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Determination of glutathione isopropyl ester in rat, dog and human blood by high-performance liquid chromatography with fluorescence detection

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Abstract: A HPLC method is developed for the determination of glutathione isopropyl ester, a drug for the treatment of cerebral vascular disease, in rat, dog and human blood. The blood is deproteinized with sulphosalicylic acid and the clear supernatant treated with a thiol-specific fluorogenic reagent, ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F) in borate buffer pH 7.5 at 30°C. The derivatization of glutathione isopropyl ester with SBD-F is markedly enhanced by the addition of dimethyl sulphoxide, and is complete in 30 min. The fluorescent derivatives of glutathione isopropyl ester and the internal standard, glutathione ethyl ester are separated from those of endogenous thiols such as cysteine and glutathione on a reversed-phase column. The method is simple and selective with a detection limit of 0.05 μg ml^{-1}. Blood concentrations of glutathione isopropyl ester in rats, dogs and humans after intravenous administration are determined using the method.

Keywords: *Glutathione isopropyl ester; reversed-phase liquid chromatography; ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate; fluorometric derivatization; blood concentration.*

Introduction

Glutathione isopropyl ester, a monoester of glutathione in which the glycine carboxyl group of glutathione is esterified, improves neurological deficits in cerebral hematoma models. It is now under clinical trial for the treatment of patients with cerebral vascular disease. Glutathione isopropyl ester significantly reduced mortality and the increase in brain edema and lipid peroxide evoked by cerebral ischemia in bilateral carotid artery-occluded rats, whereas glutathione showed no significant effect [1]. Glutathione content in this model was found to be significantly decreased. This reduction was completely abolished by glutathione isopropyl ester, but not by glutathione [2]. In autologous-applied rats, glutathione isopropyl ester significantly facilitated the recovery of neurological deficits and ameliorated the increase in lipid peroxide, while glutathione had no effect on neurological deficits [1, 3]. A sensitive method for the determination of blood concentrations was required for pharmacokinetic studies in preclinical and clinical evaluations of glutathione isopropyl ester. HPLC determination after derivatization of glutathione isopropyl ester with a thiol-specific reagent represented one possibility as it is highly selective and sensitive and prevents oxidation of the sulphhydryl group of the drug. The present study reports a LC method for determining glutathione isopropyl ester in blood using ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F) as a fluorogenic labelling reagent.

Experimental

Chemicals

Glutathione isopropyl ester and glutathione ethyl ester as an internal standard were synthesized at our central research laboratories. They were obtained as 1/2 sulphate 1/2 hydrate. Their chemical structures are shown in Fig. 1. Glutathione and γ-glutamylcysteine monohydrate were purchased from Kojin Co., Ltd (Tokyo, Japan). Cysteine hydrochloride monohydrate, 2-mercaptoethylamine hydrochloride, HPLC-grade acetonitrile and distilled water were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Tri-*n*-butylphosphate was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan) and tri-*n*-butylphosphine from

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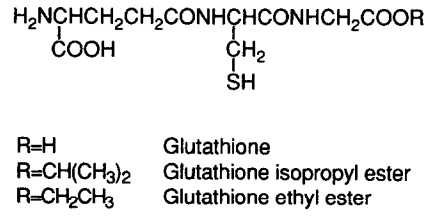

Figure 1
Chemical structures of glutathione, glutathione isopropyl ester and glutathione ethyl ester.

Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). Cysteinylglycine was obtained by reduction of cystinyl-bis-glycine (Chemical Dynamics Corp., Hardley Road, NJ, USA) with tri-*n*-butylphosphine. SBD-F was supplied by Dojindo Laboratories (Kumamoto, Japan). All other chemicals used were commercially available and of the highest purity. Chemicals were used without further purification.

Apparatus

The LC system consisted of a model LC-9A pump (Shimadzu, Kyoto, Japan), a model SIL-6B autosampler (Shimadzu), a model CTO-6A column oven, a model RF-550 spectrofluorometer (Shimadzu) and a model C-R4AX integrator (Shimadzu). A stainless steel column (25 cm × 4 mm i.d.) packed with Nucleosil$_5$C$_{18}$ (Machery–Nagel, Düren, Germany) was maintained at 40°C. The mobile phase was acetonitrile–1 N phosphoric acid–distilled water (15:5:80, v/v/v); the flow rate was 1 ml min^{-1}. The fluorescence signal was monitored at 520 nm emission with an excitation wavelength of 373 nm.

