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Status of Glutathione and Other Thiols and Disulfides in Human Plasma

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ABSTRACT. While plasma thiols, including homocysteine (HCys), glutathione (GSH), and cysteine (Cys), are being investigated as potential indicators of disease risk and health status, low levels, poor stability, and the lack of comprehensive methodologies have hampered their accurate assessment. Using our previously described HPLC with electrochemical detection method, our goal was to assess levels, stability, and distribution of biologically relevant thiols and disulfides in human plasma. In fresh plasma, processed immediately after collection, low levels of Cys, cystine, Cys-Gly, and the mixed disulfide Cys-GSH (CSSG) were consistently observed, whereas the levels of GSH and Cys-Gly disulfide were often below the limits of detection. These profiles were a consequence of poor thiol stability, as thiol standards added to human plasma were lost rapidly due to autoxidation or formation of mixed disulfides. A 75% loss of added GSH observed after 30 min was accounted for completely by the formation of GSH disulfide (24%) and CSSG (74%). Similar changes were found with other thiols when added to plasma. Thiols lost to oxidation were recovered quantitatively by reducing samples with potassium borohydride (KBH_4) prior to analysis. In a study of 106 healthy adults, mean total thiol levels in plasma were: Cys (201 μM) > Cys-Gly (101 μM) > HCys (7 μM) > γ-Glu-Cys (5 μM) > GSH (4 μM). All together, these results account for the poor stability of thiols in plasma and provide a method for their comprehensive and accurate determination. BIOCHEM PHARMACOL **60**;1:19–29, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. glutathione; cysteine; homocysteine; thiols; disulfides; human blood

Low molecular weight thiols and disulfides, including GSH, Cys,† and HCys, are critical cellular components that play numerous important roles in metabolism and homeostasis. GSH, the most abundant non-protein thiol, has many wide-ranging functions within the cell including detoxification of free radicals and peroxides, regulation of cell growth and protein function, and maintenance of immune function [1]. Cys also plays numerous important roles as a key extracellular reducing agent, critical substrate for protein synthesis, and rate-limiting precursor for GSH and taurine synthesis. HCys is a critical regulatory intermediate of the Met cycle that serves as a precursor for Cys via the transsulfuration pathway as well as for Met through remethylation.

Altered levels of thiols in plasma have been linked with specific pathological conditions. Highly elevated plasma and urine levels of HCys are a clinically relevant indicator of deficiencies in cystathionine β-synthase, methylenetetrahydrofolate reductase, or methionine synthase, a well-known but rare group of inborn errors of metabolism known commonly as homocysteinuria [2]. More recently, mildly elevated plasma HCys levels have been associated with an increased risk for cardiovascular and cerebrovascular diseases [3, 4]. Common causes of moderate homocysteinemia are deficiencies in specific vitamins including folate or vitamin B_{12}, coenzymes for HCys remethylation, and pyridoxal phosphate, a coenzyme for cystathionine β-synthase [5, 6]. While measurements of plasma Cys and GSH are not commonly used diagnostically, altered levels have been implicated in a number of pathological conditions, including Alzheimer's and Parkinson's diseases for Cys [7], and diabetes, macular degeneration, and HIV disease for GSH [8–10]. Other abundant thiols and disulfides including Cys-Gly have received little research attention in this regard.

A metabolic link among these three thiols is well established, with HCys and Cys being key components of the transsulfuration pathway and Cys being the rate-limiting substrate for the synthesis of GSH. However, there is little information on the dynamic relationship between these thiols in human plasma in either health or disease. Additionally, virtually no information is available regard-

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† *Abbreviations*: Cys, cysteine; cyst(e)ine, cysteine or cystine; CSSG, cysteine and glutathione mixed disulfide; Cys-(γ-Glu-Cys), cysteine and γ-Glu-Cys mixed disulfide; Cys-(Cys-Gly), cysteine and Cys-Gly mixed disulfide; HCys, homocysteine; $(HCys)_2$, homocystine; $(Cys\text{-}Gly)_2$, Cys-Gly disulfide; Met, methionine; DTT, dithiothreitol; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; MPA, metaphosphoric acid; and DEC, dual electrochemical detection.

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ing the levels and biological significance of other abundant low molecular weight thiols in plasma.

The comprehensive measurement of thiols and disulfides in human plasma has proven to be problematic. Most thiols, including GSH and HCys, are present in low concentrations (1–10 μM) and are very unstable in plasma. An almost immediate and complete loss of GSH [11–13] has been observed when added to plasma. A rapid decrease in plasma levels of numerous thiols has been reported when freshly prepared plasma is incubated at room temperature [12]. In the case of GSH, the loss was apparently due to the formation of an unidentified low molecular weight GSH mixed disulfide [11]. Unlike GSH, the loss of HCys is apparently due to the formation of protein mixed disulfides, since >70% of the HCys found in plasma is in that form [14]. The measurement of GSH in plasma can also be affected by the high levels of GSH found in erythrocytes (1 mM), so that even a slight degree of hemolysis could result in falsely high GSH levels. Therefore, sample processing is a critical step in the analysis. Inadequate methodology has been a major problem leading to either artificially high or low concentrations of GSH being reported [14, 15].

Because of these problems, few comprehensive methods for the accurate measurement of thiols and disulfides have been described. Two basic approaches have been used to circumvent the loss of thiols due to oxidation during processing. In one approach, plasma samples are reduced with agents such as $NaBH_4$ or DTT prior to analysis to release thiols that had been lost to oxidation [13, 16]. However, using this approach, only "total thiols" (all forms including disulfides, mixed disulfides, and free thiols) can be measured, and reduced and oxidized forms cannot be distinguished.

In another approach specific for GSH, DTNB, added to whole blood immediately after collection and prior to separation of plasma from erythrocytes, forms a conjugate with the thiol group of GSH, preventing autoxidation or formation of mixed disulfides [17]. Once GSH is stabilized in this fashion, the resultant GSH-conjugate is analyzed by the DTNB enzymatic recycling method [18]. This method requires that blood samples be treated with DTNB directly after collection, and is only suitable for the measurement of total glutathione and not other thiols and disulfides.

To determine actual *in vivo* concentrations of thiols in plasma, Mansoor *et al*. [19] added NEM or monobromobimane directly to blood collection tubes prior to blood collection. Thus, existing thiols in plasma would be "trapped" by these agents and later analyzed by HPLC with fluorescence detection. Using this method, very low levels were observed for all thiols examined: GSH (2–6 μM), HCys (0.1–0.4 μM), Cys-Gly (2–4 μM), and Cys (3–17 μM).

