol component	Mean ± SD	Range	% of total thiol components
	Females		
Cysteine	236.00 ± 26.21	197.00–283.00	84.1
Cysteinylglycine	27.55 ± 3.42	20.47–32.09	9.8
Homocysteine	10.91 ± 2.05	7.55–13.85	3.9
Glutathione	6.23 ± 1.42	4.10–9.87	2.2
	Males		
Cysteine	264.31 ± 33.31	231.07–307.90	83.8
Cysteinylglycine	31.77 ± 5.36	27.92–41.03	10.1
Homocysteine	11.85 ± 1.51	9.49–13.25	3.7
Glutathione	7.55 ± 1.27	5.45–9.04	2.4

Note. Blood was collected into evacuated tubes containing no sulfhydryl-reactive agent and immediately centrifuged at 10,000*g* for 1 min. From the plasma, aliquots were withdrawn and analyzed for the content of total plasma thiol components as described under Materials and Methods, procedure 1.

various substances agreed with those previously found (1,8,20,24).

Glutathione differ from the other thiol components in human plasma in that a large part exists in the reduced form (>60%) *in vivo*. A possible explanation is that glutathione, as opposed to the other thiols, is continuously secreted in the reduced form from the liver (39,40) and rapidly eliminated by the kidneys (half-life, 1.6 min) (37). Thus, there is a constant supply of reduced glutathione to the blood, sufficient to maintain a high level of this substance *in vivo*. This level of GSH, however, is not high enough to keep a substantial part of the other thiol components in their reduced forms.

In these experiments, 1.3 μM (females) and 1.6 μM (males) of glutathione were released from plasma proteins by reduction. These values are slightly below those (2 μM) demonstrated by others (20). Despite the rapid derivatization of the free thiol groups in our experiments, glutathione levels as high as 1.2 μM (females) and 1.5 μM (males) were found to exist in the free oxidized form. This gives a ratio between the oxidized and reduced forms of 0.3 both in females and in males, which is considerably higher than that previously reported (0.05) (16).

The predominant forms in plasma of cysteine, cysteinylglycine, and homocysteine are disulfide species, in contrast to the plasma GSH component. The amounts of reduced cysteine measured, 10 and 8 μM (females, males), are slightly above or equal to previously published values of 6 to 10 μM (20,41,42). Previous reports have demonstrated that much of the total cysteine exists in mixed disulfides with plasma protein. Therefore, our findings of 154 and 175 μM (females, males) protein-bound cysteine are consistent with published values of 145 (43) and 120 μM (20,44). Furthermore, our measurements demonstrated 80 and 85 μM (females, males) cysteine released from low-molecular-weight disulfides by reduction. These values agree well with previous published values of 90 (20) and 60 μM (41).

The level of reduced cysteinylglycine was approximately 3 μM in both sexes. Among the substances investigated, cysteinylglycine is the thiol component next to glutathione that is most abundant in its reduced form. This compound is formed from both reduced and oxidized glutathione by the action of γ-glutamyl transpeptidase present in human plasma (5). Since reduced glutathione predominates, plasma is continuously supplied with reduced cysteinylglycine. This may partly account for the relatively high fraction of the reduced form. The size of the thiols is another important factor determining this properties. Small molecules are generally more easily oxidized than larger molecules (9). Most of the cysteinylglycine is protein-bound (15 and 19 μM), and in percentages of total amount it is comparable with cysteine in this respect. Furthermore, our measurements demonstrate approximately 8 and 10 μM cysteinylglycine bound in low-molecular-weight disulfides in females and males, respectively. The values of total cysteinylglycine (28 μM in females and 32 μM in males) demonstrated in this study are slightly higher than those previously found (23 μM) (8).

A distinguishing feature of homocysteine is the low fraction of reduced form in normal human plasma. Only trace amounts (0.23 μM) could be detected. The low content of reduced homocysteine makes this sulfhydryl

TABLE 6

Distribution of the Different Forms of Four Thiol Components in Human Plasma Obtained from 10 Healthy Females and 8 Healthy Males, Expressed in Percentages

Thiol	% reduced	% free oxidized	% protein-bound
	Females		
Cysteine	4.2	32.8	63.0
Cysteinylglycine	12.1	30.1	57.7
Homocysteine	2.1	16.2	81.8
Glutathione	62.1	17.9	20.0
	Males		
Cysteine	3.1	31.6	65.3
Cysteinylglycine	9.2	30.6	60.1
Homocysteine	1.8	15.3	82.8
Glutathione	62.2	17.9	19.9

Note. The cumulative values of the reduced, oxidized, and protein-bound forms were taken as 100%. See notes to Tables 2, 3, and 4 and Materials and Methods for details.

compound different from related compounds in plasma. Previous efforts have demonstrated that human plasma contains 2–3 μM free homocyst(e)ine (the oxidized form is predominating) (1,8) and 9–12 μM total homocysteine (1,45,46), agreeing well with values obtained by us. There are consistent reports that the total values for homocysteine are higher in males than in females (1,30,47–49). This is also demonstrated in our experiments (Table 5). Our results suggest that this finding is a general phenomenon applying to all the plasma thiols studied (Table 5).

Our method was validated by comparing the total values for the various thiol components obtained by calculation with those obtained by direct analysis (procedure 1). When the values for reduced (XSH), free oxidized (XSSX + XSSR), and ProSSX were added, the sum equaled the values for the total plasma thiol components as determined by procedure 1, which involves reduction of whole plasma.

In conclusion, the present method includes four procedures established for the determination of the reduced, oxidized, and protein-bound forms together with the total amount of cysteine, cysteinylglycine, homocysteine, and glutathione in human plasma. The oxidized and protein-bound components are converted to their reduced counterparts by the employment of $NaBH_4$, and, following precolumn derivatization with mBrB, the thiol–bimane adducts are quantitated by ion-pair reversed-phase liquid chromatography and fluorescence detection. Selective determination of the oxidized forms is managed by blocking free sulfhydryl groups with NEM. Excess NEM is inactivated by the subsequent addition of $NaBH_4$. In order to obtain data reflecting the *in vivo* situation, the thiols are derivatized at the time of blood collection. This is achieved by injecting isotonic solutions of mBrB or NEM into the tubes before sampling. The assay has proven its usefulness for the determination of various forms of the four thiol components in normal human plasma. It may prove to be of value for acquiring a broader knowledge of the redox status of these moieties in plasma *in vivo* or in other biological systems, especially under certain pathological conditions and following pharmacological intervention.

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