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Increase in the Protein-Bound Form of Glutathione in Human Blood after the Oral Administration of Glutathione

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ABSTRACT: The present study examined the impact of the supplementation of glutathione (GSH), γ-L-glutamyl-L-cysteinyl-glycine, on human blood GSH levels. Healthy human volunteers were orally supplemented with GSH (50 mg/kg body weight). Venous blood was collected from the cubital vein before and after ingestion. Plasma was mixed with 3 volumes of ethanol. The supernatant and precipitate were used for the deproteinized and protein fractions of plasma, respectively. Blood cell and plasma fractions were pretreated with 5% trichloroacetic acid−2% 2-mercaptoethanol to reduce the oxidized form of GSH and liberate protein-bound GSH. The 2-mercaptoethanol-pretreated GSH was determined by precolumn derivatization with 6-aminoquinolyl-*N*-hydroxy succinimidyl carbamate and liquid chromatography−tandem mass spectrometry. There was no significant difference in GSH contents in the deproteinized fraction of plasma and blood cell fraction after GSH ingestion. However, the GSH contents in the protein-bound fraction of plasma significantly ($P < 0.01$) increased from 60 to 120 min after GSH supplementation.

KEYWORDS: *glutathione, GSH, blood, supplementation, oral administration, human trial*

INTRODUCTION

Glutathione (GSH), γ-L-glutamyl-L-cysteinyl-glycine, is a ubiquitous compound found in humans, animals, plants, and microorganisms.[1] Within cells, GSH exists in both reduced and oxidized states. In healthy cells, the majority of GSH is present in the reduced form.[2] Most cells in the human body are capable of synthesizing GSH from glutamate, cysteine, and glycine. First, γ-glutamyl-cysteine is synthesized from L-glutamate and L-cysteine by γ-glutamyl-cysteine synthetase. Second, glycine is conjugated to the C-terminaus of γ-glutamyl-cysteine by GSH synthetase.[1,3]

GSH is a cofactor or substrate for GSH peroxidase and GSH *S*-transferase (GST), which are involved in very important antioxidant and detoxification systems, respectively.[4] The main function of GSH peroxidase is to reduce hydrogen peroxide to water and to reduce lipid hydroperoxides to their corresponding alcohols.[3,5] The reduced form of GSH, which is involved in these reactions as a cofactor, is converted to the oxidized form. Thus, the ratio of the oxidized form to the reduced form of GSH has been thought to reflect oxidative status in cells, organs, and individuals.[6] However, oxidized GSH is reduced by GSH reductase. The reduced form of nicotinamide adenine dinucleotide phosphate, which is a cofactor for GSH reductase, is supplied by the pentose phosphate pathway.[7] GST catalyzes the conjugation of reduced GSH to a wide variety of exogenous chemicals through a sulfhydryl group to make the compounds more soluble and to facilitate urinary excretion.[8,9]

Decreased GSH levels in cells have been demonstrated to increase the risks of diseases and poisoning.[1,10] GSH has therefore been used to treat chronic liver diseases and poisoning through intravenous injections.[11,12] In addition to intravenous injections of GSH, oral administration has been used for medical applications. Aside from medical applications, GSH has also been implicated in skin whitening. In vitro studies have revealed that GSH inhibits melanin synthesis in the reaction of tyrosinase and L-3,4-dihydroxyphenylalanine in a dose-dependent manner.[13,14] On the basis of these findings, GSH has been used as a supplement for skin whitening and improvement of liver function.

GSH supplementation has been prevalent worldwide. However, bioavailability and the metabolic fate of orally administered GSH have not been fully understood. It has been proposed that GSH is first degraded by γ-glutamyl transferase into γ-glutamyl-X (X = other amino acids) and cysteinyl-glycine. The cysteinyl-glycine is degraded into cysteine and glycine by peptidases.[6,15] Orally administered GSH could be degraded in this system. In addition, some human studies have demonstrated no significant increases in blood GSH levels in human volunteers after the oral administration of a relatively large dose of GSH.[16,17] These findings suggest that GSH supplementation has no impact on blood and organ GSH levels. However, GSH can potentially bind to proteins by disulfide bonds. Therefore, it is possible that orally administered GSH may be present and transported as a protein-bound form in the blood, and this has not been reported in previous studies.

The objective of the present study was to elucidate the impact of GSH supplementation on human blood GSH levels in the free and protein-bound forms.