Because of the importance of biological thiols and disulfides and the emerging use of plasma levels as biomarkers of disease status, there is a need to fully understand the dynamic relationship between all of the thiol and disulfide components in human plasma. To this end, we adapted our previously described HPLC method with DEC for the simultaneous determination of thiols and disulfides in biological samples [20]. This method was used based upon its ability to detect all biologically relevant thiols and disulfides simultaneously without the need for prior derivatization of samples. It was modified to include the highly specific and sensitive analysis of the wide range of GSH-related thiols and disulfides present in plasma [21]. Our current objectives were to utilize the HPLC–DEC method to assess the levels, stability, and distribution of biologically important thiols and disulfides in human plasma. In the development and validation of these procedures, particular attention was given to the problems of low GSH concentrations, thiol instability, and extensive variability of GSH levels previously observed in human plasma.

MATERIALS AND METHODS

Reagents

GSH, GSSG, Cys, cystine, HCys, $(HCys)_2$, Cys-Gly, $(Cys\text{-}Gly)_2$, and γ-Glu-Cys were obtained from the Sigma Chemical Co. Reductacryl reagent was obtained from Calbiochem. All other chemicals of high purity or HPLC grade were obtained from the Aldrich Chemical Co., Mallinckrodt, or EM Science. Deionized water was prepared using a Millipore Milli-Q System.

Chromatography

Chromatography was carried out using a Waters 510 pump, Rheodyne 7125 injector with a 50 μL injection loop, Bioanalytical Systems dual LC-4B amperometric detector, dual Au/Hg working thin layer electrode, and Ag/AgCl reference electrodes (BAS). The working electrodes were in the series configuration with the upstream set at a potential of −1.0 V to reduce disulfides to their corresponding thiols and the downstream electrode at +0.15 V for thiol detection. A Hitachi D-2500 Chromato-Integrator was used for peak integration. Stainless steel was replaced with PEEK components (Upchurch) wherever possible, to prevent metal interactions with thiols.

Optimal separation was achieved using a 5 μm Inertsil ODS 2 silica column (250 × 4.6 mm i.d.) (GL Sciences, obtained through Alltech) with a mobile phase of 93.25% (v/v) 0.1 M monochloroacetic acid, 5% methanol, 1.75% *N*,*N*-dimethylformamide, and 2.25 mM heptanesulfonic acid, adjusted to a final pH of 2.8 with NaOH. This system was particularly effective when analyzing samples containing multiple closely eluting mixed disulfides. Samples were run isocratically at a flow rate of 1.0 mL/min. The resultant profiles were quantified with external standards based on peak areas.

Using this procedure, a standard mixture containing 14 biologically important thiols and disulfides was analyzed (Fig. 1). With the upstream electrode turned on, both thiols and disulfides are detected, whereas with the upstream electrode turned off, only thiols are detected. Although

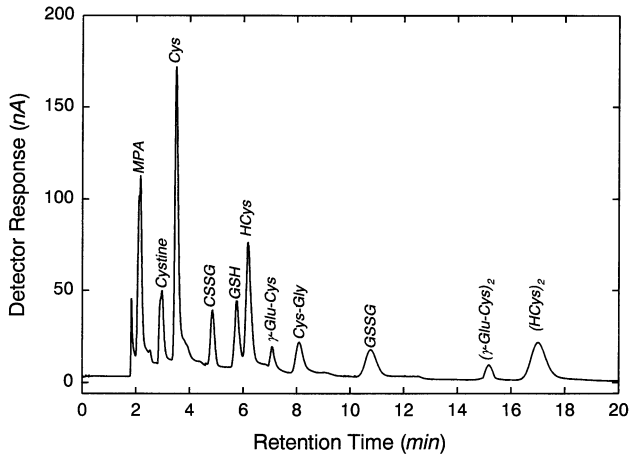

FIG. 1. HPLC–DEC chromatogram of thiol and disulfide standards. A standard mixture containing 25 μL of each thiol and disulfide was prepared and analyzed as described in the text. Sample volumes were 50 μL.

ergothioneine (3.06 min) was not resolved completely from cystine (3.17 min), it was differentiated easily by reanalyzing with the upstream electrode turned off, so that cystine would not be detected. Though not shown, $(Cys\text{-}Gly)_2$ eluted at 18.31 min, and the mixed disulfides eluted as follows: (Cys)-(γ-Glu-Cys) at 4.17 min, CSSG [(Cys)-(GSH)] at 4.52 min, and (Cys)-(Cys-Gly) at 5.56 min.

Standards

Stock solutions of standards were prepared as follows: 1.0 mM solutions of ergothioneine, Cys, GSH, HCys, γ-Glu-Cys, $(\gamma\text{-Glu-Cys})_2$, Cys-Gly, $(Cys\text{-}Gly)_2$, GSSG, and CSSG in 3 mM EDTA; 1.0 mM solutions of cystine and $(HCys)_2$ in 10 mM NaOH. Working standards were prepared daily from stock solutions by diluting in HPLC mobile phase.

Thiol and disulfide concentrations in plasma are presented in micromolar units except where comparisons between oxidized and reduced forms are made or combinations of oxidized and reduced forms are calculated. In these two latter circumstances, μequivalent (μEq)/L units are used, where μEq/L = μM for free thiols and mixed disulfides and μEq/L = 0.5 μM for all homodisulfides (GSSG, cystine, and so on).

Preparation of CSSG and Other Mixed Disulfides

CSSG standard was synthesized using a modification of a previously described method [22] as follows: solutions of 4.5 mM GSH and 22.5 mM Cys were incubated in a 32 mM sodium bicarbonate buffer containing 13.5 mM iodine (pH 8.0). The mixture was incubated for 5 days at room temperature and purified on a 50 mL Dowex 1-X2 anion exchange (Bio-Rad) column. The column was pre-washed with 8 vol. of 0.5 M NaOH, 8 vol. of 0.5 M acetic acid, and 10 vol. of H_2O. After applying the sample, the column was washed with 14 vol. of 0.02 M acetic acid, then with 0.9 vol. of 0.3 M acetic acid, and was eluted with 2 vol. of 0.3 M acetic acid. The eluate was flash-frozen, lyophilized, and stored under argon. Prior to use, a 1.0 mM stock solution of CSSG was prepared as above.

