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Metabolism, Excretion and Pharmacokinetics of S-Allyl-L-cysteine in Rats and Dogs

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**A List of Nonstandard Abbreviations**

AGE, aged garlic extract; AUC, area under the plasma concentration versus time curve; BA, bioavailability; CL, plasma clearance; CL_inh, intrinsic clearance; CL_r, renal clearance; GFR, glomerular filtration rate; GSAC, L-γ-glutamyl-S-allyl-L-cysteine; LC-MS/MS, liquid chromatography tandem mass spectrometry; NAc-SAC, N-acetyl-S-allyl-L-cysteine; NAc-SACS, N-acetyl-S-allyl-L-cysteine sulfoxide; SAC, S-allyl-L-cysteine; SACS, S-allyl-L-cysteine sulfoxide; SBC, S-1-butenyl-L-cysteine.
Abstract

The metabolism, excretion and pharmacokinetics of S-allyl-L-cysteine (SAC), an active key component of garlic supplements, were examined in rats and dogs. A single dose of SAC was administered orally or intravenously to rats (5 mg/kg) and dogs (2 mg/kg). SAC was well absorbed (bioavailabilities of > 90%) and its four metabolites, N-acetyl-S-allyl-L-cysteine (NAc-SAC), N-acetyl-S-allyl-L-cysteine sulfoxide (NAc-SACS), S-allyl-L-cysteine sulfoxide (SACS) and L-γ-glutamyl-S-allyl-L-cysteine, were identified in the plasma and/or urine. Renal clearance values (< 0.01 l/h/kg) of SAC indicated its extensive renal reabsorption, which contributed to the long elimination half-life of SAC, especially in dogs (12 h). The metabolism of SAC to NAc-SAC, principal metabolite of SAC, was studied in vitro and in vivo. Liver and kidney S9 fractions of rat and dog catalyzed both N-acetylation of SAC and deacetylation of NAc-SAC. Following the intravenous administration of NAc-SAC, SAC appeared in the plasma and its concentration declined in parallel with that of NAc-SAC. These results suggested that the rate and extent of the formation of NAc-SAC was determined by the N-acetylation and deacetylation activities of liver and kidney. Also, NAc-SACS was detected in the plasma following the intravenous administration of either NAc-SAC or SACS, suggesting that NAc-SACS could be formed via both N-acetylation of SACS and S-oxidation of NAc-SAC. In conclusion, this study demonstrated that pharmacokinetics of SAC in rats and dogs was characterized by its high oral
bioavailability, N-acetylation and S-oxidation metabolisms, and extensive renal reabsorption, indicating the critical roles of liver and kidney in the elimination of SAC.
Introduction

Garlic (*Allium sativum*) has been widely recognized as a health-promoting and disease-preventing food, and used traditionally as a complementary medicine in the treatment of several diseases (Rivlin, 2001). For this reason, different types of garlic preparations have been developed and are commercially available, including garlic powders, garlic oils and aged garlic extract (AGE). A number of clinical trials have demonstrated the beneficial pharmacological effects of AGE (Steiner et al., 1996; Budoff et al., 2004; Budoff et al., 2009; Ried et al., 2010; Nantz et al., 2012). AGE is a unique garlic product that is manufactured from garlic clove through a long extraction process for more than 10 months. This extraction process can eliminate or modify the odorous, harsh and irritating compounds included in fresh garlic and leads to the enrichment of water-soluble sulfur-containing compounds (Amagase et al., 2001). Indeed, AGE contains a variety of water-soluble organosulfur compounds such as *S*-allyl-L-cysteine (SAC), *S*-1-propenyl-L-cysteine and *S*-allylmercapto-L-cysteine. Among them, SAC has been recognized as an active key component of AGE (Amagase et al., 2001). The biological and pharmacological activities of SAC have been reported including anti-oxidation effect (Imai et al., 1994), anti-cancer effect (Chu et al., 2007), and cardio- and renal-protective effects (Chuah et al, 2007; Cruz et al, 2007).

The pharmacological activities of AGE are dependent on the pharmacokinetic properties of its
active ingredients such as SAC. To date, the pharmacokinetics of SAC has been studied in a few
animal studies. SAC was well absorbed in mice, rats and dogs with the high oral bioavailabilities
(87-103%) (Nagae et al., 1994; Chang-Kai and Fan-Dian, 2005). Excretion study of SAC has
identified its three metabolites, N-acetyl-S-allyl-L-cysteine (NAc-SAC), N-acetyl-S-allyl-L-cysteine
sulfoxide (NAc-SACS) and S-allyl-L-cysteine sulfoxide (SACS), in the urine of rats (Krause et al.,
2002). Although previous studies have reported several findings on the pharmacokinetics of SAC,
the overall pharmacokinetic profile of SAC has not been fully understood. In particular, its
metabolism including the entire metabolic pathway, presence of other metabolite(s) and primary
organ(s) responsible for the metabolism has not been clarified.

