Optimum Conditions for Derivatization of Glutathione, Cysteine and Cysteinylglycine in Human Plasma with Ammonium 7-Fluorobenzo-2-Oxa-1,3-Diazole-4-Sulfonate for Accurate Quantitation by High-Performance Liquid Chromatography

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For the accurate quantitation of glutathione (GSH), cysteine (Cys) and cysteinylglycine (CysGly) in human plasma by detection of fluorescence after derivatization with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F), a thiol-specific fluorogenic agent and high-performance liquid chromatography (HPLC) , we attempted to identify optimum conditions for the derivatization and treatment of samples. The extent of derivatization with SBD-F of endogenous thiol compounds in human plasma was affected by several factors, such as the concentration of SBD-F and the reaction time. Acidification and deproteinization of plasma immediately after the separation of plasma from whole blood were essential and prevented the determination of inappropriately low values. The method developed in the present study allowed recovery that was closed to 100% for each compound. The mean values determined for 61 healthy individuals were as follows: Cys, 50.1 μM; CysGly, 12.2 μM; and GSH, 5.3 μM.

Key words —— glutathione, cysteine, cysteinylglycine, human plasma, HPLC

INTRODUCTION

Glutathione (GSH) is a tripeptide, gamma-glutamylcysteinylglycine, that is widely distributed in animal tissues and plasma.[1)] GSH plays several essential roles in the maintenance of the integrity of thiol moieties of proteins and other compounds; the activity of some enzymes; and the protection of cells against oxidative stress, free radicals and the toxicity of certain drugs and other chemicals.[1–3)] The concentration of GSH in human plasma is altered by antioxidants (*e.g.*, ascorbate,[4)] α-tocopherol[5)]), alcoholic cirrhosis,[6)] infection by human immunodeficiency virus[7)] and strenuous exercise.[8)] However, levels in the literature of GSH in normal human plasma vary by more than one order of magnitude.[9)] Therefore, a method for the accurate determination of concentrations of GSH and other major thiol compounds, such as cysteine (Cys) and cysteinylglycine (CysGly), in human plasma is clearly necessary. In the present study, we attempted to identify the optimum conditions for derivatization of GSH, Cys and CysGly in human plasma using ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F), a thiol-specific fluorogenic agent[10)] that has been used frequently in analyses of thiol compounds by high-performance liquid chromatography (HPLC).[11)]

MATERIALS AND METHODS

Reagents —— Cys was obtained from Kyowa Hakko Kogyo (Tokyo, Japan). Both GSH and CysGly were purchased from Sigma (St. Louis, MO, U.S.A.); and tri-*n*-butylphosphine (TBP) was obtained from Nacalai Tesque (Kyoto, Japan).

Derivatization with SBD-F —— Blood was collected in tubes that contained EDTA (1 mg/ml blood) from the antecubital vein of human healthy volunteers after each had given informed consent. Immediately after the collection, blood was centrifuged at 3000 × *g* for 5 min at 4°C and then 100 μl of the re-

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sultant plasma were mixed with 100 μl of trichloroacetic acid (TCA, 10%) that contained 10 mM EDTA. After centrifugation of this mixture for 3 min at 3000 $\times$ *g*, 350 μl of potassium borate buffer (1 M, pH 10.5) that contained 5 mM EDTA and 5 μl TBP (20% in isopropanol) were added to 100-μl aliquots of the supernatant (TCA-supernatant of plasma). Each mixture was allowed to stand at 25°C for 10 min and then it was supplemented with 100 μl of a solution of SBD-F (0.6% in water). After heating for 40 min at 60°C, the mixture was put in an ice bath and 50 μl of 4 M HCl were added to terminate the reaction. Aliquots of the resultant solution (50 μl) were injected into the HPLC system.

Analysis by HPLC —— HPLC was performed with a system (LC10A) from Shimadzu (Kyoto, Japan), which include a system controller (SCL10A), a degasser (DGU3A), an automatic injector (SIL10A) and a fluorescence detector (RF10A). A Shodex C18M-4D column (150 mm $\times$ 4.6 mm i.d.; Showa Denko, Tokyo, Japan) was used for separation, and it was eluted with a mixture of 0.1 M citrate buffer (pH 3.2) and acetonitrile (97 : 3, v/v) at a flow rate of 0.7 ml/min. The fluorescence of SBD derivatives was monitored at 516 nm after excitation at 384 nm.[12]