The structure and purity of the CSSG were confirmed by mass spectrometry using a Finnigan MAT TSQ 700 tandem quadruple mass spectrometer with an ICIS data system and an ULTRIX-32 operating system. The lyophilized CSSG was diluted to 1 mg/mL in H_2O, and 1 μL was injected with a mobile phase of 20 mM diethylamine at pH 5. Analysis was carried out using electrospray ionization monitored from 200 to 500 a.m.u. at a rate of 90 a.m.u./sec. The m/z spectrum had a major peak with a mass of 427.1, representing protonated CSSG. A peak with a mass of 308.1 represents protonated GSH. Since HPLC–DEC analysis of the CSSG standard showed no GSH contaminant, the GSH in the MS analysis appeared to be fragmented from the CSSG by the electrospray source. Smaller peaks representing Na and K adducts of GSH (330.2 to 385 m/z) and CSSG (448.9 to 465.2 m/z) were also observed.

For the synthesis of (Cys)-(Cys-Gly), solutions of 0.516 mM Cys and 0.129 mM Cys-Gly were incubated in 0.97 mM sodium bicarbonate buffer containing 1.29 mM iodine (pH 12.0). The mixtures were incubated overnight, neutralized with MPA, diluted with mobile phase, and analyzed by HPLC–DEC. (Cys)-(γ-Glu-Cys) was synthesized as above, using 0.129 mM γ-Glu-Cys with 0.516 mM Cys.

Human Subjects and Blood Sample Collection

All subjects provided their informed consent approved by our Institutional Human Subjects Committee.

STUDY 1. Fifteen healthy volunteers (8 females, 7 males) ranging in age from 24 to 43 years participated in the study. Blood was collected by venipuncture into evacuated tubes containing EDTA. To obtain plasma, the whole blood was centrifuged immediately at 2000 *g* in a refrigerated centrifuge for 10 min. Within 20 sec after centrifugation, the plasma was removed, deproteinized by the addition of 1 vol. of 5% (w/v) MPA, incubated at 4° for 15 min, and centrifuged at 10,000 *g* for 2 min. The acid-soluble supernatant fractions were analyzed by HPLC–DEC. To assess stability, aliquots of unprocessed plasma samples were stored for different periods of time at 4°, −20°, or −80°.

STUDY 2. One hundred and six healthy adults (54 males and 52 females) ranging in age from 23 to 72 were recruited during an in-house screening of American Health Foundation employees. Blood was collected by venipuncture, immediately placed on ice, and, within 30 min, processed by centrifugation to obtain plasma. Acid-soluble fractions of plasma samples were obtained as above. Aliquots of plasma were also reduced with potassium borohydride (KBH_4) for analysis of protein-bound thiols (see below).

Recovery Studies

Known amounts of standards of the following thiols: GSH, Cys, Cys-Gly, and HCys, and the disulfides GSSG, cystine, and CSSG were each added to human plasma. Samples were incubated at 4° and processed as above. The incubation reaction was terminated by the addition of 1 vol. of 5% MPA to the samples.

Reduction of Plasma

Fresh plasma and plasma with added GSH were reduced with either Reductacryl (Cleland's reagent immobilized on a polyacrylamide resin) or KBH_4.

REDUCTACRYL. Reductacryl reagent (50 mg) was added to 1 mL of plasma, and samples were shaken for 20 min at room temperature on an Ames aliquot mixer. Reduced samples then were processed as above, with a final concentration of 2.5% MPA and centrifuged before being analyzed by HPLC–DEC.

KBH_4. Reductions were performed using a slight modification of a previously described method [23]. A solution of 8 M urea and 1 mM EDTA (500 μL) was added to 500 μL plasma, shaken on an Ames aliquot mixer for 10 min, and incubated for 10 min at 40°, after which 30 μL of octanol and 500 μL of 1.3 M KBH_4 were added (final KBH_4 concentration = 0.43 M). After incubating for 1 hr at 40°, 1 mL of 20% MPA was added in 250 μL aliquots to prevent excess foaming. After 15 min at room temperature, the samples were vortexed and centrifuged at 10,000 *g* for 10 min. The acid-soluble supernatants were removed and filtered through 0.45 μm Acrodisc filters (Gelman Sciences) prior to HPLC–DEC analysis.

Bound GSH Analysis

Acid-insoluble pellets from MPA extractions were reduced to release protein-bound GSH. Pellets were washed twice by resuspending in 1 mL of 5% MPA, and once in 1 mL of 1 mM EDTA with centrifugation at 14,000 *g* for 5 min after each wash. The pellets were resuspended in 1 mL of 8 M urea/1 mM EDTA, and reduced as described above by the addition of 0.5 mL of 1.3 M KBH_4. After a 45-min incubation at 40°, 1 mL of 20% MPA was added, samples were centrifuged, and supernatant fractions were removed and filtered through a 0.45 μm nylon Acrodisc syringe filter (Gelman Sciences) prior to analysis.

DTNB Stabilization of Plasma GSH

Plasma GSH was stabilized by treating blood with DTNB. Immediately after collection, 100 μL of DTNB solution (20 mg DTNB in 1 mL of 0.1 M Na_2HPO_4/0.005 M disodium EDTA, pH 7.5) was added to 1 mL of whole blood and mixed by gentle inversion for 5 min. After centrifugation at 2000 *g* for 15 min, the plasma was removed and, at various times thereafter, deproteinized by the addition of 0.5 vol. of 5% MPA and centrifuged at 14,000 *g* for 2 min. The acid-soluble fractions were removed and analyzed for GSH.

Since high concentrations of DTNB interfere with the HPLC–DEC analysis, GSH + GSSG was determined in DTNB-treated plasma by the DTNB-enzymatic recycling method [18] modified for use with 96-well plates [24].

In some experiments, cyst(e)ine levels obtained from HPLC were compared with those obtained using the acid ninhydrin method of Gaitonde [25].