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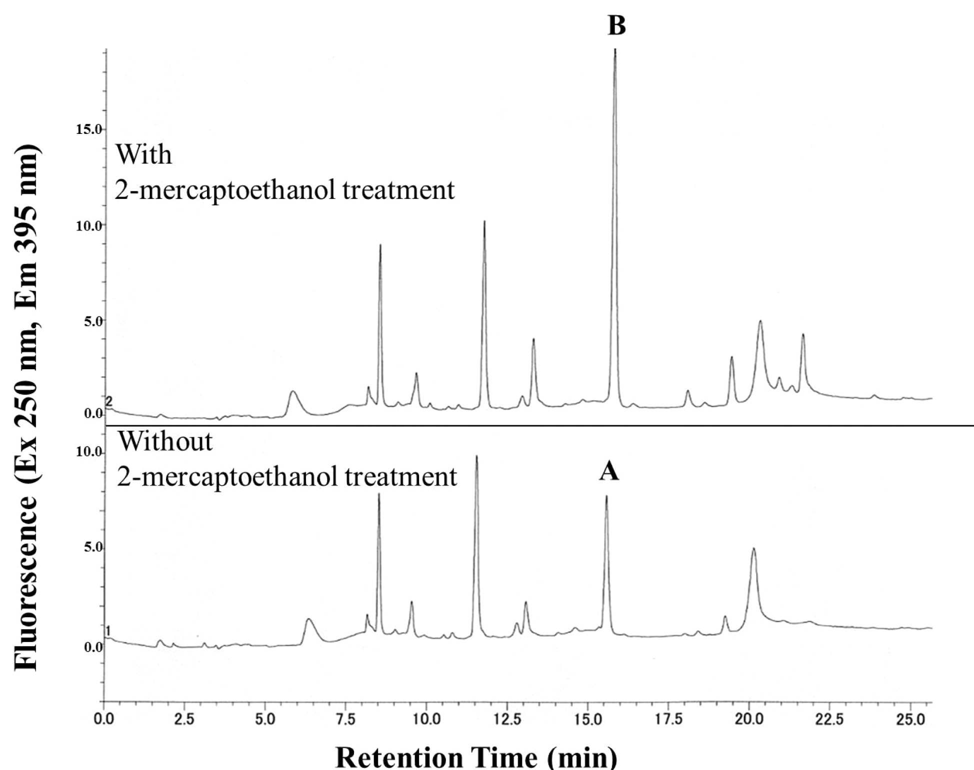

Figure 1. Elution profiles of the 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ) derivatives of 2-mercaptoethanol-pretreated or intact glutathione (GSH). Pretreated and intact GSH was dried in the presence of triethylamine (TEA) before derivatization with AccQ. Peaks marked A and B show GSH derivatives.

■ MATERIALS AND METHODS

Chemicals. The reduced form of L-glutathione was produced by fermentation with Torula yeast by KOHJIN Life Sciences, a subsidiary of Mitsubishi Corp. (Tokyo, Japan). This product has been qualified for Generally Recognized As Safe status by the U.S. Food and Drug Administration (U.S. FDA GRAS notified GRN000293). γ-L-Glutamyl-L-cysteine (γGlu-Cys) and L-cysteinyl-glycine (Cys-Gly) were obtained from Sigma-Aldrich (St. Louis, MO, USA). An amino acid labeling kit (AccQ Tag) that consisted of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate reagent (AccQ), acetonitrile, and 0.2 mM sodium borate buffer, pH 8.8, was purchased from Waters (Milford, MA, USA). Acetonitrile [high-performance liquid chromatography (HPLC) grade], triethylamine (TEA), formic acid, trichloroacetic acid (TCA), and 2-mercaptoethanol were obtained from Wako Pure Chemicals (Osaka, Japan). All other reagents were of analytical grade or higher.

Clinical Study Design. The experimental protocol was submitted to and approved by the experimental ethical committees of Kyoto Prefectural University (KPU no. 63) and KOHJIN Life Sciences. The human studies were performed according to the Helsinki Declaration and were under the control of medical doctors. The potential risks of the ingestion of GSH and the objective of the present study were revealed to the volunteers. After careful reading of a consent form, all subjects signed it. Seven healthy male subjects (mean age ± standard deviation 39.8 ± 15.1 years) were fasted for 12 h before the experiment and given the reduced form of L-glutathione at 50 mg/kg body weight and 100 mL of water, which was the same dose as used in the previous study.[17] Approximately 10 mL of venous blood was collected in the presence of heparin from the cubital vein before and 15, 30, 60, 90, and 120 min after ingestion.

