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In Vitro and ex Vivo Uptake of Glutathione (GSH) across the Intestinal Epithelium and Fate of Oral GSH after in Vivo Supplementation

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ABSTRACT: Glutathione (GSH) is the most prevalent low-molecular-weight thiol in mammalian cells and is crucial for antioxidant defense, nutrient metabolism, and the regulation of pathways essential for whole body homeostasis. GSH transport systems have been identified in the membranes of various tissues and organs, including the small intestine. However, little is known regarding GSH transport across intestinal epithelial cells. The current in vitro and ex vivo uptake study of GSH demonstrated that intact GSH can be transported across intestinal epithelial cells, suggesting that GSH uptake is not proton-dependent. It would appear that the initial uptake of GSH into cells is a rapid process. Furthermore, the visualized GSH after 60 min of transport by MALDI-MS imaging showed localization of intact GSH inside the intestinal wall. In vivo study found that ingested ^{13}C-GSH was rapidly converted to GSSG and accumulated in red blood cells and liver, but was little present in plasma. The ingested GSH has potent nutraceutical benefits for human health to improve oxidative stress and defense in human.

KEYWORDS: *glutathione, Caco-2, transport, MALDI-MS imaging, intestinal epithelial cell, LC-MS/MS*

INTRODUCTION

Glutathione (GSH) is the most prevalent low-molecular-weight thiol in mammalian cells.[1] It is crucial for antioxidant defense and the regulation of pathways essential for whole body homeostasis via catalysis by glutathione *S*-transferases (GST) and glutathione peroxidases (GPx).[2,3] Glutathione deficiency is associated with oxidative stress and, therefore, may play a key role in aging and the pathogenesis of many chronic diseases.[3]

The two cytosolic enzymes, γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase, catalyze GSH synthesis from glutamate, cysteine, and glycine. This pathway occurs in virtually all cell types, with the liver being the major producer and exporter of GSH.[3] Glutathione can be synthesized through its recycling after oxidation. Glutathione catabolism occurs extracellularly by the activity of γ-glutamyltransferase (γ-GT), which is expressed mainly on the apical surface of cells. It cleaves the γ-glutamyl moiety from GSH, transferring it to other amino acids or dipeptides and producing cysteinylglycine or cysteinylglycine conjugates. These products are further hydrolyzed by dipeptidases and taken up into the cell via specific transporters, where they can participate in GSH synthesis.[4,5]

Intact GSH may be transported across both basolateral and apical surfaces, and GSH transport systems have been identified in the membranes of various tissues and organs, including the small intestine.[6,7] It has been reported that oral administration of GSH could be absorbed with intact form in vivo and that dietary GSH or supplemental GSH might be an important source of circulating GSH.[8] However, there are controversial reports regarding the bioavailability of orally administered GSH in intact animals.[9,10] The uptake of intact GSH into plasma membrane vesicles in intestinal epithelial cells has been reported by Vinzenzinin et al.[11] However, other groups have reported that orally induced GSH could be degraded by γ-glutamyl transferase and peptidases at the brush border membrane at the intestinal epithelial cell surface into amino acids of γ-glutamyl amino acid, cysteine, and glycine.[12,13] In fact, a relatively large dose of orally administered GSH did not increase serum GSH levels in human subjects, and it has been concluded that dietary GSH does not alter serum GSH and this is because of degradation of intact GSH.[14,15] Grattagliano et al.[16] also found that GSH and GSH monoethyl ester (GSHE) do not affect the circulating concentration of GSH and cysteine unless very high doses are administered, but increased hepatic cysteine and GSH at lower doses because of the efficient extraction by the liver of cysteine originating from the breakdown of GSG and GSHE in the gut. After careful review of these past work on GSH uptake, we found that these controversial results might be due to differences of sample pretreatment and sampling for GSH analysis. For example, those who reported no effect of GSH intake orally conducted only plasma GSH analysis.[14−16] However, we found in this study that ingested GSH is rapidly converted into GSSG

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and does not exist as free GSH in plasma, but was deposited into red cells or liver for future uses.

The objective of this work was to examine whether intact glutathione (GSH) can be transported across the intestinal epithelium and to characterize its uptake, as well as to determine the fate of orally administered GSH in animal models.

MATERIALS AND METHODS

Materials. GSH was kindly provided by Kohjin Life Science Ltd., Tokyo, Japan. ^{13}C-GSH was synthesized by a conventional liquid-phase method via ^{13}C-GSSG using the disulfide bond as a thiol-protecting group. L-Cystine containing 1,1′-^{13}C isotopes (L-cystine-1,1′-$^{13}C_2$, Sigma-Aldrich, St. Louis, MO, USA) was converted into the corresponding *N*,*N*′-*tert*-butyloxycarbonyl (Boc) protected ^{13}C-cystine using di-*tert*-butyl dicarbonate. The crude ^{13}C-GSH was purified using reversed-phase HPLC on a Nakalai Tesque C18 column (COSMOSIL 5C18-AR-II, 20 × 250 mm). The final product was characterized by reversed-phase HPLC using a TSKgel ODS-100S (3.0 × 150 mm; Tosoh, Tokyo, Japan) and electrospray ionization time-of-flight mass spectrometry (ESI-TOFMS) with a Hitachi NanoFrontier LC-MS system (Tokyo, Japan). MS (ESI) *m*/*z*: 308.99 $[M + H]^+$ (calcd 309.09) (data not shown).