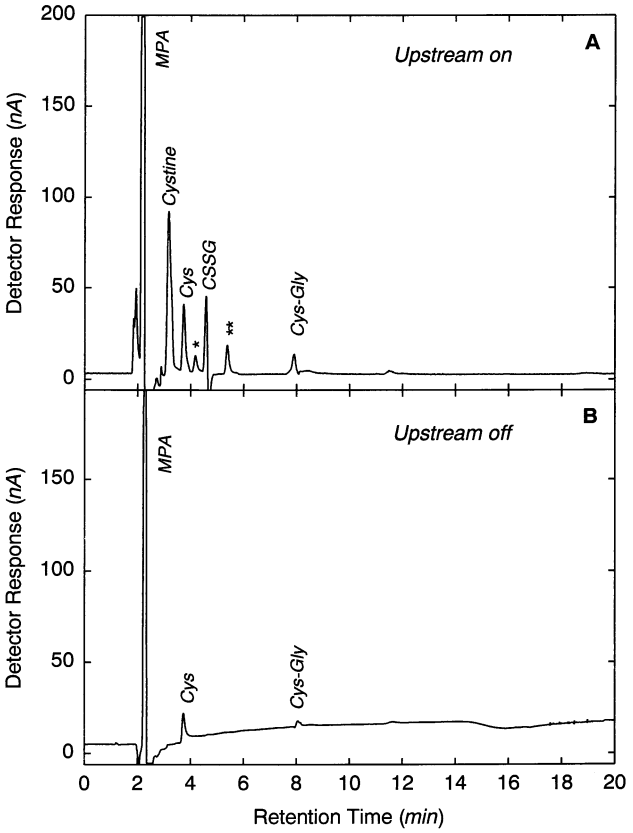

FIG. 2. Typical HPLC–DEC chromatogram of a human plasma sample. Blood samples were collected from healthy adults and centrifuged immediately at 4° to obtain plasma. Within 20 sec after centrifugation, plasma was deproteinized by the addition of MPA, and the acid-soluble fraction was isolated by centrifugation and analyzed by HPLC–DEC. Sample volumes were 50 μL. Details of sample processing and HPLC–DEC conditions are provided in the text. The HPLC–DEC profile is representative of >100 independent experiments. (A) Upstream electrode turned on; (B) upstream electrode turned off. Unknown peaks were identified based upon retention time as follows: (*) Cys-(γ-Glu-Cys); and (**) Cys-(Cys-Gly).

RESULTS

Fresh plasma samples from fifteen healthy subjects (study 1) were processed at 0–4° within 1 min of being drawn, and were analyzed by HPLC–DEC. A typical chromatogram obtained from these samples is provided in Fig. 2A. Cys and cystine were the main metabolites found, with Cys values

ranging from 4.96 to 21.1 μEq/L, cystine from 33.2 to 212 μEq/L, and cyst(e)ine (Cys + cystine) from 76.5 to 233 μEq/L. The cyst(e)ine levels obtained by HPLC–DEC were highly correlated with those obtained using the method of Gaitonde ($r = 0.992$) [25]. GSH and GSSG were generally not found in fresh human plasma samples, and, when present, the levels were low and below minimum detectable quantities (8 nM for GSH and 10 nM for GSSG). Similar results for GSH and GSSG were obtained using the DTNB enzymatic recycling assay for total glutathione. Cys-Gly was found in all plasma samples analyzed, with concentrations ranging from 1.8 to 3.2 μM. CSSG was also found in all human plasma samples analyzed, ranging in concentration from 1.4 to 12.5 μM. Cys-Gly disulfide was present in only a few samples and at low concentrations.

Two unknown peaks were found in all human plasma samples analyzed. The first eluted at about 4.2 min, and the second and larger peak at about 5.5 min. Both unknowns apparently were disulfides, as neither was observed when analyzed with the upstream electrode turned off (Fig. 2B). Based upon retention times, the peak at 4.2 min was identified as the mixed disulfide of Cys and γ-Glu-Cys and the peak at 5.5 min as a mixed disulfide of Cys and Cys-Gly. Even though the latter peak was close to that of GSH, it was distinguished easily by analysis with the upstream electrode turned off.

The effect of long-term storage of plasma on HPLC–DEC profiles was also examined. Eight plasma samples were analyzed immediately and again after 36 months of storage at −20°. After long-term storage, only one peak was detectable, eluting between cystine and Cys. This apparent disulfide did not coelute with any available standard.

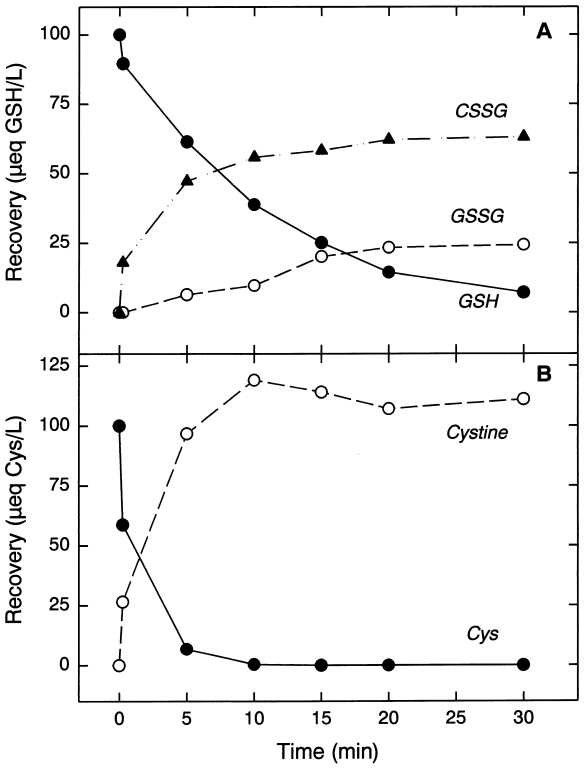

FIG. 3. Loss of GSH and Cys added to human plasma. A standard solution of (A) GSH or (B) Cys was added to fresh human plasma equilibrated at 4° to achieve a final concentration of 100 μM. Samples were incubated at 4°. Aliquots of plasma were removed immediately prior to thiol addition and at different times thereafter up to 60 min. Aliquots were processed by deproteinization with MPA, and the resulting acid-soluble fractions were analyzed by HPLC–DEC. Sample volumes were 50 μL. Details of sample processing and HPLC–DEC conditions are provided in the text. The HPLC–DEC profile is representative of >20 (GSH) and 6 (Cys) independent experiments.