Preparation of Blood Samples. One milliliter of the blood was centrifuged at 3700*g* for 5 min to prepare the plasma and blood cell fractions. Four hundred microliters of the plasma was mixed with 1.2 mL of ethanol and then centrifuged at 3700*g* for 5 min. Then, the supernatant was collected. Aliquots of the supernatant (300 μL) were dried under vacuum and added to 100 μL of 5% TCA containing 2% 2-mercaptoethanol, and the mixture was allowed to stand for 30 min at room temperature and used as the deproteinized plasma fraction in the following experiments. The ethanol precipitate of the plasma was washed with 200 μL of 75% (v/v) ethanol and then mixed with 160 μL of 10% (w/v) TCA and stirred vigorously. To this suspension were added another 320 μL of 5% (w/v) TCA and 10 μL of 10% (v/v) 2-mercaptoethanol. The mixture was allowed to stand for 30 min at room temperature and then centrifuged at 14800*g* for 5 min. The supernatant was collected and used as the protein-bound plasma fraction. The blood cell from 1 mL of blood was mixed with 500 μL of 10% TCA and 1 mL of 5% TCA and stirred for 3 min. The supernatant was collected after centrifugation at 14800*g* for 5 min. Fifty microliters of the 5% TCA extract of the blood cell was mixed with 450 μL of 5% TCA containing 2% 2-mercaptoethanol and allowed to stand for 30 min at room temperature and used as the blood cell fraction.

The weights of the blood cell and plasma ethanol precipitate were determined before the addition of TCA solutions. The samples were stored at −80 °C until use.

Derivatization of GSH with the AccQ Reagent. The reduced form of GSH was dissolved in 10 mM HCl to result in 2.5 μmol/mL and used as a stock solution. Suitable amounts of the standard stock solution were dried under vacuum in a glass tube (50 mm × 6 mm i.d.) with a KLV-CC-105 centrifugal concentrator (Tomy, Tokyo, Japan). Fifty microliters of 5% TCA containing 2% 2-mercaptoethanol was added to the tube and allowed to stand for 30 min, and the solution was then dried under vacuum in a hydrolysis/derivatization vial with a resealable valve (Waters). Twenty microliters of an alkaline solution, which consisted of methanol, water, and TEA at a ratio of 7:1:2 (v/v), was added to the tube, and the mixture was then dried under vacuum to increase the pH for derivatization with AccQ. In some cases, this treatment was deleted from the protocol. The dried standards were resolved in 20 μL of 20 mM HCl, and 20 μL of AccQ reagent and 60 μL of borate buffer were added in the AccQ Tag Kit and reacted at 50 °C for 10 min. The resultant AccQ derivatives were

diluted with 5 mM sodium phosphate buffer, pH 7.4, containing 10% (v/v) acetonitrile. The solutions were filtered through a 0.45 μm filter (column guard LCR4; Millipore, Billerica, MA, USA).

Fifty microliters of the plasma deproteinized and protein-bound fractions and blood cell fraction were also dried under vacuum in the presence of TEA and then derivatized with AccQ, as described above.

Isolation of the AccQ Derivatives. The AccQ derivatives of GSH with and without 5% TCA−2% 2-mercaptoethanol and TEA pretreatments were isolated by reversed phase HPLC (RP-HPLC) with an Inertsil ODS-3 (4.6 mm i.d. × 250 mm, GL Sciences, Tokyo, Japan). Elution was performed with a binary gradient system with 0.1% formic acid (solvent A) and 60% (v/v) acetonitrile (solvent B). The gradient profile was as follows: 0−1 min, 5−10% B; 1−10 min, 10−20% B; 10−25 min, 20−40% B; 25−30 min, 40−45% B; 30−35 min, 45−50% B; 35−40 min, 50−100% B; 40−45 min, 100% B; 45−45.1 min, 100−5% B; and 45.1−55 min, 5% B. The column was equilibrated with 5% B at 1 mL/min. The column was maintained at 45 °C, and elution was monitored by fluorescence at 395 nm that was excited at 250 nm.

Mass Spectrometry Analysis. The isolated AccQ derivatives of GSH were analyzed by electrospray ionization ion trap mass spectrometry (ESI-MS) with an LCQ (Thermo Fisher Scientific, Waltham, MA, USA). The sample was delivered to the ion source by a syringe pump at 3 μL/min. Detection was conducted in positive mode and optimized and processed by Xcalibur version 2.07 (Thermo Fisher Scientific).