Procedure

Glutathione isopropyl ester is rapidly oxidized to the disulphide form in plasma with a half life of about 5 min, requiring rapid treatment of the sample. The oxidation of glutathione isopropyl ester in plasma was completely avoided by deproteinization with sulphosalicylic acid. We therefore attempted to establish a method for determining glutathione isopropyl ester in blood which could be treated with sulphosalicylic acid immediately after sampling. To 0.5 ml of 200 mM sulphosalicylic acid–5 mM EDTA was added 0.5 ml of blood obtained using a heparinized syringe. They were mixed thoroughly and centrifuged at 10,000*g* for 30 min at 4°C for deproteinization. A portion of 0.05 ml of supernatant was transferred into a 10-ml brown glass-stoppered tube. To the glass tube were added 0.2 ml of 500 mM sodium borate–5 mM EDTA (pH 7.5) containing 0.05 μg of internal standard, 0.2 ml of dimethyl sulphoxide, 0.025 ml of 100 mM SBD-F and 5 mM EDTA to give a final volume of 0.5 ml. The mixture was kept at 30°C for 30 min. The reaction was terminated by the addition of 0.5 ml of 0.2 M hydrochloric acid, and the reaction mixture was washed twice with 6 ml of tri-*n*-butylphosphate containing 5% (v/v) of distilled water. A 0.2-ml portion of the sample was injected onto the HPLC.

Reaction rates of glutathione isopropyl ester and the internal standard with SBD-F

The effects of buffer pH, reaction temperature, time and catalysts on the reaction rates of glutathione isopropyl ester and the internal standard with SBD-F were investigated with a reaction mixture (0.5 ml) containing 10 μg ml^{-1} of glutathione isopropyl ester or the internal standard, 0.05 ml of deproteinized supernatant of control rat blood, 200 mM sodium borate buffer, 2.5 mM EDTA and SBD-F. The reactions were terminated by 0.5 ml of 0.2 M hydrochloric acid, and a 0.05-ml portion of the sample was injected onto the HPLC. The reaction rate was estimated from the peak areas of SBD derivatives of the compounds.

Calibration curve

Standard solutions of glutathione isopropyl ester were prepared in 5 mM EDTA just before use. To 0.05 ml of deproteinized supernatant from control blood, a known amount of glutathione isopropyl ester was added. Standard samples were treated as described above. The peak height ratios of SBD-glutathione isopropyl ester to the SBD-internal standard were plotted against concentrations of added glutathione isopropyl ester, and the calibration curve was constructed by the least squares method.

Drug administration

Rats and dogs. Glutathione isopropyl ester dissolved in physiological saline was administered intravenously to male Fischer rats (7 weeks) and male beagle dogs (14–15 kg) at a dose of 30 mg kg^{-1}.

Humans. Four healthy volunteers (56–64 kg) were dosed with 1600 mg of glutathione

isopropyl ester intravenously as a 1-h infusion. The dosing solution was prepared as follows: the contents of two vials of lyophilized formula for clinical study (containing 800 mg of glutathione isopropyl ester and 164.96 mg of sodium bicarbonate) were dissolved in 32 ml of distilled water, then diluted to 100 ml with physiological saline.