The primary objective of present study was to investigate the metabolism of SAC with the
clarification of its metabolic pathway. For this purpose, we conducted the excretion and
pharmacokinetic studies of SAC in rats and dogs. In vitro metabolism studies using subcellular
fractions of liver and kidney were also carried out to examine the N-acetylation metabolism of
SAC and SACS to NAc-SAC and NAc-SACS, respectively. The results of current study would be
useful to design and conduct human pharmacokinetic study of SAC in future.
Materials and Methods

General Chemicals. Formic acid of LC-MS grade was purchased from Wako Pure Chemical Industries (Osaka, Japan). Acetonitrile of LC-MS grade was purchased from Kanto Chemical (Tokyo, Japan). Acetyl-CoA and pentadecafluorooctanoic acid of LC-MS grade were purchased from Tokyo Chemical Industry (Tokyo, Japan). Liver and kidney S9 fractions of rat, dog and human were purchased from XeoTech (Lenexa, KS).

Chemicals. SAC was purchased from Tokyo Chemical Industry. The authentic standards of NAc-SAC, NAc-SACS (mixture of two diastereomers), (+)-SACS and L-γ-glutamyl-S-allyl-L-cysteine (GSAC), and the internal standard of S-1-buteny-L-cysteine (SBC) were synthesized in the Drug Discovery Laboratory of Wakunaga Pharmaceutical (Hiroshima, Japan).

Animals and Dosing Solutions. The animal studies were performed according to the protocols approved by the Wakunaga Pharmaceutical Company Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (230-280 g, Charles Liver Japan, Yokohama, Japan) and male beagle dogs (10-14 kg, Kitayama Labes, Nagano, Japan) were fasted overnight before the studies. All compounds of NAc-SAC, NAc-SACS, SAC and SACS were dissolved in distilled water for oral administration and in saline for i.v. injection.

Excretion Study in Rats. Male rats were administered a single i.v. or oral dose (5 mg/kg) of
SAC, NAc-SAC, NAc-SACS and SACS. Urine samples were collected from rats individually housed in a metabolic cage for up to 24 h after the dosing. Bile samples were collected from bile duct-cannulated rats individually kept in a Bollmann cage for 24 h after the dosing. The urine and bile samples were stored at -30°C until analysis. The extent of urinary and biliary excretion (% of dose) was calculated on a molar basis by dividing the amount of analytes excreted in biological fluids by the amount of compound administered.

**Pharmacokinetic Studies in Rats and Dogs.** Male rats and dogs were administered a single i.v. or oral dose (5 mg/kg in rats and 2 mg/kg in dogs) of SAC, NAc-SAC, NAc-SACS and SACS. In rat study, blood samples were collected through an indwelling catheter inserted in the left femoral vein at 0.083, 0.25, 0.5, 1, 2, 3, 4 and 6 h after the dosing. In dog study, blood samples were collected by venipuncture of the cephalic vein at 0.083, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 36 and 48 h after the dosing. Urine of dogs was also collected for 0 to 24 and 24 to 48 h after the dosing. The blood sampling times in the rat and dog studies were determined based on t_{1/2} values of SAC reported (Nagae et al., 1994). Blood samples were rapidly cooled on ice and centrifuged at 1000g for 10 min at 4°C to obtain the plasma. The plasma and urine samples were stored at -30°C until analysis.

**Quantitative Analysis of Plasma, Urine and Bile.** An aliquot (80-100 μl) of plasma, urine and bile samples was mixed with 1 ml of methanol and spiked with SBC (internal standard). After the
centrifugation, a portion of the supernatants was evaporated to dryness. The residues were
dissolved in 0.3 ml of solvent A (0.1% pentadecafluorooctanoic acid, 99.5% water/0.5%
acetonitrile with 0.1% formic acid), and analyzed with liquid chromatography tandem mass
spectrometry (LC-MS/MS).

**LC-MS/MS Analysis.** LC-MS/MS analysis was performed using a Waters LC-MS/MS system
(Waters, Milford, MA). Chromatographic separation was achieved, on an ACQUITY UPLC C18
BEH column (2.1 × 50 mm, 1.7 μm, Waters) kept at 45°C, using a linear gradient at 0.8 ml/min
with solvents of A and B (0.1% pentadecafluorooctanoic acid, 10% water/90% acetonitrile with
0.1% formic acid). The gradient was started at 99.9% A, and solvent B was linearly increased
from 0.1 to 2% (0-0.5 min), 2 to 20% (0.5-2.0 min), 20 to 40% (2.0-4.0 min), 40 to 99.9% (4.0-4.5
min) and maintained at 0.1% (4.5-10 min). The mass spectrometer was operated in the positive
ion mode with electrospray ionization. The ionization source parameters were capillary voltage
0.5 kV, source temperature 150°C and desolvation gas temperature 400°C, with desolvation gas
and cone gas flow rates of 1000 and 50 l/h, respectively. The MS/MS transitions (m/z) monitored
were used for analysis: m/z 162.1 > 145.1 for SAC, 204.1 > 162.1 for NAc-SAC, 220.1 > 130.1
for NAc-SACs, 178.1 > 88.1 for SACS, 291.1 > 162.1 for GSAC and 176.1 > 159.1 for SBC at
cone and collision energies of 15 and 10 V, respectively.

**Pharmacokinetic Analysis.** Pharmacokinetic parameters were determined by
non-compartmental method using a WinNonlin software (ver. 2.1, Pharsight, Mountain View, CA).