Determination of GSH by Liquid Chromatography−Tandem Mass Spectrometry (LC-MS/MS). The concentrations of GSH, γGlu-Cys, and Cys-Gly in the blood samples were determined by precolumn derivatization with AccQ that was followed by an LC-MS/MS analysis with a Q-TRAP 3200 (AB SCIEX, Framingham, MA, USA). The AccQ derivative was prepared as described above and clarified by passing through a filter (0.45 μm column guard; Millipore). The samples (10 μL) were injected onto an Inertsil ODS-3 column (2.1 mm i.d. × 250 mm; GL Sciences), and a binary gradient elution was performed with 0.1% formic acid (solvent A) and 60% acetonitrile (solvent B) at a flow rate of 0.2 mL/min. The column was equilibrated with 100% solvent A, and the gradient profile was as follows: 0−15 min, 0−50% B; 15−20 min, 50−100% B; 20−24 min, 100% B; 24−24.1 min, 100−0% B; and 24.1−30 min, 0% B. The column was maintained at 40 °C throughout. The multireaction monitoring (MRM) condition was optimized in positive mode with Analyst version 4.2 (AB SCIEX) in autoselect mode.

Statistics. Differences between the means were evaluated by analyses of variance (ANOVA) that were followed by one-way ANOVAs with StatView 4.11 (Abacus Concepts, Berkeley, CA, USA). Significant differences between the groups were evaluated by Scheffe's post hoc test.

RESULTS

Structures of the AccQ Derivatives. The AccQ derivatives of GSH, which had been pretreated with or without the 5% TCA−2% 2-mercaptoethanol solution, were resolved by RP-HPLC. In both cases, the GSH was pretreated by TEA before derivatization with AccQ. As shown in Figure 1, several peaks of fluorescence were observed. When different amounts of GSH were derivatized, only the peaks marked A and B changed in a dose-dependent manner, which indicated that these peaks corresponded to AccQ−GSH derivatives and that the other ones were reagent peaks. The derivative that had been pretreated without 2-mercaptoethanol (A) showed a similar but slightly different retention time and a smaller peak area compared to the counterpart (B). These peaks were collected and subjected to an ESI-MS analysis. As shown in Figure 2A, the AccQ derivative without 2-mercaptoethanol pretreatment showed a main peak with a m/z of 477 (full scan). A zoom scan analysis revealed that the difference in m/z between the adjacent isotopes was 0.5, which indicated that the charge

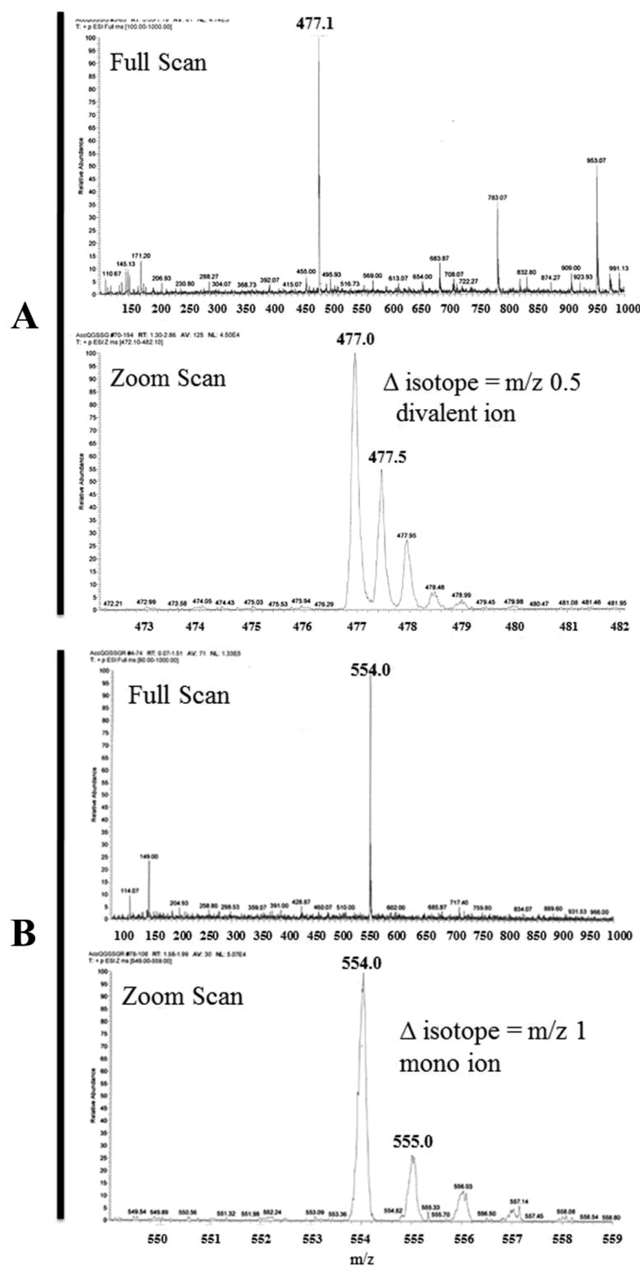

Figure 2. Mass spectrometry analyses of the GSH derivative that had been prepared without (A) and with (B) 2-mercaptoethanol pretreatment. Peaks marked A and B in Figure 1 were collected and analyzed by electrospray ionization (ESI) ion trap MS. The upper and lower panels represent full and zoom scan spectra, respectively.

