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Pharmacokinetics of Cycloalliin, an Organosulfur Compound Found in Garlic and Onion, in Rats

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Cycloalliin, an organosulfur compound found in garlic and onion, has been reported to exert several biological activities and also to remain stable during storage and processing. In this study, we investigated the pharmacokinetics of cycloalliin in rats after intravenous or oral administration. Cycloalliin and its metabolite, (3*R*,5*S*)-5-methyl-1,4-thiazane-3-carboxylic acid, in plasma, urine, feces, and organs was determined by a validated liquid chromatography−mass spectrometry method. When administered intravenously at 50 mg/kg, cycloalliin was rapidly eliminated from blood and excreted into urine, and its total recovery in urine was 97.8% ± 1.3% in 48 h. After oral administration, cycloalliin appeared rapidly in plasma, with a t_{max} of 0.47 ± 0.03 h at 25 mg/kg and 0.67 ± 0.14 h at 50 mg/kg. Orally administered cycloalliin was distributed in heart, lung, liver, spleen, and especially kidney. The C_{max} and AUC_{0-inf} values of cycloalliin at 50 mg/kg were approximately 5 times those at 25 mg/kg. When administered orally at 50 mg/kg, cycloalliin was excreted into urine (17.6% ± 4.2%) but not feces. However, the total fecal excretion of (3*R*,5*S*)-5-methyl-1,4-thiazane-3-carboxylic acid was 67.3% ± 5.9% (value corrected for cycloalliin equivalents). In addition, no (3*R*,5*S*)-5-methyl-1,4-thiazane-3-carboxylic acid was detected in plasma (<0.1 μg/mL), and negligible amounts (1.0% ± 0.3%) were excreted into urine. In in vitro experiments, cycloalliin was reduced to (3*R*,5*S*)-5-methyl-1,4-thiazane-3-carboxylic acid during anaerobic incubation with cecal contents of rats. These data indicated that the low bioavailability (3.73% and 9.65% at 25 and 50 mg/kg, respectively) of cycloalliin was due mainly to reduction to (3*R*,5*S*)-5-methyl-1,4-thiazane-3-carboxylic acid by the intestinal flora and also poor absorption in the upper gastrointestinal tract. These findings are helpful for understanding the biological effects of cycloalliin.

KEYWORDS: Cycloalliin; pharmacokinetics; metabolite; garlic; onion

INTRODUCTION

Sulfoxides are widely distributed in *Allium* vegetables, such as garlic (*Allium sativum* L.), onion (*Allium cepa* L.), leek (*Allium porrum* L.), and elephant garlic (*Allium ampeloprasum* L.). *S*-Alk(en)yl-L-cysteine sulfoxides (e.g., alliin, isoalliin, and methiin) are converted into thiosulfinates by the enzyme alliinase (EC 4.4.1.4) when *Allium* vegetables are cut or crushed (*1*, *2*). However, cycloalliin [(1*S*,3*R*,5*S*)-5-methyl-1,4-thiazane-3-carboxylic acid 1-oxide, **1**], a cyclic sulfoxide present in garlic and onion, shown in **Figure 1**, is not converted into thiosulfinate by alliinase and remains stable during processing (*3*, *4*). Recently we have reported that the contents of cycloalliin in raw garlic cloves increases during storage through a mechanism in which isoalliin produced from γ-L-glutamyl-*S*-(*trans*-1-propenyl)-L-cysteine by γ-glutamyl transpeptidase and oxidase is chemically converted to cycloalliin (*5*). Previous studies have suggested that cycloalliin might be partly responsible for the biological effects of garlic, onion, and their preparations, such as a reduction in the risk of cancer and cardiovascular disease (*6*,

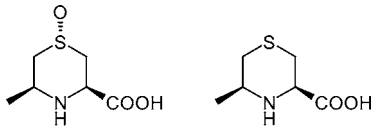

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Figure 1. Chemical structures of cycloalliin [**1**, (1*S*,3*R*,5*S*)-5-methyl-1,4-thiazane-3-carboxylic acid 1-oxide] and its metabolite [**2**, (3*R*,5*S*)-5-methyl-1,4-thiazane-3-carboxylic acid].

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7). Cycloalliin has been reported to increase significantly the fibrinolytic activity of blood in a human trial but to have no effects on platelet aggregation (*8*) and to reduce serum triacylglycerols in rats without affecting hepatic triacylglycerol synthesis (*9*). Xiao and Parkin (*10*) have reported that cycloalliin promotes reduction of quinone reductase activity in vitro. All of these studies suggest that cycloalliin could be useful as a chemical and/or biological marker for garlic, onion, and their preparations. To date, however, there have been no reports on the pharmacokinetics of cycloalliin, despite the fact that it is present in various foods, supplements, and phytomedicines containing garlic or onion.

In the present study, we established a liquid chromatography−mass spectrometry (LC-MS) method to determine the concentration of cycloalliin and its reduced metabolite, (3*R*,5*S*)-5-methyl-1,4-thiazane-3-carboxylic acid (**2**, **Figure 1**), in rat plasma, urine, feces, and organs. We then investigated the stability of cycloalliin and its oral bioavailability and pharmacokinetics in rats. Here we report the pharmacokinetics of cycloalliin compared with other organosulfur compounds in garlic, and we further discuss the relationship to its biological activity.

MATERIALS AND METHODS

Chemicals. Cycloalliin [(1*S*,3*R*,5*S*)-5-methyl-1,4-thiazane-3-carboxylic acid 1-oxide, **1**] and (3*R*,5*S*)-5-methyl-1,4-thiazane-3-carboxylic acid (**2**) were synthesized as described previously (*11*). (1*R*,3*R*,5*S*,6*R*)-5,6-Dimethyl-1,4-thiazane-3-carboxylic acid 1-oxide was synthesized as described below and used as an internal standard (IS) in LC-MS analysis for quantitative determination of cycloalliin and **2**. The internal standard solution was prepared at a concentration of 0.1 μg/mL in ethanol. Saline (0.9% NaCl injectable solution) was obtained from Fuso Pharmaceutical Co., Ltd. (Osaka, Japan). Acetonitrile for HPLC was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals of reagent grade were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), Wako Pure Chemical Industries, Ltd., and Yoneyama Yakuhin Kogyo Co., Ltd. (Osaka, Japan). Gifu anaerobic medium (GAM) broth was obtained from Nissui Seiyaku Co., Ltd. (Tokyo, Japan). Water purified with a Milli-Q Labo system (Millipore, Bedford, MA) was used for mobile-phase preparation.