Maximum plasma concentration and time to reach the maximum concentration were determined directly from the experimental data. The area under the plasma concentration versus time curve (AUC) was calculated by the trapezoidal rule. Bioavailability (BA) was calculated by the ratio of AUC values following oral and i.v. administration of SAC.

**In Vitro Drug Metabolism Study.** Drug metabolism study was carried out using liver and kidney S9 fractions of rat (male), dog (male), and human (mix of male and female). In N-acetylation metabolism studies of SAC and SACS, the reaction mixtures (100 µl) containing 0.1 M phosphate buffer (pH7.4), 5 µM SAC or SACS, 2 mM Acetyl-CoA and each S9 fraction (0.25-1 mg protein/ml) were incubated for 10 to 240 min at 37°C. In deacetylation metabolism studies of NAc-SAC and NAc-SACS, the reaction mixtures (100 µl) containing 0.1 M phosphate buffer (pH7.4), 5 µM NAc-SAC or NAc-SACS, and each S9 fraction (0.1-1 mg protein/ml) were incubated for 10 to 120 min at 37°C. All reaction mixtures were added with 1 ml of methanol to terminate the reactions and spiked with SBC. After the centrifugation, a portion of the supernatants was evaporated to dryness. The residues were dissolved in solvent A and analyzed with LC-MS/MS.

**Determination of Intrinsic Clearance (CL_{int}).** Intrinsic clearance for deacetylation (CL_{int, deacetylation}) of NAc-SAC and NAc-SACS in each S9 fraction was determined by *in vitro* half-life
method (Obach, 1999). The intrinsic clearance for $N$-acetylation ($\text{CL}_{\text{int, acetylation}}$) of SAC and SACS was calculated using the following formula by nonlinear curve regression fitting (Kyplot ver. 5.0, Keyence, Osaka, Japan): $C_t = \frac{A}{a} \times (1 - \exp^{-at})$, $a = P \times (\text{CL}_{\text{int, acetylation}} + \text{CL}_{\text{int, deacetylation}})$, $\frac{A}{a} = S \times \frac{\text{CL}_{\text{int, acetylation}}}{(\text{CL}_{\text{int, acetylation}} + \text{CL}_{\text{int, deacetylation}})}$, where $P$, $S$ and $C_t$ are the protein concentration, initial concentration of SAC and SACS, and the concentration of their $N$-acetylated metabolites at the incubation time, $t$, respectively.
Results

Metabolism, Excretion and Pharmacokinetics in Rats. Following oral or i.v. administration of SAC (5 mg/kg) to rats, the amount of SAC and its metabolites excreted in urine and bile was quantified and expressed as percentage of the dose given (Table 1 and 2). SAC was little excreted in both urine and bile in the unchanged form. Almost the entire SAC dose administered was excreted as its N-acetylated metabolites, NAc-SAC and NAc-SACS, in the urine; the urinary excretion of SAC, NAc-SAC, SACS and NAc-SACS was 0.95 (i.v.) and 1.6% (oral), 84 and 83%, 0.01 and 0.01%, and 11 and 11% of the SAC dose, respectively. The total recovery of SAC and its three metabolites in the urine was 96 and 96% of the oral and i.v. dose, respectively.

The pharmacokinetic profile of SAC in rats (5 mg/kg, oral and i.v.) is summarized in Table 3. SAC was well absorbed with the BA of 92.1%. The pharmacokinetic parameters of $t_{1/2}$, plasma clearance (CL) and volume of distribution were 1.1-1.2 h, 0.91 l/h/kg and 1.0 l/kg, respectively. The renal clearance (CLr, 0.0086 l/h/kg) of SAC was much smaller than glomerular filtration rate (GFR, approximately 0.3 l/h/kg) of rat reported (Davies and Morris, 1993), indicating that SAC undergoes extensive renal reabsorption. The mean plasma concentration-time curves of SAC are shown in Figs. 1A (oral) and 1B (i.v.). Following the administration of SAC, its three urinary metabolites, NAc-SAC, SACS and NAc-SACS, were detected in the plasma. LC-MS/MS analysis also identified GSAC as a novel metabolite of SAC. The plasma concentration of SAC was
considerably higher than those of the metabolites at all time-points analyzed.

The metabolism, excretion and pharmacokinetics of NAc-SAC, SACS and NAc-SACS (5 mg/kg, i.v.) were also studied. A large portion of NAc-SAC administered was excreted in the urine as the unchanged form; the urinary excretion of NAc-SAC, SAC, SACS and NAc-SACS was 84, 1.8, 0.02 and 12% of the NAc-SAC dose, respectively (Table 1). Almost the entire SACS dose administered was excreted into the urine as NAc-SACS; the urinary excretion of SACS and NAc-SACS was 0.10 and 85% of the SACS dose, respectively. Almost the entire NAc-SACS dose administered was recovered in the urine as the unchanged form (96% of the dose).