Results and Discussion

Reaction condition of glutathione isopropyl ester with SBD-F

The reaction rate of thiols with SBD-F increased with increasing pH and temperature [4]. Cysteine, homocysteine, glutathione and captopril were derivatized completely within 60 min with 0.5–1 mM SBD-F in borate buffer (pH 9.5) at 60°C [5–9]. The same reaction conditions were applied to the derivatization of glutathione isopropyl ester with SBD-F and the reaction rate was investigated by HPLC. As shown in Fig. 2, HPLC responses of SBD derivatives of glutathione isopropyl ester and the internal standard reached maximum at 20 and 10 min, respectively, and decreased thereafter. On the contrary, SBD-glutathione increased up to 60 min, suggesting that the ester bond of glutathione isopropyl ester and the internal standard were hydrolysed during the reaction. In order to prevent the undesirable hydrolysis of the ester bond, the reaction was carried out under mild conditions, namely pH 7.5 at 30°C. As shown in Fig. 2, hydrolysis of the compounds was slowed under these conditions, but derivatization was still not complete at 60 min. These results indicated the necessity of an appropriate catalyst which enhances the reaction of the compounds without causing hydrolysis of the ester bond. Andrews *et al.* reported that the reaction of glutathione with SBD-Cl, a prototype of SBD-F in which fluorine is substituted by chloride as the leaving group, was markedly enhanced with dimethyl sulphoxide [10]. The effects of several aprotic solvents on the reaction of the compounds were therefore examined. As shown in Fig. 2, the addition of dimethyl sulphoxide (40%, v/v) greatly improved the reaction of glutathione isopropyl ester and the internal standard with SBD-F; the reaction plateaued after 30 min with no significant degradation of the compounds at up to 60 min. However, other aprotic solvents such as dimethyl formamide and sulpholane decreased

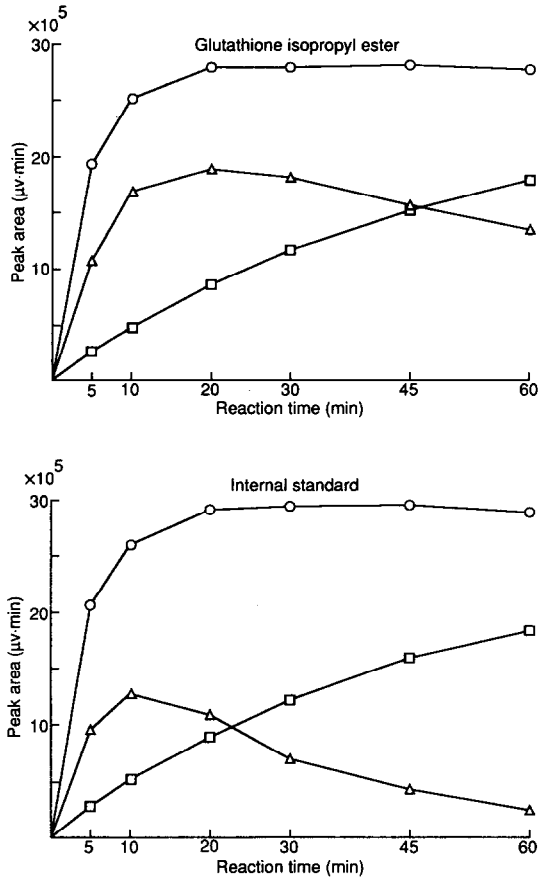

Figure 2
Time courses for derivatization of glutathione isopropyl ester and the internal standard with SBD-F. pH 9.5, 1 mM SBD-F, 60°C (△); pH 7.5, 5 mM SBD-F, 30°C (□); pH 7.5, 5 mM SBD-F, 30°C, 40% dimethyl sulphoxide (○).

the reaction rate. The effects of water-miscible solvent and crown ether also were investigated. The reaction rate decreased in the presence of methanol, ethanol, acetonitrile and 18-crown-6. Thus, fundamentally the same conditions for the derivatization of other thiols with SBD-F were applied to this method. Hydrolysis of the ester bond of glutathione isopropyl ester during derivatization, which was not seen in the derivatization of other thiols was restrained under mild conditions, and the reaction rate was increased with dimethyl sulphoxide as a catalyst.

HPLC separation of SBD-glutathione isopropyl ester and SBD-internal standard from interference peaks

Because of the high water solubilities of SBD-thiols, a reversed-phase column was used for the separation of SBD derivatives of glutathione isopropyl ester and the internal

standard from those of endogenous thiols. The SBD derivative of glutathione isopropyl ester showed high fluorescence intensity in an acidic medium. Considering this, a mixture of water, acetonitrile and phosphoric acid was used as the mobile phase. The retention times for SBD derivatives are listed in Table 1. SBD derivatives of glutathione isopropyl ester and the internal standard were eluted at 8.8 and 5.5 min, respectively. SBD derivatives of endogenous thiols tested were eluted at about 3.2 min. Figure 3 shows chromatograms of blood from rats, dogs and humans. Blood contains high concentration of glutathione. However, the SBD derivative of glutathione was coeluted with those of the other endogenous thiols, which were completely separated from SBD derivatives of glutathione isopropyl ester and the internal standard. Only a few unidentified peaks were observed as illustrated by representative chromatograms. However, no interference peaks were found at the retention times of SBD-glutathione isopropyl ester or SBD-internal standard, showing the high selectivity of this method. About five samples could be analysed per hour, and the samples were stable for at least 3 days at room temperature.

