Metabolism and Pharmacokinetics of Orally Administered Saikosaponin b₁ in Conventional, Germ-Free and Eubacterium sp. A-44-Infected Gnotobiote Rats

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The metabolic fate of saikosaponin b₁ (1) was investigated using conventional, germ-free and Eubacterium sp. A-44-infected gnotobiote rats. After the oral administration of 1 to germ-free rats at a dose of 50 mg/kg, no metabolite was detected in the plasma, the cecal contents or the cumulative feces through the experiment. On the other hand, when 1 was orally given to the Eubacterium sp. A-44-infected gnotobiote rats, considerable amounts of its metabolites, prosaikogenin A (2) and saikogenin A (3), were detected in the rat plasma with the respective AUC₁₀₈ values of 17424 and 22260 pmol·min/ml, similar to the case of its oral administration to conventional rats (AUC₁₀₈ values of 9936 and 12414 pmol·min/ml for 2 and 3, respectively). Furthermore, significant amounts of both metabolites were detected in the cecal contents and the cumulative feces of the gnotobiote and conventional rats, but not in those of the germ-free rats, within 10 h after the administration.

Fecal and cecal activities of hydrolyzing 1 and 2 were found in the gnotobiote and conventional rats, though there were no detectable activities in the germ-free rats. Accordingly, both hydrolyzing activities in the intestinal bacteria, such as Eubacterium sp. A-44, are essential for the appearance of 2 and 3 in the rat plasma and cumulative feces, since orally administered 1 was poorly absorbed from the gastrointestinal tract.

Key words: intestinal bacteria; saikosaponin b₁; Eubacterium sp. A-44; germ-free rat; gnotobiote rats

The prescriptions used in traditional medicine are mainly admixtures of various herbs, in which some glycosides are considered to be main components in manifestation of their medicinal effects. Since these prescriptions are orally administered to patients but the glycosides seem to be poorly absorbed from the gastrointestinal tract, in general, it is most likely that these glycosides are first hydrolyzed to their respective aglycons by intestinal bacteria, and the aglycons are subsequently absorbed into the body fluid. Some of these glycosides, such as paeniniflorin from peony roots and glycyrrhizin from licorice, have been demonstrated to be transformed by intestinal bacteria to pharmacologically active forms.1–3)

We have previously reported that saikosaponins a, b₁, b₂ and d, the main glycosides in Bupleuri Radix (Saiko in Japanese), are metabolized in vitro by a bacterial mixture of human feces (but not by a rat liver homogenate) to the respective saikogenins via their corresponding prosaikogenins.5) Shimizu et al. have reported that saikosaponins are metabolized by a mouse intestinal bacterial mixture.5) Furthermore, we clarified that only a few bacterial strains were capable of hydrolyzing saikosaponins when 31 defined strains of human intestinal bacteria and 60 colonies from human feces were examined.6) The hydrolysis of these glycosides was effectively achieved by Eubacterium sp. A-44, an anaerobe isolated from human feces. From this strain, we isolated two novel glycosidases responsible for the hydrolysis of saikosaponins: saikosaponin-hydrolyzing β-D-glucosidase and prosaikogenin-hydrolyzing β-D-fucosidase (Chart 1).6) In the in vivo study, saikosaponin a and its metabolites, prosaikogenin F and saikogenin F, were detected in the blood stream7) and cumulative feces,5) after the oral administration of saikosaponin a to conventional rats, suggesting that the metabolites were absorbed after the bacterial transformation of saikosaponin a in the rat alimentary tract. However, further in vivo studies are necessary to define the site responsible for the production of these metabolites.

Saikosaponin b₁ (1), one of the major saikosaponins in the decoction of traditional Chinese medicine8) (Dai-saiko-to and Sho-saiko-to), was reported to show anti-allergic9) and anti-tumor effects.10) Moreover, Shibata et al. demonstrated that an orally administered crude saikosaponin mixture has the same spectrum of pharmacological effects as saikogenin A, the aglycone of 1.11)

In the present paper, we report direct evidence that saikosaponin-metabolizing bacteria in the intestine are requisite for the appearance of prosaikogenin A (2) and saikogenin A (3) in the plasma after the oral administration of 1, through pharmacokinetic studies using conventional, germ-free and Eubacterium sp. A-44-infected gnotobiote rats.

MATERIALS AND METHODS

Apparatus A Shimadzu LC-6A liquid chromatograph (Shimadzu Co., Kyoto, Japan) equipped with a spectrophotometer detector S-310A model-II (Soma Optics, Ltd., Tokyo, Japan), and a dual wavelength chromatoscanner CS-9300 (Shimadzu Co., Kyoto, Japan) were used for measuring 1 and its metabolites (2 and 3). A sonicator, Sonifer 250 (Branson Co., Danbury, CT, U.S.A.) was used to disrupt the bacterial cells.