In Vitro Transport of GSH across Human Intestinal Epithelial Cell Monolayers. In vitro transport experiments were performed using Caco-2 or HT-29 human intestinal epithelial cells (ATCC). Caco-2 or HT-29 cell monolayers were grown in Costar transwell inserts (1.0 cm^2, 0.4 μm pore size) (Corning, Cambridge, MA, USA) in Eagle's minimal essential medium (MEM) (Invitrogen, Burlington, ON, Canada) containing 2 mM L-glutamine, 1 mM sodium pyruvate (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 50 U/mL penicillin−streptomycin (Invitrogen), and 20% FBS (HyClone) (Fisher Scientific, Ottawa, ON, Canada). The cells were seeded at a density of 0.6 × 10^5 cells/cm^2, and the culture medium was changed every second day until confluent monolayers with transepithelial electrical resistance (TEER) values >500 Ω·cm^2 were obtained. TEER values were measured using a Millicell-ERS Volt-Ohm Meter (Millipore, Bedford, MA, USA). Cell monolayers were rinsed with Hanks' balanced salt solution (HBSS) adjusted to pH 7.4 with 10 mM HEPES, and the same buffer was added to both apical and basolateral chambers. After equilibration in buffer for 30 min, GSH (1 mM) was added to the apical chamber. Cells were incubated at 37 °C, and aliquots were removed from the basolateral chamber at the indicated time points. Apparent permeability coefficients (P_{app}) were calculated using the formula

$$P_{app} = V_R/A \times C_0 \, dC/dt$$

where V_R is the volume in the basolateral chamber, A is the filter surface area, C_0 is the initial GSH concentration in the apical chamber, and dC/dt is the slope of the curve of basolateral GSH concentration versus time. To examine the effect of pH on GSH uptake, 1 mM GSH was prepared in HBSS buffer adjusted to pH 6.0 with 10 mmol/L MES and was added to the apical chamber.

To examine if GSH could be transported intact, Caco-2 cells were first incubated with 0.25 mM Acivicin (AT-125; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min and/or 0.5 mM buthionine sulfoximine (BSO) (Sigma-Aldrich), to inhibit GSH degradation and synthesis, respectively. Probenecid (PB) (Sigma-Aldrich) (1 mM), a known organic anion transport inhibitor and inhibitor of GSH transport, was added 30 min before the addition of GSH.

Cell Viability Assay. Viability of the treated cells was measured using the WST-1 Cell Proliferation Reagent (Roche Applied Sciences, Laval, Quebec, Canada) according to the manufacturer's instructions. Briefly, 20 μL of WST-1 was added to the basolateral chamber, and cells were incubated for 1−2 h. Absorbances were read at 450 nm and converted to percent viability relative to untreated control cells.

Ex Vivo Transport of GSH across Rat Intestinal Epithelial Tissue. Sections of small intestine were removed from 8-week-old male Sprague−Dawley rats (SPF/VAF Crj:SD; Charles River Japan., Kanagawa, Japan) and mounted into an Ussing chamber for transport. Krebs−Ringer bicarbonate (KRB) buffer, adjusted to pH 6.0 and 7.4, was added to apical and basolateral chambers, respectively. After equilibration for 30 min, 10 mM GSH was added to the apical chamber, and transport was allowed to proceed for 60 min. After the 60 min incubation, the intestinal tissue membrane was removed and frozen using powdered dry ice. The frozen tissue was cryosectioned by a cryostat (Leica) with 12 μm thickness to prepare cross sections of intestinal wall and thaw-mounted to an indium−tin oxide (ITO)-coated glass slide followed by drying under N_2 gas for analysis by mass spectrometry.[17]

In Vivo Supplementation with ^{13}C-GSH in Mice. Six 8-week-old female BALB/c mice (16−20 g) (Charles River Laboratories Inc., Montreal, Quebec, Canada) were group housed on a 12 h light−dark cycle and allowed unrestricted access to standard mouse chow and water. All animal studies were approved by the University of Guelph Animal Care Committee and carried out in accordance with the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals. Balb/c mice were administered ^{13}C-GSH (10 mg/mouse) by oral gavage. Control mice received vehicle alone. The dose of 10 mg ^{13}C-GSH per mouse was determined on the basis of in vitro assay and actual human subject trial in the past. Mice (5 mice/group) were euthanized at the time points indicated, and blood and liver samples were collected. Liver samples were flash-frozen (−80 °C) for further analysis. Blood samples were immediately flushed with N_2 gas under heparin-treated clinical tube (Fisher Sci) and centrifuged at 4 °C (3500*g* for 10 min). The red blood cells (RBCs) were treated by lysate buffer (Sigma-Aldrich) and subjected to further analysis. The plasma and RBC samples were immediately subjected to GSH and GSSH measurement. A reference GSH was used to confirm that GSH was not converted to GSSG during these sample pretreatments (data not shown).

Enzymatic Detection of Transported GSH. GSH was quantified in the Caco-2 cells and HT-29 basolateral chamber using an enzymatic assay for the detection of GSH/GSSG, according to the manufacturer's instructions (Oxford Biomedical Research, Rochester Hills, MI, USA). Briefly, a GSH/GSSG assay was designed to measure total, reduced, and oxidized glutathione using an Ellman's reagent (DTNB) and glutathione reductase (GR). This method can be used to measure oxidized (GSSG) by using a specific protocol, which first scavenges all GSH with 1-methyl-2-vinylpyridinium triflate. GSH concentrations were calculated from a GSSG standard curve, and results are expressed as micromolar total GSH (GSH_t) in the basolateral solution. The total GSH, reduced GSH, and GSSG in mouse plasma, red cells, and liver were quantified using the same protocol.

Detection of GSH and ^{12}C-GSH by Mass Spectrometry. To confirm transport of intact GSH across Caco-2 monolayers, basolateral solutions were analyzed by LC/TOF-MS for the presence of GSH or GSH degradation products. Samples were derivatized using ThioGlo3 reagent (Covalent Associates, Inc., Corvallis, OR, USA) by incubating 20 μL of basolateral solution with 20 μL of 0.2 mM reagent and incubating for 1 h at room temperature. LC-MS was carried out using a Cosmosil 5C_{18}-MS-II column (i.d. 2.0 mm × 150 mm, Nacalai Tesque, Kyoto, Japan) with a linear gradient of 0−100% methanol containing 0.1% formic acid at a flow rate of 0.2 mL/min. MS conditions were as follows: mode, negative-low, expert mode; nebulizer, 1.6 bar; dry gas, 8.0 L/min; mass range, 100−1000; hexapole RF, 200 Vpp; dry temperature, 200 °C.

