ANALYTICAL BIOCHEMISTRY **184**, 338–346 (1990)

Determination of Reduced, Oxidized, and Protein-Bound Glutathione in Human Plasma with Precolumn Derivatization with Monobromobimane and Liquid Chromatography

Asbjørn M. Svardal, Mohammad A. Mansoor, and Per M. Ueland
Department of Pharmacology and Toxicology, University of Bergen, 5021 Bergen, Norway

Received August 7, 1989

This assay measures reduced (GSH), oxidized (GSSG, GSSR), and protein-bound (glutathione–protein mixed disulfides, ProSSG) glutathione in human plasma. Oxidized glutathione and ProSSG are converted to GSH in the presence of $NaBH_4$, and, after precolumn derivatization with monobromobimane, GSH is quantitated by reversed-phase liquid chromatography and fluorescence detection. The $NaBH_4$ concentration is optimized so that total recovery of oxidized glutathione is obtained and no interference with the formation/stability of the GSH–bimane adduct occurs. The presence of 50 μM dithioerythritol prevents reduced recovery at low concentrations of GSH, and the standard curve for GSH is linear over a wide concentration range and is superimposed upon that obtained with GSSG. Selective determination of oxidized glutathione exploits the fact that *N*-ethylmaleimide (NEM) blocks free sulfhydryl groups and excess NEM is inactivated by the subsequent addition of $NaBH_4$. To measure total glutathione including the protein-bound forms, the protein is solubilized with dimethyl sulfoxide, which is compatible with the other reagents and slightly increases the yield of the fluorescent GSH derivative. The assay is characterized by a sensitivity (<2 pmol) sufficiently high to detect the various forms of glutathione in plasma, by an analytical recovery of GSH and GSSG close to 100%, and by a within-day precision corresponding to a coefficient of variation of 7%. The assay was used to determine the dynamic relationships among various glutathione species in human plasma.

The tripeptide glutathione has been assigned an important role in the cellular defense against oxidative injury, in detoxification processes, and in the protection of the cell against radiation damage (1–3). It is the most abundant intracellular nonprotein thiol and the cellular content amounts to 0.5–10 mM. Most intracellular glutathione is maintained in the reduced form (GSH)[1] which in most tissues accounts for at least 90% of the total amount of GSH equivalents. The intracellular content of glutathione disulfide (GSSG) is low but may increase upon oxidative stress (4).

The concentration of GSH and GSSG in extracellular media like plasma is orders of magnitude lower than the intracellular content. However, plasma glutathione may play an important role in and may reflect the overall glutathione homeostasis. The plasma level is a function of a balance between secretion from the liver and elimination through the kidneys. The liver delivers GSH to plasma and may in this way furnish cysteine sulfur to other tissues. Oxidative stress may enhance the formation of GSSG which is exported into the extracellular compartment (5).

The glutathione content in plasma may be altered under several clinical conditions including myocardial oxidative stress, renal insufficiency (6), chronic alcohol abuse (7), γ-glutamyltransferase deficiency (8), cirrhosis (9), and malignant diseases (10).

Because of the low concentration and instability of glutathione in plasma (11), its determination is a more difficult task than in tissues. Numerous procedures have been published including chromatographic and enzymatic (11–18), but no method fulfills all requirements for a convenient and comprehensive plasma assay. The

[1] Abbreviations used: CV, coefficient of variation; DMSO, dimethyl sulfoxide; DTE, dithioerythritol; EGTA, ethylene glycol bis(β-aminoethyl ether)*N*,*N*′-tetraacetic acid; GSH, reduced glutathione; GSSG, glutathione disulfide; GSSR, soluble glutathione mixed disulfide; ProSSG, mixed disulfide between glutathione and protein; mBrB, monobromobimane; NEM, *N*-ethylmaleimide; MOS, dimethyloctylsilyl.

0003-2697/90 $3.00

latter should be of sufficient sensitivity and specificity, including determination of GSH, GSSG, and glutathione forming mixed adducts with protein thiols and various low-molecular-weight plasma thiols.

The present method for the determination of all these GSH species in plasma is based on the derivatization of free thiol with monobromobimane (mBrB), which has previously been used in assays for reduced GSH in plasma (12,17). Our method exploits the ability of $NaBH_4$ to reduce glutathione disulfides, of *N*-ethylmaleimide (NEM) to block free thiols, and of dimethyl sulfoxide (DMSO) to solubilize protein-bound glutathione and stabilize the assay. Conditions under which these reagents may be sequentially combined in a single reaction vial were carefully worked out and are described.

MATERIALS AND METHODS

Materials

N-Ethylmaleimide, *N*-ethylmorpholine, dithioerythritol, GSH, and GSSG were obtained from Sigma Chemical Co. (St. Louis, MO) and $NaBH_4$ was from Fluka Chemie AG (Switzerland). Dimethyl sulfoxide, hydrogen bromide (HBr), 5-sulfosalicylic acid (dihydrate), perchloric acid, acetic acid, and methanol (for chromatography) were purchased from Merck AG (Darmstadt, FRG), and monobromobimane was from Calbiochem-Behring Diagnostics (La Jolla, CA). MOS-Hypersil (3-μm) was obtained from Shandon Southern Ltd. (Chesire, UK). Columns for reversed-phase liquid chromatography (3-μm MOS-Hypersil, 150 × 4.6) were slurry packed at 9000 psi using a Shandon column packer.

Some frequently used solutions are: Solution A: DMSO containing 50 μM DTE and 5% sulfosalicylic acid. Solution B: Physiological salt solution containing 140 mM HBr and 44% DMSO. Solution C: Same as B but containing also 1% sulfosalicylic acid.

Methods

Standards. GSH and GSSG were dissolved at a concentration of 500 μM in 5% sulfosalicylic acid containing 50 μM DTE. These solutions were diluted to known concentrations in the same solvent.

