Determination of L-Buthionin (SR)-Sulfoximine, γ-Glutamylcysteine Synthetase Inhibitor in Rat Plasma with HPLC after Prelabeling with Dansyl Chloride

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Abstract

L-(SR)-Buthionin sulfoximine (L-(SR)-BSO) is a potent and specific inhibitor of γ-glutamylcysteine synthetase, which catalyzes the first reaction of glutathione biosynthesis. A selective, sensitive, and simple high-performance liquid chromatographic method was developed for the determination of L-(SR)-BSO in rat plasma. After the compound was labeled with dansyl chloride (Dns-Cl) under optimal conditions, it was separated in a Zolbax-ODS column with a mobile phase that consisted of 0.01M phosphate buffer, methanol, and acetonitrile (8:1:3, v/v). The compound was detected with a fluorescence detector at an excitation wavelength of 335 nm and an emission wavelength of 525 nm using a xenon lamp. The coefficients of variation (CV) from the interassay in the low and high concentrations (10 and 500 μg/mL of L-(SR)-BSO in rat plasma) were 2.5 and 4.8%, respectively. The CVs from the intra-assay in the low and high concentrations were 3.2 and 5.6%, respectively. The minimum concentration of L-(SR)-BSO that could be determined was 10 μg/mL when 100-μL serum samples were used. The detection limit was 50 ng per injection volume. This method enables pharmacokinetic and pharmacodynamic studies in rats.

Introduction

Buthionin sulfoximine (BSO) is a potent and specific inhibitor of γ-glutamylcysteine synthetase. This enzyme catalyzes the first reaction of glutathione biosynthesis. Glutathione synthesis is blocked in animals or cultured cells exposed to BSO, and glutathione is substantially depleted in cells or tissues with moderate to high rates of glutathione utilization (1,2).

BSO has diastereomers and enantiomers. Campbell and co-workers (1) reported that the use of L-(S)-BSO and L-(SR)-BSO significantly decreased cellular glutathione levels in the kidney, liver, and pancreas of mice in comparison with the use of the other BSO isomers.

BSO is used as a chemosensitizer in tumor chemotherapy; it increases the sensitivity of tumorous tissue to antitumor agents and radiation (3–6). Mitchell and co-workers (3) reported that the depletion of cellular glutathion caused by BSO sensitized tumor cells to irradiation. However, BSO depletes glutathione not only from tumorous tissues and cells but also from normal cells. Normal tissues or cells are similarly damaged because of the cytotoxicity of the antitumor agents used in combination with BSO. Ishikawa and co-workers (5) reported that the toxicity of cisplatin in the renal system of mice increased when used in combination with BSO, and this suggests that glutathione plays an important role in protecting cells from these various antitumor drugs.

To selectively deplete glutathione in tumorous tissues or cells, we are developing a thermosensitive liposome that contains L-(SR)-BSO (TL-BSO). We expect to achieve the selective death of tumorous tissues or cells with hyperthermia in combination with chemotherapy. To investigate the effect of drug delivery and the pharmacokinetics of L-(SR)-BSO after administration in the liposomal form, a specific and sensitive assay is required. This paper describes a high-performance liquid chromatographic (HPLC) assay for L-(SR)-BSO in rat plasma.

Experimental

Chemicals and reagents

L-(SR)-BSO was purchased from Sigma (St. Louis, MO). 5-Sulfosalicylic acid dihydrate, dansyl chloride (Dns-Cl), ethylamine hydrochloride, and L-norvaline were obtained from Nacalai Tesque (Kyoto, Japan). All reagents were of reagent grade. Acetonitrile, methanol, and water, which were used as the mobile phase, were of chromatographic grade.

The stock acetone solution that contained Dns-Cl (3 mg/mL) and the L-norvaline solution, which was used as an internal standard, were stored at –20°C until use.

Apparatus and HPLC conditions

The HPLC system (Shimadzu; Kyoto, Japan) consisted of an LC-10A liquid delivery module, an RF-530 spectromonitor (Shimadzu), a CTO-10A column oven (Shimadzu), and a

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Zolbax-ODS column (4.6 × 150-mm i.d.). Samples were injected with a SIL-10A automatic injector. The system was controlled with an SCL-10A system controller under the following conditions: flow rate, 1.0 mL/min; column temperature, 40°C; excitation wavelength, 335 nm; emission wavelength, 525 nm using a xenon lamp; injection volume, 5 µL. The area under each peak was calibrated with a Shimadzu data processor (CR-5A Chromatopac).

The mobile phase consisted of 0.01M phosphate buffer (pH 2.1), methanol, and acetonitrile (8:1:3, v/v). This mixture was filtered and degassed by vacuum and sonication.