Detection of ^{12}C-GSH transported by rat small intestine was carried out using matrix-assisted laser desorption/ionization time-of-flight imaging mass spectrometry (MALDI-IMS) as described by Hong et al. with modifications.[17] Briefly, the matrix reagent and ionization enhancer (20 mg/mL 2,4,6-trihydroxyacetophenone (THAP) with 10 mM diammonium citrate) were dissolved in 50% ACN/0.1% TFA and uniformly sprayed using an ImagePrep automatic matrix sprayer (Bruker-Daltonics, Tokyo, Japan). MALDI-MS imaging was preformed using autoflex III equipped with smart beam (Bruker-Daltonics) with the following analytical parameters: mode, positive; ion source 1, 20.00 kV; ion source 2, 18.80 kV; lens, 7.50 kV; pulsed ion extraction, 0 ns; mass range, 120−1000 Da; laser frequency, 200.0 Hz; number of shots, 500 shots/spot; spatial resolution (spot distance), 50 μm; laser attenuator, 1 minimum; laser power, 100%.

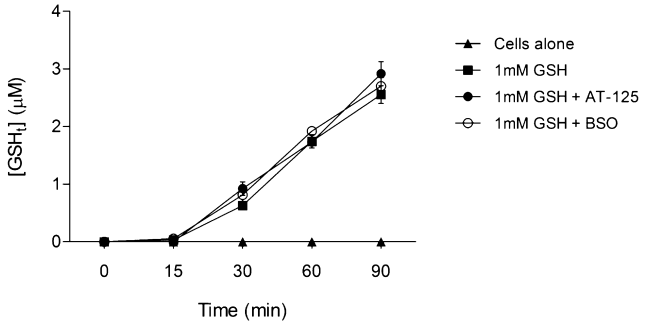

Figure 1. Time-dependent transport of 1 mM GSH across Caco-2 cell monolayers in the presence or absence of 0.25 mM AT-125 or 0.5 mM buthionine sulfoximine (BSO).

Table 1. Apparent Permeability Coefficients (*Papp*) for the Transport of 1 mM GSH across Caco-2 Cell Monolayers in the Presence or Absence of 0.25 mM AT-125 or 0.5 mM Buthionine Sulfoximine (BSO)

treatment	$P_{app} \times 10^{-6}$ (cm/s)
1 mM GSH	1.28 ± 0.11[a]
1 mM GSH + AT-125	1.23 ± 0.35[a]
1 mM GSH + BSO	1.20 ± 0.15[a]

[a]Similar letters following entries indicate no statistically significant difference.

Determination of ^{13}C-GSH and ^{13}C-GSSG by Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS). The concentrations of ^{13}C-GSH and ^{13}C-GSSG in the blood, red cells, and liver samples were determined with a slight modification of a previously reported ^{13}C-stable isotope labeled dipeptide detection method.[18] Sample preparation followed the method described by Nakashima et al.[18] To prevent oxidation of GSH, all samples were flushed by N_2 gas at 4 °C. The derivates of GSH were prepared by the same method described above using ThioGlo3 reagent. A chromatographic separation was performed on a Biosuite C18 PA-A column (Waters, Milford, MA, USA), and a mobile phase of CH_3CN-water containing 0.1 FA) and a flow rate (0.20–0.30 mL/min) were applied. LC-MS/MS was performed as described above. Three separate replicates of ^{13}C-GSH with different concentrations between 0.1 and 5.0 ng/mL were conducted for obtaining the calibration curve. We detected about 7% of endogenous ^{13}C-GSH against ^{12}C-GSH in vehicle mouse tissue samples (data not shown); therefore, we determined the actual exogenous ^{13}C-GSH by subtracting the endogenous one. The ^{13}C-GSSG and reduced ^{13}C-GSH were quantified on the basis of a combination of the LC-MS/MS and an enzymatic assay.

Statistical Analysis. Data are expressed as means ± SEM. Statistical analyses were performed with GraphPad Prism version 5.0 (GraphPad, San Diego, CA, USA) using one-way ANOVA followed by Tukey's multiple-comparison test. Student's *t* test was used when comparisons between only two groups were carried out. Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Several mechanisms may be involved in the uptake and utilization of luminal GSH by brush-border membrane enzymes in the intestine, including (1) cleavage of GSH into cysteinyl glycine, γ-glutamyl amino acids, or its constituent amino acids (Glu, Cys, Gly) by γ-glutamyltransferase, followed by their transport and intracellular GSH synthesis; and (2) transport of intact GSH directly by specific transporters.[19] GSH transport systems have been identified in the membranes of various tissues and organs, including renal cells, pulmonary alveolar