RESULTS AND DISCUSSION

For the accurate quantitation of endogenous thiol compounds in human plasma, such as GSH, Cys and CysGly, by HPLC with fluorescence detection, we attempted to identify the optimum conditions for derivatization of thiol compounds in human plasma with SBD-F. We first examined the effects of the reaction time on derivatization of these thiol compounds with SBD-F using human plasma as experimental samples. We measured the areas of peaks corresponding to the SBD derivatives of GSH, Cys and CysGly (SBD-GSH, SBD-Cys and SBD-CysGly) after HPLC and found that the areas were increased with increases in the reaction time up to 30 min and then remained constant for up to 100 min in each case (Fig. 1). Figure 2 shows the effects of the concentration of the solution of SBD-F in the reaction mixture on the area of the peak of each derivative after HPLC. The areas of the peaks of SBD derivatives of each of the three thiol compounds in human plasma reached maximum values when SBD-F was added to the reaction mixture at a concentra-

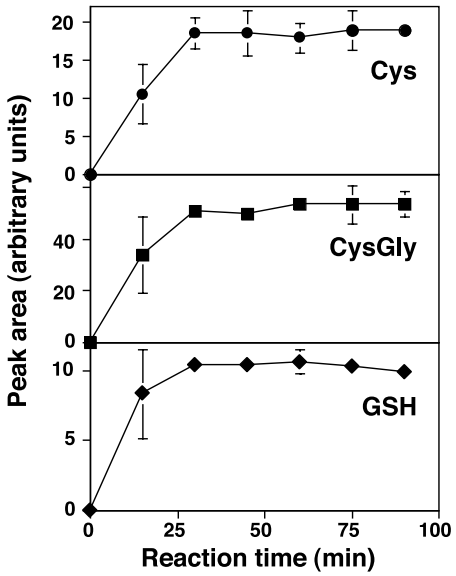

Fig. 1. The Relationship between the Duration of the SBD-Derivatization Reaction and Peak Areas of Derivatives of Cys, CysGly and GSH after HPLC

Deproteinized human plasma (100 μl) was mixed with 20% TBP (5 μl) and 1 M borate buffer (pH 10.5) that contained 5 mM EDTA (350 μl). Then reaction with SBD-F (0.6%, 50 μl) was allowed to proceed at 60°C for the indicated times. After each reaction, the reaction mixture was analyzed by HPLC with fluorometric detection, as described in the text.

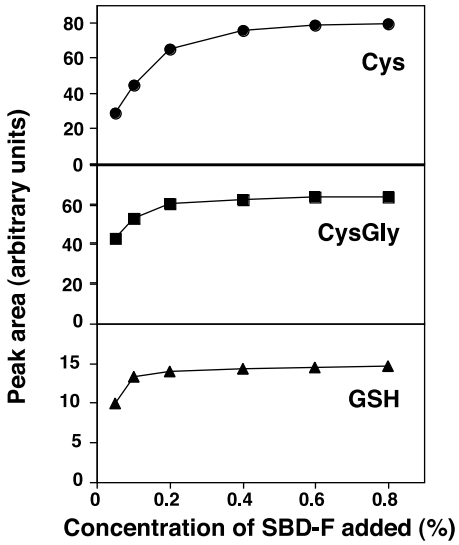

Fig. 2. Effects of the Concentration of SBD-F on the Peak Areas of Fluorescent Derivatives of Cys, CysGly and GSH

Deproteinized human plasma (100 μl) was mixed with 20% TBP (5 μl) and 1 M borate buffer (pH 10.5) that contained 5 mM EDTA (350 μl). Then it was allowed to react with SBD-F (50 μl) at various concentrations for 40 min at 60°C. After each reaction, the reaction mixture was analyzed by HPLC with fluorometric detection.

tion equal to or greater than 0.6%(v/v). In the SBD-derivatization reaction, TBP must be added as a reducing agent for quantitation of total GSH (reduced GSH plus oxidized GSH).[12,13] Addition of 5 μl of a 20% solution of TBP was sufficient to generate maximum peak area for each derivative (data not shown). The SBD-derivatization reaction also required heating the reaction mixture at 60°C or a higher temperature and a high pH (10.5) for generation of maximum peak areas (data not shown). Our results indicated that all the GSH, Cys and CysGly in human plasma was converted to the respective derivatives in a solution of 1 M borate buffer (pH 10.5) that contained 5 mM EDTA (350 μl), 20% TBP in isopropanol (5 μl), and 0.6% SBD-F in water (50 μl) during incubation for 40 min at 60°C.