Table 1
Retention time of SBD derivatives of glutathione isopropyl ester, internal standard, glutathione and glutathione-related thiols

Thiols	Retention time (min)
Cysteine	3.2
Mercaptoethylamine	3.2
Homocysteine	3.2
Cysteinylglycine	3.2
γ-Glutatmylcysteine	3.3
Glutathione	3.2
Internal standard	5.5
Glutathione isopropyl ester	8.8

Operating conditions as given in the text.

Linearity, sensitivity, accuracy and precision of the method

Calibration curves showed good linearity at the concentrations up to at least 100 μg ml^{-1}. The detection limit was 0.05 μg ml^{-1} at a signal to noise ratio of 3:1. The accuracy and precision of the method were examined using control blood from rats, dogs and humans spiked with glutathione isopropyl ester at concentrations of 0.5, 2.5 and 10 μg ml^{-1}. Five samples were determined for each concentration. As shown in Table 2, the method has adequate accuracy and precision.

Application of the method

The utility of the method was demonstrated by monitoring blood concentrations of unchanged drug after intravenous administration of glutathione isopropyl ester to experimental animals and humans. Blood concentrations could be determined for the pharmacokinetic evaluation of the drug after a bolus injection of

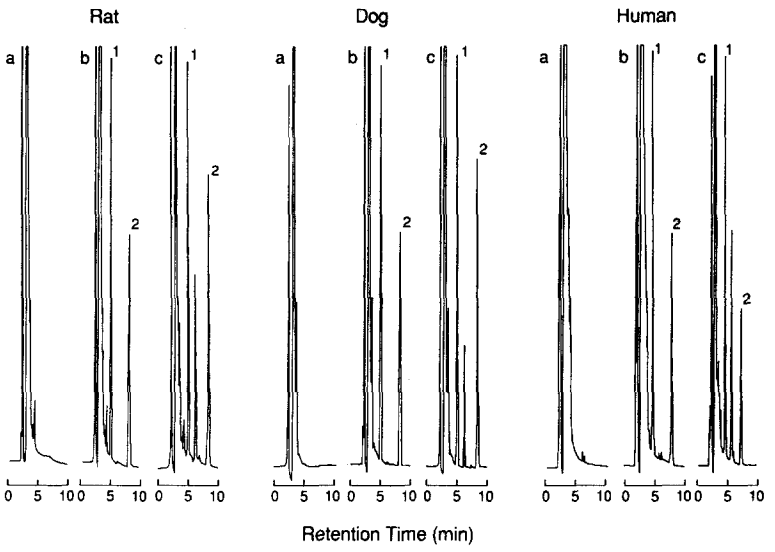

Figure 3
Typical chromatograms of glutathione isopropyl ester. (a) Blank blood; (b) spiked with glutathione isopropyl ester and the internal standard; (c) blood samples obtained after intravenous administration of glutathione isopropyl ester. Peaks: 1 = internal standard; 2 = glutathione isopropyl ester.

Table 2
Accuracy and precision of methods in the determination of glutathione isopropyl ester in rat, dog and human blood

Conc. prepared ($\mu g\ ml^{-1}$)	Species	Conc. measured (mean ± SD, $n = 5$) ($\mu g\ ml^{-1}$)	Percentage difference (%)	Relative standard deviation (%)
0.5	Rat	0.471 ± 0.003	−5.8	0.6
	Dog	0.481 ± 0.004	−3.9	0.8
	Human	0.489 ± 0.005	−2.3	1.1
2.5	Rat	2.51 ± 0.02	0.2	0.7
	Dog	2.50 ± 0.02	0.0	0.9
	Human	2.50 ± 0.02	−0.2	0.7
10	Rat	9.9 ± 0.1	−1.0	1.1
	Dog	10.3 ± 0.1	2.6	1.3
	Human	10.0 ± 0.2	0.2	1.7

$$\text{Percentage difference} = 100 \times \frac{\text{conc. prepared} - \text{conc. measured}}{\text{conc. prepared}}.$$