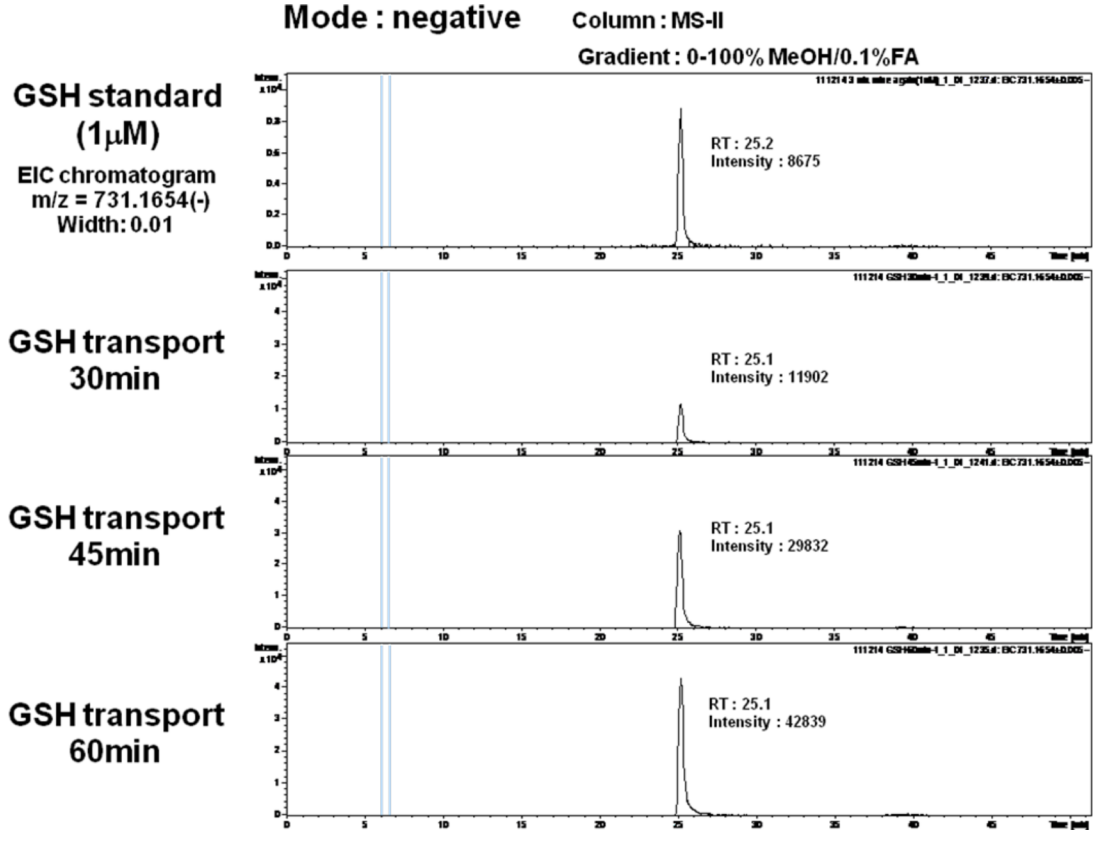

Figure 2. LC-MS analysis of transported GSH over 60 min. Transported samples were compared to a known GSH standard (top panel).

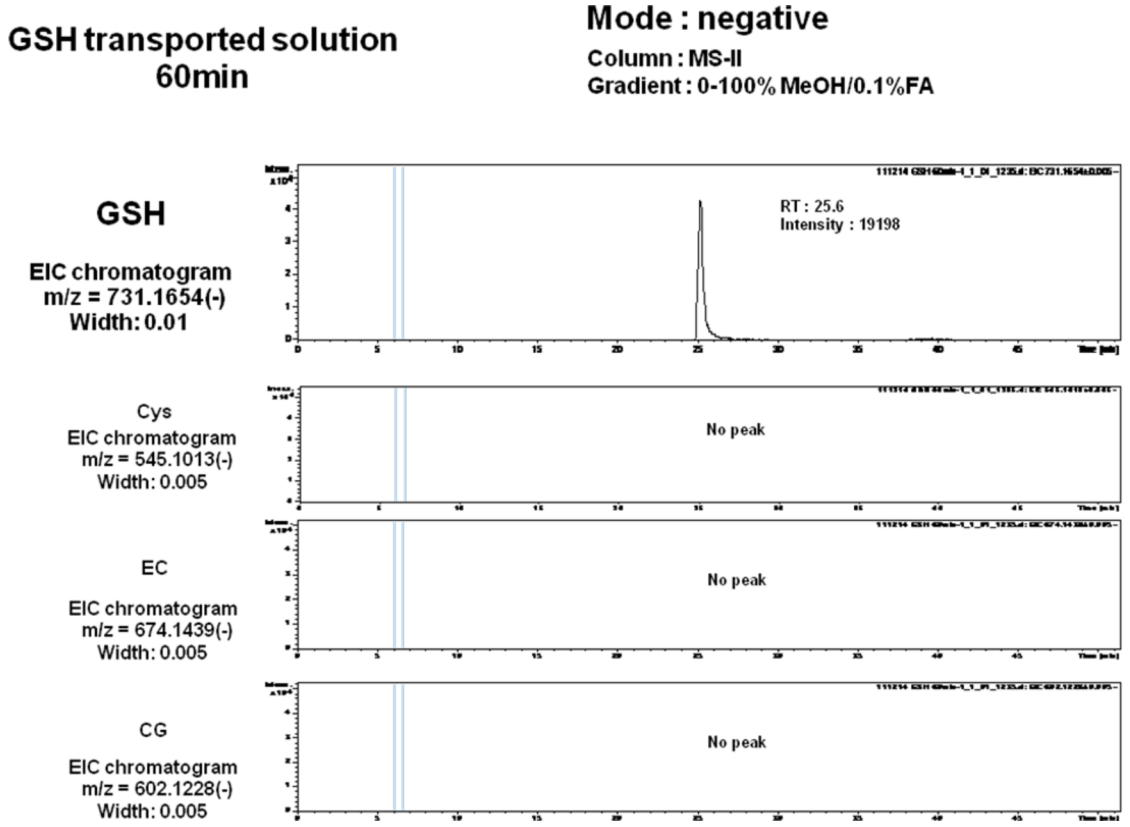

Figure 3. LC-MS analysis of transported GSH (top panel), showing absence of GSH degradation products.

cells, retinal pigment epithelial cells, brain endothelial cells, but less is known about its transport across the intestinal epithelium. Several studies examining GSH uptake in the intestine have been carried out using different animal species and techniques including pig and rabbit intestinal brush-border membrane vesicles (BBMV),[5,11,20,21] vascularly perfused rat small intestine,[8] and rat intestinal epithelial cells.[22] However, little is known regarding GSH transport across intestinal epithelial cells. Here, the transport of intact GSH across human intestinal epithelial cells was examined in vitro using Caco-2 cell monolayers. GSH was detected in the basolateral transport chamber of the cell monolayers using a GSH/GSSG enzyme assay and the structure of intact GSH confirmed by mass spectrometry (LC-MS).

Caco-2 cell monolayers were treated with exogenous GSH (1 mM), and the amount of GSH present in the basolateral transport chamber of Caco-2 cell monolayers was measured at different time points using a GSH/GSSG enzyme assay. To confirm that the GSH detected in the basolateral chamber was not as a result of GSH degradation by brush-border membrane enzymes and subsequent resynthesis within the cells, cells were treated with Acivicin (AT-125) and/or buthionine sulfoximine (BSO). AT-125 can prevent GSH degradation by irreversibly inhibiting γ-glutamyltransferase, and BSO can prevent GSH synthesis through inhibition of γ-glutamylcysteine synthetase. To test whether intact GSH is transported across Caco-2 cell monolayers, cells were pretreated with AT-125 or BSO, and GSH concentration in the basolateral chamber was measured as a function of time during incubation with 1 mM exogenous GSH.