The influence of pH and temperature on L-(SR)-BSO dansylation

To investigate the influence of pH on dansylation, 50 µL Dns-Cl–acetone solution was added to 50 µL L-(SR)-BSO aqueous solution (50 µg/mL) dissolved in a phosphate buffer of various pH values: 4.49, 5.91, 6.98, 7.18, 7.38, 8.04, 9.18, 9.53, 9.96, and 10.5. After incubation for 10 min, 50 µL of 0.2% ethylamine hydrochloride aqueous solution was added to stop the reaction. Then, 5 µL of the mixture was injected into the HPLC system.

To investigate the influence of temperature and analysis time on dansylation, the samples were reacted at 30°C, 40°C, and 50°C for 0.5, 1, 5, 10, 30, and 60 min. The dansyl products obtained under each set of conditions were analyzed.

To investigate the stability of the reaction products stored at –20°C, Dns-L-(SR)-BSO was stored in a freezer and was analyzed at 0, 1, 2, 3, and 20 h after dansylation.

Prelabeling of L-(SR)-BSO in rat plasma

Exactly 50 µL L-norvaline, which was used as the internal standard (100 µg/mL), was added to rat plasma that contained L-(SR)-BSO. The plasma protein was precipitated with 10% sulfosalicylic acid. After centrifugation at 13,000 rpm for 5 min, the supernatant was transferred to another tube and dried under a stream of nitrogen gas. The residue was dissolved in 50 µL 0.4M phosphate buffer (pH 9.53). Then,50 µL Dns-Cl–acetone solution was added to this mixture and incubated at 40°C for 10 min. The reaction mixture was stored at –20°C until analysis.

Calibration curve of L-(SR)-BSO in rat plasma

The calibration standard was prepared by adding known amounts of L-(SR)-BSO to rat plasma that contained the liposome component.

Table I. Reproducibility of HPLC Assay of L-(SR)-BSO in Rat Plasma

Plasma concentration (µg/mL)	Interassay variation (%)	Intra-assay variation (%)
10	2.5	3.2
500	4.8	5.6

*n = 10.

Results

Figure 1 shows the relationship between the peak area of Dns-L-(SR)-BSO and the pH value of the reaction mixture. As shown in this figure, we found that the reaction progressed from pH 7.73 to 10.5.

Figure 2 shows the plot of Dns-L-(SR)-BSO versus reaction time for three different reaction temperatures. The maximum yields of Dns-L-(SR)-BSO were observed, in all the reactions, about 10 min into the reaction. The yield of Dns-L-(SR)-BSO at 50°C was the greatest. There was a statistically significant difference between the yield of dansyl product at 30°C and the other temperatures. However, there was no statistically significant difference between the yields of dansyl product at 40°C and 50°C after 10 min. A slow but measurable decrease in each yield occurred as the reaction proceeded. Decomposition of Dns-L-(SR)-BSO observed at 40°C after 10 min was less than that observed at 50°C.

The reaction mixtures that contained Dns-L-(SR)-BSO were stored at –20°C. Dns-L-(SR)-BSO was significantly stable under these conditions for 20 h.

The typical chromatogram of Dns-L-(SR)-BSO and Dns-L-norvaline is shown in Figure 3. L-(SR)-BSO and the internal standard were separated on the chromatogram within 15 min. There were no interference peaks on the chromatogram.

The calibration curve was linear over the range of 10 to 500 µg/mL L-(SR)-BSO in rat plasma, and the correlation coefficient was higher than .999.

Table I shows the reproducibility of this method based on the analysis of 10 rat plasma samples. The coefficients of variation from the interassays in the low and high concentrations (10 and 50 µg/mL of L-(SR)-BSO in rat plasma) were 2.5% and 4.8%, respectively. The coefficients of variation from the intra-assays in the low and high concentrations were 3.2% and 5.6%, respectively. The minimum determinable concentration of L-(SR)-BSO was 10 µg/mL when 100 µL rat serum was used. The detection limit was 50 ng per injection volume.

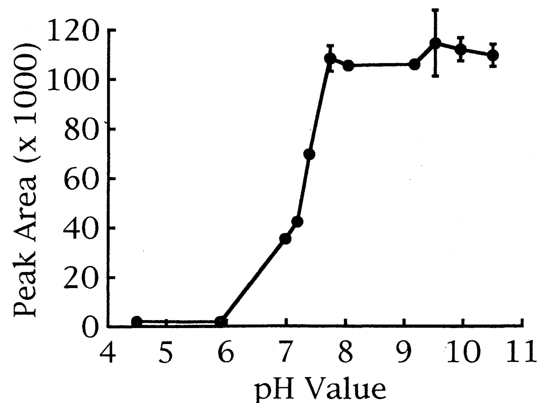
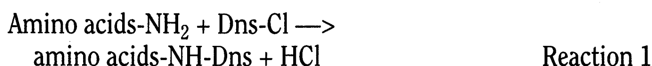


Figure 1. Relationship between the yield of Dns-L-(SR)-BSO and various pH values.