Synthesis of (1*R*,3*R*,5*S*,6*R*)-5,6-Dimethyl-1,4-Thiazane-3-Carboxylic Acid 1-Oxide. L-Cysteine methyl ester hydrochloride (3 g) was dissolved in 10 mL of methanol at 0−5 °C, and then triethylamine (3.54 g) and 3-chlorobutan-2-one (2.1 g) were added to the mixture, followed by stirring for 1 h. The reaction mixture was acidified with 7.5 mL of 4 M hydrochloric acid−dioxane at 0−5 °C. Sodium borohydride (1 g) was added in small portions to the mixture below 5 °C. After the mixture had been stirred for 30 min below 5 °C, excess sodium borohydride was decomposed with 10% HCl. The reaction mixture was made alkaline with sodium hydrogen carbonate and then extracted with methylene chloride. The extract was dried with sodium sulfate and concentrated in vacuo. The residue was purified by silica gel column chromatography with methylene chloride−methanol (50:1 v/v), and the 5,6-dimethyl-1,4-thiazane-3-carboxylic acid methyl ester fraction was recovered. After removal of the solvent in vacuo, the residue (1.42 g) was dissolved in 20 mL of 50% methanol. Sodium metaperiodate (0.5 mM) in methanol (30 mL) was added dropwise to the preparation below 5 °C. The reaction mixture was filtered after stirring for 2 h, and 3 mM NaOH (5 mL) was added to the filtrate at 0−5 °C. After being stirred for 4 h, the reaction mixture was neutralized with 10% HCl. The mixture was applied to a Dowex 50W-X4 column (H^+ form) (Dow Chemical Co., Midland, MI), eluted with 2 M ammonium hydroxide, and the 5,6-dimethyl-1,4-thiazane-3-carboxylic acid 1-oxide fraction was recovered (0.54 g). Further purification of 5,6-dimethyl-1,4-thiazane-3-carboxylic acid 1-oxide (0.40 g) was performed by use of a semipreparative HPLC system (LC-10A system, Shimadzu, Kyoto, Japan) under the following conditions: column, 250 × 4.6 mm Shodex Asahipak NH2P-50 4E (Showa Denko, Tokyo, Japan); mobile phase, 0.1% formic acid/acetonitrile (1:9 v/v); flow rate, 2 mL/min; detection, 210 nm. Finally, 98 mg of (1*R*,3*R*,5*S*,6*R*)-5,6-dimethyl-1,4-thiazane-3-carboxylic acid 1-oxide was isolated as a white-yellowish powder. The melting range of this compound was 275−280 °C (decomp). NMR experiments were performed on a JNM-ECP500 instrument (JEOL, Tokyo, Japan) operating at 500 MHz for ^{1}H and 126 MHz for ^{13}C, respectively. The NMR data [D_2O, sodium 3-(trimethylsilyl)propanesulfonate as an internal standard] for this compound are follows: ^{1}H NMR δ 4.30 (dd, 1H, J = 13, 3 Hz, H3), 4.14 (m, 1H, H5), 3.53 (m, 1H, H2), 3.35 (m, 1H, H6), 3.10 (dd, 1H, J = 16, 13 Hz, H2), 1.47 (d, 3H, J = 7 Hz, C5-CH_3), 1.29 (d, 3H, J = 8 Hz, C6-CH_3); ^{13}C NMR δ 173.6 (C3-*C*OOH), 52.5 (C6), 51.7 (C3), 48.5 (C5), 41.4 (C2), 18.0 (C5-*C*H$_3$), 9.3 (C6-*C*H$_3$). Mass spectrometry was carried out on a ThermoElectron LTQ linear ion trap mass spectrometer fitted with an electrospray ionization (ESI) source run by Xcalibur 2.0 SUR1 software (ThermoElectron, San Jose, CA). The mass spectrum showed a base peak at m/z 192, corresponding to the $[M + H]^+$ ion. High-resolution mass spectrometry (HR-MS) was performed with a JEOL SX102A mass spectrometer (JEOL, Tokyo, Japan) equipped with a fast atom bombardment ion source. The high-resolution mass spectrum showed a protonated molecule peak at m/z 192.0668 (calculated for $C_7H_{14}O_3NS$, 192.0694).

Animals. Seven-week-old male Sprague-Dawley rats were purchased from Charles River Japan Inc. (Shiga, Japan). The rats were acclimated for 7 days in a laboratory animal facility maintained at constant temperature (23 ± 3 °C) and humidity (50% ± 20% relative humidity) with a 12-h light/dark cycle and free access to food and water, and then administration experiments were performed. All animal treatments in this study were approved by the institutional Animal Care and Use Committee of Wakunaga Pharmaceutical Co., Ltd.

Tests of Cycloalliin Stability in Aqueous Solution and Biological Fluids. The stability of cycloalliin was evaluated in chemical and biological fluids. Tests of stability in acidic and basic solution were performed as follows: cycloalliin was dissolved in 0.1 M HCl or 0.1 M NaOH at 14 mM, and these solutions (1 mL) were poured into a 1.5-mL test tube, followed by incubation at 70 °C. After 3 h of incubation, these solutions were collected and diluted with methanol at a final concentration of 1 mM. Five microliters of the solution was added to 200 μL of the internal standard solution, mixed, and then the solution was analyzed by LC-MS to determine cycloalliin. Tests of stability in biological fluids were performed as follows: cycloalliin dissolved in saline at 0.28 mM was added to rat blood or plasma at a final concentration of 0.01 and 0.1 mM and kept at room temperature for 30 min. The mixture (5 μL) was then pipetted into a 0.5-mL plastic tube, and 50 μL of the internal standard solution was added, followed by thorough mixing. The mixture was centrifuged at 11000*g* for 5 min, and the supernatant was analyzed by LC-MS for determination of cycloalliin. Tests of stability in rat liver homogenates were performed according to the method reported previously (*12*). Seven-week-old rats were fasted for 18 h, and the liver was removed under ether anesthesia. The liver was homogenized in PBS and the final concentration was adjusted to 10% or 17% (w/v). Cycloalliin was added to the homogenate at a final concentration of 0.1 or 1 mM, and these mixtures (0.9 mL) were poured into a 5-mL test tube, followed by incubation for 7 h at 37 °C under 5% CO_2 in air. The resulting mixture (5 μL) was added to 200 μL of the internal standard solution, mixed, and then centrifuged at 11000*g* for 5 min. The supernatant was analyzed by LC-MS to determine cycloalliin and its metabolite.

Administration to Animals. Cycloalliin was administered to rats in a single dose of 25 or 50 mg/kg body weight. Cycloalliin was dissolved in distilled water at 5 mg/mL for oral administration and in saline at 50 mg/mL for intravenous administration.