Transported GSH was measured at various time points (Figure 1), and the apparent permeability coefficients, P_{app}, were calculated. There was no significant difference between the concentration of GSH detected in the presence or absence of the inhibitors AT-125 or BSO, indicating that the GSH present in the basolateral chamber was likely due to the uptake/transport of intact GSH across the Caco-2 monolayers, and not GSH resynthesis. No GSH was detected from untreated control cells (cells alone). Likewise, there was no difference in the permeability (P_{app}) of GSH in the presence or absence of either inhibitor (Table 1). Cell viability did not appear to be affected by the treatments (data not shown). Analysis by LC-MS confirmed that the detected sample was indeed intact

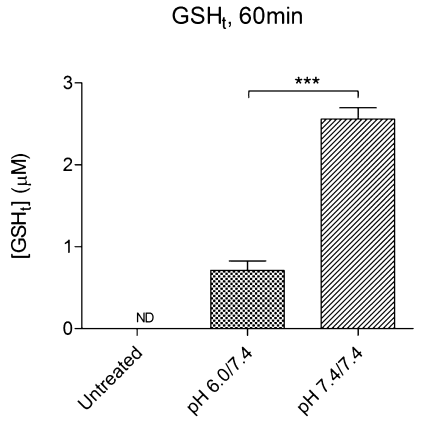

Figure 4. pH-dependent uptake of 1 mM GSH across Caco-2 cell monolayers in the presence of 0.25 mM AT-125 after 60 min at pH 6.0/7.4 indicates an apical pH of 6.0 and a basolateral pH of 7.4. pH 7.4/7.4 indicates apical and basolateral pH values of 7.4. ND, none detected. (***) $P < 0.001$. Untreated denotes 60 min of incubation in the absence of GSH at pH 7.4.

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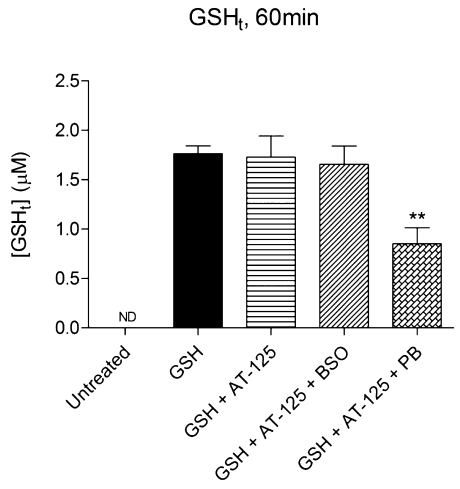

Figure 5. Uptake of 1 mM GSH across Caco-2 cell monolayers in the presence or absence of 0.25 mM AT-125 and 0.5 mM buthionine sulfoximine (BSO) or 1 mM Probenecid (PB) after 60 min. ND, none detected. (**) $P < 0.01$ relative to GSH, GSH + AT-125, and GSH + AT-125 + BSO. Untreated denotes 60 min of incubation in the absence of GSH at pH 7.4.

GSH by comparison to a known GSH standard (Figure 2). Moreover, no products of GSH degradation (e.g., cysteine, cysteinyl-glycine) were observed in the transported samples (Figure 3). These results indicated that intact GSH can cross intestinal epithelial cells without degradation, in contrast to previous reports.[14−16] Previously, it was speculated that intestinal epithelial cells also have the same mechanistic transport of GSH with other tissues of renal cells, pulmonary alveolar cells, retinal pigment epithelial cells, and brain endothelial cells,[19] whereas no-one has reported clearly with direct evidence until the current work. This is the first study to demonstrate direct uptake of GSH using human intestinal epithelial cells.

To examine whether GSH uptake is pH dependent or not, we carried out a proton-dependent study using different pH gradients at apical and basolateral sides (both apical and basolateral pH values of 7.4 or an apical pH of 6.0 and a basolateral pH of 7.4, typical of proton-dependent transporters such as the di/tripeptide transporter PepT1). The amount of GSH detected in the basolateral solution was significantly higher when an apical pH of 7.4 was used when compared to an apical pH of 6.0 (Figure 4), suggesting that GSH uptake is not proton-dependent. These results are in line with previous reports that uptake of GSH into brush-border membrane vesicles was not proton-driven and occurred optimally around pH 7.5.[21] To further characterize GSH uptake across the Caco-2 monolayers, transport was carried out in the presence of the inhibitor Probenecid (PB), a known organic anion transport inhibitor and inhibitor of GSH transport in basolateral membrane vesicles.[23] The amount of GSH detected in the basolateral solution of PB-treated cells was significantly reduced when compared to all other samples (Figure 5), suggesting transport of intact GSH.

It was notable when we used HT-29 cell monolayer, which does not express the human PepT1 transporter with the same conditions. We observed similar results (data not shown) and