Sample collection and processing. Blood was routinely collected into cooled evacuated tubes containing heparin as an anticoagulant and was immediately centrifuged at 10,000*g* for 1 min at room temperature to remove cells and platelets. From the plasma thus obtained aliquots were withdrawn and further treated as described below.

Principles for glutathione determination. GSSG, GSSR, or ProSSG were reduced to GSH by $NaBH_4$. The free sulfhydryl group of GSH was subsequently derivatized with mBrB. It is important that the various components are added in the order stated.

Total plasma glutathione (GSH + GSSG + GSSR + ProSSG, procedure 1). To 30 μl of plasma was added 30 μl of 2.0 M $NaBH_4$, 30 μl of solution A, 130 μl of solution B, 50 μl of 1.0 M *N*-ethylmorpholine (final pH 9.0), and 10 μl of 0.1 M mBrB in 100% acetonitrile. After a 20-min incubation at room temperature (20°C) in the dark, 20 μl of 8.15 M perchloric acid was added. After having stood at 4°C for 2 h to allow protein precipitation, the samples were centrifuged at 10,000*g* for 2 min and were then ready for analysis by HPLC. This method is referred to as procedure 1.

Free oxidized glutathione (GSSG + GSSR, procedure 2). Samples of plasma were deproteinized by addition of a 50% solution of sulfosalicylic acid (final concentration 5%) containing 500 μM DTE (final concentration 50 μM). Precipitated protein was removed by centrifugation. Two hundred microliters of the supernatent was neutralized to pH 6.8 by the addition of 37 μl of 1.2 N $KHCO_3$/1.44 N KOH and then supplemented with 20 μl of 154 mM NEM (final concn 12 mM) to trap GSH. After 20 min at room temperature the samples were ready for reduction with $NaBH_4$ and subsequent derivatization with mBrB using the following procedure: To 30 μl of the NEM-treated samples was added 30 μl of 1.4 M $NaBH_4$, 160 μl of solution C, 50 μl of 1.0 M *N*-ethylmorpholine (final pH 9.0), and 10 μl of 0.1 M mBrB in 100% acetonitrile. After a 20-min incubation at room temperature (20°C) in the dark, 20 μl of 5.82 M perchloric acid was added. This method is referred to as procedure 2.

Total free glutathione (GSH + GSSG + GSSR, procedure 3). To 30 μl of the protein free (acid treated) plasma supernatant described above was added 160 μl of solution B, 50 μl of 1.0 M *N*-ethylmorpholine, 10 μl of 0.1 M mBrB in 100% acetonitrile, and 30 μl of 1.4 M $NaBH_4$ (final pH 9.0). After a 20-min incubation at room temperature (20°C) in the dark, 20 μl of 5.82 M perchloric acid was added. This method is referred to as procedure 3.

Protein-bound glutathione (ProSSG, procedure 4). To 30 μl of plasma was added 150 μl of 5% sulfosalicylic acid/50 μM DTE. Following precipitation of the protein by centrifugation, 30 μl of 2.0 M $NaBH_4$ was added. To the dissolved pellet was added 30 μl of solution A, 160 μl of solution B, 50 μl of 1.0 M *N*-ethylmorpholine (final pH 9.0), and 10 μl of 0.1 M mBrB in 100% acetonitrile. After a 20-min incubation at room temperature (20°C) in the dark, 20 μl of 8.15 M perchloric acid was added. After having stood at 4°C for precipitation for 2 h the samples were centrifuged at 10,000*g* for 2 min. This method is referred to as procedure 4.

Reduced glutathione (GSH, procedure 5). To 30 μl of the protein free (acid treated) plasma supernatant described above was added 30 μl of 5% sulfosalicylic acid/50 μM DTE, 160 μl distilled water, 50 μl of 1.0 M *N*-ethylmorpholine (final pH 8.5), and 10 μl of 0.1 M mBrB

in 100% acetonitrile. After a 20-min incubation at room temperature (20°C) in the dark, 20 μl of 5.82 M perchloric acid was added. This method is referred to as procedure 5.

Chromatography. The HPLC system was programmed to inject 25-μl samples into the 150 × 4.6-mm column packed with 3-μm particles of MOS-Hypersil (C8). The column was used at ambient temperature and a flow rate of 1.5 ml/min. The elution solvent B was 2.5 ml acetic acid, diluted to 1 liter with distilled water, pH 3.9, and solvent C was 200 ml methanol diluted to 1 liter with distilled water. The solutions were filtered through an 0.2-μm Millipore filter. The elution profile was as follows: 0–8 min, 40% C isocratic; 8–12 min, 40–80% C, linear gradient. After each injection, the column was washed for 5 min with 90% methanol to remove late-eluting fluorescent material. The retention time for the monobromobimane derivative of GSH was 12 min.

Instrumentation. The HPLC analyses were carried out with a Spectra-Physics SP 8700 solvent delivery system coupled to a Perkin–Elmer ISS 100 autosampler. 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. GSH or GSSG dissolved in water containing DTE was added to plasma which had been frozen and thawed once or to an equal volume of water to give a final concentration of exogenously added glutathione of 5 μM (GSH equivalents). The final concentration of DTE was 50 μM. From the plasma values obtained was subtracted the amount of endogenous GSH/GSSG calculated by analyzing parallel samples of plasma. Net values of exogenously added GSH/GSSG were thus achieved. The recovery of GSH/GSSG was calculated as the percentage recovered from plasma relative to the amount detected in the pure water matrix. To determine the within-run precision (CV) of the assay, we assayed 10 replicates of plasma supplemented with GSH or GSSG each at a concentration of 5 μM (GSH equivalents).

Assay standard curve and detection limit. We prepared a standard curve by adding known concentrations of GSH to 5% sulfosalicylic acid containing 50 μM DTE, ranging from 0.1 to 10 μM. From this experiment the detection limit was also determined.