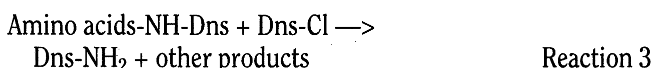
Discussion

For the determination of amino acid concentrations in biological fluids, a precolumn is generally used with HPLC. Several reports (7–16) on the determination of amino acid concentrations with fluorescence derivatization have been published. Benson and co-workers (16) reported that the fluorometric detection of amino acids with *o*-phthalaldehyde or fluorescamine was more sensitive than that with ninhydrin. Fürst and co-workers (13) assessed four precolumn methods for determination of amino acids with *o*-phthalaldehyde (OPA), 9-fluorenylmethyl chloroformate (FMOC-Cl), phenyl isothiocyanate (PITC), and 1-dimethylaminonaphthalene-5-sulfonyl chloride (Dns-Cl). In their report, the OPA method proved to be useful for routine analysis of free primary amino acids (except cystein) in biological fluids, the FMOC-Cl method was useful for the determination of secondary amino acids, the PITC method was used to analyze clinical samples, and the Dns-Cl method was useful for the determination of free cystein or cystein-containing short-chain peptides. BSO, which has an α-amino group and an α-carboxyl group, is considered to be a primary amino acid. The OPA method would be suitable for the determination of BSO in biological fluids. However, the Dns-Cl method was selected because of its detection limit, reproducibility, and analysis conditions. Dns-Cl is a well known fluorogenic reagent used for the determination of primary and secondary amines. Moreover, in contrast to the PITC method, the Dns-Cl method, which is used for the determination of amino acids, showed excellent linearity in the low range.

Dansylation of amino acids is explained by three competing reactions (9):


Amino acids-NH_2 + Dns-Cl —>
amino acids-NH-Dns + HCl   Reaction 1

H_2O + Dns-Cl —> Dns-OH + HCl   Reaction 2


Amino acids-NH-Dns + Dns-Cl —>
Dns-NH_2 + other products   Reaction 3

The dansylation of BSO, which has an α-amino group, is thought to occur in a similar fashion. Dansylation of amino acids was investigated in detail by Tapuhi and co-workers (9). They suggested that environmental factors such as pH and reaction temperature influenced the yields of Dns–amino acids. They reported that the desirable reaction, reaction 1, was accelerated by a high pH value, but high pH values also favored reaction 2. Reaction 3 led to the decomposition of Dns–amino acids. Reaction conditions had to be selected to minimize reaction 3. In our experiment, when the pH value ranged between 7.73 and 10.5, the largest amounts of dansyl products were obtained. We decided to use a pH 9.53 phosphate buffer for two reasons: (*a*) dansylation could not occur in acidic condition that had resulted from the addition of sulfosalicylic acid to precipitate rat plasma proteins and (*b*) it is not desirable to use high pH conditions in a reversed-phase column such as Zolbax-ODS. Tapuhi and co-workers determined amino acids with Dns-Cl by applying a pH 9.5 lithium carbonate buffer, which suppressed reaction 3. The optimum pH value in our experiment agreed with their optimal pH value for dansylation. In addition, we found that reaction 3 did not occur if the dansyl products were stored by the methods described previously.

Dansylation of amino acids at 60°C and 95°C was also performed by Tapuhi and co-workers, and lower yields were obtained at 95°C with a 5-min reaction time. They reported that the hydrolysis of Dns-Cl rapidly progressed at the higher temperature, and this reaction could be conveniently performed up to temperatures of approximately 60°C with short reaction

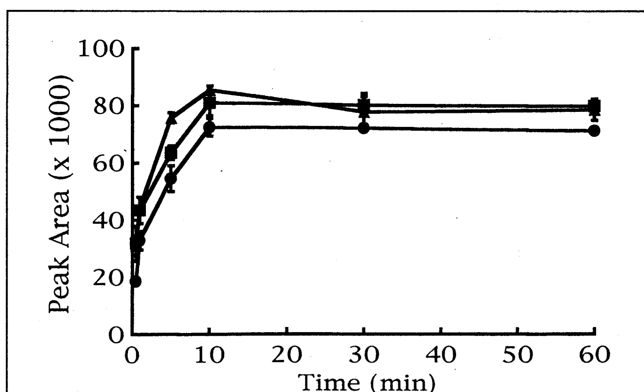


Figure 2. Reaction kinetics of the derivatization of L-(SR)-BSO with Dns-Cl. Reaction temperatures: ●, 30°C; ■, 40°C; ▲, 50°C.

