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Effect of ageing on human plasma glutathione concentrations as determined by high-performance liquid chromatography with fluorimetric detection

Chung-Shi Yang[a,*], Su-Tze Chou[b], Lin Liu[a], Pi-Ju Tsai[a], Jon-Son Kuo[a]

[a]*Department of Medical Research, Taichung Veterans General Hospital, Taichung, 40705, Taiwan*
[b]*Department of Food and Nutrition, Providence University, Taichung, 40705, Taiwan*

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Abstract

A convenient method for the determination of reduced glutathione (GSH) and oxidized glutathione (GSSG) in human plasma by high-performance liquid chromatography with fluorescence detection is reported. This assay involves direct addition of human plasma to methanolic monobromobimane. for simultaneous protein precipitation and thiol derivatization. The assay was validated by addition of authentic GSH and GSSG to plasma samples. Plasma glutathione levels in Chinese male and female volunteers were found to decrease with increasing age (age groups 20–30, 30–40, 40–50, 50–60, and >60; mean ± S.E.M. 0.95 ± 0.03, 0.77 ± 0.02, 0.67 ± 0.03, 0.51 ± 0.02, 0.48 ± 0.02 μM for male volunteers and 1.11 ± 0.06, 0.76 ± 0.03, 0.61 ± 0.03, 0.53 ± 0.04 and 0.43 ± 0.04 μM for female volunteers). GSSG levels, in both males and females, did not show a correlation with age. There were no significant differences in GSH or GSSG levels among male and female volunteers of the same age group. These results suggest that elderly persons might be more susceptible to oxidative injury due to decreased plasma glutathione levels.

1. Introduction

Glutathione (γ-glutamylcysteinylglycine) (GSH) is an important tripeptide that is present in most mammalian tissues at concentrations of 0.1–10 mM. GSH is involved in various physiologically important events, and acts as a major reducing agent. GSH is an important physiological antioxidant which protects cells from oxidative damage [1,2]. In addition, GSH serves as a reservoir for cysteine [3] and participates in the detoxification of xenobiotics and in the metabolism of numerous cellular compounds [4–6]. GSH is required for the synthesis of some prostaglandins [2], and is involved in the cell cycle regulation [7–9]. Results of previous studies indicate that tissue or cellular GSH is related to drug-resistance mechanisms of certain tumours [10,11].

GSH concentrations in plasma, usually in the micromolar range, are much lower than those in tissues. Thus, high sensitivity is required to assay both reduced and oxidized GSH concentrations in plasma. Several HPLC methods employing either fluorescence or electrochemical detection have been successfully applied to the measure-

* Corresponding author.

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ment of GSH and oxidized GSH (GSSG) concentrations. The electrochemical detector, involving serial Au–Hg dual electrodes [12], is capable of the simultaneous determination of GSH and GSSG in physiological samples. However, frequent redeposition of Hg on the Au surface is needed to achieve an optimum electrochemical response. A widely used alternative is fluorescence detection, with monobromobimane (BBr) [13–15] and *o*-phthaldehyde (OPA) [16,17] as labelling reagents. However, plasma must be treated with deproteinizing reagents such as perchloric acid, sulfosalicyclic acid, metaphosphoric acid or trifluoroacetic acid prior to analysis. Additionally, when fluorescence detection is used for the determination of GSH, the deproteinized solutions must be neutralized prior to derivatization with labelling reagents. Loss of plasma GSH might occur due to oxidation or to consumption by reaction with thiols of the biological matrix [18]. Other derivatizing reagents such as 1-fluoro-2,4-dinitrobenzene could also be used and the derivative could be determined by HPLC with UV–visible detection [19,20]. However, the use of a UV–visible detector might be limited for the determination of plasma GSH levels owing to its low sensitivity in HPLC.

We therefore investigated the possibility of minimizing the steps and time needed for sample preparation. This paper describes a convenient and accurate method for the determination of plasma thiols that involves the use of methanolic BBr to accomplished derivatization and protein precipitation in one step. GSSG concentrations were determined by subtraction of GSH levels from total non-bound GSH levels obtained by the treatment of dithiothreitol as the reducing reagent. The validity of this method was also examined. Since plasma levels of GSH and GSSG are often used as indices of physiological stress [21], the effect of age on plasma GSH concentrations was also investigated.