The pharmacokinetic parameters of NAc-SAC, SACS and NAc-SACS (5 mg/kg, i.v.) are summarized in Table 3. The CLr values of NAc-SAC (1.5 l/h/kg) and NAc-SACS (1.2 l/h/kg) were higher than GFR of rat (Davies and Morris, 1993), suggesting that the N-acetylated metabolites of SAC were eliminated by active renal secretion. Similar to SAC, SACS had extremely lower CLr value (0.0014 l/h/kg). The mean plasma concentration-time curves of NAc-SAC and SACS (5 mg/kg, i.v.) are shown in Figs. 1C and 1D, respectively. Following the administration of NAc-SAC, SAC, NAc-SACS and GSAC were detected in the plasma. The plasma concentration of SAC exceeded that of NAc-SAC in 30 min or later, and declined in parallel with that of NAc-SAC (Fig. 1C). Following the administration of SACS, its plasma level rapidly decreased accompanying the appearance of NAc-SACS (Fig. 1D).
Metabolism, Excretion and Pharmacokinetics in Dogs. The urinary excretion and pharmacokinetics of SAC in dogs (2 mg/kg, oral and i.v.) are summarized in Table 4 and 5, respectively. SAC was also well absorbed in dogs with the BA of 92.0%. Different from in rats, NAc-SAC was little excreted in the urine of dogs; the urinary excretion of SAC, NAc-SAC, SACS and NAc-SACS was 0.32 (i.v.) and 0.34% (oral), 0.89 and 0.91%, 1.6 and 1.7%, and 12 and 10% of the SAC dose, respectively. The CL value (0.048 l/h/kg) of SAC in dogs was significantly smaller than that (0.91 l/h/kg) in rats, which contributed to the long t1/2 value (12 h) in dogs. As observed in rats, the CLr value (0.0002 l/h/kg) of SAC was extremely lower than the GFR of dog (Davies and Morris, 1993), indicating extensive renal reabsorption of SAC. All metabolites of SAC identified in the rat plasma, NAc-SAC, SACS, NAc-SACS and GSAC, were also detected in the dog plasma following the oral and i.v. administration of SAC (Figs. 2A and 2B).

The urinary excretion and pharmacokinetics of NAc-SAC, SACS and NAc-SACS (2 mg/kg, i.v.) were also studied. In addition to NAc-SAC, all of SAC, SACS, NAc-SACS and GSAC were detected in the plasma of dogs given NAc-SAC (Fig 2C) with the urinary excretion of SAC, NAc-SAC, SACS and NAc-SACS being 0.49, 1.8, 1.8 and 12% of the NAc-SAC dose, respectively (Table 4). The small CLr value (0.0019 l/kg/h) of NAc-SAC suggested that it apparently underwent renal reabsorption (Table 5). Following the administration of SACS, NAc-SACS was detected in the plasma and excreted in the urine (Table 4 and Fig. 2D); the
urinary excretion of SACS and NAc-SACS was 5.6 and 22% of the SACS dose, respectively. Different from in rats, SACS was detected in the plasma of dogs given NAc-SACS (Fig. 2E) with the urinary excretion of NAc-SACS and SACS being 82 and 2.5% of the NAC-SACS dose, respectively (Table 4).

**In Vitro Drug Metabolism.** In vitro metabolism study was carried out using liver and kidney S9 fractions of rat, dog and human. The $\text{CL}_{\text{int}}$ values for SAC $N$-acetylation and NAc-SAC deacetylation in each S9 fraction were determined and compared (Table 6). The $\text{CL}_{\text{int}}$ values for SAC $N$-acetylation were 5 to 7 times higher than those for NAc-SAC deacetylation in rat liver and kidney S9 fractions. The $\text{CL}_{\text{int}}$ values for SAC $N$-acetylation and NAc-SAC deacetylation were similar in dog liver S9 fraction; however, the $\text{CL}_{\text{int}}$ value (460 μl/min/mg protein) for NAc-SAC deacetylation was approximately 80 times higher than that (5.9 μl/min/mg protein) for SAC $N$-acetylation in dog kidney S9 fraction. In human liver and kidney S9 fractions, the $\text{CL}_{\text{int}}$ values for NAc-SAC deacetylation were more than 10 times higher than those for SAC $N$-acetylation.

The $\text{CL}_{\text{int}}$ values for SACS $N$-acetylation and NAc-SACS deacetylation were also determined (Table 6). In liver and kidney S9 fractions of rat, dog and human, the $\text{CL}_{\text{int}}$ values for SACS $N$-acetylation were one or two magnitude lower than those for SAC $N$-acetylation, while all S9 fractions had little activity for NAc-SACS deacetylation except for dog kidney S9 fraction.
Discussion

To date, there is little study examining the metabolism of SAC in animals and humans. Nagae et al. have reported that SAC was little excreted in the unchanged form in rats and also little excreted in the urine of dogs (Nagae et al., 1994). Krause et al. have identified three metabolites of SAC, NAc-SAC, SACS and NAc-SACS, in the urine of rats given SAC (Krause et al., 2002). In that study, the total recovery of SAC and its three metabolites in the urine was 40-50% of the SAC dose; however, the residual 50-60% was uncertain. In the present study, we aimed to clarify the metabolism of SAC in rats and dogs by conducting excretion and pharmacokinetic studies in vivo and metabolism study in vitro.

At first, as reported previously (Nagae et al., 1994; Chang-Kai and Fan-Dian, 2005), our studies confirmed the high oral absorption of SAC in rats and dogs with its BA values of 92.1 and 92.0%, respectively.