Collection of Plasma Samples. Rats were fasted for 16 h prior to administration. Blood samples were collected from the orbital vein under ether anesthesia into heparinized capillary tubes (75 μL) at several different time points (5, 10, 20, and 30 min and 1, 2, 4, and 6 h) after administration of cycloalliin. The blood was centrifuged at 1000*g* for 10 min, and plasma was collected and stored at −35 °C until analysis.

Collection of Urine and Feces Samples. The animals were acclimated individually in metabolic cages with free access to food and water for 4 days. Food was completely withdrawn 16 h prior to

administration and then returned 4 h after dosing. Urine and feces were collected at 0−4, 4−8, 8−12, 12−24, 24−48, and 48−78 h after administration, respectively. The urine samples were immediately centrifuged at 1000*g* for 10 min to remove solid contaminants, and the supernatant was stored at −35 °C until analysis. Feces samples were immediately freeze-dried and crushed to powder with a pestle and mortar and were stored at −35 °C until analysis.

Collection of Organ Samples for Study of Distribution. Rats were fasted for 16 h prior to administration. Cycloalliin (50 mg/kg) was administered orally to rats, and then 40 min and 4 and 24 h later, the animals were anesthetized by inhalation of diethyl ether. The rats were exsanguinated via the abdominal aorta and then blood was collected in a heparin-containing tube and centrifuged at 1000*g* for 10 min. The plasma was collected and stored at −35 °C until analysis. After blood withdrawal, the organs were rapidly extirpated, weighed, frozen in liquid nitrogen, and stored at −35 °C until analysis.

Sample Preparations for LC-MS Analysis. A plasma sample (5 μL) was transferred to a 0.5-mL plastic tube and 50 μL of the internal standard solution was added, followed by thorough mixing. The powdered fecal sample (100 mg) was added to 5 mL of 80% methanol and the mixture was sonicated for 10 min, followed by centrifugation at 11000*g* for 5 min. A 5 μL aliquot of the fecal supernatant or urine diluted 25-fold with water was transferred to a 0.5-mL plastic tube and then 100 μL of the internal standard solution was added, followed by thorough mixing. The frozen organ samples were homogenized with a 20-fold volume of methanol at the highest speed for 1 min in a model 7012 laboratory blender (Waring Products, Inc., Torrington, CT). Then 50 μL of the homogenate was transferred to a 0.5-mL plastic tube, 50 μL of the internal standard solution was added, and the mixture was stirred well. All the solutions were centrifuged at 11000*g* for 5 min, and the supernatant was analyzed by LC-MS to determine cycloalliin and its metabolite.

Sample Preparations for Analysis of Changes of Cycloalliin in Cecal Contents. Cecal contents were obtained from adult male rats fasted for 18 h. The cecum was removed under ether anesthesia and the entire contents were suspended in GAM broth at a final concentration of 10% or 17% (w/v) in the presence of CO_2. Cycloalliin was added to the suspension at 0.08 or 0.8 mM, and the resulting mixtures (0.9 mL) were poured into 5-mL test tubes, each of which was flushed with CO_2 and stoppered. The tubes were anaerobically incubated at 37 °C by use of an AnaeroPack (Mitsubishi Gas Chemical Co., Ltd., Tokyo, Japan). Samples were collected after 0, 1, 2, 4, 7, and 24 h of incubation and were stored at −35 °C before analysis. The thawed sample (5 μL) was mixed with 200 μL of the internal standard solution and then centrifuged at 11000*g* for 5 min. The supernatant was analyzed by LC-MS to determine cycloalliin and **2**.

LC-MS Conditions. LC-MS analysis was carried out by coupling a Shimadzu HPLC system (LC-20A Prominence, Shimadzu, Kyoto, Japan) to a ThermoElectron LTQ linear ion trap mass spectrometer fitted with an ESI source. HPLC separation was performed on a 150 × 2-mm (5-μm) Shodex Asahipak NH2P-50 2D column (Showa Denko, Tokyo, Japan). The mobile phase consisted of a mixture of 0.1% formic acid and acetonitrile (2:8 v/v) delivered at a rate of 0.2 mL/min. Positive ion collision-induced dissociation mass spectrometry (MS/MS) was conducted to detect cycloalliin, **2**, and IS with selective reaction monitoring (SRM). The mass spectrometric conditions were as follows: electrospray voltage, 5.5 kV; heated capillary temperature, 350 °C; sheath gas (nitrogen gas) pressure, 45 arbitrary units; auxiliary gas (nitrogen gas) pressure, 15 arbitrary units. In the MS/MS experiments, the protonated precursor molecular ions $[M + H]^+$ of cycloalliin (*m*/*z* 178), **2** (*m*/*z* 162), and IS (*m*/*z* 192) were selected and fragmented by helium gas collision in the ion trap at a relative collision energy of 30%. The mass spectra resulting from their fragmentations were acquired in the SRM mode at *m*/*z* 88 for cycloalliin, *m*/*z* 116 for **2**, and *m*/*z* 105 for IS, and these product ions were extracted for quantification to achieve higher sensitivity and selectivity.

Validation of the LC-MS Method To Determine Cycloalliin and Its Metabolite in Biological Samples. *Specificity*. Specificity was assessed by analyzing three different sources of biological matrices (plasma, urine and feces, and organs). Chromatograms were visually inspected for interfering chromatographic peaks from endogenous substances.

Linearity. To examine the linearity of cycloalliin and **2**, calibration standards with more than three concentrations were prepared and assayed. The final concentration ranges of cycloalliin are 0.01−20 μg/mL for plasma, 0.02−20 μg/mL for feces and urine, and 0.02−2.5 μg/mL for organs, respectively. The final concentration ranges of **2** are 0.01−17 μg/mL for plasma and feces, 0.04−4.1 μg/mL for urine, and 0.02−2.1 μg/mL for organs, respectively. A calibration curve was derived from the peak area ratio (analyte/internal standard) versus the known concentrations of the calibration samples. A weighting of $1/x^2$ (where x is the concentration of a given standard) was used for curve fitting.

Accuracy and Precision. To evaluate the accuracy and precision of the method, recovery experiments ($n = 3$) were carried out by adding different concentrations of cycloalliin and **2** to biological matrices. The added concentrations of cycloalliin are 0.5−200 μg/mL for plasma, 50−10 000 μg/mL for feces and urine, and 0.5−50 μg/mL for organs, respectively. The added concentrations of **2** are 0.413−165 μg/mL for plasma, 41.3−8254 μg/mL for feces, 20.6−330 μg/mL for urine, and 0.413−41.3 μg/mL for organs, respectively. The accuracy was expressed in terms of relative error, and the precision was evaluated in terms of the relative standard deviation (RSD).