Recovery Studies

Known amounts of GSH standard were added to either fresh plasma or plasma that had been stored at −20°. At different times thereafter, samples were analyzed by HPLC–DEC. In all cases, a rapid loss of the added GSH was observed. When 100 μM GSH was added, a 35% decrease in GSH levels was observed by 5 min at room temperature (Fig. 3A). When 300 μM GSH was added (100–300 times the normal reported plasma concentration), a 50% loss occurred by 15 min at room temperature. This loss of GSH was not due to the activity of GSH-metabolizing enzymes such as γ-glutamyltranspeptidase, since similar decreases in added GSH were observed when plasma was pretreated by heating to 99° for 5 min or passed through a MW 10,000 cut-off filter. However, the rapid disappearance of added GSH was not observed when incubations were performed in distilled H_2O or phosphate-buffered saline solutions.

HPLC–DEC profiles of plasma samples spiked with GSH demonstrated that the loss of GSH was accompanied by a slight increase in GSSG and a decrease in Cys. Concurrent with these changes was a proportional increase in a peak co-eluting with CSSG at about 4.88 min, which disappeared when analyzed with the upstream electrode off. Identification of this peak as CSSG was confirmed by GC–MS. When both CSSG and GSSG levels were quantitated, their combined accumulation completely accounted for the loss of GSH upon incubation (Table 1). Thirty minutes after 100 μM GSH was added to fresh plasma, only 20–25% remained as GSH, about 20% was accounted for by GSSG, and almost 60% was accounted for by conversion to CSSG.

Similar to GSH, when 100 μM Cys was added to fresh plasma, a rapid loss in recovery was observed (Fig. 3B). About 40% of the added Cys was lost by 0.25 min and > 90% by 5 min. At each time point examined, the loss of Cys was accounted for completely by an increase in cystine.

When 100 μM Cys-Gly was added to plasma, a decrease of 25% within 0.25 min and 50% within 5 min was observed in Cys-Gly recovery (Fig. 4A). Concurrent with the decrease in Cys-Gly were increases in both $(Cys\text{-}Gly)_2$ and the mixed disulfide Cys-(Cys-Gly). As observed for GSH, autoxidation of Cys-Gly to $(Cys\text{-}Gly)_2$ accounted for about 30% of the loss of Cys-Gly, while oxidation with existing Cys to form Cys-(Cys-Gly) accounted for about 60% of the loss at each time point.

TABLE 1. Recovery of glutathione added to fresh human plasma

	Plasma concentration (μEq/L)								
Time (min)	Cys	Cystine	Cyst(e)ine*	GSH	GSSG	GSH + GSSG*	CSSG	Total GSH equivalents* (GSH + GSSG + CSSG)	% Recovery†
0	57.3	12.3	126.8		0		5.4	5.4	
0.25	70.6	11.5	152.7	96.2	0	96.2	5.6	101.6	95.8
5	38.0	21.0	97.0	64.7	2.6	69.9	26.4	96.3	91.1
10	39.5	26.5	105.0	54.7	6.8	68.3	50.7	119.0	113
15	35.4	24.1	94.9	39.4	10.9	61.2	53.0	114.2	108
20	40.0	28.5	108.0	35.4	18.7	73.3	60.0	133.3	126
30	38.6	23.5	100.0	24.8	21.0	66.8	58.5	125.3	118
60	44.0	13.7	101.0		23.8	47.0	61.5	108.5	102

GSH (100 μM) was added to fresh plasma and incubated at 4°. Samples were removed immediately prior to GSH addition (0 min) and at various times thereafter and analyzed for thiol and disulfide content. Data are representative of 6 independent experiments.

*Calculated values.

†% Recovery = total GSH equivalents/[total GSH equivalents at 0 min (5.4) + added GSH (100)] × 100.

When 100 μM HCys was added to human plasma, a decrease of 88% was observed within 5 min, and 100% within 10 min (Fig. 4B). $(HCys)_2$ levels increased from undetectable levels at baseline to 9.2 μmol/mL after 5 min and remained unchanged thereafter. Thus, autoxidation accounted for only about 20% of the loss of HCys from plasma. Concurrent with the loss of HCys, a new unknown (retention time = 7.38 min) appeared after 5 min, which increased through 10 min and decreased slightly thereafter. This new peak was identified as the mixed disulfide Cys-HCys, based upon its retention time and its disappearance when the upstream electrode was turned off. The formation of Cys-HCys accounted for about 35% of the loss of HCys. The remaining 40–50% was apparently in the form of protein mixed disulfides.

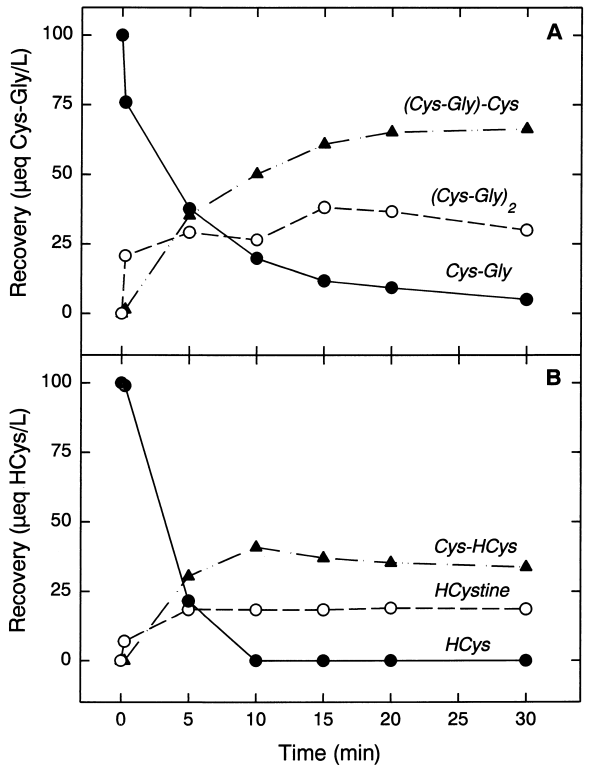

FIG. 4. Loss of HCys and Cys-Gly added to human plasma. A standard solution of (A) Cys-Gly or (B) HCys was added to fresh human plasma equilibrated at 4° to achieve a final concentration of 100 μM. Samples were incubated at 4°. Aliquots of plasma were removed immediately prior to thiol addition and at different times thereafter up to 60 min. Aliquots were processed by deproteinization with MPA, and the resulting acid-soluble fractions were analyzed by HPLC–DEC. Sample volumes were 50 μL. Details of sample processing and HPLC–DEC conditions are provided in the text. The HPLC–DEC profile is representative of 6 (Cys-Gly) and 5 (HCys) independent experiments.