In rats, following the administration of SAC (5 mg/kg, oral and i.v.), SAC was little excreted in the unchanged form but as its N-acetylated metabolites, NAc-SAC and NAc-SACS. The urinary excretion of SAC, NAc-SAC, SACS and NAc-SACS was 0.95, 84, 0.01 and 11% of the i.v. dose of SAC, respectively (Table 1). We also identified GSAC in the plasma as a novel metabolite of SAC in addition to its three urinary metabolites, NAc-SAC, SACS and NAc-SACS (Figs 1A and 1B). The plasma concentration of SAC was considerably higher than those of NAc-SAC and
NAc-SACS despite the fact that SAC was excreted as its N-acetylated metabolites. It is well established that amino acids are actively reabsorbed from the urine via several types of transporter in kidney (Silbernagl et al., 1975; Bröer, 2008). The CLr values of SAC (0.0086 l/h/kg) and its N-acetylated metabolites (>1.2 l/h/kg) indicated that SAC undergoes extensive renal reabsorption, whereas NAc-SAC and NAc-SACS are eliminated by active renal secretion (Table 3). These results clearly explained why SAC was eliminated as its N-acetylated metabolites; SAC was ultimately metabolized to NAc-SAC and NAc-SACS, both of which were actively excreted in the urine, through the cycle of its urinary excretion, renal reabsorption and systemic recirculation. The majority of the administered amount of NAc-SACS (5 mg/kg, i.v.) was recovered in urine as the unchanged from (96% of the dose) indicating that this metabolite does not undergo further metabolism in rats (Table 1).

Following the administration of SAC (2 mg/kg, oral and i.v.) to dogs, all metabolites of SAC identified in rats were detected in the plasma and/or urine (Table 4, Figs. 2A and 2B). In fact, NAc-SAC was the major plasma metabolite of SAC even in dogs. The very small CLr value of SAC (0.0002 l/h/kg) indicated its extensive renal reabsorption in dogs as in rats. However, in contrast to that in rats, NAc-SAC was little excreted in the urine of dogs; the urinary excretion of SAC, NAc-SAC, SACS and NAc-SACS was 0.32, 0.89, 1.6 and 12%, respectively, of the i.v. dose of SAC (Table 4). In addition, the pharmacokinetic study of NAc-SAC (2 mg/kg, i.v.)
demonstrated the small CLr value (0.0019 l/h/kg) of NAc-SAC, suggesting its apparent renal reabsorption (Table 5). As described above, the metabolism, excretion and pharmacokinetics of SAC were similar between in rats and dogs except for the large difference in the urinary excretion of its principal plasma metabolite, NAc-SAC.

Of four metabolites of SAC, NAc-SAC and SACS are produced via N-acetylation and S-oxidation metabolism of SAC, respectively. The formation of NAc-SACS could occur via two pathways, S-oxidation of NAc-SAC and N-acetylation of SACS. We detected NAc-SACS in the plasma of rats and dogs following the i.v. administration of either NAc-SAC or SACS (Figs. 1C, 1D, 2C and 2D). These results suggested that both of S-oxidation of NAc-SAC and N-acetylation of SACS could be operative for the formation of NAc-SACS in rats and dogs.

Our excretion and pharmacokinetic studies in rats and dogs demonstrated that NAc-SAC was also metabolized back to SAC. Following the i.v. administration of NAc-SAC, SAC rapidly appeared in the plasma and its plasma concentration declined in parallel with that of NAc-SAC (Figs. 1C and 2C). These results suggested that the formation of NAc-SAC from SAC was dependent on the metabolic activities of organs responsible for SAC N-acetylation and NAc-SAC deacetylation. SAC is one of cysteine S-conjugates that are formed in a series of drug metabolism of glutathione S-conjugates and furthermore undergo N-acetylation metabolism to form mercapturic acids in the liver and kidney of mammalian species (Dekant et al., 1988;
Hinchman and Ballatori, 1994). Based on these works, we carried out metabolism study *in vitro* to examine both metabolism of SAC N-acetylation and NAc-SAC deacetylation using liver and kidney S9 fractions of rat, dog and human. In our preliminary experiments, it was observed that the enzymatic activities of the S9 fractions for SAC N-acetylation and NAc-SAC deacetylation were present in different subcellular fractions, microsomes and cytosol, respectively (data not shown). Therefore, we used the S9 fractions for the direct comparison of both metabolisms. Our study demonstrated that liver and kidney S9 fractions of rat, dog and human had both activities for SAC N-acetylation and NAc-SAC deacetylation although a large species difference was observed (Table 6). In rat liver and kidney S9 fractions, the CL_int values for SAC N-acetylation were 5-7 times higher than those for NAc-SAC deacetylation, suggesting that NAc-SAC could be formed in liver and kidney of rats. On the other hand, both S9 fractions had little activity for NAc-SACS deacetylation. This result was consistent with our finding *in vivo* that SACS was not detected in the plasma following the administration of NAc-SACS to rats (Table 3). In dog study, it was of special note that the kidney S9 fraction had extremely high activity for NAc-SAC deacetylation; the CL_int value (460 µl/min/mg protein) for NAc-SAC deacetylation was approximately 80 fold higher than that for SAC N-acetylation. The metabolism of SAC to NAc-SAC seems to be the primary elimination pathway of SAC in rats and dogs, judging from the AUC ratios (1/2-1/3) of NAc-SAC to SAC. However, non-renal CL value (0.048 l/h/kg) of SAC in
dogs was significantly smaller than that in rats (0.90 l/h/kg). Together with little urinary excretion of NAc-SAC in dogs, these results suggested that NAc-SAC was converted to SAC before its urinary excretion by the strong renal activity for NAc-SAC deacetylation and excreted in the urine as SAC. As a result, it was estimated that almost all of SAC administered circulated repeatedly as the unchanged form through its renal reabsorption and ultimately excreted in bile. This view could explain why SAC had the long t\textsubscript{1/2} value in dogs, but the biliary excretion of SAC should be confirmed in future study. Among all S9 fractions tested, only dog kidney S9 fraction had the activity for NAc-SAC deacetylation, which was consistent with the \textit{in vivo} result that SACS was detected in the plasma of dogs given NAc-SACS (Fig 2E). In human S9 fractions, the Cl\textsubscript{int} values for NAc-SAC deacetylation were more than 10 times higher than those for SAC N-acetylation, suggesting a short residence time for NAc-SAC in human.

