Optimized Method for Determining Free L-Cysteine in Rat Plasma by High-Performance Liquid Chromatography with the 4-Aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole Conversion Reagent

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The analytical method was optimized for l-cysteine (Cys) in rat plasma with co-existing l-cysteine (Cyss). We observed that more than 100% Cyss in rat plasma was converted to Cys under typical conditions for the conversion with 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F). Another conversion reagent, 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F), was then employed, with which the reaction could be carried out at a low temperature without the use of a reducing reagent. Under the optimized conditions of 4°C and pH 8.3, the conversion ratio of Cyss to Cys in rat plasma was as low as 5–7%. We determined the Cys concentration in plasma of the portal vein of rats that had been orally administered with Cys and Cyss by applying this method. The result indicated that Cyss administration effectively increased the concentration in plasma of the portal vein of rats that had been orally administered with rats that had been orally administered with Cys and Cyss by applying this method. The result indicated that Cyss administration and also Cyss administration effectively increased the plasma Cys level. The method developed in this study is well suited for determining the thiol compounds in biological samples.

Key words: cysteine; cystine; reduced thiol; 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole; rat plasma

Cysteine (Cys) is an amino acid with an important functional thiol (–SH) group. Cys can be oxidized to a disulfide called cystine (Cyss). The reduced-form thiol/oxidized-form disulfide ratio plays a key role in the maintenance of redox status in the whole body. Cys is also the rate-limiting substrate for glutathione (GSH) synthesis that determines the major defense against oxidation mediated by reactive oxygen species (ROS).1,2) ROS, an essential player in mediating biological samples has therefore been complicated by the presence of oxidized-form thiols.

Numerous methods for the determination of thiols have been reported, including those employing high-performance liquid chromatography (HPLC),3,4) gas-chromatography-mass spectrometry (GC-MS),5) and capillary electrophoretic separation.6) Among these, methods using HPLC with fluorescence detection are the most popular because of their high sensitivity and reasonable simplicity. The thiols in a sample are detected by converting with such fluorescence reagents as ortho-phthalaldehyde (OPA),7) monobromobimane (mBBr),8) N-(1-pyrenyl)-maleimide (NPM),9) and benzfuranazan reagents including 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F)10) and 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F).11) SBD-F among these methods is the most commonly used fluorescence reagent because it selectively reacts with thiol compounds and emits no fluorescence in itself. Conversion with the fluorescence reagents of thiol compounds (Cys, homocysteine, and reduced-form GSH) usually follows a pretreatment with such reducing agents as tris-(2-carboxyethyl) phosphine (TECP)12) and tri-n-butylphosphine (TBP).13) However, the use of reducing agents can interfere with the determination of reduced-form thiol with a biological sample in which the oxidized-form thiols also exist. Omitting the reducing step on the other hand leads to oxidation of the reduced-form thiols during the conversion reaction. In addition, co-existence of the reduced- and oxidized-form thiols results in an exchange reaction of thiols in different compounds during the conversion procedure. The accurate determination of the reduced-form Cys concentration in biological samples has therefore been complicated by the presence of oxidized-form thiols.

Cys and Cyss both play a major role in redox state regulation which is associated with proliferation, cell adhesion and apoptosis.14) A previous study has shown decreased plasma Cys concentration concomitant with increased plasma Cyss in elderly subjects and cancer patients;15) in contrast, Jacob N. et al. have shown an

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Abbreviations: Cys, l-cysteine; Cyss, l-cystine; SBD-F, 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate; ABD-F, 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole; ROS, reactive oxygen species; HPLC, high-performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry; OPA, ortho-phthalaldehyde; mBBr, monobromomimane; NPM, N-(1-pyrenyl)-maleimide
increased Cys level in patients with cardiovascular disease.16) However, no precise method for measuring reduced-form thiols has been described.

We propose in the present study an optimized HPLC-fluorescence method for determining reduced-form thiols in biological samples by converting with ABD-F. ABD-F has high reactivity, implying that the thiol-exchange reaction can be minimized since conversion can be carried out at a relatively low temperature (4°C) and low pH value (8.3). This method was applied to determining the plasma Cys concentration of rats orally administered with Cys and Cyss, leading to the finding that the plasma Cys level was effectively increased after a single Cyss administration.