Limit of Detection. The limit of detection (LOD) was determined as the analyte concentration that gave a signal equal to 3 times the noise level.

Pharmacokinetic Analysis. Pharmacokinetic parameters were calculated by use of the MOMENT computer program (*13*). The peak plasma concentration (C_{max}) and time to reach C_{max} (t_{max}) after oral administration were read directly from the experimental data. The total area under the plasma concentration−time curve from time zero to infinity (AUC_{0-inf}) was calculated by the trapezoidal rule-extrapolation method. The elimination rate constant (K_{el}) was calculated from the slope of the line by regression analysis of the log plasma concentration versus time, and the half-life ($t_{1/2\beta}$) was obtained by $0.693/K_{el}$. The mean residence time (MRT) was calculated as the ratio of the area under the first moment curve from time zero to infinity ($AUMC_{0-inf}$) to AUC_{0-inf}. The apparent total body clearance (CL_{tot}) was calculated from the equation $CL_{tot} = \text{dose}/AUC_{0-inf}$. The apparent volume of distribution at steady state (Vd_{ss}) was calculated from the equation $Vd_{ss} = (MRT)\text{-}CL_{tot}$. The bioavailability was calculated from the equation $F = \{[AUC_{0-inf}(po)][\text{dose (iv)}]\}/\{[AUC_{0-inf}\text{ (iv)}][\text{dose (po)}]\}$. Renal clearance ($CL_r$) was determined as Ae/AUC_{0-inf}, where Ae is the total amount of cycloalliin excreted into urine. Nonrenal clearance (CL_{nr}) was determined by subtracting CL_r from CL_{tot}.

Statistical Analyses. The results are expressed as means ± standard error (SE) of at least four rats. Differences at $p < 0.05$ were considered to be statistically significant by the *t*-test for paired or unpaired data.

RESULTS

Tests of Cycloalliin Stability in Aqueous Solution and Biological Fluids. Stability of cycloalliin in 0.1 M HCl, 0.1 M NaOH, rat blood, plasma, and liver homogenates was evaluated. Cycloalliin was stable in 0.1 M HCl or 0.1 M NaOH at 70 °C for 3 h and its remaining amount was more than 98%. Almost all (>96%) of the spiked cycloalliin was recovered from rat blood and plasma. In experiments with rat liver homogenates, more than 97% of cycloalliin was not degraded after incubation at 37 °C for 7 h, and furthermore no metabolite of cycloalliin was detected.

LC-MS Analysis of Cycloalliin and (3*R*,5*S*)-5-Methyl-1,4-thiazane-3-carboxylic Acid (2). Cycloalliin and its main metabolite, **2**, in biological matrices were determined quantitatively by LC-MS analysis, which has high specificity and selectivity. The ESI spectra mainly showed the protonated molecular ions $[M + H]^+$ of cycloalliin (*m*/*z* 178), **2** (*m*/*z* 162), and IS (*m*/*z* 192), as shown in **Figure 2A−C**, respectively. The