2. Experimental

The following chemicals were purchased from Sigma (St. Louis, MO, USA): a 40% aqueous solution of tetrabutylammonium hydroxide (TBA), L-cysteine, glutathione, glutathione disulfide, 5,5′-dithiobis(2-nitrobenzoic acid) and dithiothreitol. BBr was obtained from Calbiochem (LaJolla, CA, USA). HPLC-grade methanol and N-ethylmaleimide were purchased from Merck (Darmstadt, Germany).

2.1. Chromatographic analysis system

The HPLC system consisted of a 1050 Series quaternary pump, a 1050 Series autosampler, a 1050 Series on-line degasser and a Model 1462A HPLC fluorescence monitor with two holographic diffraction monochromators, all obtained from Hewlett-Packard (Waldbronn, Germany). Optimum responses of bimane derivatives was observed when the excitation and emission wavelengths were set at 260 and 474 nm, respectively. Peak areas and concentrations were determined using a Hewlett-Packard Chem Station Chromatographic Management System.

Separations were achieved using an Econosphere C_{18} cartridge column (150 × 4.6 mm I.D., particle size 5 μm) obtained from Alltech (Deerfield, IL, USA). Prior to the preparation of mobile phases containing TBA, a commercially available 40% aqueous solution of tetrabutylammonium hydroxide was neutralized to pH 6.0 with 85% phosphoric acid. All mobile phases containing TBA were 30 m*M* in TBA. After addition of TBA, the pH of the mobile phases was adjusted to 3.1–3.4 (apparent) as measured with a glass electrode. In all cases, the mobile phases were filtered through filters (pore size 4.5 μm) and degassed. Binary gradient elution at a flow-rate of 1.5 ml/min was used. Mobile phase A consisted of 30 m*M* TBA in 25% methanol, while mobile phase B consisted of 30 m*M* TBA in 100% methanol. The elution profile was 0–10 min isocratic with mobile phase A, followed by a column wash with 90% mobile phase A for 5 min to remove late-eluting bimane derivatives. Then the column was re-equilibrated with mobile phase A for 5 min before the next injection.

2.2. Preparation of plasma samples

Blood samples from normal volunteers were drawn and immediately centrifuged at 2000 *g* for

20 min at 4°C to remove blood cells. Two volumes of 100% methanol containing 2 m*M* BBr were added to the plasma sample. The mixtures were stored at 4°C for 20–30 min for completion of protein precipitation, followed by storage at room temperature for 20 min for derivatization. The derivatized materials were centrifuged twice (20 min each) at 13 500 *g*. The derivatized samples were either analysed immediately or stored at −20°C until analysed.

2.3. Determination of GSSG concentration

An equal volume of methanol was added to plasma and incubated at 4°C for 30 min, to precipitate the proteins, followed by centrifugation for 20 min at 13,000 *g*. An equal volume of 50 m*M* sodium phosphate buffer (pH 7.4) containing 2 m*M* EDTA and dithiothreitol (final concentration 500 μM) was added to the supernatant. The mixture was heated at 50°C for 10 min, with subsequent cooling and addition of an equal volume of 2 m*M* methanolic BBr. The derivatized materials were incubated for 20 min prior to HPLC analysis. To determine the recovery, GSH and GSSG at various concentrations were added to the plasma at a ratio of 1:2. An equal volume of sodium phosphate buffer containing 2 m*M* EDTA and dithiothreitol (final concentration 500 μM) was added to the GSH- or GSSG-spiked plasma samples before derivatization and subsequent HPLC analysis.