Chemicals and Media 1 was isolated from the dried roots of Bupleurum falcatum L. as reported by Shimao et al.12) 2 and 3 were obtained as described previously.13) p-Nitrophenyl β-D-glucopyranoside and methyl benzoxate were purchased from Nakalai Tesque (Kyoto, Japan). p-Nitro-
phenyl β-D-fucopyranoside was a product of Sigma Chemical Co. (St. Louis, U.S.A.). Acetoni trile (HPLC grade) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Heparin sodium salt was a product of Wako Pure Chemical Industries (Osaka, Japan). General anaerobic medium (GAM) was purchased from Nissui Seiyaku Co. (Tokyo, Japan). A pentobarbital sodium solution was a product of Abbott Laboratories (North Chicago, U.S.A.).

**Bacterial Strains** *Eubacterium* sp. A-44[13] was isolated from human feces and maintained in GAM broth. The bacterium was cultured in GAM broth for 20 h before use.

**Animal Treatment and Sampling** Male Wistar rats (6 weeks old) were purchased from SLC Co. (Hamamatsu, Japan), and male Wistar germ-free rats (WAlJic, 6 weeks old) were from Clea Japan, Inc. (Tokyo, Japan). Conventional rats were maintained in a wire-bottomed cage for the experiments; water and standard laboratory chow (CE-2, Clea Japan, Inc.) were freely available. Germ-free rats were maintained in metabolic cages under germ-free conditions, and autoclaved water and germ-free CE-2 chow were freely available. Six germ-free rats were infected with *Eubacterium* sp. A-44 (2 ml medium) on the first day and third day to ascertain and to establish the gnotobiotic rats. A solution of 1 (50 mg/kg dissolved in 30% ethanol) was filtered through a sterile membrane filter (0.22 µm, Millipore Co., Tokyo, Japan) and orally given on the seventh day. The same dose of 1 was orally administered to two groups of conventional and germ-free rats. Fresh feces were sampled before drug administration to measure the enzyme activities. Cumulative feces for 10 h after the administration were collected, and blood samples (400 µl) were taken with a heparin-flushed syringe from the tail vein at time intervals (0.5, 1.5, 6 and 8 h). Plasma was prepared by centrifugation of the blood samples and stored at -20°C until analysis. Contents of the gastrointestinal and blood from the abdominal vein were taken under pentobarbital anesthesia at 4 and 10 h after the administration.

**HPLC** Saikosaponin b1 (1) and its metabolites in the plasma (150 µl) were extracted with ethanol (15 ml) containing methyl benzoate (75 nmol) as an internal standard. After centrifugation, the supernatant was collected and evaporated to dryness under a vacuum. The residue was dissolved in 50% ethanol (30 µl), and filtered through a 0.5 µm membrane filter (Millipore Co., Tokyo, Japan). A 20 µl portion of the solution was applied to a Develosil ODS-5 column (4.6 mm i.d. x 150 mm, Nomura Chemical Co., Seto, Japan). The mobile phase was 38% acetonitrile containing 0.1% trifluoroacetic acid, and the flow rate was 1.0 ml/min. 1 and its metabolites were monitored at a wavelength of 250 nm. The calibration lines for 1 and its metabolites 2 and 3 were linear in a range of 8-500 ng/ml.

**Determination of the Intestinal Permeability by an in Vitro Everted Sac Method** Everted sacs of the small intestine were prepared by the method of Wilson and Wiseman.[14] Each sac (10 cm) was filled with 1.5 ml of incubation medium [modified Krebs Ringer bicarbonate phosphate buffer (pH 7.4) composed of 113.3 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 17.0 mM NaHCO₃, 10.2 mM NaHPO₄ and 0.6 mM CaCl₂] containing 0.1% glucose, and placed in 15 ml of the incubation medium containing 0.1% glucose and 250 µM of either 1 or its metabolites (2 and 3) in a test tube through which air was bubbled. The test tube was incubated at 37°C in a water bath. A 100 µl portion of the incubation medium was sampled from the serosal side at intervals (10, 25 and 45 min), and then each compound was quantitatively analyzed by HPLC, as mentioned above.