Materials and Methods

Reagents. L-Cysteine (Cys) as a standard, L-cystine (Cyss) for measurements of the conversion ratio, N-acetyl-L-cysteine (NAC), EDTA-2Na (ethylenediamine-N,N,N',N' tetraacetic acid, disodium salt, dehydrate), tri-n-butylphosphine (TBP) and acetic acid were purchased from Wako (Osaka, Japan). Methanol (HPLC grade) was from Kousuka Chemical (Tokyo, Japan), and 7-fluoro-2,1,3-benzoazines (SBD-F) and 4-amino-3-methyl-7-fluoro-2,1,3-benzoazines (ABD-F) were obtained from Dojindo (Kumamoto, Japan). All other reagents used were of analytical reagent grade. Cys from Kokusan Chemical (Tokyo, Japan), and 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F) and 4-amino-3-methyl-7-fluoro-2,1,3-benzoazines (ABD-F) were obtained from Dojindo (Kumamoto, Japan). All other reagents used were of analytical reagent grade. Cys and Cyss for administration to the rats were provided by Protein Chemical Co. (Tokyo, Japan).

HPLC. The HPLC system consisted of a PU-2080 pump (Jasco, Tokyo, Japan), an AS-2057 autosampler (Jasco) and an FP-2025 fluorescence detector (Jasco). The analyses were performed in a TSKgel ODS-80T (4.6 × 150 mm, Tosoh, Tokyo, Japan) at a flow rate of 1.0 mL/min. The mobile phase for the SBD-F fluorescence reagent was a 0.1 M acetic acid containing 4% methanol (adjusted to pH 4.0) in the isocratic condition. The fluorescence intensity was measured at 515 nm with excitation at 385 nm. The mobile phase for the ABD-F fluorescence reagent was composed of 0.1 M acetic acid under linear gradient elution with 10–25% (v/v) methanol. The fluorescence intensity was measured at 516 nm with excitation at 376 nm.

Plasma sample preparation and processing. Eight-week-old male Wistar rats were purchased from Charles River Laboratories (Kanagawa, Japan). The rats were kept at room temperature of 23 ± 1°C under a 12-h light-dark cycle (lighting from 0800 to 2000). The rats were fed with an MF-2 commercial diet before the experiments. After 16 h of overnight fasting, the rats were orally administered with the vehicle (0.08 N hydrochloric acid in physiological saline) for the control group, a Cys solution (150 μL) after mixing and centrifugation (10,000 × g for 1 min), and the resulting supernatant was transferred to new tubes. The plasma and samples from the small intestinal contents were immediately used for the measurements.

Conversion procedure using SBD-F. The conversion of Cys was based on the method of Durand et al. (1996) and carried out with 120 μL of plasma and 30 μL of an internal standard (2.5 mmt NAC to a final concentration of 0.5 mmt and 4 mmt EDTA in 0.9% saline) which were mixed for 30 min at 4°C with 15 μL of 0.6 N TBP as the reducing agent dissolved in N,N-dimethylformamide. Proteins were then precipitated with 0.6 N per chloric acid containing 1 mmt EDTA (150 μL) after mixing and centrifugation (10,000 × g for 10 min). A 50 μL volume of the clear supernatant was taken, and 10 μL of 1.55 M NaOH and 125 μL of a 125 mmt borate buffer (pH 9.5) containing 4 mmt EDTA were added. A 50 μL amount of a 1 mg/mL solution of SBD-F in the borate buffer was then added. Conversion with SBD-F was carried out for 60 min at 60°C and pH 9.5.

Conversion procedure using ABD-F. The method of Santa et al. (2006) was applied with modifications. A 30 μL amount of the internal standard was added to 120 μL of the plasma sample and quickly mixed. After adding 0.6 mtt perchloric acid containing 1 mmt EDTA (150 μL), the sample was mixed and centrifuged at 10,000 × g for 10 min. A 50 μL volume of the clear supernatant was taken, and 2.7 μL of 5 m NaOH and 100 μL of a 400 mmt borate buffer containing 2 mmt EDTA (pH 8.3) were then added. A 50 μL amount of a 1 mg/mL solution of ABD-F in the borate buffer was then added. Conversion in ABD-F was carried out for 120 min at 4°C and pH 8.3. Preliminary experiments indicated that thiol exchange occurred effectively with the conversion reaction under alkaline conditions in a pH range of 8.3–9.5 (data are not shown), the efficiency of fluorescence labeling being reduced under more acidic conditions (pH < 8.3).