The stability of various disulfides was also examined and in each case was found to be much greater than that observed with thiols (Fig. 5). When 100 μM GSSG was added to plasma, a 13% decrease was observed after 1 hr and a 50% decrease after 12 hr of incubation. With cystine, no loss was observed after 1 hr, and only a 25% decrease in recovery occurred after 12 hr. When 100 μM CSSG was added to plasma, 25% was lost by 1 hr, slightly more than that observed for GSSG. Finally, $(HCys)_2$ recovery was similar to those of cystine and GSSG (data not shown). After 3 days of incubation, 15% CSSG remained. At the same time, cyst(e)ine levels increased from 7 to 29 μM. In addition, a large (5-fold) increase occurred in the size of an unknown oxidized peak that eluted just prior to GSH (4.2 min). This peak coeluted with the mixed disulfide of Cys-(Cys-Gly).

Reduction

Fresh plasma samples, to which 100 μM GSH, Cys, or HCys had been added, were reduced with Reductacryl or with KBH_4. Typical chromatographic profiles obtained before and after reduction are provided in Fig. 6. As before, in untreated plasma, no GSH or HCys was observed. After reduction, GSH, HCys, Cys, and Cys-Gly were the major peaks observed. Results with KBH_4 reduction consistently

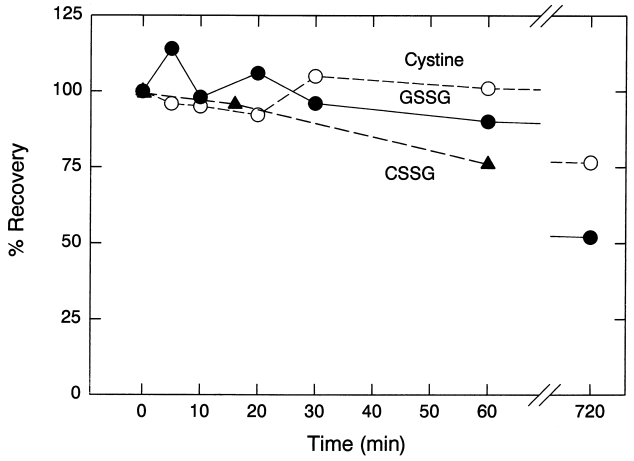

FIG. 5. Stability of disulfides added to human plasma. A standard solution of GSSG or cystine or CSSG was added to fresh human plasma equilibrated at 4° to achieve a final concentration of 100 μM. Samples were incubated at 4°. Aliquots of plasma were removed immediately prior to thiol addition and at different times thereafter up to 60 min. Aliquots were processed by deproteinization with MPA, and the resulting acid-soluble fractions were analyzed by HPLC–DEC. Sample volumes were 50 μL. Details of sample processing and HPLC–DEC conditions are provided in the text. The HPLC–DEC profile is representative of 3 independent experiments.

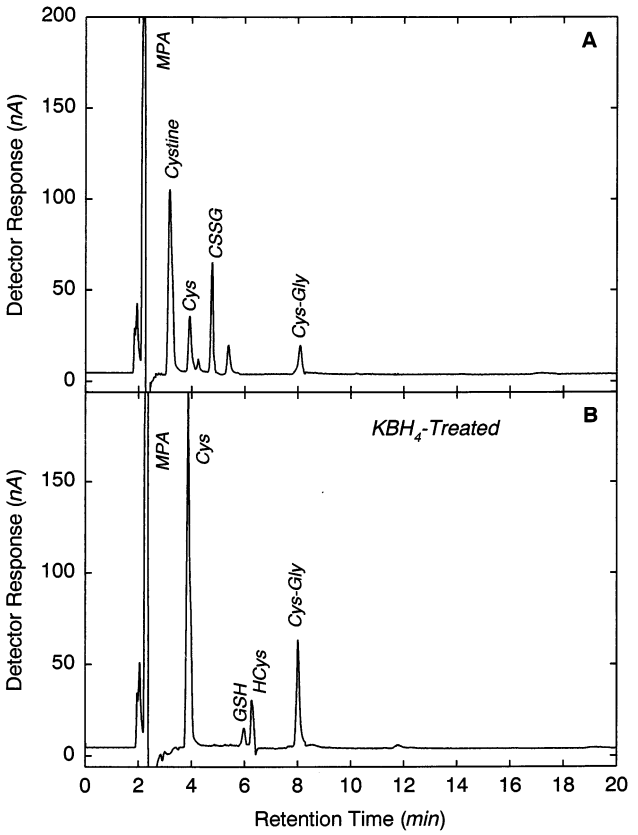

FIG. 6. HPLC–DEC chromatogram of a human plasma sample before and after KBH_4 reduction. Blood samples were collected from healthy adults and centrifuged within 1 hr to obtain plasma. Within 30 min of centrifugation, aliquots of plasma were processed either by (A) deproteinization with MPA or (B) KBH_4 reduction followed by deproteinization. Acid-soluble fractions were obtained by centrifugation and analyzed by HPLC–DEC. Sample volumes were 50 μL. Details of sample processing and HPLC–DEC conditions are provided in the text. The HPLC–DEC profiles are representative of >100 independent experiments.

yielded higher thiol levels and greater recovery values than those with Reductacryl. Thus, in all subsequent experiments, KBH_4 was used.

Recovery studies were performed in fresh plasma spiked with known amounts of thiol followed by KBH_4 reduction. Recovery values for thiols ranged from 101 to 104% when samples were processed 4 hr after thiol addition (Table 2). Similar high recovery levels were obtained when spiked plasma was stored for periods up to 6 months.

Based upon the high recovery obtained, we utilized this reduction method to assess the total plasma thiol status of a sample of 106 healthy adults (Study 2) (Table 3). Cys was the most abundant thiol observed (mean = 201 μM), followed by Cys-Gly (mean = 101 μM), HCys (mean = 7.48 μM), γ-Glu-Cys (mean = 5.17 μM), and GSH (mean = 3.60 μM). Substantial interindividual variability was observed, with coefficient of variation (CV) values ranging from 29 to 35% for all thiols except Cys, which was significantly lower (% CV = 21). No gender differences were observed.