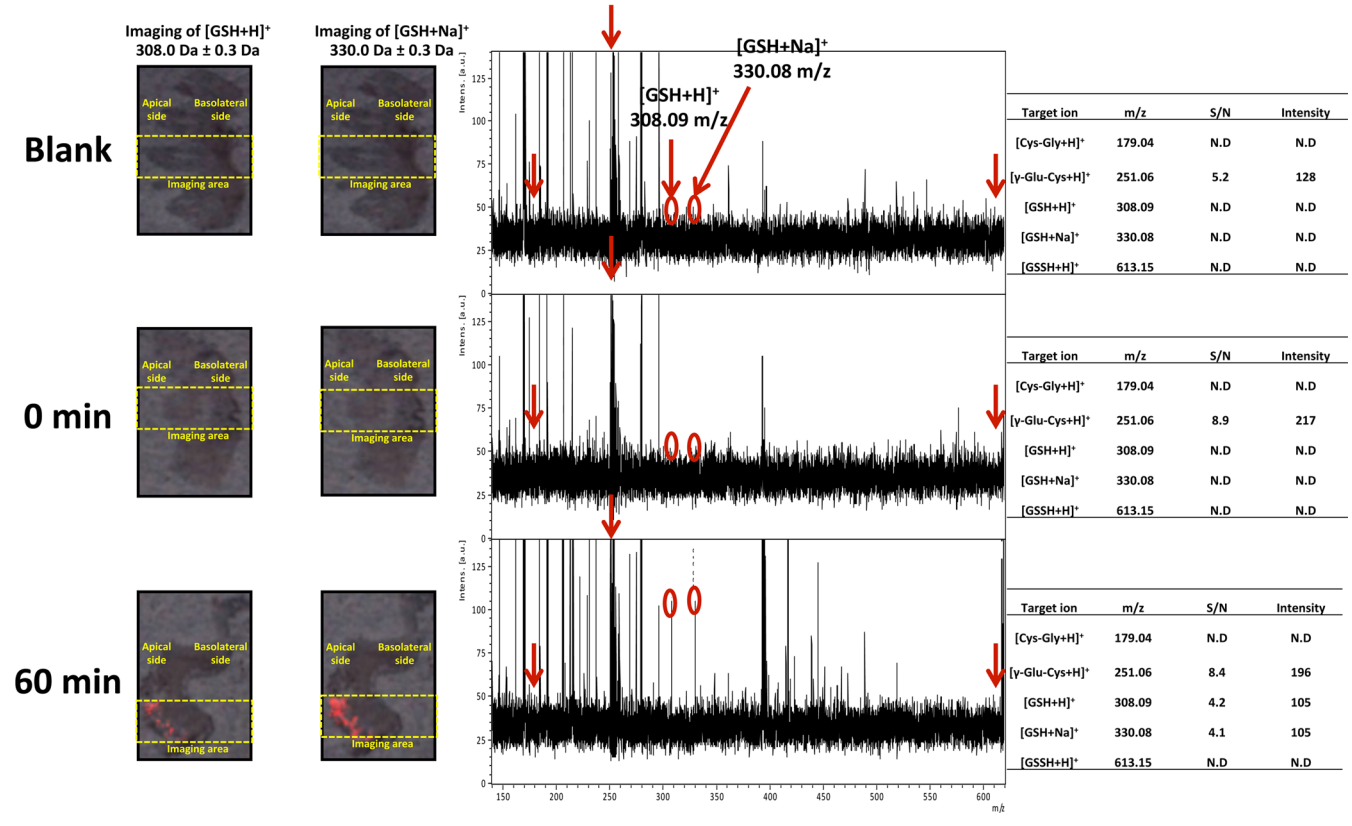

Figure 6. MALDI-MS imaging and spectra of transported GSH using rat intestinal membrane.

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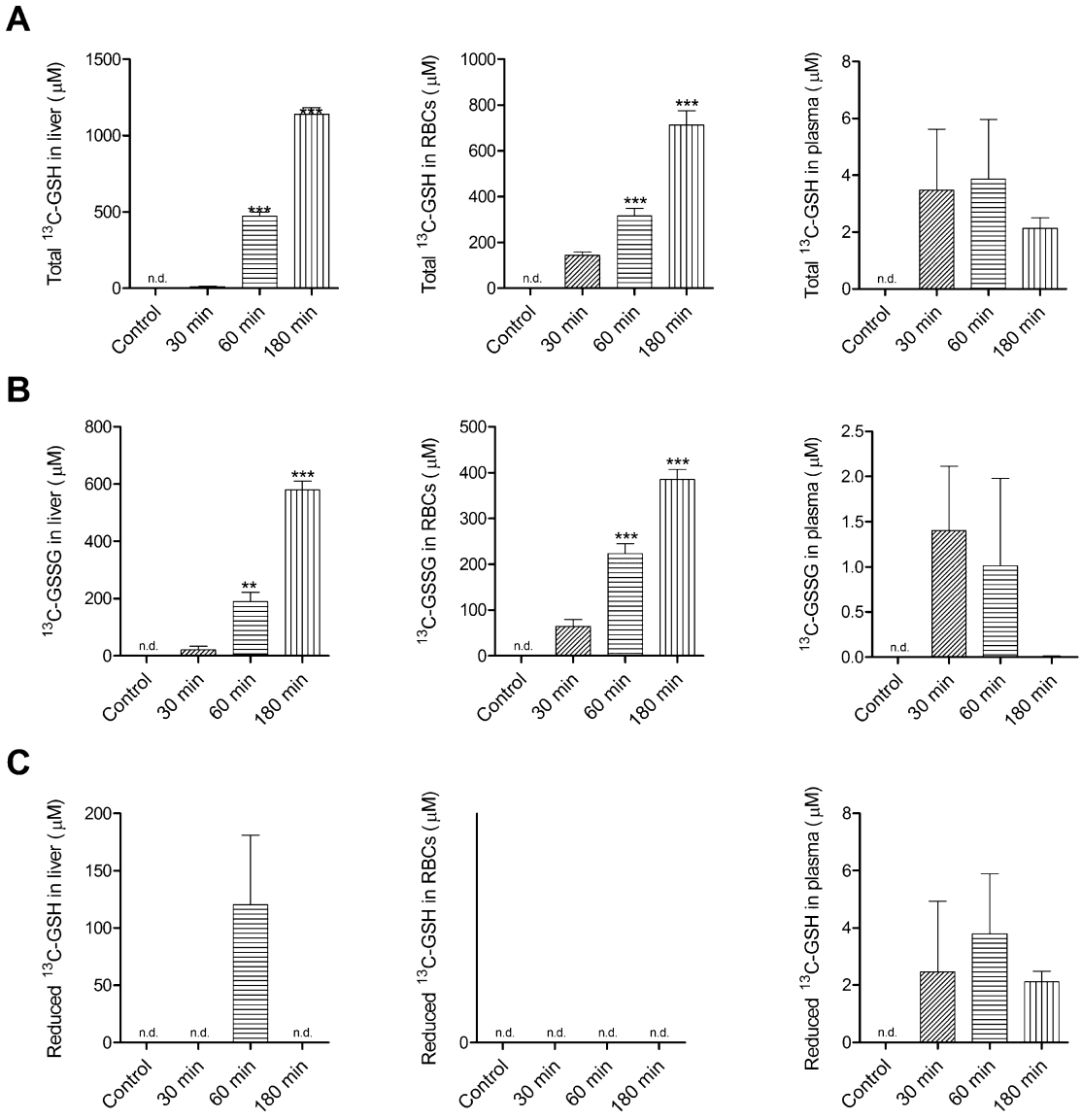

Figure 7. Measurement of (A) total ^{13}C-GSH, (B) ^{13}C-GSSG, and (C) reduced ^{13}C-GSH in liver, red blood cells (RBCs), and plasma of mice orally administered 10 mg of ^{13}C-GSH. n.d., none detected. (**) $P < 0.01$ and (***) $P < 0.001$ relative to vontrol.