RESULTS

Reduction of GSSG and Formation of a Bimane Derivative of GSH in the Presence of $NaBH_4$

GSH but not GSSG incubated in the presence of mBrB forms a glutathione–bimane adduct, which can be isolated and quantitated by reversed-phase liquid chromatography (19) (Fig. 1). When we treated a standard sample of GSSG with $NaBH_4$ before derivatization, a peak which cochromatographed with the GSH adduct was observed (Fig. 1).

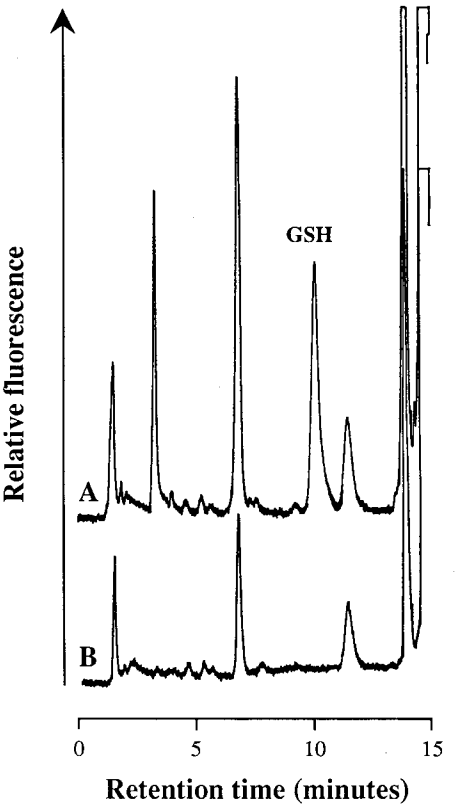

FIG. 1. Reversed-phase high-performance liquid chromatogram of a mBrB-derivatized solution of 3 μM GSSG in 5% sulfosalicylic acid/50 μM DTE. (A) Pretreated with 1.4 M $NaBH_4$ (the concentration refers to the standard solution and procedure 3 was used as described under Materials and Methods. (B) No treatment.

We investigated the concentration–response curve for $NaBH_4$ to obtain the concentration required to fully reduce GSSG but which was below the level interfering with the formation/stability of the GSH–bimane adduct. The formation of fluorescent material from GSSG was dependent on the concentration of $NaBH_4$ and increased in a dose-dependent manner up to about 1.4 M and then the curve declined (Fig. 2). A similar curve was obtained when equivalent amounts of GSH were treated in the same way. However, the latter curve started at a higher level compared to that for GSSG but yet considerably lower than would be expected from the amount of GSH. This unexpected finding may be explained by the fact that GSH and GSSG were both assayed by procedure 1 (see Materials and Methods), i.e., in the presence of DMSO. The presence of DMSO may lead to oxidative conditions and therefore some of the originally reduced glutathione is oxidized at low concentrations of $NaBH_4$.

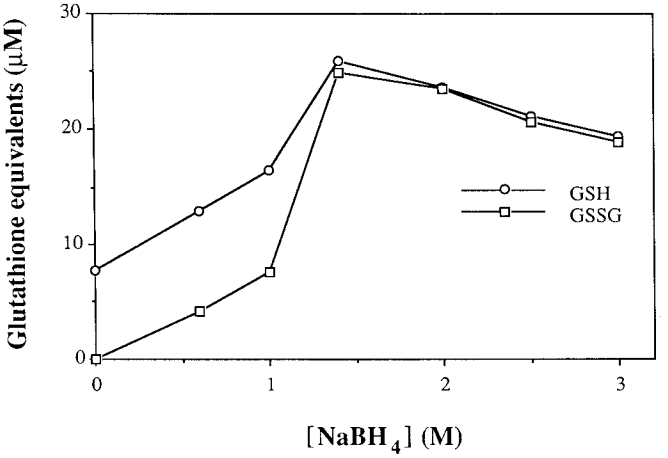

FIG. 2. The effect of the concentration of $NaBH_4$ on the reduction of GSSG in a standard solution. Solutions of GSSG (12.5 μM) and GSH (25 μM) in 5% sulfosalicylic acid/50 μM DTE were pretreated with $NaBH_4$ at concentrations indicated and then derivatized with mBrB using procedure 1 (as described under Materials and Methods). The concentrations of $NaBH_4$ refer to the standard solution.

Under appropriate reducing conditions GSH and GSSG gave exactly the same yield of the GSH–bimane adduct, which also equals the yield obtained when GSH was assayed by the procedure (procedure 5) developed for GSH (see Fig. 5). Given these findings, we routinely used a $NaBH_4$ concentration of 1.4 M (refers to the standard solution), corresponding to a final concentration of 0.15 M.

Reduction of Total Oxidized Glutathione in Plasma

HPLC of stored plasma derivatized with mBrB showed a very small peak corresponding to trace amounts of GSH in the sample (Fig. 3). Following $NaBH_4$ treatment, larger amounts could be demonstrated in the mBrB-derivatized plasma, probably originating from oxidized glutathione (Fig. 3). Figure 3 also shows that the GSH–bimane peak is well separated from other peaks.

We determined the formation of the GSH–bimane adduct in whole plasma and in whole plasma spiked with 5 μM GSSG as a function of increasing concentration of $NaBH_4$. The two curves showed maxima at the same $NaBH_4$ concentration, and a total recovery of GSSG was obtained (Fig. 4). Notably, the optimal concentration of $NaBH_4$ required to obtain maximal yield of GSH from plasma was slightly above that observed for GSSG dissolved in 5% sulfosalicylic acid/50 μM DTE (standard solution) (Fig. 4). Given these results we used 2 M $NaBH_4$ (final concentration of 0.21 M) when total plasma glutathione was measured (procedure 1).