number was 2. Assuming that this derivative had two protons, its molecular weight (x_1) can be calculated with the following formula: $(x_1 + 2)/2 = 477$, $x_1 = 952$. This value coincided with the molecular weight of the oxidized form of GSH that was coupled with two AccQ molecules through two amino groups, as shown in Figure 3A. However, the AccQ derivative with 2-mercaptoethanol pretreatment showed a main peak with a m/z of 554 (Figure 2B). The difference in m/z between the adjacent isotopes was 1.0. Then, its molecular weight (x_2) can be calculated as follows: $(x_2 + 1)/1 = 554$, $x_2 = 553$. This value did not coincide with the reduced form of AccQ-GSH that was conjugated through an amino group, but it corresponded to the AccQ−GSH−2-mercaptoethanol conjugate through an amino

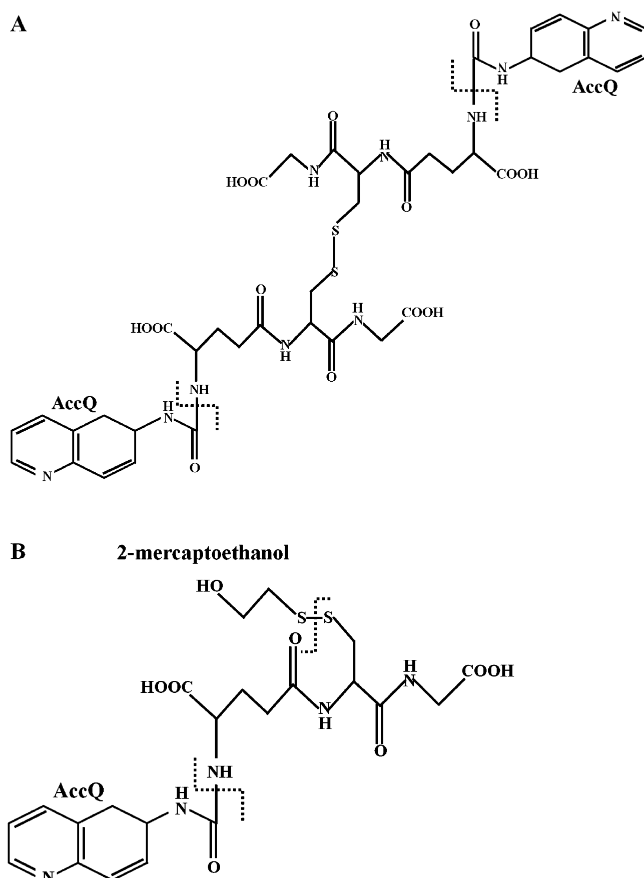

Figure 3. Estimated chemical structures of GSH derivatives that were prepared without (A) and with 2-mercaptoethanol pretreatment (B).

group and a sulfhydryl group, respectively, as shown in Figure 3B. The other peaks in Figure 1 could not be assigned to GSH derivatives, such as the AccQ–GSH conjugate with a free sulfhydryl group and the AccQ–GSH–AccQ conjugate through an amino and a sulfhydryl group. For preparation of these derivatives, the 5% TCA–2% 2-mercaptoethanol solution was removed by vacuum in the presence of TEA before reaction with the AccQ reagent. When this drying treatment with TEA was deleted, a similar chromatogram was obtained (Figure 4, upper) with a larger peak of the derivative (C) compared to the derivative with the drying treatment (A). The ESI-MS analysis of the derivative without the drying treatment with TEA (peak C) showed no significant peak of m/z 554, which was the protonated ion of the AccQ–GSH–2-mercaptoethanol conjugate (Figures 2B and 3B). However, two main peaks with m/z 171 and 249 were observed, and these corresponded to the AccQ fragment ion and the AccQ–2-mercaptoethanol protonated ion, respectively. These results indicated that the amino group of GSH was dissociated to the $-NH_3^+$ ion in the presence of a strong acid (TCA) and that it then could not react with the AccQ reagent. However, an excess amount of 2-mercaptoethanol reacted with AccQ through the sulfhydryl group under this condition. Therefore, the drying treatment with TEA was critical for the derivatization of GSH when the sample was pretreated with 5% TCA–2% 2-mercaptoethanol to liberate the protein-bound GSH.