2.4. Validity of the assay

Stock solutions of 1 m*M* GSH were prepared in 50 m*M* phosphate buffer (pH 7.4) containing 2 m*M* EDTA and 50 μM dithiothreitol. Solutions with various GSH concentrations were obtained by dilution of the stock solutions with dithiothreitol (50 μM)–phosphate buffer. For validation of the assay, standard GSH solutions of different concentrations were added to human plasma in a 1:4 ratio, to which an equal volume of methanolic BBr was subsequently added. One volume of N-ethylmaleimide (NEM) dissolved in 50 m*M* phosphate buffer (pH 7.4) yielding a final concentration of 24 *M* was added to four volumes of human plasma or standard GSH solutions, and the mixtures were allowed to incubate for 10 min prior to the addition of methanolic BBr. This serves to identify the peaks of GSH and GSSG in the HPLC trace (see Results and Discussion).

2.5. Determination of glutathione concentration

The concentrations of reduced GSH in human plasma were calculated from calibration graphs, established by repeated injection of standard GSH solutions of various concentrations. For every sample series a new calibration graph was used. Standard GSH solutions were prepared by dissolving GSH in 50 m*M* phosphate buffer containing 50 μM dithiothreitol.

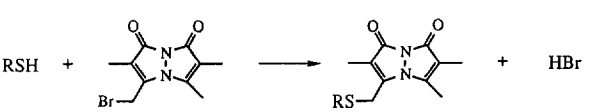
2.6. Human subjects

The human subjects enrolled in this study were volunteers undergoing physical examination at Taichung Veterans General Hospital. The volunteers were divided into five different age groups (21–30, 31–40, 41–50, 51–60 and >60 years of age).

3. Results and discussion

3.1. Chromatographic determination of glutathione

Direct addition of plasma to methanolic BBr effected both protein precipitation and derivatization in one step. The generalized reaction of a thiol with BBr is shown in Fig. 1. Derivatized samples could be directly injected into the HPLC system. Fig. 2a illustrates a typical chromatogram of human plasma treated with methanolic BBr. GSH was eluted within 10 min and no interfer-



Thiol monobromobimane

Fig. 1. Generalized reaction of a thiol with monobromobimane.