Stabilization of Plasma with DTNB

To prevent loss of thiols to oxidation, blood samples were treated with DTNB immediately after collection. At various times between 10 min and 6 hr, plasma was separated and analyzed for GSH. Since DTNB interferes with the HPLC–DEC analysis, total glutathione was analyzed by the DTNB/GSSG reductase-recycling assay. GSH levels ranged between 0 and 2 μM for samples without DTNB treatment and between 4 and 7 μM with DTNB treatment. Levels in DTNB-treated plasma were stable for up to 6 hr (Fig. 7). The recovery of GSH added to fresh plasma samples at concentrations ranging from 4.5 to 15 μM after 1 hr ranged from 94 to 97%.

DISCUSSION

In recent years, there has been an increasing interest in the measurement of thiols in human plasma. Intense effort has been directed at HCys as an important indicator of cardiovascular and cerebrovascular disease [3]. As a result, much has been learned regarding the status and regulation of HCys in blood, and numerous methods have been developed for its measurement, as reviewed by Ueland *et al.* [14]. Research has also suggested that plasma GSH is a potentially important biomarker of disease [26–28] and other conditions such as oxidative stress [29]. However, there is a critical and recognized need to fully understand the dynamic relationships among HCys, GSH, and other biologically important thiols and disulfides in plasma [12, 30]. In

TABLE 2. Recovery of thiols in plasma after KBH_4 reduction

Thiol	Unreduced, before addition (μM)	Reduced, before addition (μM)	Concn added (μM)	Reduced, after addition (μM)	% Recovery*
Cys	11.7	150	50	209	104
GSH	0	5.19	50	56.2	101
Cys-Gly	1.8	122	50	174	101
HCys	0	8.6	50	61	104

Thiol standards were added to fresh plasma to a concentration of 50 μM. Plasma thiols were measured in either KBH_4-reduced or unreduced samples prior to and 2–4 hr after thiol addition. Data are means of 2–4 independent experiments.

*% Recovery = (reduced, after addition/reduced, before addition + concn added) × 100.

addition, while attention has focused on HCys and GSH, little attention has been given to Cys and Cys-Gly, the two most abundant thiols in plasma.

We observed very low levels of thiols in both fresh and stored plasma using either HPLC–DEC or the DTNB enzymatic recycling method for GSH, or the acid/ninhydrin method for Cys. These low levels appear to be due, in part, to the poor stability of thiols in plasma, which severely limits their accurate assessment. Indeed, rapid losses for all thiols were observed when added to fresh plasma samples. While similar problems regarding stability have been noted previously for certain thiols in plasma [11–13, 16], numerous investigators continue to report on the measurement of plasma thiols without taking this lack of stability into account.

In this study, we identified the oxidative reactions responsible for the loss of thiols in plasma. For both GSH and Cys-Gly, reaction with endogenous Cys to form the corresponding mixed disulfide was the major reaction responsible for 60–70% of the thiol loss. In both cases, autoxidation accounted for about 25–30% of the loss. Thus, it is not surprising that CSSG and Cys-(Cys-Gly) were major components found in untreated plasma. Previously, CSSG was found to be an abundant GSH-containing component in rat plasma [13]. In the case of Cys, all of the loss in recovery could be accounted for by autoxidation to cystine. Since Cys is the most abundant thiol found in human plasma, it was not unexpected that the major products formed from GSH, Cys-Gly, or Cys were the corresponding Cys-containing disulfides. This is consistent with a previous report suggesting that the loss of GSH in plasma was due to formation of an unidentified low molecular weight mixed disulfide [11]. Unlike the other thiols, HCys preferentially reacted with plasma proteins to form protein mixed disulfides, accounting for 40–50% of the loss in added HCys. Formation of the HCys-Cys mixed disulfide accounted for 35% and autoxidation for about 20% of the loss. These results are consistent with other reports of HCys in plasma, where 70–80% was in the form of protein mixed disulfides and the remaining 20–30% was predominately in the form of HCys-Cys mixed disulfide [31].

In our studies, the loss of added thiols from plasma was exponential with respect to time, based upon the linear relationship between the logarithm of thiol concentration and time as determined by the high degree of linearity observed by plotting the logarithm of thiol concentration and time ($r > 0.91$). Thus, the oxidation reactions responsible for thiol loss followed pseudo first order kinetics, in accordance with previous reports [12]. The resulting half-lives at 4° were 1.45 min for Cys, 2.43 min for HCys, 6.54 min for Cys-Gly, and 7.29 min for GSH. These short half-lives are consistent with data reported by Anderson and Meister [11] for endogenous GSH in plasma as well as with time–course data for endogenous GSH in plasma reported by Svardal *et al.* [16]. However, other investigators have observed much longer half-lives. Andersson *et al.* [12] reported plasma half-lives for endogenous thiols at 4° ranging from 38 min for GSH to 80 min for Cys. Much longer values (1–2 hr) were also found for thiols added to plasma [32]. The causes for these discrepancies are not known.

Unlike thiols, disulfides were quite stable in plasma. In particular, cystine levels remained unchanged with long-term storage. It is of interest to note that, whereas CSSG was stable for at least 1 day, by 3 days a significant amount of degradation to (Cys)-(Cys-Gly) occurred, suggesting the

TABLE 3. Plasma total thiol levels in healthy adults

Group	N	GSH (μM)	HCys (μM)	Cys (μM)	γ-Glu-Cys (μM)	Cys-Gly (μM)
Males	54	3.72 ± 1.31 (1.00–7.02)	8.30 ± 2.29 (3.29–13.4)	202 ± 44.7 (82.7–274)	5.36 ± 1.63 (1.45–10.2)	107 ± 33.5 (43.6–220)
Females	52	3.47 ± 1.22 (1.37–7.63)	6.59 ± 1.90 (2.74–10.9)	199 ± 40.1 (94.7–266)	4.96 ± 1.42 (2.67–8.17)	93.6 ± 27.0 (39.7–145)
All	106	3.60 ± 1.27 (1.00–7.63)	7.48 ± 2.27 (2.74–13.4)	201 ± 42.0 (82.7–274)	5.17 ± 1.54 (1.45–10.2)	101 ± 31.2 (39.7–220)

Values are means ± SD (range is given in parentheses).