**TLC** TLC was carried out with silica gel plates 60 F₂₅₄ (thickness 0.25 mm, Merck Co., Darmstadt, Germany) to determine 1 and its metabolites (2 and 3) in cumulative feces and cecal contents. Each sample was extracted with 5 volumes of ethanol and centrifuged at 1000×g for 10 min. Twenty microliters of the supernatant were applied to a TLC plate, which was developed with a solvent system of CHCl₃-MeOH-H₂O (30 : 10 : 1). 1 and its metabolites (2 and 3) were simultaneously determined with a Shimadzu chromatograph scanner CS-9300 (λs=262 nm, λr=400 nm), as reported previously.[10]

**Enzyme Assay** Fecal and bacterial suspensions were sonicated to obtain maximal activities and were used as enzyme sources. β-D-Glucosidase and β-D-fucosidase activities were determined using p-nitrophenyl β-D-glucoside and p-nitrophenyl β-D-fucoside, respectively, as reported previously.[15] Saikosaponin β₁ and prosaikogenin A-hydrolyzing activities were determined as reported previously.[6]

**Determination of Protein** Protein was determined by the Lowry method using BSA as a standard sample.[16]

**Pharmacokinetic Analysis** Areas under the plasma con-
RESULTS

Permeability of 1 and Its Metabolites Measured by an in Vitro Everted Sac Method Figure 1 shows the time courses of the intestinal permeability of 1 and its metabolites (2 and 3) from the mucosal side to the serosal side. The concentrations of the tested compounds in the serosal side increased almost linearly with incubation time. Of these compounds, 3 passed most readily through the intestinal everted membrane, and the permeability decreased in the order of 3, 2, and 1.

Plasma Concentration of 1 and Its Metabolites after the Oral Administration of 1 to Conventional Rats Figure 2 shows the plasma concentration–time course of 1 and its metabolites (2 and 3) after oral administration of 1 at a dose of 50 mg/kg to conventional rats. The maximal plasma concentration ($C_{\text{max}}$) of 1 was reached 30 min after the administration (129 pmol/ml), and 1 disappeared after 4 h. In contrast, 2 and 3 could be detected after 4 and 6 h, respectively. The plasma concentrations of both metabolites (2 and 3) reached maximal values of 49.6 and 54.7 pmol/ml at 6 and 8 h, respectively. Ten hours after the administration of 1, 2 disappeared from the rat plasma, but 3 (22.3 pmol/ml) was still detected. The pharmacokinetic parameters are listed in Table 1. In the conventional rats, the sum of each $AUC_{0-\text{t}}$ value of metabolites (2 and 3) (22350 pmol·min/ml) was larger than that of 1 (12654 pmol·min/ml).

Plasma Concentration of 1 and Its Metabolites after Oral Administration of 1 to Germ-Free Rats and Eubacterium sp. A-44-Infected Gnotobiote Rats Figure 3A shows the plasma concentration–time course of 1 after oral administration of 1 at a dose of 50 mg/kg to germ-free rats. The $C_{\text{max}}$ of 1 (113 pmol/ml) was reached at 30 min after the administration, and 1 (15.4 pmol/ml) was still detected in the plasma at 10 h (the $AUC_{0-\text{t}}$ value of 34308 pmol·min/ml), while 2 and 3 were not detected (Table 1).

On the other hand, 2 and 3 were detected in the plasma of gnotobiote rats after the oral administration of 1 at a dose of 50 mg/kg (Fig. 3B), similar to the case of conventional rats (Fig. 2). However, the $AUC_{0-\text{t}}$ values of 2 and 3 (17424 and 22260 pmol·min/ml) were 2.0–2.6 fold larger than that of 1 (8652 pmol·min/ml) (Table 1).

1 and Its Metabolites in the Cecal Contents and Cumulative Feces of Conventional, Germ-Free and Gnotobiote Rats after the Oral Administration 1, 2 and 3 were recovered from the cecal contents of conventional rats 4 h (3.81, 0.32 and 0.51 μmol, respectively) and 10 h (0.28, 1.89 and 0.76 μmol, respectively) after the administration (Table 2). Similarly, 1 and its metabolites (2 and 3) were also detected in the cumulative feces of conventional rats within 10 h (1.01, 1.15 and 0.91 μmol, respectively) after the administration (Table 3).

However, only 1 (4.93 and 2.73 μmol, respectively) was recovered from the cecal contents of the germ-free rats 4 and 10 h after the administration (Table 2). Similarly, 1 (1.80 μmol) was also detected in the cumulative feces of the germ-free rats within 10 h after the administration (Table 3). How-

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Table 1. Pharmacokinetic Parameters after Oral Administration of Saikosaponin b₁ (1) at a Dose of 50 mg/kg to Conventional, Germ-Free and Gnotobiote Rats