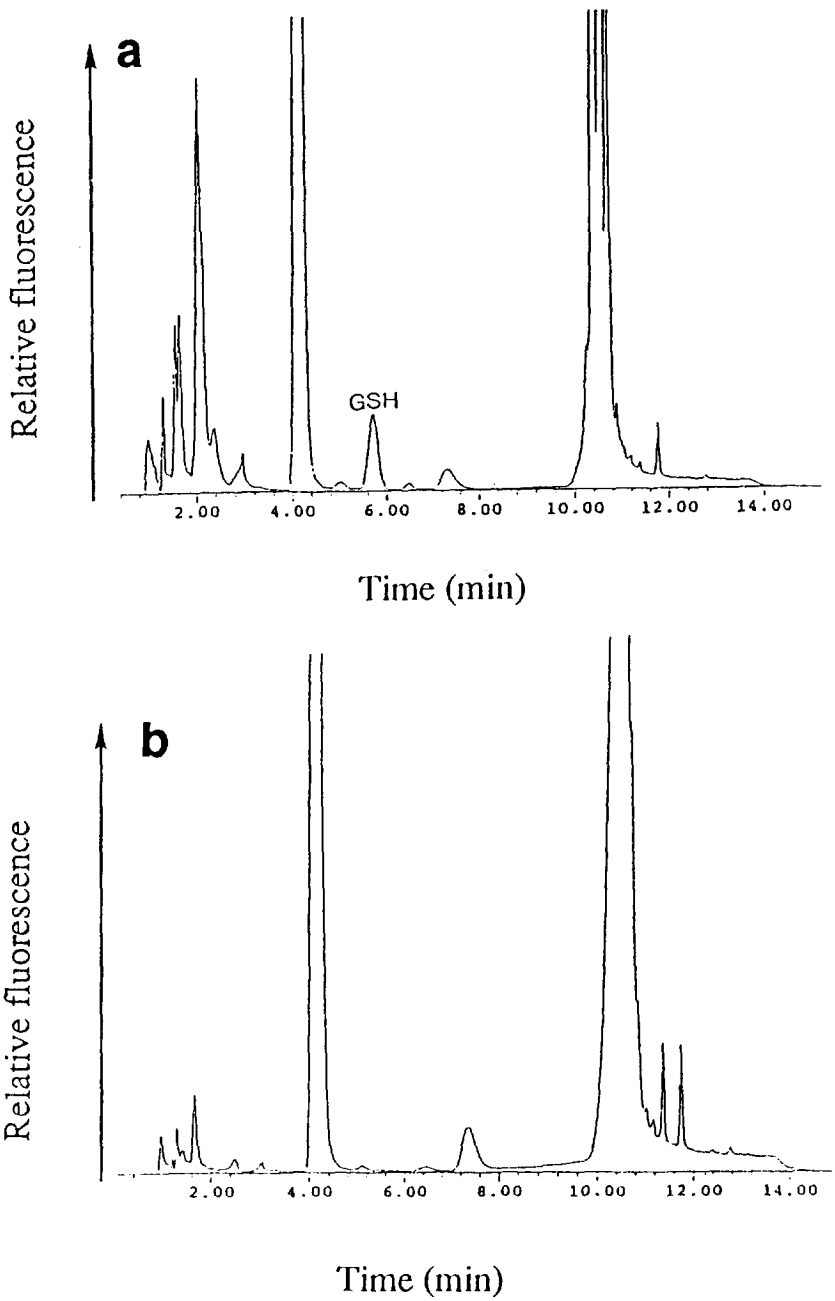


Fig. 2. Typical chromatogram of (a) BBr-derivatized plasma sample and (b) plasma samples treated with NEM. Experimental procedures and HPLC eluting conditions are described under Experimental.

ence signals were observed. Identification of the GSH peak was confirmed by either injection of plasma which had been spiked with the authentic components or by the disappearance of both signals after treatment with NEM, a well known thiol-blocking agent (Fig. 2b).

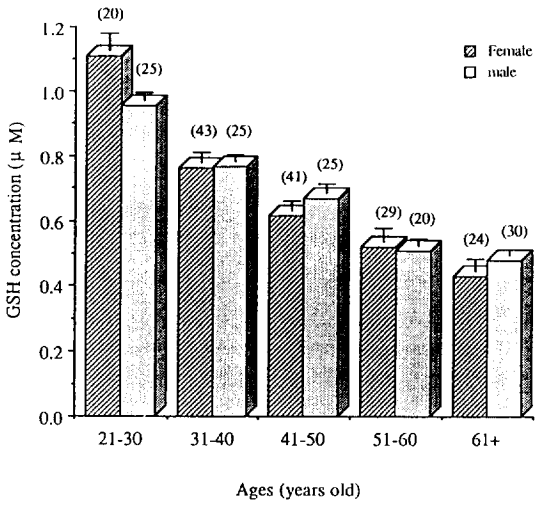


Fig. 3. Effect of age on plasma glutathione concentrations in normal Chinese subjects. Data are presented as means ± S.E.M. The numbers in parentheses represent the number of subjects undergoing blood withdrawal.

3.2. Recovery of GSH added to plasma samples

For the recovery of authentic GSH and GSSG added to plasma, three different amounts of GSH or GSSG were added to the biological matrix prior to sample processing and analysis.

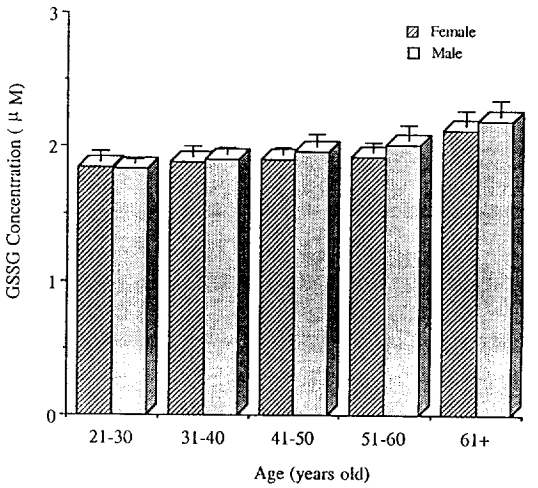


Fig. 4. Effect of age on plasma glutathione disulfide concentrations in normal Chinese subjects. Data are presented as means ± S.E.M. The number of subjects in each age group was the same as in Fig. 2.