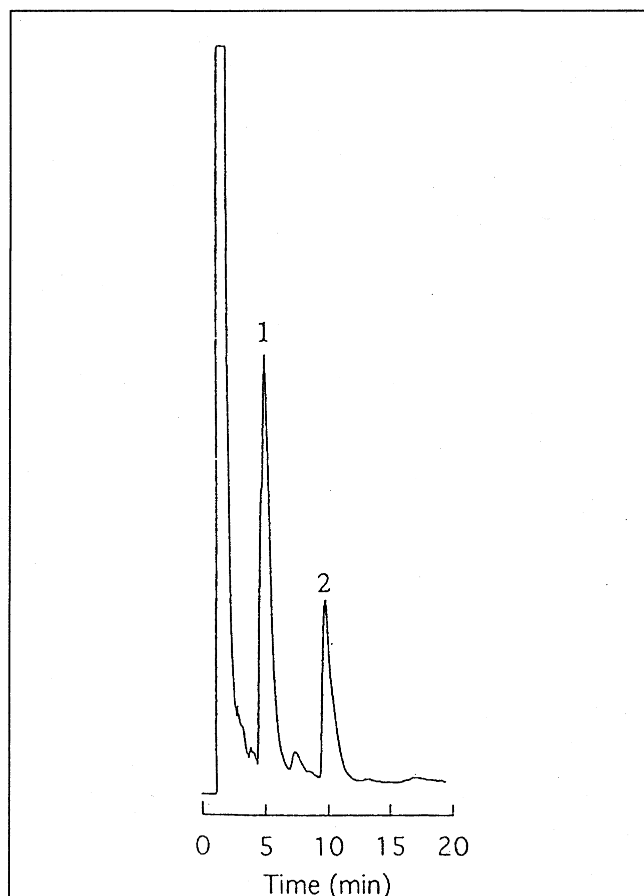


Figure 3. Typical chromatograms of Dns-L-(SR)-BSO and Dns-L-norvaline. Peaks: 1, Dns-L-(SR)-BSO; 2, Dns-L-norvaline.

times. We searched for an optimum reaction temperature below 60°C. In our experiment, very slow but measurable decreases in yield occurred as the reaction proceeded over long periods of time. Tapuhi and co-workers also observed this. Presumably, it occurs as a consequence of the decomposition reaction. Tapuhi and co-workers selected Li_2CO_3 in the hope that Li^+ would reduce the rate of reaction 3 because there would be some ion pairing to the carboxylate anion. Although we used a phosphate buffer for the dansylation of L-(SR)-BSO, we managed to significantly decrease the rate of reaction 3. From these results, the optimum reaction condition, which yielded the most Dns-L-(SR)-BSO, was 40°C for 10 min.

It has been reported that dansyl derivatives are photosensitive. Tapuhi and co-workers investigated the influence of ultraviolet irradiation on the yields of dansyl products from five amino acids. They reported significant decreases in the Dns-amino acid peaks, and another unidentified decomposition product was detected in the chromatograms. In these experiments reaction tubes were wrapped with aluminum foil to exclude light.

Conclusion

In regard to BSO, several reports have been published. To determine BSO concentration, Griffith and co-workers used an amino acid analyzer, and Campbell and co-workers (1) used HPLC. The method used by Campbell and co-workers separated BSO enantiomers and diastereomers but was complicated. They determined BSO in aqueous solutions with both methods. To our knowledge, few groups have investigated methods for the quantitation of L-(SR)-BSO in rat plasma by HPLC. In this paper, to investigate the pharmacokinetics of L-(SR)-BSO in rats, we developed a method to quantitate L-(SR)-BSO in rat plasma by HPLC.

This precolumn derivatization method with Dns-Cl required optimum reaction conditions. Our data suggested that the optimization of reaction conditions with Dns-Cl helped to maximize dansyl product yields. Phosphate buffer is a suitable buffer for the production of dansyl products, and it reduces the rate of reaction 3, which decomposes these products. This assay is highly sensitive for the detection of L-(SR)-BSO in rat plasma, and was not influenced by the addition of liposomes. To investigate the pharmacokinetics of L-(SR)-BSO in rats after administration of TL-BSO, this prelabeling method which involves Dns-Cl and HPLC is very useful for the quantitation of L-(SR)-BSO in rat plasma.

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Manuscript accepted February 12, 1996.