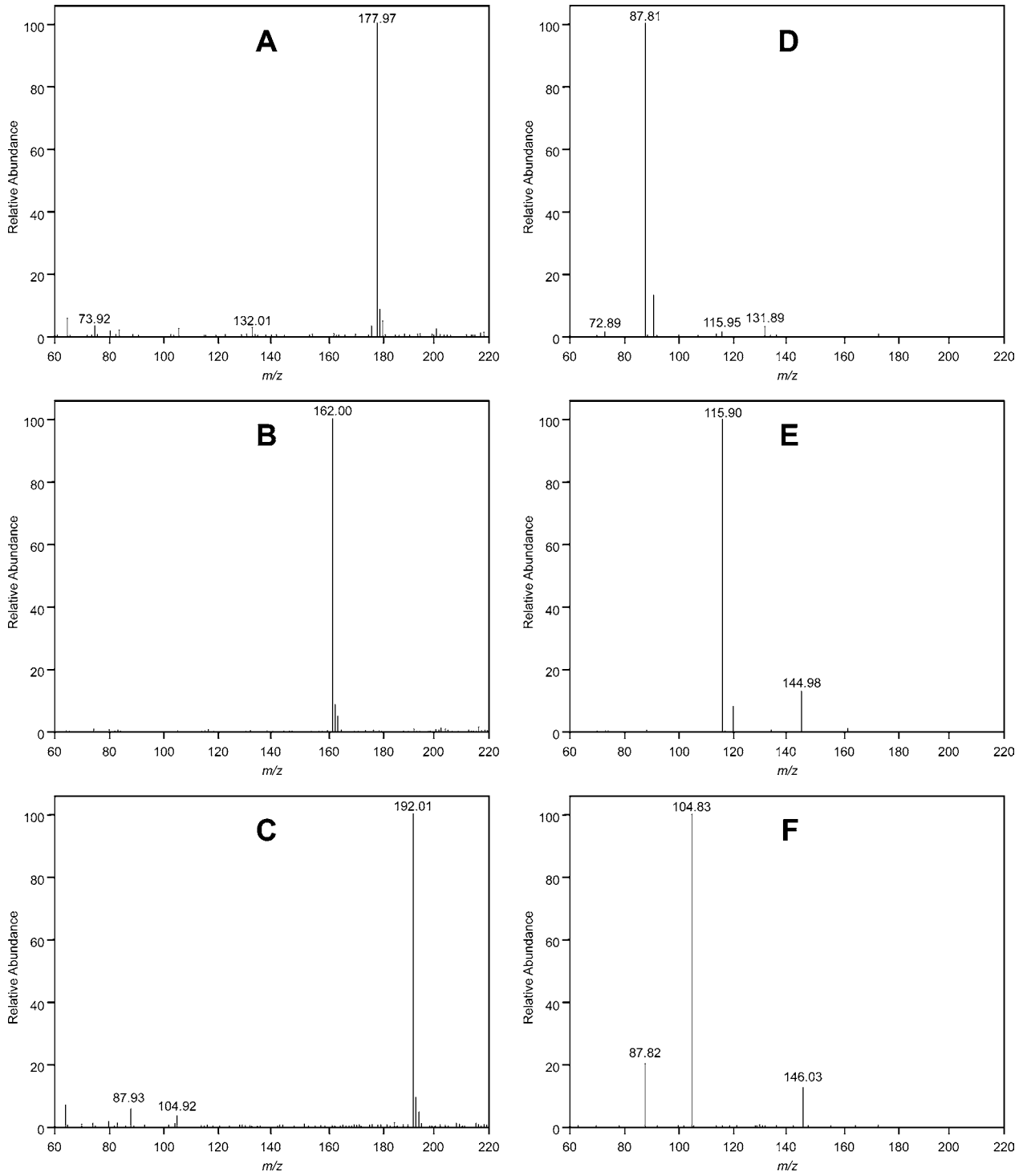

Figure 2. Positive ion electrospray mass spectra of (**A**) cycloalliin, (**B**) (3*R*,5*S*)-5-methyl-1,4-thiazane-3-carboxylic acid, and (**C**) (1*R*,3*R*,5*S*,6*R*)-5,6-dimethyl-1,4-thiazane-3-carboxylic acid 1-oxide and their product ion mass spectra with precursors of (**D**) *m*/*z* 178, (**E**) *m*/*z* 162, and (**F**) *m*/*z* 192.

product ions at *m*/*z* 88 (HR-MS found, 88.0370; calculated for $C_3H_6O_2N$, 88.0399) for cycloalliin, *m*/*z* 116 (HR-MS found, 116.0534; calculated for $C_5H_{10}NS$, 116.0534) for **2**, and *m*/*z* 105 (HR-MS found, 105.0375; calculated for C_4H_9OS, 105.0374) for IS were observed in the MS/MS experiments (**Figure 2D**−**F**). Therefore, these product ions were monitored in SRM mode in order to quantify them. Typical SRM chromatograms of a rat plasma sample prepared by spiking it with cycloalliin at 0.1 μg/mL, **2** at 0.08 μg/mL, and IS at 0.1 μg/mL are shown in **Figure 3**. The retention times of cycloalliin, **2**, and IS were 14.4, 8.4, and 14.7 min, respectively.

Validation of the LC-MS Method To Determine Cycloalliin and Its Metabolite in Biological Samples. Specificity was assessed by analyzing three different sources of plasma, urine and feces, and organs. No endogenous interfering sources were observed at the retention times of cycloalliin, **2**, and IS on the chromatograms obtained from blank biological matrices. The calibration curves of cycloalliin and **2** in the respective spiked samples had correlation coefficients of >0.991 and >0.992, respectively. Accuracy and precision were determined by use of three replicates of the spiked samples. The relative error ranged from −13.1% to 7.3% for cycloalliin and from −16.6%

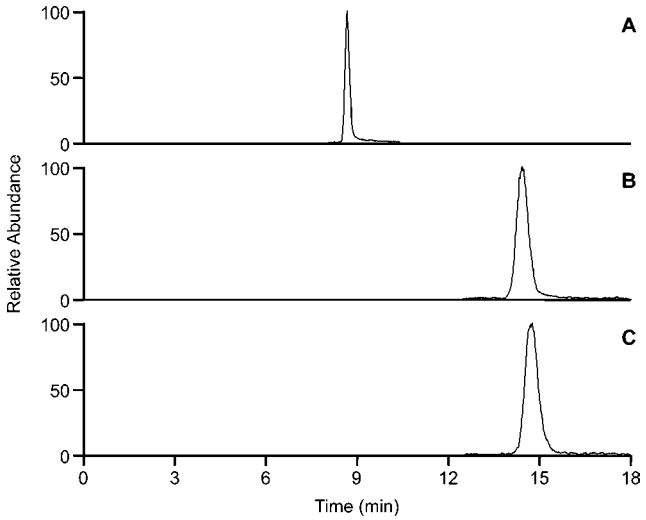

Figure 3. Selective reaction monitoring chromatograms of rat plasma sample spiked with (**A**) (3*R*,5*S*)-5-methyl-1,4-thiazane-3-carboxylic acid, (**B**) cycloalliin, and (**C**) (1*R*,3*R*,5*S*,6*R*)-5,6-dimethyl-1,4-thiazane-3-carboxylic acid 1-oxide. The product ions were monitored at *m*/*z* 162 → 116 for (3*R*,5*S*)-5-methyl-1,4-thiazane-3-carboxylic acid, *m*/*z* 178 → 88 for cycloalliin, and *m*/*z* 192 → 105 for (1*R*,3*R*,5*S*,6*R*)-5,6-dimethyl-1,4-thiazane-3-carboxylic acid 1-oxide.

to 13.0% for **2**, whereas intraday relative standard deviation (RSD) did not exceed 13.1% for cycloalliin and 16.0% for **2**. The limit of detection (LOD) of cycloalliin was 0.1 μg/mL for plasma, 20 μg/mL for urine, 20 μg/g for feces, and 0.1 μg/g for organs, respectively. The LOD of **2** was 0.1 μg/mL for plasma, 20 μg/mL for urine, 10 μg/g for feces, and 0.1 μg/g for organs, respectively.

Study of Cycloalliin Pharmacokinetics. After intravenous administration at 50 mg/kg to rats, the plasma level of cycloalliin declined rapidly (**Figure 4A**) and the concentration−time curve exhibited a monophasic decline. The AUC_{0-inf}, $t_{1/2\beta}$, *MRT*, Vd_{ss}, and CL_{tot} values were 63.17 ± 2.53 μg·h mL^{-1}, 0.84 ± 0.03 h, 0.65 ± 0.01 h, 0.52 ± 0.02 L kg^{-1}, and 0.80 ± 0.03 L h^{-1} kg^{-1}, respectively (**Table 1**). The plasma concentration−time profiles after oral administration are shown in **Figure 4B**, and some relevant pharmacokinetic parameters are listed in **Table 1**. Cycloalliin was rapidly absorbed after oral administration, and the concentration in plasma showed peaks at 0.47 ± 0.03 and 0.67 ± 0.14 h at 25 and 50 mg/kg, respectively. At 25 mg/kg, the values of C_{max} and AUC_{0-inf} were 0.33 ± 0.03 μg/mL and 1.18 ± 0.17 μg·h mL^{-1}, respectively. However, at 50 mg/kg, these values did not increase proportionally (1.59 ± 0.20 μg/mL and 6.10 ± 0.55 μg·h mL^{-1}, respectively). The values of $t_{1/2\beta}$ and *MRT* did not differ significantly between 25 and 50 mg/kg. However, the Vd_{ss} value at 25 mg/kg was significantly higher than that at 50 mg/kg because the clearance (CL_{tot}) at 25 mg/kg was 2.7-fold higher than that at 50 mg/kg. Consequently, the bioavailability (*F*) of cycloalliin at 50 mg/kg was 2.6-fold higher than that at 25 mg/kg.