In this study, metabolic pathway of SAC was investigated \textit{in vitro} and \textit{in vivo}. S-oxidation metabolism of SAC \textit{in vitro} by flavin-containing monooxygenases of mammalian species has been reported (Ripp et al., 1999; Krause et al., 2003). Our \textit{in vitro} studies demonstrated that N-acetylation of SAC and SACS was catalyzed by liver and kidney S9 fractions of rat and dog. The pharmacokinetic studies of SACS and NAc-SAC suggested that NAc-SACS was formed via both N-acetylation of SACS and S-oxidation of NAc-SAC \textit{in vivo}. Collectively, the entire metabolic pathways of SAC in rats and dogs are postulated in Fig.3, displaying each metabolite
formation that we confirmed in the present studies. We also identified GSAC as the novel metabolite of SAC for the first time (Figs. 1A, 1B, 2A and 2B). Gamma-glutamyl transpeptidase catalyzes the transfer of gamma-glutamyl group from glutathione to peptides, amino acids and water (Griffith et al., 1979). The metabolism of SAC to GSAC by this enzyme will be investigated in our future study.

In conclusion, this study demonstrated that the pharmacokinetics of SAC in rats and dogs was characterized by the high oral bioavailability, N-acetylation and S-oxidation metabolisms, and extensive renal reabsorption. This study also emphasizes the critical roles of liver and kidney in the elimination of SAC; the metabolism of SAC to its N-acetylated metabolites in liver and kidney facilitates the elimination of SAC, whereas renal reabsorption of SAC delays its elimination.
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Authorship Contributions

Participated in research design: Amano.

Conducted experiments: Amano, Kazamori, Itoh and Kodera.

Performed data analysis: Amano and Kazamori.

Wrote or contributed to the writing of the manuscript: Amano
References


Nantz MP, Rowe CA, Muller CE, Creasy RA, Stanilka JM, and Percival SS (2012) Supplementation with aged garlic extract improves both NK and γδ-T cell function and reduces the severity of cold and flu symptoms: a randomized, double-blind, placebo-controlled nutrition...


Figure Legends

Figure 1 Plasma concentration-time profiles of SAC, NAc-SAC, SACS, NAc-SACS and GSAC in rats after administration of a single dose (5 mg/kg) of SAC (A, oral; B, i.v.), NAc-SAC (C, i.v.) and SACS (D, i.v.). Each point represents mean ± S.D. of three to four rats.

Figure 2 Plasma concentration-time profiles of SAC, NAc-SAC, SACS, NAc-SACS and GSAC in dogs after administration of a single dose (2 mg/kg) of SAC (A, oral; B, i.v.), NAc-SAC (C, i.v.), SACS (D, i.v.) and NAc-SACS (E, i.v.). Each point represents mean ± S.D. of three dogs.

Figure 3 The postulated metabolic pathways of SAC in rats and dogs.
Table 1 Urinary excretion of SAC, NAc-SAC, SACS and NAc-SACS in rats after oral and i.v. administration.

Data represent mean ± S.D. of three rats.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Urinary excretion (% of dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SAC</td>
</tr>
<tr>
<td>SAC</td>
<td></td>
</tr>
<tr>
<td>i.v.</td>
<td>0.95 ± 0.14</td>
</tr>
<tr>
<td>oral</td>
<td>1.6 ± 0.92</td>
</tr>
<tr>
<td>NAc-SAC</td>
<td></td>
</tr>
<tr>
<td>i.v.</td>
<td>1.8 ± 0.53</td>
</tr>
<tr>
<td>SACS</td>
<td></td>
</tr>
<tr>
<td>i.v.</td>
<td>n.d.</td>
</tr>
<tr>
<td>NAc-SACS</td>
<td></td>
</tr>
<tr>
<td>i.v.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d., not detected.

* A single oral or i.v. dose (5 mg/kg) of SAC, NAc-SAC, SACS and NAc-SACS was administered to rats.

* Amount (% of dose, on a molar basis) of SAC, NAc-SAC, SACS and NAc-SACS excreted in urine for 24 h postdose.
Table 2 Biliary excretion of SAC, NAc-SAC, SACS and NAc-SACS in rats after oral and i.v. administration.