Calibration. The Cys solution for calibration was freshly prepared by dissolving an appropriate amount of Cys in 0.08 N HCl. Standard solutions were prepared with six standard concentrations (0.10–0.60 mmt) of Cys diluted in saline. The calibration standards were spiked into saline or plasma. A 500 μt final concentration of NAC as an internal standard was added to each standard solution. The calibration standard was subjected to Cys measurements, calibration curves being obtained by plotting the ratio of the peak area of Cys to that of NAC. The endogenous plasma cysteine concentrations were subtracted.

Conversion ratio of Cys to Cyss. The Cyss solutions used to determine the conversion ratio was freshly prepared by dissolving an appropriate amount of Cys in 0.08 N HCl; these solutions were prepared for six concentrations (0.05–0.30 mmt) of Cys diluted in saline. These solutions were spiked into saline or plasma. A 500 μm final concentration of NAC was added as an internal standard to each standard solution, these solutions then being subjected to Cys measurements. The conversion ratio (CR) was calculated as:

\[ CR(%) = \frac{Cyss - Cys}{Cyss} \times 100 \]

where Cys denotes the concentration of Cys in the spiked plasma, Cyss the concentration of Cyss in the non-spiked plasma, and Cyss the concentration of Cyss added to the plasma.

Statistical analysis. All data are presented as the mean ± SD. Data were statistically analyzed with the Eksu-eru-Touket 2008 software package (Social Survey Research Information Co., Tokyo, Japan) by a one-way analysis of variance (ANOVA) with the Bonferroni and Tukey post hoc test. A probability value of p < 0.05 was considered statistically significant.

Results and Discussion

Separation of the SBD-F and ABD-F derivatives of Cys

HPLC chromatograms of the standard solutions for SBD- and ABD-Cys and for the derivatives of NAC are shown in Fig. 1A and C. Typical chromatograms obtained from rat plasma are shown in Fig. 1B and D. The derivatives were separated in the reversed-phase column by isocratic (SBD-F) and linear gradient (ABD-F) elution. The elution was in the order of SBD-Cys and SBD-NAC (Fig. 1A and B), and ABD-Cys and ABD-NAC (Fig. 1C and D). SBD-Cys and ABD-Cys produced well-separated peaks in saline and plasma. The respective retention times for Cys and NAC with conversion by SBD-F were 3.2 and 8.9 min, and those with conversion by ABD-F were 5.3 and 11.1 min.
Fig. 1. Representative Chromatograms for Cys Converted with SBD-F (A and B) and ABD-F (C and D) in Saline and Plasma. Cys (0.1 mM) and NAC (0.5 mM) were converted, separated, and detected as described in the Materials and Methods section.

Fig. 2. Calibration Curves for Different Chols Added to Saline and Rat Plasma. Saline (A) and plasma (B) were spiked with Cys and converted with SBD-F. Saline (C) and plasma (D) were spiked with Cys and converted with ABD-F. Endogenous Cys in the plasma was subtracted.
Evaluation of the method using SBD-F for conversion

Calibration curves for the SBD-F derivatives of Cys with spiked saline (Fig. 2A) and plasma (Fig. 2B) exhibited high linearity with correlation coefficients greater than 0.995 in the range of 0.10–0.60 mM (Fig. 2A and B). SBD-F is the most commonly used fluorescence reagent owing to its selective reaction with thiol compounds and no intrinsic fluorescence. The conversion of Cys with SBD-F was performed at 60 °C in an alkaline borate buffer (pH 9.5). It has been pointed out that reduced-form thiols were easily oxidized during the pre-treatment procedure on biological samples.\(^{17-19}\) In addition, thiol-exchange reactions occur easily and generate mixed disulfides in a solution where reduced and oxidized thiols coexist.\(^{5}\) It is therefore usually recommended to pre-treat a sample with a reducing agent to prevent such reactions.