On the basis of these findings, all of the blood samples were pretreated with 5% TCA–2% 2-mercaptoethanol, which was followed by the drying treatment with TEA before derivatization with AccQ for the determination of GSH by LC-MS/MS in the MRM mode. The fragment ions with the first and second high intensities (m/z 554.1 → 171.3 and m/z 554.1 → 145.2) were automatically selected.

Blood GSH Levels. Figure 5a shows the GSH contents in the deproteinized fraction of plasma before and after the ingestion of GSH. There was no significant difference in the GSH contents after the ingestion of GSH in this fraction. Much higher contents of GSH were contained in the blood cell fraction than in the deproteinized fraction of the plasma (Figure 5b). However, no significant difference was observed in the GSH contents in the blood cell fraction during the experimental period. However, the contents of GSH, γGlu-Cys, and Cys-Gly in the protein-bound fraction of plasma significantly increased 60–120 min after the supplementation of GSH (Figure 6). The protein-bound GSH and related compounds in plasma did not return to the initial level 2 h after supplementation.

DISCUSSION

GSH is present in high concentrations in liver and blood cells.[3,18] Generally, GSH concentrations have been determined in such samples with Ellman's reagent, which contains 5,5′-dithiobis(2-nitrobenzoic acid). To increase sensitivity, an enzyme recycling reaction using GSH reductase has been developed.[19,20] The GSH content in the blood cells, hepatic tissues, and other tissues has been determined with these methods. Allen et al. have reported that oral GSH supplementation did not improve blood cell GSH levels.[16] However, plasma GSH levels are lower (low μM levels) than those in blood cells. The plasma GSH levels have been determined by RP-HPLC with the precolumn derivatization of the sulfhydryl group or the enzyme recycling reaction method. Witschi et al. have observed no increase in plasma GSH levels after a single oral supplementation of GSH to healthy human volunteers at 0.15 mmol/kg body weight.[17] The present study confirmed these results (Figure 5). On the basis of these results, it has been suggested that the oral supplementation of GSH does not affect blood GSH levels.

It has been demonstrated that plasma proteins, including albumin, can bind to low molecular weight thiol compounds through a disulfide bond.[21,22] Therefore, there is the possibility that supplemented GSH may be transported as a conjugate of protein in the blood, and this has not been examined. In the present study, the effects of the supplementation of GSH on plasma protein-bound GSH levels were examined. To liberate GSH from protein conjugate, a reducing reagent is necessary. For this purpose, dithiothreitol has been frequently used,[23,24] but it is incompatible with the enzyme recycling reaction method. The dithiothreitol also reacts with labeling reagents for sulfhydryl and amino groups, which can interfere with the precolumn labeling reaction. In the present study, 2-mercaptoethanol was used to liberate GSH from the conjugates. The excess amount of 2-mercaptoethanol can be removed by vacuum in the presence of TEA. This drying treatment is, therefore, indispensable in the reaction between AccQ and an amino group of GSH. The final GSH derivative was double conjugated with AccQ and 2-mercaptoethanol through amino and sulfhydryl groups, respectively. As the final GSH derivative had no free sulfhydryl groups, it could not be oxidized to form a disulfide bond. A single GSH derivative was obtained, as shown by the MS analysis of the derivative peaks