Data represent mean ± S.D. of three rats.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Biliary excretion (% of dose)</th>
<th>SAC</th>
<th>NAc-SAC</th>
<th>SACS</th>
<th>NAc-SACS</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAC</td>
<td>i.v.</td>
<td>0.51 ± 0.09</td>
<td>0.92 ± 0.47</td>
<td>0.01 ± 0.01</td>
<td>0.06 ± 0.04</td>
<td>1.5 ± 0.59</td>
</tr>
<tr>
<td></td>
<td>oral</td>
<td>0.45 ± 0.28</td>
<td>0.77 ± 0.58</td>
<td>n.d.</td>
<td>0.05 ± 0.03</td>
<td>1.3 ± 0.89</td>
</tr>
<tr>
<td>NAc-SAC</td>
<td>i.v.</td>
<td>0.49 ± 0.14</td>
<td>0.72 ± 0.21</td>
<td>n.d.</td>
<td>0.08 ± 0.04</td>
<td>1.3 ± 0.34</td>
</tr>
<tr>
<td>SACS</td>
<td>i.v.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.10 ± 0.02</td>
<td>0.21 ± 0.06</td>
<td>0.32 ± 0.07</td>
</tr>
</tbody>
</table>

n.d., not detected.

a A single oral or i.v. dose (5 mg/kg) of SAC, NAc-SAC and SACS was administered to bile duct-cannulated rats.

b Amount (% of dose, on a molar basis) of SAC, NAc-SAC, SACS and NAc-SACS excreted in bile for 24 h postdose.
Table 3  Pharmacokinetic parameters of SAC, NAc-SAC, SACS and NAc-SACS in rats following oral and i.v. administration.

Data represent mean ± S.D. of three to four rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SAC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NAc-SAC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SACS&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NAc-SACS&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>i.v.  oral</td>
<td>i.v.</td>
<td>i.v.</td>
<td>i.v.</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (mg/l)</td>
<td>6.2 ± 0.56</td>
<td>3.9 ± 0.64</td>
<td>13 ± 2.7</td>
<td>15 ± 1.8</td>
</tr>
<tr>
<td>t&lt;sub&gt;max&lt;/sub&gt; (h)</td>
<td>-</td>
<td>0.27 ± 0.17</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>1.1 ± 0.27</td>
<td>1.2 ± 0.19</td>
<td>1.0 ± 0.25</td>
<td>0.99 ± 0.13</td>
</tr>
<tr>
<td>CL (l/h/kg)</td>
<td>0.91 ± 0.035</td>
<td>-</td>
<td>1.8 ± 0.34</td>
<td>1.5 ± 0.13</td>
</tr>
<tr>
<td>CL&lt;sub&gt;r&lt;/sub&gt; (l/h/kg)</td>
<td>0.0086</td>
<td>-</td>
<td>1.5</td>
<td>0.0014</td>
</tr>
<tr>
<td>V&lt;sub&gt;ss&lt;/sub&gt; (l/kg)</td>
<td>1.0 ± 0.072</td>
<td>-</td>
<td>0.94 ± 0.21</td>
<td>0.75 ± 0.056</td>
</tr>
<tr>
<td>BA (%)</td>
<td>-</td>
<td>92.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AUC (mg.h/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAC</td>
<td>5.50 ± 0.21</td>
<td>5.07 ± 0.58</td>
<td>1.87 ± 0.33</td>
<td>n.d.</td>
</tr>
<tr>
<td>NAc-SAC</td>
<td>1.80 ± 0.19</td>
<td>1.96 ± 0.39</td>
<td>2.89 ± 0.66</td>
<td>n.d.</td>
</tr>
<tr>
<td>SACS</td>
<td>0.057 ± 0.013</td>
<td>0.062 ± 0.014</td>
<td>n.d.</td>
<td>3.47 ± 0.32</td>
</tr>
<tr>
<td>NAc-SACS</td>
<td>0.11 ± 0.025</td>
<td>0.11 ± 0.013</td>
<td>0.13 ± 0.073</td>
<td>1.29 ± 0.090</td>
</tr>
<tr>
<td>GSAC</td>
<td>0.063 ± 0.011</td>
<td>0.059 ± 0.011</td>
<td>0.010 ± 0.001</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

C<sub>max</sub>, maximum plasma concentration; t<sub>max</sub>, time to reach C<sub>max</sub>; V<sub>ss</sub>, distribution volume at steady state; n.d., not detected.

<sup>a</sup>A single oral or i.v. dose (5 mg/kg) of SAC, NAc-SAC, SACS and NAc-SACS was administered to rats.
Table 4 Urinary excretion of SAC, NAc-SAC, SACS and NAc-SACS in dogs after oral and i.v. administration.