The recoveries of GSH added to plasma are shown in Table 1. The GSH recovery ranged from 95% to 103%. The results imply quantitative derivatization of GSH with BBr and thus provide evidence that the precipitation procedure also quantitatively releases GSH from plasma proteins as a consequence of the denaturing effect of methanol.

3.3. Precision of GSH assay

The precision of the assay for GSH was determined using a human plasma sample and an authentic GSH solution. The intra- ($n = 13$) and inter-assay ($n = 13$) variabilities were assessed and expressed as coefficients of variation (C.V.). The intra-assay variabilities for a human plasma sample and a standard solution (1.25 μM), assessed at intervals of 1 h, were 1.15% and 1.25%, respectively. The inter-assay variabilities for a human plasma sample and a standard solution (1.25 μM), assessed on three consecutive working days, were 0.41% and 4.90%, respectively. These results demonstrate that the precision of this assay is acceptable.

3.4. Determination of GSSG in plasma samples

Determination of GSSG was accomplished by subtracting the GSH level from the total non-bound glutathione level. Dithiothreitol (final concentration 500 μM) was used as the reducing reagent. Three different incubation times (5, 10 and 15 min) and three different temperatures (30, 50 and 100°C) were used to determine the optimum reducing conditions (results not shown). Authentic GSSG added to plasma samples, with subsequent DTT treatment, was optimally recovered when incubated at 50°C for 10 min (results not shown). For the determination of the recoveries of GSSG, authentic GSSG at various concentrations was added to plasma. Results of the GSSG recovery experiments are summarized in Table 2. Two different concentrations of GSSG added to plasma were successfully recovered (94–110%), which is indicative

Table 1
Recovery of glutathione (GSH) added to blood samples

Trial No.	Amount added (nmol)	Amount detected (nmol)	Amount expected (nmol)	Recovery (%)
1	0	2.25		
	5	6.87	7.25	94.77
	10	11.98	12.25	97.78
	20	21.61	22.25	97.12
2	0	2.44		
	5	7.27	7.44	97.64
	10	12.30	12.44	98.81
	20	22.19	22.44	98.88
3	0	2.34		
	5	7.28	7.39	98.75
	10	12.63	12.39	101.64
	20	23.31	22.37	104.22
4	0	0.99		
	5	6.53	5.99	109.13
	10	11.17	10.99	101.64
	20	21.06	20.99	100.36
5	0	0.84		
	5	5.72	5.84	98.01
	10	10.63	10.84	98.08
	20	20.45	20.84	98.14

Blood samples were withdrawn from different subjects in the different trials. The average recoveries for 5, 10 and 20 nmol of reduced glutathione added to 1 ml of plasma were (mean ± S.D.) 99.67 ± 5.50, 99.68 ± 2.04 and 99.75 ± 2.76%, respectively.

that derivatization is quantitative, as also observed for GSH.

3.5. Limit of detection

Based on a signal-to-noise ratio of 2, the detection limit for the GSH determined as the BBr derivative was 0.2 pmol per 20 μl injected. The calibration graphs showed good linearity for concentrations ranging from 0.1 to 250 μM.

3.6. Effect of age on plasma glutathione concentrations in normal Chinese subjects

Male and female volunteers were divided into five different age groups (20–30, 30–40, 40–50, 50–60 and >60 years). Plasma GSH levels in both males and females decreased with increasing age (mean ± S.E.M. 0.95 ± 0.25, 0.77 ± 0.21, 0.67 ± 0.29, 0.51 ± 0.19 and 0.48 ± 0.15 μM for male volunteers and 1.11 ± 0.06, 0.76 ± 0.03, 0.61 ± 0.03, 0.53 ± 0.04 and 0.43 ± 0.04 μM for female volunteers). Glutathione disulfide levels showed no significant correlation with age for either males or females. Neither GSH nor GSSG levels showed significant differences among male and female volunteers of the same age.