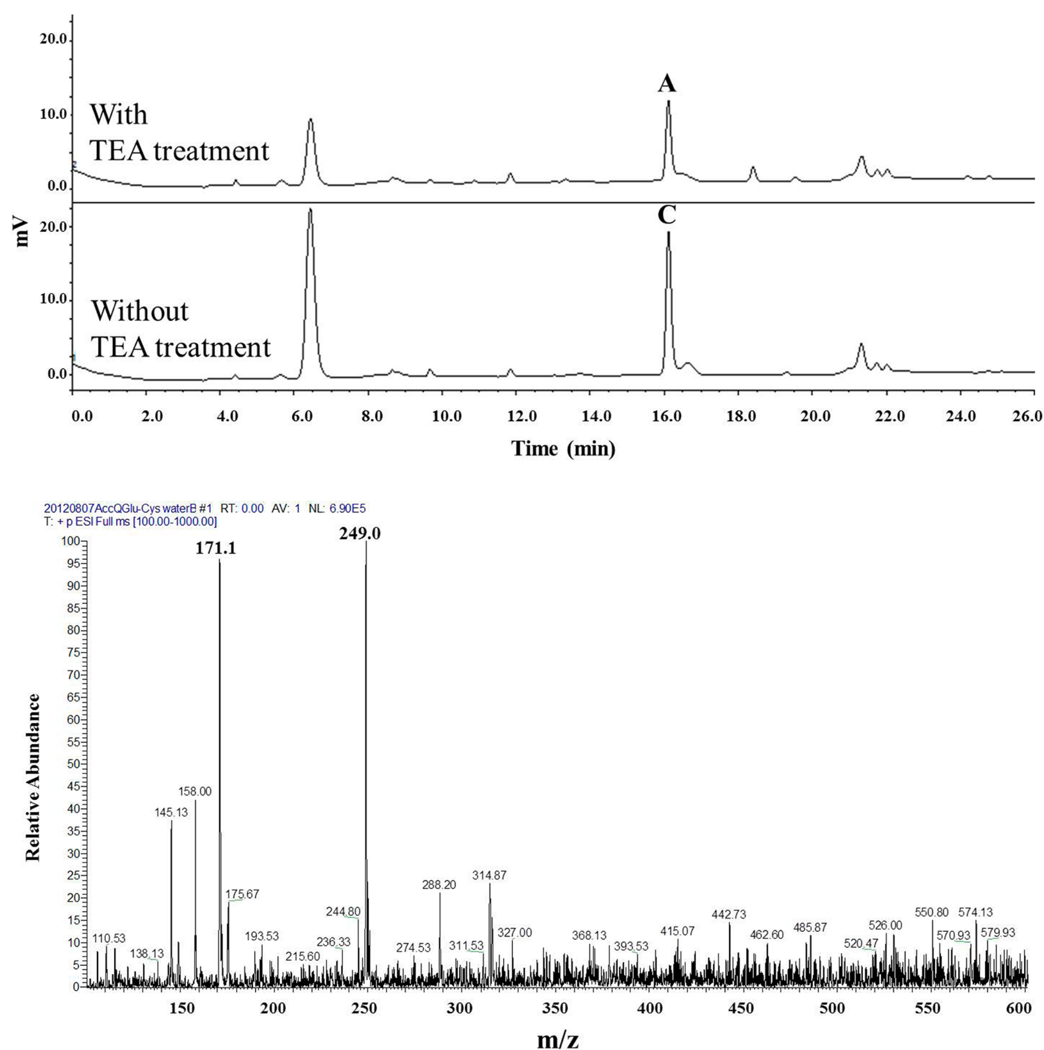

Figure 4. Effects of the drying pretreatment with TEA on the derivatization of 2-mercaptoethanol-pretreated GSH with AccQ: elution profiles of the derivatives with and without the TEA treatment (upper); ESI-MS analysis of the derivative without TEA treatment (lower).

(Figure 1). The GSH derivative could be resolved by RP-HPLC and detected by fluorescence. Other derivatives of thiol compounds, such as γGlu-Cy and Cys-Gly, however, show similar retention times (data not shown). These peaks may interfere with the determination of GSH by fluorescence detection.

Therefore, the MRM was used for the specific detection of the GSH and its fragment peptides (γGlu-Cys, Cys-Gly) by monitoring m/z 554 → 171.3 and m/z 554 → 145.2 (GSH), m/z 497 → 171.3 and m/z 497 → 145.2 (γGlu-Cys), and m/z 425 → 171.2 and m/z 425 → 116.1 (Cys-Gly), respectively, in the present study. By using this method, protein-bound GSH and its fragment peptides at a concentration of <1 μM in the plasma could be successfully determined.

Ikegaya et al. have demonstrated that cysteine and cysteinyl-glycine bind to human serum albumins through disulfide bonds, whereas no significant amount of GSH is bound to serum albumin that is obtained from human blood.[22] The present study also demonstrated that only a negligible amount of GSH was bound to plasma protein before the supplementation of GSH. However, the protein-bound GSH significantly ($P < 0.01$) increased from 60 to 120 min after the oral supplementation of GSH. This is the first report to demonstrate an increase in GSH in the human blood fraction by the oral supplementation of GSH. The protein-bound form GSH level in plasma after supplementation of GSH is much higher (>1000 times) than that of other food-derived peptides such as Val-Tyr[25] and Ile-Pro-Pro[26] but less than that of the food-derived collagen peptides in human blood.[27]

It has been thought that orally administered GSH is successively degraded to cysteinyl-glycine, cysteine, and glycine by γ-glutamyl-transferase and peptidase.[17,28,29] Cysteine could be used for GSH synthesis in cells. Increased levels of protein-bound GSH might be derived from the newly synthesized GSH. The present study also detected fragment peptide (Cys-Gly) and precursor peptide (γGlu-Cys) as protein-bound forms in human blood, which suggests some GSH is synthesized from degradation products of GSH.