Data represent mean ± S.D. of three dogs

<table>
<thead>
<tr>
<th>Compound</th>
<th>SAC</th>
<th>NAc-SAC</th>
<th>SACS</th>
<th>NAc-SACS</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.v.</td>
<td>0.32 ± 0.07</td>
<td>0.89 ± 0.18</td>
<td>1.6 ± 0.87</td>
<td>12 ± 1.7</td>
<td>14 ± 2.3</td>
</tr>
<tr>
<td>oral</td>
<td>0.34 ± 0.19</td>
<td>0.91 ± 0.44</td>
<td>1.7 ± 1.4</td>
<td>10 ± 4.9</td>
<td>13 ± 6.7</td>
</tr>
<tr>
<td>i.v.</td>
<td>0.49 ± 0.06</td>
<td>1.8 ± 0.54</td>
<td>1.8 ± 0.47</td>
<td>12 ± 3.0</td>
<td>16 ± 3.6</td>
</tr>
<tr>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>5.6 ± 2.3</td>
<td>22 ± 4.0</td>
<td>27 ± 4.3</td>
</tr>
<tr>
<td>i.v.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>2.5 ± 1.1</td>
<td>82 ± 5.8</td>
<td>85 ± 4.7</td>
</tr>
</tbody>
</table>

n.d., not detected.

*a* A single oral or i.v. dose (2 mg/kg) of SAC, NAc-SAC, SACS and NAc-SACS was administered to dogs.

*b* Amount (% of dose, on a molar basis) of SAC, NAc-SAC, SACS and NAc-SACS excreted in urine for 48 h postdose.
Table 5 Pharmacokinetic parameters of SAC, NAc-SAC, SACS and NAc-SACS in dogs following oral and i.v. administration.

Data represent mean ± S.D. of three dogs

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SAC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NAc-SAC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SACS&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NAc-SACS&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>i.v.</td>
<td>oral</td>
<td>i.v.</td>
<td>i.v.</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (mg/l)</td>
<td>3.2 ± 0.025</td>
<td>2.1 ± 0.14</td>
<td>9.7 ± 2.1</td>
<td>12 ± 3.3</td>
</tr>
<tr>
<td>t&lt;sub&gt;max&lt;/sub&gt; (h)</td>
<td>-</td>
<td>1.3 ± 0.58</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>12 ± 0.39</td>
<td>12 ± 1.2</td>
<td>11 ± 1.5</td>
<td>6.0 ± 0.28</td>
</tr>
<tr>
<td>CL (l/h/kg)</td>
<td>0.048 ± 0.006</td>
<td>-</td>
<td>0.11 ± 0.031</td>
<td>0.074 ± 0.010</td>
</tr>
<tr>
<td>CLr (l/h/kg)</td>
<td>0.0002</td>
<td>-</td>
<td>0.0019</td>
<td>0.0042</td>
</tr>
<tr>
<td>V&lt;sub&gt;ss&lt;/sub&gt; (l/kg)</td>
<td>0.83 ± 0.10</td>
<td>-</td>
<td>1.3 ± 0.10</td>
<td>0.51 ± 0.059</td>
</tr>
<tr>
<td>BA (%)</td>
<td>-</td>
<td>92.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AUC (mg h/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAC</td>
<td>42.1 ± 4.96</td>
<td>38.7 ± 3.46</td>
<td>27.6 ± 4.46</td>
<td>n.d.</td>
</tr>
<tr>
<td>NAc-SAC</td>
<td>24.4 ± 6.09</td>
<td>22.7 ± 2.82</td>
<td>19.4 ± 4.87</td>
<td>n.d.</td>
</tr>
<tr>
<td>SACS</td>
<td>5.90 ± 0.59</td>
<td>4.92 ± 0.26</td>
<td>3.52 ± 1.14</td>
<td>27.2 ± 3.43</td>
</tr>
<tr>
<td>NAc-SACS</td>
<td>2.85 ± 0.56</td>
<td>2.27 ± 0.31</td>
<td>1.95 ± 0.53</td>
<td>3.70 ± 0.18</td>
</tr>
<tr>
<td>GSAC</td>
<td>0.34 ± 0.064</td>
<td>0.28 ± 0.024</td>
<td>0.23 ± 0.059</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

C<sub>max</sub>, maximum plasma concentration; t<sub>max</sub>, time to reach C<sub>max</sub>; V<sub>ss</sub>, distribution volume at steady state; n.d., not detected.

<sup>a</sup>A single oral or i.v. dose (2 mg/kg) of SAC, NAc-SAC, SACS and NAc-SACS was administered to dogs.
Table 6  CL_{int} values (μl/min/mg protein) for N-acetylation of SAC and SACS and for deacetylation of NAc-SAC and NAc-SACS in liver and kidney S9 fractions of rat, dog, and human.

<table>
<thead>
<tr>
<th>Species</th>
<th>S9</th>
<th>N-acetylation of</th>
<th>Deacetylation of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SAC</td>
<td>SACS</td>
</tr>
<tr>
<td>Rat</td>
<td>Liver</td>
<td>31</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>190</td>
<td>15</td>
</tr>
<tr>
<td>Dog</td>
<td>Liver</td>
<td>0.58</td>
<td>0.056</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>5.9</td>
<td>0.13</td>
</tr>
<tr>
<td>Human</td>
<td>Liver</td>
<td>1.1</td>
<td>0.041</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>3.1</td>
<td>0.24</td>
</tr>
</tbody>
</table>

n.d., not detected.
SAC NAc-SAC
SACS NAc-SACS

demonstrated in rats and dogs
demonstrated in dogs

GSAC

N-acetylation
Deacetylation

N-acetylation
Deacetylation

γ-glutamylation

S-oxidation
S-oxidation

S-oxidation
S-oxidation

O

O

O

O

Figure 3