Using the optimized conditions for the derivatization reaction, we next examined the appropriate duration of deproteinization (addition of TCA; see Methods) of the plasma after it had been separated from whole blood. In this experiment, plasma was separated from whole blood immediately after collection and kept at room temperature. Then it was treated with TCA, with subsequent derivatization with SBD-F. As shown in Fig. 3, the peak areas of the fluorescent derivatives of GSH, Cys and CysGly decreased with time after the separation of plasma from whole blood. When the plasma was treated with TCA immediately after the separation of plasma from whole blood, peak areas did not decrease for at least 120 min (Fig. 4). These results indicate that plasma should be treated with TCA immediately after its separation from whole blood to prevent errors in quantitation.

To examine the accuracy of our method for the quantitation of thiol compounds in human plasma, we examined the rates of recovery of GSH, Cys and CysGly from human plasma. As shown in Table 1, these rates indicated that recovery was complete in each case. We determined the average concentrations of GSH, Cys and CysGly in plasma from healthy Japanese individuals using our optimized method and the results are shown in Table 2. The availability of a method for the accurate quantitation of thiol compounds in human plasma now allows us to examine the relationship between several pathophysiologic states and the plasma concentration of thiol compounds.

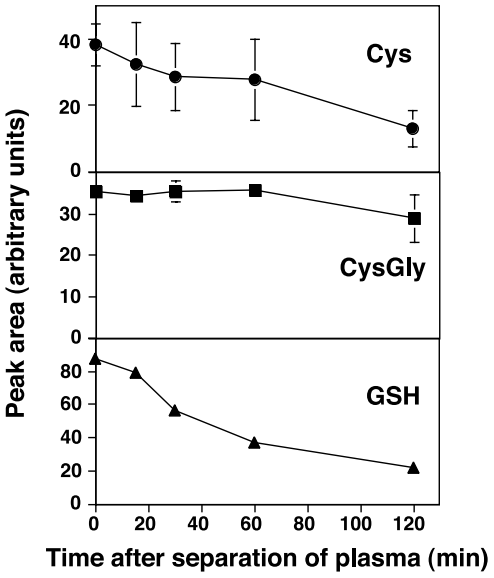

Fig. 3. Effects of Delaying the Deproteinization of Plasma Prior to Derivatization with SBD-F

Human plasma, obtained from whole blood, was kept at room temperature for zero to 120 min. Then it was deproteinized with TCA and thiols were reacted with SBD-F. After derivatization, each reaction mixture was analyzed by HPLC with fluorometric detection.

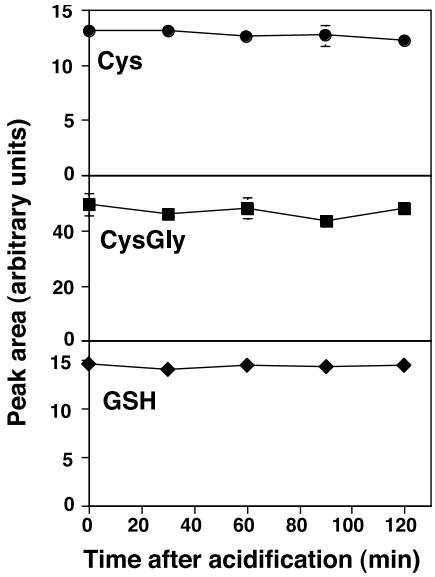

Fig. 4. Effects of Delaying the Derivatization of Thiols in Deproteinized Plasma

Human plasma, obtained from whole blood, was deproteinized with TCA and then kept at room temperature for zero to 120 min. After subsequent derivatization with SBD-F, each reaction mixture was analyzed by HPLC with fluorometric detection.

Table 1. Rates of Recovery of Cys, CysGly and GSH from Human Plasma

	Level in human plasma (μM)	Added (μM)	Expected (μM)	Found (μM)	Recovery rate (%)
Cys	51.25	20.00	71.25	71.73	100.7 ± 9.6
CysGly	21.27	5.00	26.27	26.31	100.2 ± 3.9
GSH	5.88	3.00	8.88	8.90	100.2 ± 2.0

Cys, CysGly and GSH were added to fresh human plasma, and then the concentration of each thiol compound was determined by HPLC after derivatization with SBD-F. Values are means of the results of six determinations.

Table 2. Average Concentrations of Cys, CysGly and GSH in the Plasma of Healthy Individuals

	Number of samples	Mean $\pm$ S.D. (μM)	Maximum (μM)	Minimum (μM)
Cys	61	51.09 ± 34.70	197.21	18.30
CysGly	61	12.23 ± 3.51	21.83	6.66
GSH	61	5.32 ± 1.43	8.98	3.27

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