We next examined the conversion ratio of Cyss into Cys during conversion with SBD-F in combination with pre-treatment when using the TBP reducing agent (Table 1). The conversion ratio of Cyss to Cys was 73.4 ± 15.5\% in saline containing only Cyss and was 112.2 ± 24.0\% in plasma with endogenous Cys and Cyss coexisting. These results demonstrate that standard protocols using SBD-F are not suitable for measuring reduced-form thiols, especially those in biological samples.

Evaluation of the method using ABD-F for conversion

Reduced-form thiols were next determined with ABD-F conversion without any pretreatment with a reducing agent. The calibration curves for the ABD-F derivatives of Cys with spiked saline (Fig. 2C) and plasma (Fig. 2D) exhibited high linearity with correlation coefficients greater than 0.998 in the range of 0.10–0.60 mM. The conversion reaction of Cys with ABD-F proceeded at 4 °C in the borate buffer with a relatively lower pH (pH 8.3). Santa et al. have reported that the

| Table 1. Conversion Ratios of Cyss to Cys in Raline and Rat Plasma Using the SBD-F or ABD-F Methods |
|------------------|------------------|
|                   | SBD-F            | ABD-F            |
|                   | Saline Plasma    | Saline Plasma    |
| Conversion ratio (\%) | 73.4 ± 15.5      | 112.2 ± 24.0     |
|                   | 2.6 ± 1.5        | 12.8 ± 3.1       |

Data are the means ± SD.

![Fig. 3](image-url)
thiol-exchange reaction between the reduced- and the oxidized-form thiols occurred during conversion at a high temperature (60 °C). In contrast, as conversion with ABD-F proceeded at a low temperature, they concluded that ABD-F would be the preferred conversion reagent for the accurate determination of reduced-form thiols. Table 1 shows that the conversion ratio of Cyss to Cys during the reaction with ABD-F without pretreatment of the reducing agent was 2.6 ± 1.5% in saline and 12.8 ± 3.1% in plasma in which endogenous Cys and Cyss were both present. This method provides a more accurate estimate of reduced thiols, since the conversion ratio of Cyss to Cys when using ABD-F in the absence of reducing agents was relatively low when compared with the SBD-F method.

Cys concentrations in plasma of rats after oral administration of Cys or Cyss

The reduced-form Cys concentration was measured in rat plasma 60 min after orally administering Cys or Cyss. Cys in the plasma was converted with ABD-F without any pretreatment with a reducing agent. The Cys concentrations in the plasma from the portal vein (Fig. 3A) and abdominal artery (Fig. 3B) were markedly increased in rats that had been administered with Cys. In the rats administered with Cyss, the Cys concentrations in the plasma from the portal vein and abdominal artery were also increased to levels comparable with those in the Cyss-administered rats. The total amounts of the reduced form (Cys) and oxidized form (Cyss) were both present. This method provides a more accurate estimate of reduced thiols, since the conversion ratio of Cyss to Cys when using ABD-F in the absence of reducing agents was relatively low when compared with the SBD-F method.

These results suggest that Cys and Cyss were delivered to the body with almost equal efficiency when orally administered. Cys also seems to have been converted to Cys during the process of absorption. Figure 3C shows the Cys concentration in the contents of the small intestine of the rats. The Cys level in the intestine was far lower for the Cyss-administered group than for the Cys-administered group, indicating that most of the administered Cyss remained in the oxidized form in the intestinal tract. This result, together with the fact that the activity of glutathione-Cyss trans-hydrogenase is high in the intestine, further supports the foregoing assumption that Cyss was converted to Cys in intestinal epithelial cells. The plasma Cys level in the abdominal artery was consistently lower than that in the portal vein. Since the liver has a large free Cyss pool and regulates the plasma Cyss level, excessive Cys in the blood may be catabolized in the liver. It is known that a significant amount of plasma cysteine is present in protein-bound forms such as those forming S-S bonds with cysteine residues in albumin and globulins. The plasma samples in the present study were de-proteinized prior to measurement, so the amount in the protein-bound form can be neglected.

In conclusion, the method presented in this study proved suitable for determining reduced-form Cys and other thiol compounds in biological samples. The application of this method will further facilitate understanding the dynamic state of reduced/oxidized thiol compounds in organisms, as has been demonstrated by the analysis of the Cys/Cyss state in rat plasma described in this paper.

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