Figure 5 shows the cumulative excretion of cycloalliin and its metabolite **2** (value corrected for cycloalliin equivalents) in urine and feces after intravenous and oral administration at 50 mg/kg. Approximately 90% was excreted into urine within 8 h after intravenous administration, and the cumulative amount excreted was 97.8% ± 1.3% of the dosage by 48 h (**Figure 5A**). Consequently, renal clearance (CL_r) and nonrenal clearance (CL_{nr}) were 0.77 ± 0.01 and 0.03 ± 0.01 L h^{-1} kg^{-1}, respectively (**Table 1**). Also, no cycloalliin was excreted into feces after intravenous administration. On the other hand, after oral administration, 17.6% of the dosage was excreted into urine by 48 h but not into feces (**Figure 5B**).

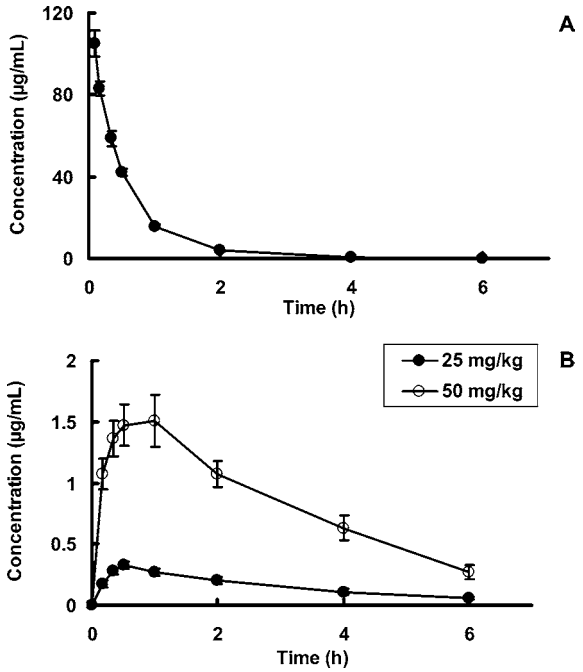

Figure 4. (**A**) Plasma concentration–time profile of cycloalliin following intravenous administration at 50 mg/kg to rats. Each value represents the mean ± SE of five animals. (**B**) Plasma concentration–time profile of cycloalliin following oral administration at 25 mg/kg (n = 6) and 50 mg/kg (n = 5) to rats. Each value represents the mean ± SE.

Table 1. Pharmacokinetic Parameters following Oral (po) and Intravenous (iv) Administration of Cycloalliin to Rats[a]

parameter	25 mg/kg (po)	50 mg/kg (po)	50 mg/kg (iv)
t_{max} (h)	0.47 ± 0.03	0.67 ± 0.14	
C_{max} (μg mL^{-1})	0.33 ± 0.03[b]	1.59 ± 0.20	
AUC_{0-inf} (μg·h mL^{-1})	1.18 ± 0.17[b]	6.10 ± 0.55	63.17 ± 2.53
$t_{1/2\beta}$ (h)	2.40 ± 0.38	2.19 ± 0.49	0.84 ± 0.03
MRT (h)	3.62 ± 0.54	3.48 ± 0.70	0.65 ± 0.01
Vd_{ss} (L kg^{-1})	78.16 ± 8.82[b]	27.70 ± 3.13	0.52 ± 0.02
F (%)	3.73	9.65	
CL_{tot} (L h^{-1} kg^{-1})	23.15 ± 3.35[b]	8.47 ± 0.76	0.80 ± 0.03
CL_r (L h^{-1} kg^{-1})			0.77 ± 0.01
CL_{nr} (L h^{-1} kg^{-1})			0.03 ± 0.01

[a] Each value represents the mean ± SE of five or six rats. [b] Significant difference (p < 0.05) between 25 and 50 mg/kg.

Distribution in organs at 40 min and 4 and 24 h after oral administration at 50 mg/kg in rats is listed in **Table 2**. Cycloalliin was widely distributed in most organs, and the concentrations in kidney were the highest. The organ to plasma concentration ratio in kidney at 4 h (9.00 ± 0.40) was significantly greater than that at 40 min (5.28 ± 0.77). In addition, cycloalliin could not be detected in any of the organs tested at 24 h after administration (<0.1 μg/g).

Identification of Metabolite. After cycloalliin had been administered orally, the rat fecal excreta were analyzed by LC-MS, and the assumed metabolite was chemically synthesized. (3*R*,5*S*)-5-Methyl-1,4-thiazane-3-carboxylic acid (**2**) was identified as the metabolite by comparison of its retention time and mass spectrum with those of authentic compounds. The mass spectrum of the metabolite gave protonated molecular ions [M + H]$^+$ at *m*/*z* 162 (**Figure 2B**), which was 16 atomic mass units less than cycloalliin, representing the elimination of oxygen. The structure of the metabolite derived from cycloalliin, **2**, is

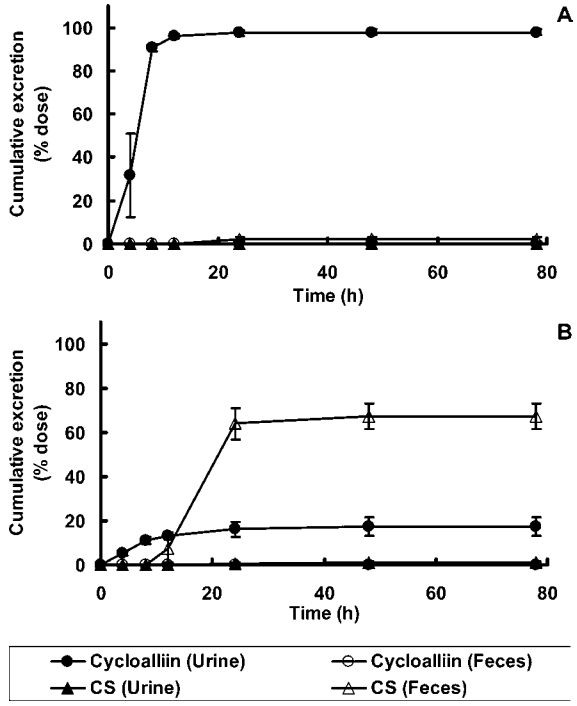

Figure 5. Cumulative excretion of cycloalliin and its metabolite (CS, value corrected for cycloalliin equivalents) in urine and feces following (**A**) intravenous and (**B**) oral administration of 50 mg/kg cycloalliin to rats. Each value represents the mean ± SE of five animals. CS, (3*R*,5*S*)-5-methyl-1,4-thiazane-3-carboxylic acid.