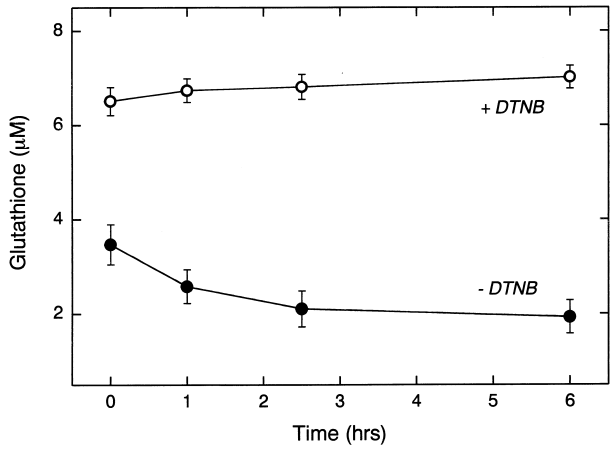

FIG. 7. Stabilization of GSH in human plasma by DTNB pretreatment. Blood samples were collected from healthy adults. Immediately after collection, 1 mL aliquots of blood were incubated for 5 min with 100 mL buffer containing either 0 or 20 mg DTNB. Plasma was removed by centrifugation and, at various times thereafter, deproteinized prior to analysis. GSH was determined using the DTNB-enzymatic recycling method. Details of sample treatments, processing, and analyses are provided in the text. Results are means ± SD (N = 3).

possible involvement of enzymatic degradation by γ-glutamyltranspeptidase. This was supported by the finding that (Cys)-(Cys-Gly) was also observed as a significant component in plasma samples subjected to long-term storage.

All together, these results emphasize the lack of stability of thiols and disulfides in plasma as a major problem in their measurement, and they identify the products of thiol and disulfide degradation. Typically, in fresh plasma, GSH and HCys were not observed, while other thiols, such as Cys-Gly and Cys, were found in low concentrations. In contrast, a number of disulfides were found, including cystine, CSSG, and the mixed disulfides (Cys)-(Cys-Gly) and (Cys)-(γ-Glu-Cys). It is apparent that, due to oxidation reactions, these profiles do not represent accurately the levels of thiols and disulfides that occur *in vivo*. These reactions are nonenzymatic and occur immediately upon separation of plasma from freshly drawn blood. Others have also found that delays in separation of plasma from erythrocytes can result in increases in the levels of HCys in plasma due to export from erythrocytes [12].

The determination of actual *in vivo* concentrations of free thiols has been addressed in previous reports. In a study by Mansoor *et al.* [19], low levels of thiols were measured in plasma by adding the thiol-blocking agents NEM or monobromobimane directly to blood collection tubes. However, we have demonstrated previously that NEM itself could cause artificial changes in the redox status of biological thiols [23]. Due to the very low levels of free thiols in plasma and the potential problems encountered in their measurement, we focused our investigations on the determination of total thiol levels in reduced samples. Indeed, in the case of HCys, it is the levels of "total" and not "free" HCys that are associated with cardiovascular disease status. Also, although many have used the GSH:GSSG ratio in plasma as an index of oxidative stress or damage [17], the present results on GSH instability suggest that such measurements could be inaccurate.

We examined two previously described approaches to account for or prevent the loss of thiol in plasma due to oxidation. Mansoor *et al.* [19] obtained complete recovery for several thiols in plasma by using KBH_4 in combination with DTT to reduce oxidized and protein-bound thiols and NEM to trap free thiols. In another study, DTT was used to reduce human plasma samples, and total GSH concentrations ranging from 1.7 to 3.3 μM were observed [27]. However, no information on validation or recovery was provided using this methodology. In the present studies, we observed complete recovery for GSH, Cys, Cys-Gly, and HCys when samples were reduced with KBH_4 but not when reduced with Reductacryl, an immobilized form of DTT. Reductacryl was tested because free DTT interferes with the HPLC analysis of thiols and an additional extraction step is required for its removal.

In another approach, complete recovery of added GSH was obtained when DTNB was added to whole blood prior to separation of plasma and the resulting DTNB conjugates were determined. This methodology had been used previously to measure GSH in plasma as a potential index of myocardial oxidative stress [17]. Whereas this procedure provides complete recovery of added GSH, it does not provide information on the proportion of GSH that was originally present *in vivo* as the free thiol.

With long-term storage of samples, nearly all thiol and disulfide peaks disappeared. However, complete or nearly complete recovery was still obtained after reduction with KBH_4. Thus, it is likely that during long-term storage, exchange reactions of disulfides and protein thiols occur, resulting in the loss of low molecular weight disulfides and the formation of protein mixed disulfides.

Our results and those of others bring into question the possible diagnostic importance of GSH levels in plasma. Unlike Cys or HCys, which are found principally in plasma and in relatively high concentrations either as a free thiol or as an oxidized product, greater than 99% of GSH in blood is found in erythrocytes [33]. Despite the low GSH levels, plasma has been considered an important pool for GSH and a key component in its interorgan transport [34]. In numerous studies, plasma GSH concentrations have been measured as an indication of overall body status [26, 27], and GSH:GSSG ratios have been used as an indicator of oxidative stress [29]. However, as described above, poor stability due to rapid oxidation severely limits the assessment of GSH as well as GSH:GSSG ratios in plasma. Further, the results of two studies have called into question the importance of plasma GSH as a delivery source for other tissues. In a systematic study of renal and hepatic output of GSH in rats, erythrocytes rather than plasma were found to be the major site of export of GSH from the liver [35]. In another study in human subjects, the GSH content of arterial blood plasma was found to be actually lower than

that found in the inferior vena cava, providing further evidence that plasma is not a carrier for GSH in humans [36].

In the review of studies on plasma GSH, it is important to note that major differences between rats and humans may severely limit the value of rat models in this regard. Reported levels of GSH found in rat plasma are 5- to 10-fold higher than levels found in human plasma [36]. Whereas in the rat about 90% of GSH in the plasma is thought to be derived from efflux from the liver, evidence for a similar translocation from liver to plasma has not been observed in humans [36]. Unlike human plasma, rat plasma is often laden with bacteria, which may affect GSH concentrations. Finally, unlike humans, rats can synthesize ascorbic acid and achieve plasma levels that are several-fold greater than those found in humans. Since an apparent relationship between the metabolism and activities of ascorbic acid and GSH has been observed [37], it is likely that these species differences in ascorbic acid levels are important.

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