GSH is an important tripeptide that is involved in a variety of physiological events. Several methods are available for the determination of GSH in tissues [14–17]. However, assays of GSH in plasma have to be highly sensitive, and sample preparation must be rapid since GSH levels in plasma are low. A prolonged sample preparation time might increase the conjugation of GSH with other sulfhydryls. The determination of GSH is further complicated by a loss of GSH, since GSH may react with other thiols or become oxidized. The loss of GSH can occur under some assay conditions [12,18]. BBr is a commonly used

Table 2
Recovery of glutathione disulfide (GSSG) added to blood samples

Trial No.	Amount added (nmol)	Amount detected (nmol)	Amount expected (nmol)	Recovery (%)
1	0	1.06		
	2.45	3.56	3.51	101.3
	3.79	4.63	4.84	95.5
2	0	1.12		
	2.45	3.80	3.67	106.4
	3.79	4.62	4.90	94.2
3	0	1.12		
	2.45	3.79	3.57	106.0
	3.79	4.79	4.91	97.5
4	0	0.73		
	2.45	3.26	3.18	102.5
	3.79	4.61	4.52	102.0
5	0	0.60		
	2.45	3.38	3.05	110.6
	3.79	4.73	4.39	107.8
6	0	0.98		
	2.45	3.79	3.43	110.3
	3.79	5.12	4.78	107.1

Blood samples were withdrawn from different subjects in the different trials. The average recoveries for 2.75 and 3.79 nmol of glutathione disulfide added to 1 ml of plasma were 106 ± 4.0, $100.7 \pm 6.0\%$, respectively.

fluorescent reagent for thiols, and its derivatives are stable for a reasonable period of time when stored at 4°C. Additionally, this reagent provides high sensitivity for fluorescence detection. We tested the possibility of combining protein precipitation and BBr derivatization into one step. We chose to use methanol as the solvent for BBr and as the deproteinizing reagent. The result was a shortened sample processing time. This procedure was advantageous since prolonged incubation of plasma, even at 4°C, can cause a decrease or variation of GSH level. Additionally, this assay circumvented the neutralization process for fluorescence detection, after acid precipitation avoiding loss of GSH. Although methanolic BBr was convenient and useful for the accurate determination of plasma GSH, repeated centrifugation at 13 000 *g* was required for complete protein precipitation. However, repeated centrifugation did not affect the accuracy of GSH analysis.

When plasma was treated with methanol to precipitate the proteins, followed by dithiothreitol to reduce disulfide components, the total non-bound glutathione level could be determined. Dithiothreitol proved to be superior compared with the commonly used sodium tetraborohydride and the reaction can be performed in phosphate buffer of approximately neutral pH (7.4). Subtraction of the GSH level from the total non-bound glutathione yielded the GSSG level. The validity of GSSG determinations was demonstrated by the successful recovery of authentic GSSG.

Glutathione is one of the major antioxidants that is physiologically present. Lowered tissue and plasma GSH levels have been reported to be associated with increasing age in various animals [22,23]. Our results showed that plasma GSH levels decreased with increasing age in both male and female Chinese subjects. Two possible causes might account for the lower GSH levels in

aged people. One is that formation of free radicals tends to increase with increasing age, and the elevated amount of free radicals might result in a decreased GSH level. The other possibility is that hepatic GSH levels are lowered in older people. Various studies on mice and rats have demonstrated that liver GSH levels are lowered in older animals [24–27]. Additionally, a positive linear correlation between plasma GSH and hepatic GSH concentrations has been found [28]. As hepatic efflux of GSH constitutes about 85% of the plasma GSSG content, lowered hepatic GSH concentrations in older people might result in lower plasma GSH concentrations.

In conclusion, we have developed a convenient method for the rapid and accurate determination of plasma GSH levels, and found that plasma GSH concentrations in normal Chinese subjects decreased with increasing age.

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