Table 2. Organ Distribution after Oral Administration of 50 mg/kg Cycloalliin to Rats[a]

	cycloalliin concn (μg g^{-1} or μg mL^{-1})		
	40 min	4 h	24 h
heart	0.64 ± 0.05	<LOD[b]	<LOD
lung	0.97 ± 0.15	0.15 ± 0.03	<LOD
kidney	11.81 ± 1.99	2.09 ± 0.43	<LOD
spleen	0.57 ± 0.07	0.16 ± 0.02	<LOD
liver	0.90 ± 0.12	0.12 ± 0.02	<LOD
plasma	2.24 ± 0.20	0.23 ± 0.04	<LOD

[a] Each value represents the mean ± SE of four rats. [b] Limits of detection (LOD) were 0.1 μg/g for organ and 0.1 μg/mL for plasma.

shown in **Figure 1**. No other metabolite could be detected in plasma, urine, and feces after oral administration of cycloalliin.

(3*R*,5*S*)-5-Methyl-1,4-thiazane-3-carboxylic Acid (2) in Biological Samples after Cycloalliin Administration. In all animal experiments, **2** in plasma was not detectable after cycloalliin had been administered intravenously and orally (<0.1 μg/mL). After intravenous administration at 50 mg/kg, **2** was excreted into feces at 2.0% ± 0.9% and 0.1% ± 0.1% of the dosage (value corrected for cycloalliin equivalents) at 12−24 h and 24−48 h, respectively, but not into urine (**Figure 5A**). After oral administration of cycloalliin at 50 mg/kg, **2** was detected in the feces after 8 h, and 67.3% ± 5.9% of the dosage (value corrected for cycloalliin equivalents) was excreted into feces within 48 h after administration, while the cumulative amount of **2** excreted into urine by 48 h was only 1.0% ± 0.3% of the dosage (value corrected for cycloalliin equivalents) (**Figure 5B**). In addition, **2** was not detected in organs after oral administration of cycloalliin at 50 mg/kg (<0.1 μg/g).

Changes of Cycloalliin in Cecal Contents. In order to assess the metabolism of cycloalliin in the gastrointestinal tract, cycloalliin was incubated in suspensions of rat cecal content at

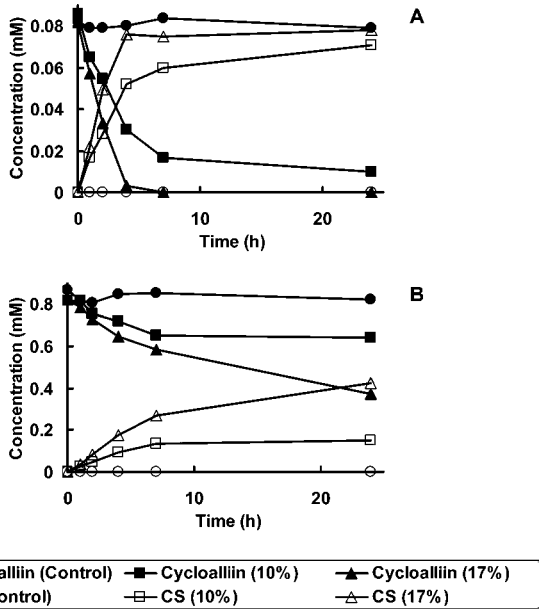

Figure 6. In vitro metabolism of cycloalliin at (**A**) 0.08 mM and (**B**) 0.8 mM by rat cecal contents. Cycloalliin was incubated anaerobically at 37 °C with 10% and 17% (w/v) rat cecal content suspensions and without cecal content suspensions (control). CS, (3*R*,5*S*)-5-methyl-1,4-thiazane-3-carboxylic acid.

37 °C under anaerobic conditions. Decreases in cycloalliin and increases in **2** were observed, as shown in **Figure 6**. When 0.08 mM cycloalliin was incubated with 10% (w/v) cecal content suspension, this compound was reduced by 81% (from 0.086 to 0.016 mM) in 7 h, and finally declined by 88% (from 0.086 to 0.010 mM) after 24 h (**Figure 6A**). On the other hand, cycloalliin disappeared completely within 7 h upon incubation with 17% (w/v) cecal content suspension. The amounts of increased **2** almost coincided with that of decreased cycloalliin at cecal content suspensions of both 10% and 17% (w/v). At a higher concentration (0.8 mM), cycloalliin decreased and **2** simultaneously increased in both 10% and 17% (w/v) cecal content suspensions, being particularly marked in the latter case (**Figure 6B**). Cycloalliin at 0.8 mM diminished during the first 7 h with the 10% (w/v) cecal content suspension and reached a plateau at about 0.65 mM. On the other hand, cycloalliin in the 17% (w/v) cecal content suspension decreased continuously and finally declined by 54% (from 0.82 to 0.37 mM) in 24 h. However, no changes in cycloalliin were observed at 24 h when this compound was incubated with 10% (w/v) cecal content suspension heated at 100 °C for 5 min (data not shown).

DISCUSSION

Cycloalliin is a cyclic organosulfur compound present in garlic and onion. This compound has been reported to show some biological properties, including fibrinolytic (*8*), lipid-lowering (*9*), and quinone reductase activities (*10*), and to be stable during the storage and processing of garlic and onion (*3*−*5*). These findings suggest that cycloalliin could be useful as a chemical marker in garlic, onion, and their preparations. However, its chemical stability and pharmacokinetics in vivo are not well-known. In this study, we assessed the stability, oral bioavailability, and pharmacokinetics of cycloalliin using LC-MS, which is an excellent tool for determining minor constituents in complex mixtures. Before we examined the stability, oral bioavailability, pharmacokinetics, and metabolism of cycloalliin, we established a method for determining cycloalliin

and **2** in biological fluids, plasma, urine, feces, heart, lung, kidney, spleen, and liver. Our validation data demonstrated that the established method was quite suitable for their quantitative analysis of biological samples. By using this system, the stability of cycloalliin in acidic and basic solutions and biological fluids (whole blood, plasma, and liver homogenate) was examined initially. Our data indicated that cycloalliin was stable in all of these solutions and biological fluids, suggesting that chemical degradation, binding to plasma proteins, and liver metabolism of cycloalliin are negligible. Furthermore, as no conversion of cycloalliin to the sulfone and/or sulfide by liver enzymes was evident, as reported previously (*14*−*16*), we consider that cycloalliin itself, not its metabolite, is responsible for its biological activities.