DTE and Linearity of the Assay for GSH and GSSG

The assay for GSH was not linear but upward concave, consistent with loss of a significant fraction of GSH at low concentrations (Fig. 5). This deviation from linearity was partly prevented by inclusion of 50 μM cysteine and abolished by 50 μM DTE in the standard solution (5.4 μM final in the assay mixture) (Fig. 5). With this concentration of DTE present in the assay, the standard curves for both GSH and GSSG were linear over the range 0.1 to 10 μM (GSH equivalents). For this reason plasma samples were routinely supplemented with 50 μM DTE prior to analysis. This low concentration of DTE does not interfere with the red./ox. relationships of glutathione or the chromatographic resolution of the GSH adduct (results not shown).

The Effect of DMSO

When the assay for GSSG was performed in an aqueous phase lacking organic solvents the yield of the GSH–bimane adducts obtained from a standard sample of GSSG was always lower than the yield from the same amount of GSH assayed by the procedure developed for reduced glutathione (procedure 5). This problem can be overcome by preparing separate standard curves for GSH and GSSG. However, we decided to develop condi-

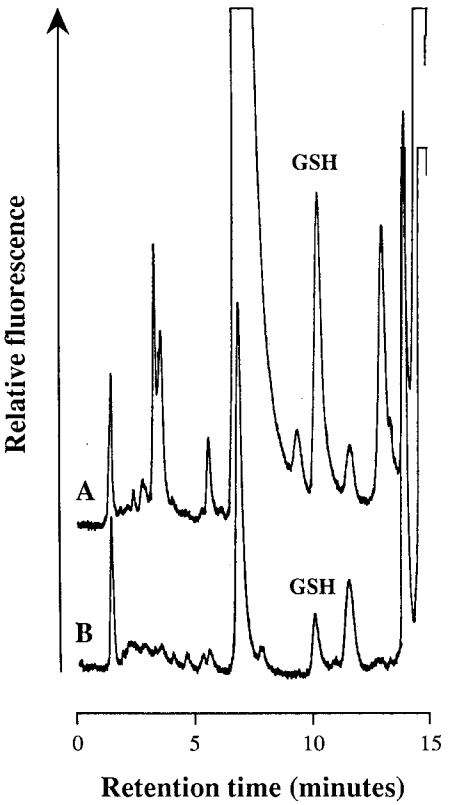

FIG. 3. Reversed-phase high-performance liquid chromatogram of mBrB-derivatized plasma (frozen and thawed once). (A) Pretreated with 2.0 M $NaBH_4$ (the concentration refers to the standard solution and procedure 1 was used as described under Materials and Methods). (B) No treatment.

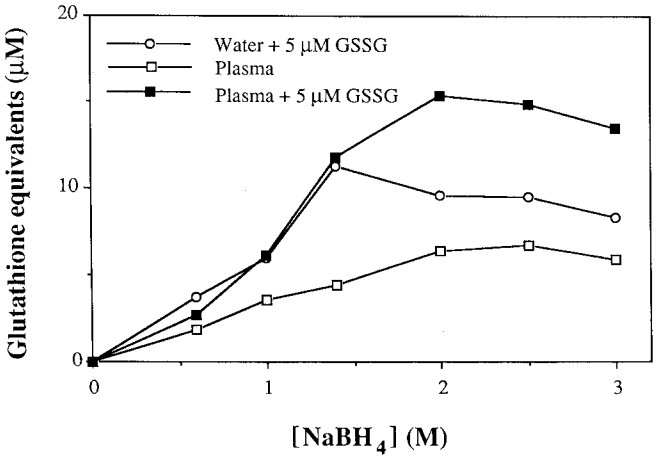

FIG. 4. The effect of the concentration of $NaBH_4$ on the reduction of GSSG in plasma. GSSG was added to water or plasma to a final concentration of 5 μM. Aliquots of these solutions together with plasma not supplemented with GSSG were pretreated with $NaBH_4$ at the concentrations indicated (refers to the standard solution) and then derivatized with mBrB using procedure 1.

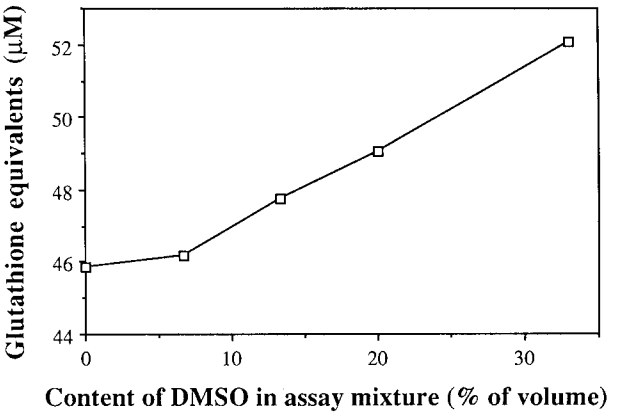

FIG. 6. The effect of the concentration of DMSO on the yield of GSSG. GSSG (25 μM) in 5% sulfosalicylic acid/50 μM DTE was assayed using procedure 1. The concentration of DMSO was increased at the expense of water. The final volume (280 μl) of the incubation mixture and the concentrations of all other components were held constant.

tions so that the yield for GSH equalled that for GSSG. We found that various substances may increase the yield, for example sodium chloride (results not shown), but DMSO gave the most favorable results. Figure 6 shows a typical curve obtained from the analysis of GSSG in a standard solution, in the presence of increasing concentrations of DMSO in the incubation mixture. The figure demonstrates that the inclusion of approximately 25% DMSO (final) in the assay mixture for GSSG gives a yield which is the same as that obtained when an equivalent amount of GSH was assayed by procedure 5. In addition, DMSO has outstanding properties as to solubilizing acid-precipitated protein and making disulfides in native/denatured proteins accessible to reductants.