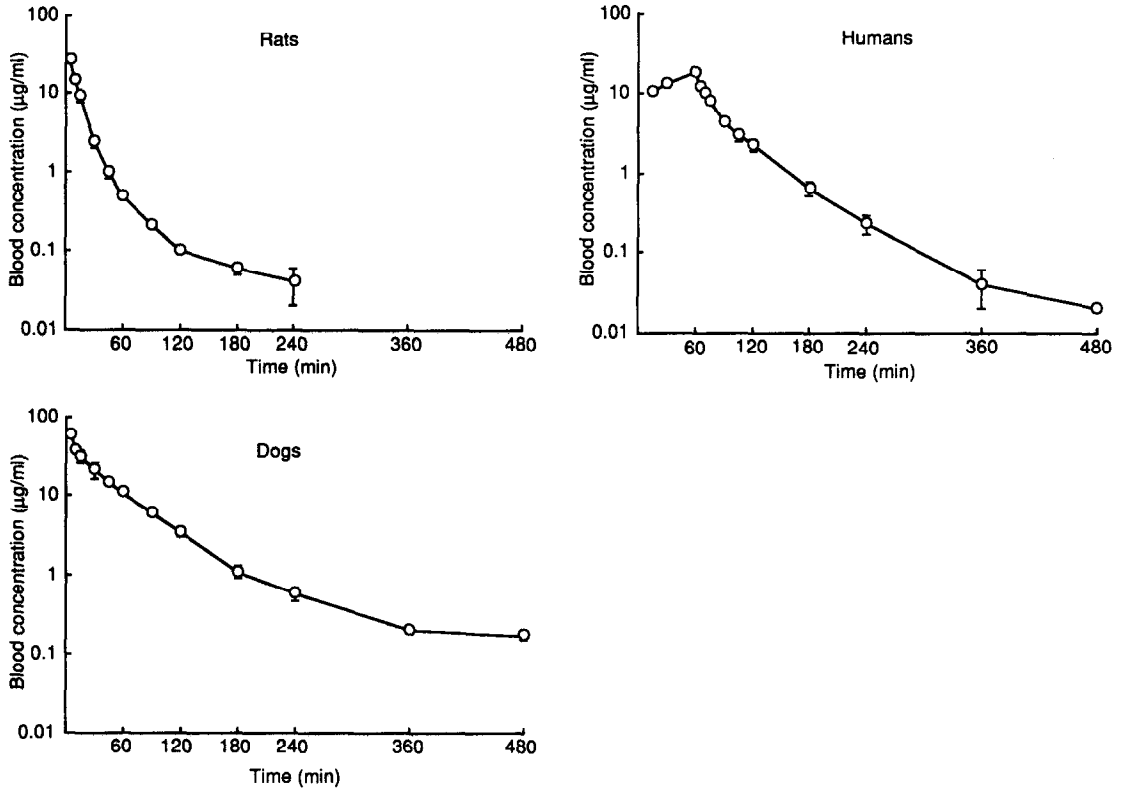

Figure 4
Blood concentration of unchanged drug after intravenous administration of glutathione isopropyl ester to rats, dogs and humans. Rats and dogs were dosed 30 mg kg^{-1} of glutathione isopropyl ester intravenously as a bolus. Values are expressed as mean ±S.E.M. of three animals. Humans were dosed 1600 mg of glutathione isopropyl ester as a 1-h infusion. Values are expressed as mean ±S.E.M. of four volunteers.

glutathione isopropyl ester 30 mg kg^{-1} to rats and dogs. The drug was eliminated with the distribution half-lives of 8.3 and 7 min and terminal half-lives of 71 and 45 min in rats and dogs, respectively (Fig. 4). Blood concentrations of glutathione isopropyl ester during and after intravenous infusion of 1600 mg of the drug to healthy volunteers over 60 min also could be determined (Fig. 4). The maximum concentration of 18 ± 0.9 $\mu g\ ml^{-1}$ was

achieved at the end of the infusion, decreasing thereafter with the distribution half-life of 9 min and the terminal half-life of 35 min.

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