could confirm that intact GSH is likely being taken up or transported across the HT-29 monolayers, and not GSH resynthesis. GSH transportation across human epithelial cells is not PepT1-mediated transport. The GSH-specific transport system in human small intestine epithelial cells was reported as a Na^+-independent transport system[24] or Na^+-coupled GSH transport.[25] However, its detailed transport mechanism is still not well understood. Further characterization of GSH transport may be required, including using the Caco-2 BB (C2BBe1) cell line or examining the effect of mono- and bivalent cations on GSH uptake, and using a more sensitive detection method to detect GSH uptake at earlier time points to gain a better understanding of uptake kinetics as from the literature it would appear that the initial uptake of GSH into cells is a rapid process.[20,21]

Rat intestinal epithelial tissues were treated with exogenous GSH, and transported ^{12}C-GSH was visualized using MALDI-IMS, which has been similarly used to detect small transportable peptides in rat small intestine.[17] MALDI-MS imaging of GSH in the intestinal tissue sections showed clear localization of transported ^{12}C-GSH as $[GSH + H]^+$ and $[GSH + Na]^+$ in the apical side of the intestinal wall after 60 min of incubation with 10 mM GSH (Figure 6). The presence of endogenous GSH in the intestinal tissue was not visualized in blank intestinal wall subjected to 60 min of incubation with KBR buffer without GSH. Neither endogenous nor transported GSSH (oxidized form of GSH), Cys-Gly, or γ-Glu-Cys was visualized in any of the intestinal samples tested (blank, 0 and 60 min GSH treated intestines). The absence of GSH on the apical surface of intestinal tissue after 0 min (actually ∼10 s of incubation) of incubation with GSH would strongly suggest that the visualized GSH after 60 min of transport showed localization of intact GSH inside the intestinal wall. To our knowledge, this is the first report of visualized intact GSH in the intestinal wall and demonstrates its presence in the tissues with intact form. Matrix-assisted laser desorption/ionization time-of-flight imaging mass spectrometry (MALDI-IMS) has been developed to visualize the target molecules in tissues, and this technique is a powerful tool for bioactive metablomics study. However, it was limited for only biological compounds with molecular weight of 1−50 kDa, and no small bioactive peptides (<500 Da) were visualized. Recently,

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there has been much attention to the study of small di/tripeptides metabolomics, and we reported a new technique of small peptide MALDI-IMS.[17] The current MALDI-IMS visualization of GSH in intestinal wall can provide new insights of intestinal absorption behavior of bioactive small compounds as foods and drugs and omics analysis of small metabolites in various tissues.

To determine the fate of orally ingested GSH, mice were supplemented with ^{13}C-GSH (10 mg/mouse), and total ^{13}C-GSH, ^{13}C-GSSG, and reduced ^{13}C-GSH in liver, RBCs, and plasma were quantified. Total ^{13}C-GSH in liver and RBCs was significantly elevated 60 min after ingestion when compared to control mice, but no change in plasma total GSH was observed (Figure 7A). Interestingly, we found that ingested ^{13}C-GSH was rapidly converted to GSSG and accumulated in RBCs and liver, but was not present in plasma (Figure 7B). Reduced ^{13}C-GSH was low or negligible in all samples tested (Figure 7C), suggesting that ^{13}C-GSH was predominantly deposited in the form of GSSG in the red cells and liver tissues after ingestion of GSH. Previously, it has been reported that dietary GSH does not increase serum GSH level after ingestion and its bioavailability is low, but increases only liver cysteine originating from the breakdown of ingested GSH in the gut.[14−16] This is in line with orally ingested GSH not altering plasma total, GSSG, and reduced GSH level from the current work as reported previously. However, the previous study overlooked analyzing GSH or GSSG level from red cells and liver. In this study, we found mostly orally ingested GSH was immediately converted into GSSG and deposited into red cells and predominantly carried into liver tissues. We speculate some of the ingested ^{13}C-GSH could be degraded during the circulation of intact GSH into γ-glutamyl amino acid, cysteine, and glycine or γ-glutamyl cysteine by endogenous enzymes. However, the current LC-MS/MS technique could not detect notable degradation of ingested GSH. This must be further clarified in the future by developing a more sensitive method. Our recent small-scale work on human subjects indicated that there was no significant change of plasma GSH after ingestion of GSH. However, the GSH contents in the protein-bound fraction of plasma significantly ($P < 0.01$) increased from 15 to 120 min after GSH supplementation.[26] This is a very unique biological system because reduced GSH has a free thiol group (−SH) in cysteine, and an excess presence of reduced GSH in the plasma or tissues might have some harmful effects for the body due to the potential risk of reactive oxygen species (ROS) generation. If ingested GSH is deposited in circulating red cells or liver tissue as a GSSG form, it could be safer and used for detoxification in the body or liver.

GSH is the major intracellular nonprotein thiol compound and plays a major role in the protection of cells and tissue structures.[12] GSH allows the detoxification of free radicals and oxygen-reactive species involved in such diseases as atherosclerosis, rheumatoid arthritis, adult respiratory distress syndrome, or reoxygenation injury; consequently, its concentration decreases in such diseases.[27] Hepatic GSH concentrations have been observed in alcoholic[2829] and nonalcoholic[30,31] liver diseases. Plasma GSHt concentrations also decrease in such diseases. Therefore, supplemental ingested GSH can benefit the treatment of these diseases and increase liver GSH concentration for detoxification. The ingested GSH has potent nutraceutical benefits for human health to improve oxidative stress and defense in humans. These data indicated that there are several unknown pathways for GSH deposit in the circulating system and transfer into the liver. Further studies will unveil the exact mechanisms of orally administered GSH transport pathway and biological roles in the body.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Franco, R.; Schoneveld, O. J.; Pappa, A.; Panaviotidis, M. I. The central role of glutathione in the pathophysiology of human diseases. *Arch. Physiol. Biochem.* **2007**, *113*, 234−258.

(2) Townsend, D. M.; Tew, K. D.; Tapiero, H. The importance of glutathione in human disease. *Biomed. Pharmacother.* **2003**, *57*, 145−155.

(3) Wu, G.; Fan, Y.-Z.; Yang, S.; Lupton, J. R.; Turner, N. D. Glutathione metabolism and its implications for health. *J. Nutr.* **2004**, *134*, 489−492.

(4) Paolicchi, A.; Dominici, S.; Pieri, L.; Maellaro, E.; Pompella, A. Glutathione catabolism as a signaling mechanism. *Biochem. Pharmacol.* **2002**, *64*, 1027−1035.