However, an early study by Kubo that used ^{35}S-labeled GSH and paper electrophoresis has suggested that GSH could be directly absorbed from the small intestine into rat portal blood.[30] Therefore, there is a possibility that supplemented GSH is directly absorbed into human blood and bound to plasma protein. To solve these problems, further studies on the metabolic fate of supplemented GSH that use ^{13}C-labeled GSH are in progress. The procedure that was used in the present study for the determination of protein-bound GSH that was based on LC-MS/MS and precolumn label techniques can

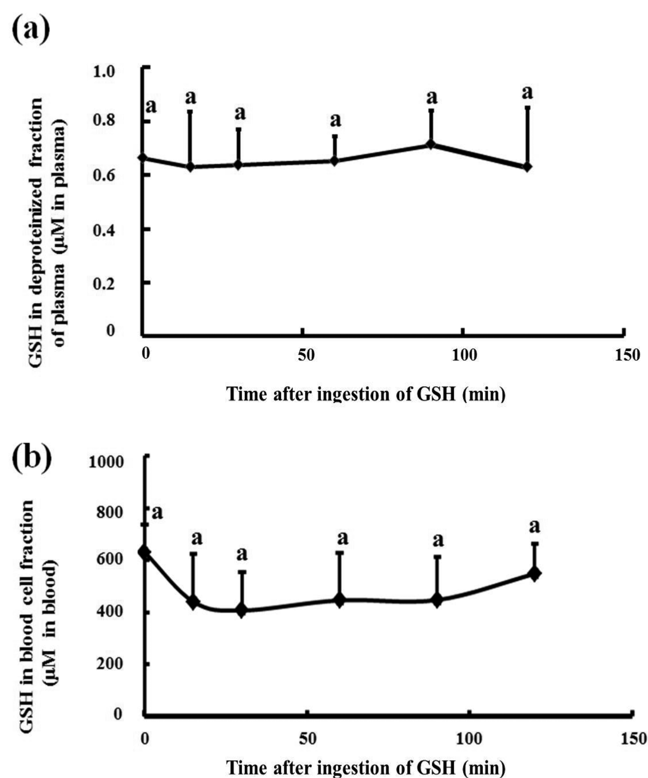

Figure 5. Total GSH contents in the deproteinized (ethanol-soluble) plasma fraction (a) and blood cell fraction (b) after the ingestion of GSH. No significant difference was observed between groups ($P < 0.01$).

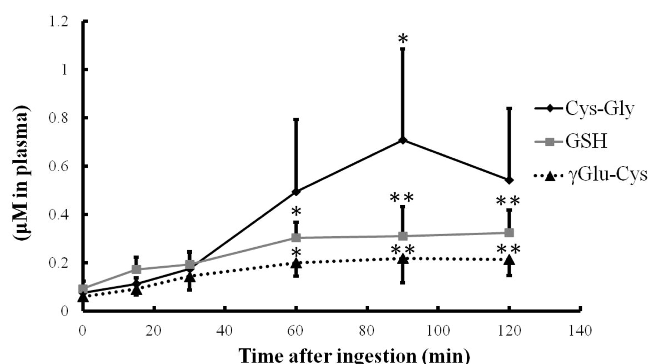

Figure 6. Contents of glutathione (GSH), γ-glutamyl-cysteine (γGlu-Cys), and cysteinyl-glycine (Cys-Gly) in the ethanol precipitate fraction of plasma after ingestion of GSH. (*, **) Significantly different from baseline $P < 0.05$ and 0.01, respectively, according to Scheffe's post hoc test.

distinguish food-derived (^{13}C-labeled) and endogenous GSH and their metabolites, such as γGlu-Cys, Cys-Gly, and cysteine, and this is a powerful tool for the elucidation of the absorption, metabolism, and transportation of food-derived GSH.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

GSH, glutathione; γGlu-Cys-Gly, γ-L-glutamyl-L-cysteinyl-glycine; Cys-Gly, L-cysteinyl-glycine; γGlu-Cys, γ-L-glutamyl-L-cysteine; AccQ, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate; TEA, triethylamine; TCA, trichloroacetic acid; RP-HPLC, reversed phase high-performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; LC-MS/MS, liquid chromatography−tandem mass spectrometry; ESI-MS/MS, electrospray ionization tandem mass spectrometry

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