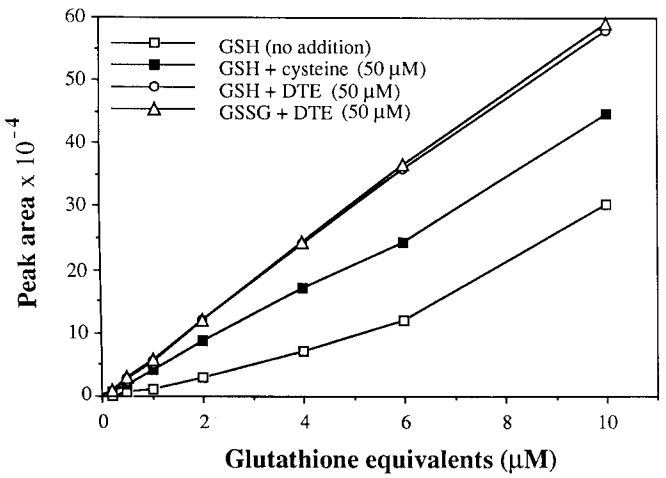

FIG. 5. Standard curves for GSH and GSSG. The standards were dissolved in 5% sulfosalicylic acid with or without coaddition of 50 μM DTE or cysteine. GSH and GSSG were analyzed by procedures 5 and 3, respectively.

Differential Determination of GSH and GSSG by Sequential Addition of NEM and $NaBH_4$

NEM blocks free thiol groups (20), and we observed that this reagent completely abolished assayable GSH over a wide concentration range in a standard solution (Fig. 7A). When we added NEM and $NaBH_4$ to the assay mixture in that order, assayable GSSG was equal to the amount added to the sample (Fig. 7B). Notably, both GSH and GSSG were assayed by procedure 2 in this experiment, i.e., in the presence of $NaBH_4$. Consequently, the results of Fig. 7A imply that the GSH–NEM adduct is resistant to the $NaBH_4$ treatment and no liberation of free GSH occurs.

Recovery and Precision of the Assay

The recovery was close to 100% for both GSH and GSSG added to stored plasma samples (Table 1). The within-day precision (CV) of the assay of reduced (at 5 μM) and oxidized (at 2.5 μM) glutathione in plasma was 7.6 and 7.4%, respectively (Table 1).

Various Species of Glutathione in Plasma from Healthy Subjects

We measured the amount of GSH, acid soluble (free) oxidized glutathione (GSSG + GSSR), ProSSG, and total glutathione (GSH + GSSG + GSSR + ProSSG) in plasma from 10 healthy subjects, and the data are listed in Table 2. The GSH level was 1.93 ± 0.37 μM, GSSG + GSSR was 1.74 ± 0.59 μM, and ProSSG was 2.06

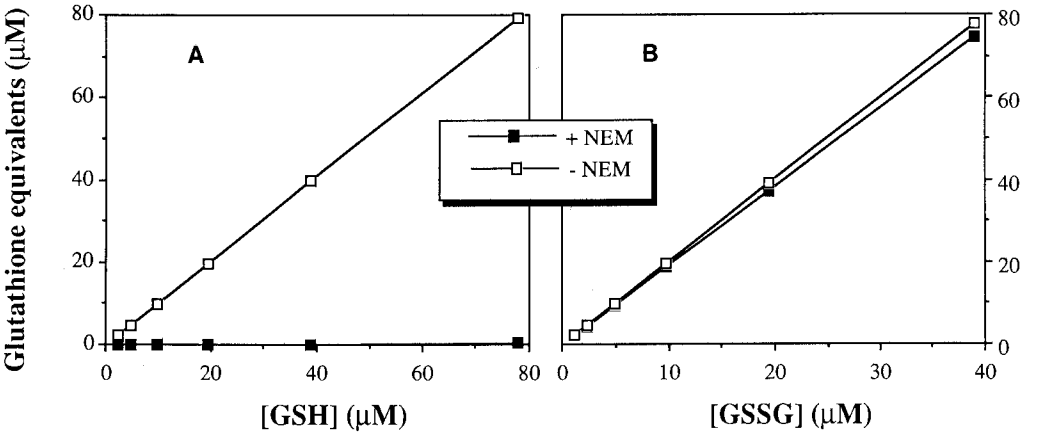

FIG. 7. Blockage of the thiol group of GSH with NEM (A) and the effect of NEM on the assay for GSSG (B). Solutions of GSH and GSSG in 5% sulfosalicylic acid/50 μM DTE were neutralized with the base as specified under Materials and Methods and treated with 12 mM (final) NEM for 20 min at room temperature. In parallel samples, the NEM solution was replaced by an equal volume of water. After incubation, 30 μl of the mixture was withdrawn and derivatized with mBrB by procedure 2. (A) GSH. (B) GSSG.

± 0.62 μM. Notably, for each individual we observed that measured total glutathione was equal to the sum of GSH, GSSG + GSSR, and ProSSG, i.e., the calculated total glutathione (Table 2).

Dynamic State between Glutathione Species in Freshly Prepared Plasma

We determined the time-dependent changes in the amount of GSH, oxidized (GSSG + GSSR), and protein-bound glutathione in freshly prepared plasma. There was a rapid decline in GSH within the first 5–10 min, and most glutathione was recovered as oxidized or protein-bound glutathione. Notably, the sum of all these species equals total glutathione, which slowly decreased by about 20% within 2 h (Fig. 8).

DISCUSSION

In a series of papers Fahey and co-workers (19,21,22) have reported a sensitive and specific method for the determination of biological thiols. This method is based upon conversion of the thiols to fluorescent derivatives by reaction with mBrB and separation of the derivatives by reversed-phase high-performance liquid chromatography. This method has recently been modified and procedures for analysis of thiols in cells, tissues (23–25), and plasma (17,24,26) have been described.

TABLE 1

Analytical Recovery of GSH and GSSG in Plasma

	Mean	SD	CV (%)
GSH	103	7.8	7.6
GSSG	105	7.8	7.4

Note. GSH or GSSG were added to plasma at concentrations of 5 and 2.5 μM respectively. $n = 10$ each. Values are percentages of added GSH or GSSG that were measured.