As a next step, we studied the pharmacokinetics of cycloalliin in rats using the same LC-MS system. After intravenous administration, cycloalliin was rapidly eliminated from blood and excreted mostly into urine (CL_r, 0.77 L h^{-1} kg^{-1}; CL_{nr}, 0.03 L h^{-1} kg^{-1}), indicating that renal excretion is the major elimination route of this compound. Also, at least 67.3% of cycloalliin was not absorbed after oral administration, as demonstrated by the amounts of **2** excreted into feces. These data indicated that cycloalliin had poor bioavailability (<10%) due to its low absorption in the intestine and/or a first-pass effect. It is known that both absorptive and secretory transporters in the intestine influence the oral absorption of drugs (*17*−*22*). The low absorption of cycloalliin in the intestine could be ascribed to the membrane transport systems of the intestinal mucosa. Also, the nonlinear increases in the C_{max} and AUC_{0-inf} values may be due to saturation of intestinal efflux transporters such as P-glycoprotein. Tamai et al. (*17*) reported that the absorption of azasetron was increased at higher dosages due to saturation of the efflux transport system. Therefore, the intestinal efflux transporters may play an important role in the intestinal absorption of cycloalliin.

Previous studies have reported the pharmacokinetics of organosulfur compounds in garlic and its preparations, such as *S*-allylcysteine (*23*), vinyldithiins (*24*), allicin (*25*), and alliin (*26*). The AUC_{0-inf} of intravenously administered cycloalliin was about 50% lower than that of *S*-allylcysteine (*23*), which includes the prevention of cardiovascular diseases (*27*−*29*) and cancer (*30*, *31*), and antioxidant properties (*32*−*34*). This difference might be considered due to the low reabsorption of cycloalliin in the kidney compared to that of *S*-allylcysteine, which disappear from blood in a biexponential manner after intravenous administration (*23*). On the other hand, with oral administration, the AUC_{0-inf} of cycloalliin was slightly larger than that of 2-vinyl-4*H*-1,3-dithiin (0.4 μg·h mL^{-1} at a dosage of 27 mg/kg), which is a cyclic sulfur compound (*24*). Although ^{35}S-labeled allicin is at least 65% absorbed within 72 h (*35*), it is rapidly metabolized to other compounds, such as allyl mercaptan, allyl methyl sulfide, and acetone (*25*). In contrast to allicin, the absorbed cycloalliin is stable. The low bioavailability of cycloalliin is similar to that of alliin, the sulfoxide-containing compound present in garlic (*26*). These data suggest that the cysteine sulfoxide derivatives may be poorly absorbed from the gastrointestinal tract due to their low intestinal absorption properties and/or first-pass effect.

It is reported that compounds containing a sulfoxide group are reduced to thioether analogues in animals (*36*−*41*). The reduction occurs in tissues (*36*−*38*) and/or the intestinal flora (*39*−*41*). In the former case, reduction to the thioether is catalyzed by methionine sulfoxide reductase (*36*), sulindac reductase (*37*), and dimethyl sulfoxide reductase (*38*) in liver and kidney. In the latter case, reduction is carried out by intestinal flora, such as *Escherichia coli* (*39*), and their related enzymes, such as dimethyl sulfoxide reductase (*42*, *43*) and biotin sulfoxide reductase (*44*). In the present study, the reduced metabolite (**2**) of cycloalliin was observed in feces after oral administration to rats, but the **2** concentrations in plasma and urine were extremely low. Furthermore, in vitro experiments with rat cecal contents indicated that cycloalliin was reduced to **2** by the intestinal flora. These data suggest that cycloalliin is reduced in the gastrointestinal tract but not in the liver and kidney. It has been reported that the sulfoxide reducing activity present in the contents of the cecum and colon is higher than that in other parts of the intestine (stomach, duodenum, jejunum, and ileum) in rats (*40*) and that the reduction of sulfoxides occurs under both aerobic and anaerobic conditions (*39*). Therefore, we speculate that cycloalliin might be reduced to **2** in the lower gastrointestinal tract by the intestinal flora containing obligate and facultative anaerobes. Although the reduction of cycloalliin to **2** depended on the concentration of rat cecal contents in the suspensions tested, the reducing activity reached a plateau at higher concentrations of cycloalliin. These data suggest that saturation of the reducing activity might occur at higher dosages of sulfoxide compounds. Therefore, the intestinal absorption of cycloalliin could be enhanced by intake of garlic, onion, and their preparations, which contain abundant sulfoxide compounds, because the reduction of cycloalliin is inhibited by saturation of the reducing activity in the gastrointestinal tract. Although large amounts of **2** were observed in feces, it was not detected in plasma, suggesting that absorption from the gastrointestinal tract is markedly low.

In spite of its low bioavailability (<10%), cycloalliin has been shown to be effective in in vivo studies (*8*, *9*). When rats were fed a cholesterol-enriched atherogenetic diet with or without cycloalliin for 14 days, the cycloalliin groups showed significantly reduced serum triacylglycerol levels (*9*). The dosages used in our study were similar to normal daily levels of dietary cycloalliin, corresponding to approximately 20 and 60 mg/day. On the other hand, cycloalliin has been shown to significantly increase blood fibrinolytic activity at 250 mg per subject in a human trial (*8*). This amount cannot be easily acquired by eating garlic and onion, in which the cycloalliin content of the bulbs ranges from 0.5 to 1.5 mg/g (*1*) and from 0.1 to 0.3 mg/g (*4*), respectively. Together with data on the biological benefits of cycloalliin, our results suggest that its bioavailability needs to be improved by modifying its chemical structure. However, due to its chemical stability and also stability during storage or processing of garlic and onion, as reported previously (*3*−*5*), cycloalliin could be more useful as a chemical marker for quality control of garlic, onion, and their products.

In conclusion, the present study has investigated the chemical stability and pharmacokinetics of cycloalliin, which is considered to be one of the biologically active compounds present in garlic, onion, and their preparations. After oral administration, cycloalliin appeared rapidly in plasma and was distributed to the tissues of the heart, lung, kidney, liver, and spleen. The low bioavailability of cycloalliin in rats was due mainly to reduction to **2** by the intestinal flora and also poor absorption in the upper gastrointestinal tract. These findings are helpful for understanding the biological effects of cycloalliin.

Supporting Information Available: Method validation results; linearity, accuracy, and precision of the method for quantitative determination of cycloalliin and **2** in biological samples; and scheme of (1*R*,3*R*,5*S*,6*R*)-5,6-dimethyl-1,4-thia-

zane-3-carboxylic acid 1-oxide synthesis. This material is available free of charge via the Internet at http://pubs.acs.org.

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