(5) Linder, M.; De Burlet, G.; Sudaka, P. Transport of glutathione by intestinal brush border membrane vesicles. *Biochem. Biophys. Res. Commun.* **1984**, *123*, 929−936.

(6) Lash, J. H. Measurement of glutathione transport. In *Current Protocols in Toxicology*; Wiley: Hoboken, NJ, USA, 1999; pp 6.3.1−6.3.14.

(7) Bachhawat, A. K.; Thakur, A.; Kaur, J.; Zulkifli, M. Glutathione transporters. *Biochim. Biophys. Acta* **2013**, *1830*, 3154−3164.

(8) Hagen, T. M.; Jones, D. P. Transepithelical transport of glutathione in vascularly perfused small intestine of rat. *Am. J. Physiol.* **1987**, *252*, G607−G613.

(9) Vina, J. C.; Perez, T.; Furukawa, M.; Palacin, M.; Vina, J. R. Effect of oral glutathione on hepatic glutathione levels in rats and mice. *Br. J. Nutr.* **1989**, *62*, 683−691.

(10) Hagen, T. M.; Wierzbicka, A. H.; Sillau, B. B.; Bowman, B. B.; Jones, P. Bioavailability of dietary glutathione-effect on plasma concentration. *Am. J. Physiol.* **1990**, *259*, G524−G529.

(11) Vincenzini, M. T.; Favilli, F.; Iantomasi, T. Intestinal glutathione transport system: possible detxication role. *Biochim. Biophys. Acta* **1991**, *1073*, 571−579.

(12) Meister, A.; Anderson, M. E. Glutathione. *Annu. Rev. Biochem.* **1983**, *52*, 711−760.

(13) Meister, A. Glutathione deficiency produced by inhibition of its synthesis, and its reversal; applications in research and therapy. *Pharmacol. Ther.* **1991**, *51*, 155−194.

(14) Witschi, A.; Reddy, S.; Stofer, B.; Lauterburg, B. H. The systemic availability of oral glutathione. *Eur. J. Clin. Pharmacol.* **1992**, *43*, 667−669.

(15) Allen, J.; Bradley, R. D. Effects of oral glutathione supplementation on systemic oxidative stress biomarkers in human volunteers. *J. Altern. Complement. Med.* **2011**, *17*, 827−833.

(16) Grattagliano, I.; Wieland, P.; Schranz, C.; Lauterburg, B. H. Effect of oral glutathione monoethyl ester and glutathione on circulating and hepatic sulfhydryls in the rat. *Pharmacol. Toxicol.* **1994**, *75*, 343−347.

(17) Hong, S.-M.; Tanaka, M.; Yoshii, S.; Mine, Y.; Matsui, T. Enhanced visualization of small peptides absorbed in rat small intestine

by phytic-acid-aided matrix-assisted laser desorption/ionization-imaging mass spectrometry. *Anal. Chem.* **2013**, *85*, 10033−10039.
(18) Nakashima, E. M. N.; Kudo, A.; Iwaihara, Y.; Tanaka, M.; Matsumoto, K.; Matsui, M. Application of ^{13}C-stable isotope labeling liquid chromatography-multiple reaction monitoring-tandem mass spectrometry method for determining intact absorption of bioactive dipeptides in rats. *Anal. Biochem.* **2011**, *414*, 109−116.
(19) Vincenzini, M. T.; Favilli, F.; Iantomasi, T. Intestinal uptake and transmembrane transport systems of intact GSH; characteristics and possible biological role. *Biochim. Biophys. Acta* **1992**, *1113*, 13−23.
(20) Vincenzini, M. T.; Favilli, F.; Iantomasi, T. Glutathione-mediated transport across intestinal brush-border membranes. *Biochim. Biophys. Acta* **1988**, *942*, 107−114.
(21) Vincenzini, M. T.; Iantomasi, T.; Favilli, F. Glutathione transport across intestinal brush-border membranes: effects of ions, pH, $\Delta\psi$, and inhibitors. *Biochim. Biophys. Acta* **1989**, *987*, 29−37.
(22) Lash, L. H.; Hagen, T. M.; Jones, D. P. Exogenous glutathione protects intestinal epithelial cells from oxidative injury. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 4641−4645.
(23) Hagen, T. M.; Aw, T. Y.; Jones, D. P. Glutathione uptake and protection against oxidative injury in isolated kidney cells. *Kidney Int.* **1988**, *34*, 74−81.
(24) Iantomasi, T. Glutathione transport system in human intestinal epithelial tract. *Biochim. Biophys. Acta* **1997**, *1330*, 274−283.
(25) Gukasyan, H. J.; Lee, V. H. L.; Simityan, H.; Kim, K.-J.; Kannan, R. Thermodynamic stoichiometry of Na^+-coupled glutathione transport. *Can. J. Physiol. Pharmacol.* **2006**, *84*, 1223−1227.
(26) Park, E. Y.; Shimura, N.; Sauchi, Y.; Wada, S.; Aoi, W.; Higashi, A.; Nakamura, Y.; Sato, K. Increase in the protein-bound form of glutathione in human blood after glutathione supplementation. *J. Agric. Food Chem.* **2014**, *62*, 6183−6189.
(27) Uhlig, S.; Wendel, A. The physiological consequences of glutathione variations. *Life Sci.* **1992**, *51*, 1083−1094.
(28) Beutler, E.; Moroose, R.; Kramer, L.; Gelbart, T.; Forman, L. γ-Glutamylcysteine synthetase deficiency and hemolytic anemia. *Blood* **1990**, *75*, 271−273.
(29) Lauterburg, B. H.; Veley, M. E. Glutathione deficiency in alcoholics: risk factor for paracetamol hepatotoxicity. *Gut* **1988**, *29*, 1153−1157.
(30) Shaw, S.; Kenneth, P. R.; Lieber, C. S. Depressed hepatic glutathione and increased diene conjugates in alcoholic liver disease. *Dig. Dis. Sci.* **1993**, *28*, 585−589.
(31) Summer, K. H.; Eisenburg, J. Low content of hepatic reduced glutathione in patients with Wilson's disease. *Biochem. Med.* **1985**, *34*, 107−111.