Thiols are present *in vivo* mainly in the reduced form (2) but in certain pathological conditions (8,13) and during exposure to some agents (27,28) a significant fraction of the thiol may become oxidized. Thiols may also become associated with proteins, and enzymes may be involved in the attachment/detachment (1,29).

Attempts have been made to quantitate the free oxidized and protein-bound forms of biological thiols, including glutathione using sulfhydryl reagents (DTE, mercaptoethanol, etc.) as reducing remedy and subsequent derivatization with mBrB. These procedures suffer from several inconveniences (22). First the high concentration of sulfhydryl reagent required may consume mBrB and second large interfering fluorescent peaks may arise together with multiple minor peaks resulting from impurities.

To avoid these problems we chose a nonthiol reducing agent, $NaBH_4$, which is one of the strongest reductants usable in aqueous solvent (30). Two problems caused by $NaBH_4$ could be foreseen. One is related to the effervescence when $NaBH_4$ is exposed to acids, and the second is that $NaBH_4$ may reduce ketone groups which are present in the mBrB molecule. The effervescence was reduced by the presence of acetonitrile in which mBrB was dissolved and the problem was also controlled by using a small assay volume. Reduction of the fluorescent yield through chemical modification of the bimane derivative was prevented by optimizing the $NaBH_4$ concentration (Figs. 2 and 4). The presence of optimal amounts of $NaBH_4$ also during the derivatization process prevented reoxidation of GSH which otherwise readily occurs at neutral or basic pH (1) (Fig. 2).

TABLE 2

Concentration (μM) of Different Glutathione Forms in Plasma Obtained from 10 Healthy Volunteers

Subject	Sex	Age	Free reduced	Free oxidized	Reduced/oxidized	Protein-bound	Total (calculated)	Total (measured)
R.A.	Female	29	1.75	1.43	1.224	2.00	5.18	5.11
G.K.	Female	38	1.41	1.00	1.410	1.72	4.13	4.20
E.B.	Female	40	1.62	1.48	1.095	1.38	4.48	4.50
P.U.	Male	40	2.06	2.10	0.981	3.06	7.22	6.93
A.H.	Male	43	2.19	2.39	0.916	2.17	6.75	6.65
H.B.	Male	42	1.96	1.75	1.120	1.92	5.63	6.07
B.C.	Female	28	2.42	1.45	1.669	1.29	5.16	4.78
E.L.	Male	48	1.46	1.23	1.187	2.26	4.95	5.18
E.T.	Female	51	1.99	1.57	1.268	1.71	5.27	5.56
O.S.	Male	33	2.43	2.97	0.818	3.10	8.50	8.43
Mean ± SD			1.93 ± 0.37	1.74 ± 0.59	1.169 ± 0.248	2.06 ± 0.62	5.73 ± 1.36	5.74 ± 1.30

Note. Blood sampled by venipuncture was immediately centrifuged at 10,000*g* for half a minute. From the plasma, aliquots were withdrawn and instantly analyzed for the various glutathione forms by the respective procedures (acid or sodium borohydride was added to plasma samples exactly 2.5 min after the blood was collected, see Materials and Methods for details). The values for total measured glutathione are obtained directly by using procedure 1 (see Materials and Methods) while the values for total calculated glutathione are the sum of the free reduced, free oxidized, and protein-bound forms.

The ability of $NaBH_4$ to reduce GSSG to GSH is shown in Fig. 1 and the effect of $NaBH_4$ treatment on whole plasma in Fig. 3. Optimal concentrations of the reducing agent were used. Only small amounts of GSH are present in frozen and thawed plasma (Fig. 3). After $NaBH_4$ treatment a GSH peak which was well separated from other fluorescent peaks appeared. Some fluorescent peaks appearing after $NaBH_4$ treatment of the sample stem from the mBrB reagent or impurities therein (Fig. 1) whereas others originate from reduced disulfides other than GSSG (Fig. 3).

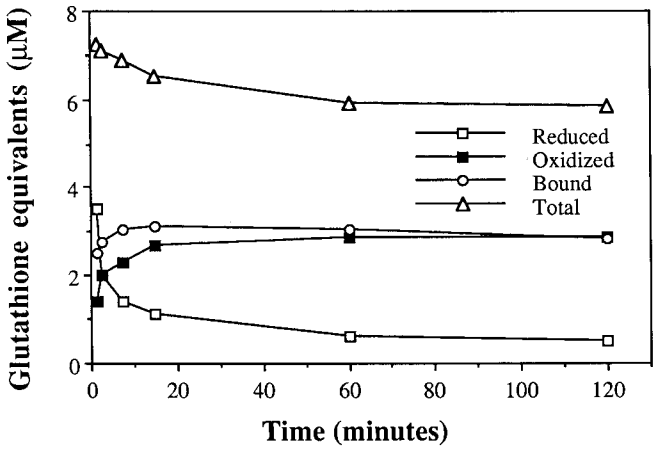

FIG. 8. Determination of reduced, oxidized, protein-bound, and total glutathione in human blood plasma allowed to stand at 4°C. Blood sampled by venipuncture was immediately centrifuged at 10,000*g* for 1 min. From the plasma obtained, aliquots were withdrawn and analyzed for the different glutathione forms at once (2 min after collection) and after they stood in ice for the times indicated. Reduced glutathione (GSH) was assayed by procedure 5, oxidized (GSSG + GSSR) by procedure 2, bound (ProSSG) by procedure 4, and total (GSH + GSSG + GSSR + ProSSG) by procedure 1. See Materials and Methods for details.

A $NaBH_4$ concentration of 1.4 M (final concentration at the time of derivatization is 0.15 M) was optimal for the reduction of free oxidized glutathione, i.e., glutathione present in deproteinized plasma (Fig. 2). A somewhat higher concentration of $NaBH_4$ (2.0 M) was required to obtained maximal yield of total glutathione (Fig. 4) and ProSSG (results not shown). This may be related to the high protein content which may interfere with the $NaBH_4$ effect, or to the reduction or liberation of protein-bound glutathione being dependent on a higher concentration of reductant.

The concentration of glutathione in plasma (<10 μM) is low compared to that found in most tissues. In this concentration range we were not immediately able to obtain linear standard curves. This is a problem noted by others using mBrB to quantify low concentrations of thiols (24). We solved this problem by adding 50 μM DTE to the solvent in which the GSH or GSSG standards were dissolved and also to plasma (Fig. 5). The DTE concentration is not critical, but 50 μM gives linear curves but is not sufficiently high to reduce GSSG (data not shown).

Our goal was that equivalent amounts of GSH and GSSG should give the same peak areas when measured by the different procedures. In an aqueous phase lacking organic solvents the yield of GSSG (assayed by procedure 1) was lower than an equivalent amount of GSH (assayed by procedure 5) (Fig. 6). The yield could be somewhat increased by addition of sodium chloride to the assay mixture (results not shown) and further increased to 100% by addition of appropriate amounts of

DMSO (Fig. 6). This modification and the mere presence of DMSO in the assay mixture bring several advantages. First, separate standard curves for GSH and GSSG are not necessary. Second, total plasma or acid-precipitated protein is easily dissolved in DMSO and this solvent has outstanding properties for making disulfides in intact proteins accessible to reductants (31). Thus, DMSO is a suitable agent for inter alia exposing disulfide groups to reduction.

Direct determination of GSSG can be done by first blocking the free sulfhydryl groups with NEM at neutral pH. This reaction goes to completeness as illustrated in Fig. 7A. Then, the GSSG is reduced by $NaBH_4$. The addition of $NaBH_4$ immediately inactivates excess NEM, but does not liberate GSH from the GSH–NEM adduct (Fig. 7A). This NEM treatment has essentially no effect on the determination of GSSG (Fig. 7B).

One main problem when measuring different glutathione forms in plasma is the rapid oxidative formation of both acid-soluble (free) and protein-bound disulfides (8,13,17,32). This can be prevented by acidification with, for example, 5-sulfosalicylic acid, but not by adding chelating agents like EDTA or EGTA (8,17,33). The formation of free oxidized glutathione is most likely due to a nonenzymatic transhydrogenation between GSH and cysteinylbisglycine, a product of γ-glutamyltranspeptidase (8,34).

We investigated the dynamic relationships among various species of glutathione in freshly prepared plasma (Fig. 8). There was a very rapid decrease in the amount of GSH together with a concomitant increase in both the free oxidized and the protein-bound forms. A similar increase in free oxidized glutathione in plasma exposed to air has been shown previously (8,13), but to our knowledge the time course of protein binding of glutathione in plasma has not previously been assessed. We found that concurrent with a rapid oxidation of GSH there is also a slow decrease in total glutathione (Fig. 8), probably due to degradation catalyzed by the enzyme γ-glutamyltranspeptidase (8). The implications of these results are that plasma samples should be cooled and rapidly prepared and derivatized, especially when comparing ratios between the different glutathione forms. We took such precautions when assaying the various forms in plasma from 10 healthy volunteers (Table 2). In these experiments the time interval between blood collection and start of analysis (addition of acid or $NaBH_4$) was exactly 2.5 min. At this time the amount of free oxidized glutathione equalled the amount of GSH in most samples. Oxidation of a considerable fraction of GSH during 2.5 min could be expected from Fig. 8. Extrapolation of the time curves for the oxidation of GSH (Fig. 8) also suggests that most glutathione in plasma *in vivo* exists as GSH.

The sum of GSH plus free oxidized glutathione (approximately 4 μM) which represents the free glutathione agrees well with values (4–6 μM) for plasma glutathione published by others (13,35). Also our finding that about 2 μM GSH could be released from plasma protein by reduction is in agreement with a previous report (35).

To evaluate our method we compared the values for total plasma glutathione obtained by calculation with those obtained by direct analysis (procedure 1). Notably, when adding up the values for GSH, free oxidized (GSSG + GSSR), and ProSSG, the sum equalled the values for total glutathione as determined by procedure 1, which involved reduction of whole plasma.

The sensitivity of the method allows detection of quantities > 2 pmol. The detection limit is in fact determined by the presence of reagent impurities which results in multiple, small interfering peaks. We optimized the HPLC system to avoid such interference, and the resulting high chromatographic resolution together with the linearity of the standard curve at low concentrations are the major reasons for the low detection limit compared to that published by others (>10 pmol) (22,35).

Satisfactory analytical precision was obtained (Table 1) without including an internal standard. There are objections to the inclusion of thiols as internal standard to compensate for oxidation or other chemical reactions (36). Differing rates of reaction for different thiols may be a source to erratic results.

The present method is limited to the detection of glutathione either as the free thiol or disulfides (free or protein-bound) plus most likely also glutathione thiol esters (1) and does not differentiate between specific disulfides or mixed disulfides. The method of Reed *et al.* (37,38) is probably still the method of choice for the identification and determination of various acid-soluble, oxidized forms of glutathione.

In conclusion, the present method includes five procedures designed for the determination of free reduced glutathione (GSH), free oxidized glutathione (GSSG + GSSR), total free glutathione (GSH + GSSG + GSSR), protein-bound glutathione (ProSSG), or total glutathione (GSH + GSSG + GSSR + ProSSG). All procedures are based on derivatization of the free thiol group with the fluorescent agent mBrB, and quantitation of the GSH–bimane derivative by reversed-phase liquid chromatography. The selective determination of these glutathione forms was obtained by sequential addition of chemicals, including mBrB, DTE, $NaBH_4$, NEM, and DMSO. $NaBH_4$ reduces disulfides to GSH, NEM blocks free thiols, and DMSO solubilizes proteins and exposes disulfides in proteins to reduction. The conditions are optimized to obtain maximal effect from one reagent without interfering with the thiol-directed effect of a previously added reagent. This assay has proven reliable for the determination of various forms of glutathione in human plasma, and the principles may be adopted for the determination of other thiols and disulfides in plasma as well as in other biological specimens.

ACKNOWLEDGMENTS

This work was supported by grants from the Norwegian Cancer Society and the Norwegian Research Council for Science and Humanities.

REFERENCES

1. Meister, A., and Anderson, M. E. (1983) *Annu. Rev. Biochem.* **52,** 711–760.
2. Meister, A. (1988) *J. Biol. Chem.* **263,** 17,205–17,208.
3. Mitchell, J. B. (1988) *ISI Atlas Sci. Pharmacol.* **2,** 155–160.
4. Ross, D. (1988) *Pharmacol. Ther.* **37,** 231–249.
5. Ishikawa, H., and Sies, H. (1984) *J. Biol. Chem.* **259,** 383.
6. Mimic-Oka, J., Djukanovic, L., and Markovic, B. (1988) *Biochem. Med. Metab. Biol.* **39,** 48–54.
7. Lauterburg, B. H., and Velez, M. E. (1988) *Gut* **29,** 1153–1157.
8. Magnani, M., Novelli, G., and Palloni, R. (1984) *Clin. Physiol. Biochem.* **2,** 287–290.
9. Chawla, R. K., Lewis, F. W., Kutner, M. H., Bate, D. M., Roy, R. G. B., and Rudman, D. (1984) *Gastroenterology* **87,** 770–776.
10. Beutler, E., and Gelbart, T. (1985) *J. Lab. Clin. Med.* **105,** 581–584.
11. Lash, L. H., and Jones, D. P. (1985) *Arch. Biochem. Biophys.* **240,** 583–592.
12. Anderson, M. (1985) *in* Methods in Enzymology (Meister, E., Ed.), Vol. 113, pp. 548–555, Academic Press, San Diego.
13. Curello, S., Ceconi, C., Cargnoni, A., Cornacchiari, A., Ferrari, R., and Albertini, A. (1987) *Clin. Chem.* **33,** 1448–1450.
14. Adams, J. D., Johannessen, J. N., and Bacon, J. P. (1987) *Clin. Chem.* **33,** 1675–1676.
15. Mårtensson, J. (1987) *J. Chromatogr.* **420,** 152–157.
16. Richie, J. P. J., and Lang, C. A. (1987) *Anal. Biochem.* **163,** 9–15.
17. Velury, S., and Howell, S. B. (1988) *J. Chromatogr.* **424,** 141–146.
18. Johansson, M., and Lenngren, S. (1988) *J. Chromatogr.* **432,** 65–74.
19. Newton, G. L., Dorian, R., and Fahey, R. C. (1981) *Anal. Biochem.* **114,** 383–387.
20. Sacchetta, P., Di Cola, D., and Federici, G. (1986) *Anal. Biochem.* **154,** 205–208.
21. Fahey, R. C., Newton, G. L., Dorian, R., and Kosower, E. M. (1980) *Anal. Biochem.* **107,** 1–10.
22. Fahey, R. C., Newton, G. L., Dorian, R., and Kosower, E. M. (1981) *Anal. Biochem.* **111,** 357–365.
23. Minchinton, A. I. (1984) *Int. J. Radiat. Oncol. Biol. Phys.* **10,** 1503–1506.
24. Burton, N. K., and Aherne, G. W. (1986) *J. Chromatogr.* **382,** 253–257.
25. Anderson, M. E., and Meister, A. (1989) *FASEB J.* **3,** 1632–1636.
26. Burton, N. K., Aherne, G. W., and Marks, V. (1984) *J. Chromatogr.* **309,** 409–414.
27. Collison, M. W., Beidler, D., Grimm, L. M., and Thomas, J. A. (1986) *Biochim. Biophys. Acta* **885,** 58–67.
28. Kramer, R. A., Zakher, J., and Kim, G. (1988) *Science* **241,** 694–697.
29. Lou, M. F., Poulsen, L. L., and Ziegler, D. M. (1987) *in* Methods in Enzymology (Jakoby, W. B., and Griffith, O. W., Eds.), Vol. 143, pp. 124–129, Academic Press, San Diego.
30. Fieser, L. F., and Fieser, M. (1967) Reagents for Organic Synthesis, Wiley, New York.
31. Kosower, N. S., and Kosower, E. M. (1987) *in* Methods in Enzymology (Jakoby, W. B., and Griffith, O. W., Eds.), Vol. 143, pp. 76–84, Academic Press, San Diego.
32. Anderson, M. E., and Meister, A. (1980) *J. Biol. Chem.* **255,** 9530–9533.
33. Wendel, A., and Cikryt, P. (1980) *FEBS Lett.* **120,** 120.
34. Griffith, O. W., and Tate, S. S. (1980) *J. Biol. Chem.* **255,** 5011–5014.
35. Cotgreave, I. A., and Moldéus, P. (1986) *J. Biochem. Biophys. Methods.* **13,** 231–249.
36. Fahey, R. C., and Newton, G. L. (1987) *in* Methods in Enzymology (Jakoby, W. B., and Griffith, O. W., Eds.), Vol. 143, pp. 85–96, Academic Press, San Diego.
37. Reed, D. J., Babson, J. R., Beatty, P. W., Brodie, A. E., Ellis, W. W., and Potter, D. W. (1980) *Anal. Biochem.* **106,** 55–62.
38. Fariss, M. W., and Reed, D. J. (1987) *in* Methods in Enzymology (Jakoby, W. B., and Griffith, O. W., Eds.), Vol. 143, pp. 101–109, Academic Press, San Diego.