<table>
<thead>
<tr>
<th>Rats</th>
<th>$C_{\text{max}}$ (pmol/ml)</th>
<th>$t_{\text{max}}$ (min)</th>
<th>$AUC_{0-10h}$ (pmol·min/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>129±17.7</td>
<td>30</td>
<td>12654</td>
</tr>
<tr>
<td>2</td>
<td>49.6± 5.15</td>
<td>360</td>
<td>9936</td>
</tr>
<tr>
<td>3</td>
<td>54.7± 5.69</td>
<td>480</td>
<td>12414</td>
</tr>
<tr>
<td>Germ-free rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>113± 6.07</td>
<td>30</td>
<td>34308</td>
</tr>
<tr>
<td>2</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>3</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Gnotobiote rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>100±14.0</td>
<td>30</td>
<td>8652</td>
</tr>
<tr>
<td>2</td>
<td>59.4± 5.72</td>
<td>240</td>
<td>17424</td>
</tr>
<tr>
<td>3</td>
<td>65.6± 8.72</td>
<td>360</td>
<td>22260</td>
</tr>
</tbody>
</table>

N.D.: not detected. Mean±S.E. are given (n=3).
ever, no metabolite was detected in either the cecal contents or cumulative feces.

In the case of the gnotobiotic rats, 1, 2 and 3 (3.3, 0.78 and 0.87 μmol, respectively) were recovered from the cecal contents 4 h after the administration (Table 2). However, only the two metabolites, 2 and 3, were recovered after 10 h (0.82 and 1.86 μmol, respectively). Furthermore, 1, 2 and 3 (0.85, 0.84 and 0.90 μmol, respectively) were also detected in the cumulative feces of gnotobiotic rats within 10 h. (Table 3).

**Enzyme Activities of Fresh Feces and Cecal Contents**

Fresh feces and cecal contents of the germ-free rats showed no β-D-glucosidase, β-D-fucosidase, saikosaponin b₁- or prosaikogenin A-hydrolyzing activity (Table 4). In the case of the conventional and gnotobiotic rats, each enzyme activity was found. However, both the cecal 1- and 2-hydrolyzing activities of the gnotobiotic rats (11.59 and 4.34 pmol/min/mg, respectively) were about 2-fold higher than those of conventional rats (5.48 and 1.88 pmol/min/mg, respectively).

**DISCUSSION**

For the purpose of investigating the metabolic fates of saikosaponins, a pharmacokinetic study of 1 was performed as a representative of the saponins using conventional, germ-free and *Eubacterium* sp. A-44-infected gnotobiotic rats.

Considerable amounts (about 83% of the administered dose) of the unabsorbed 1 remained in the cecal contents of the germ-free rats 4 h after the oral administration. These findings indicate that orally administered saikosaponins are poorly absorbed from the gastrointestinal tract (GIT). This was further confirmed by the low intestinal permeability of 1 assessed by the *in vitro* everted sac method. On the other hand, 2 and 3, in addition to 1, were detected in the blood stream, cecal contents and feces after oral administration of 1 to the conventional and gnotobiotic rats, though neither metabolite (2 or 3) was detected in the case of the germ-free rats (Fig. 3, Tables 2 and 3). These findings confirmed that unabsorbed 1 reached the cecum, and was hydrolyzed by intestinal bacteria to these metabolites (2 and 3), which were subsequently absorbed. This was supported by the observation that the $t_{\text{max}}$ (time to $C_{\text{max}}$) of each metabolite was appreciably delayed (approx. at 4–8 h), relative to that of 1 (30 min) when orally administered to the conventional or gnotobiote rats. Accordingly, these findings established that the rat cecum, which contains the highest bacterial population, was the site responsible for the appearance of these metabolites (2 and 3).

After the oral administration of 1 to germ-free rats, less
Table 4. β-d-Glucosidase, β-d-Fucosidase, Saikosaponin b2- and Prosaikogenin A-Hydrolyzing Activities in the Feces and Cecal Contents of Conventional, Germ-Free and Gnotobiote Rats

<table>
<thead>
<tr>
<th>Enzymatic activity (pmol/min/mg)</th>
<th>Conventional rats</th>
<th>Germ-Free rats</th>
<th>Gnotobiote rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β-d-Glucosidase</td>
<td>β-d-Fucosidase</td>
<td>Saikosaponin b2</td>
</tr>
<tr>
<td>Cecum</td>
<td>8480±1287</td>
<td>1840±159</td>
<td>5.48±0.46</td>
</tr>
<tr>
<td>Feces</td>
<td>8060± 65</td>
<td>3360±346</td>
<td>8.16±0.88</td>
</tr>
<tr>
<td></td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cecum</td>
<td>8810± 889</td>
<td>2200± 90</td>
<td>11.59±1.18</td>
</tr>
<tr>
<td>Feces</td>
<td>6670± 774</td>
<td>2840±184</td>
<td>10.58±1.34</td>
</tr>
</tbody>
</table>

Quantity of protein was determined by Lowry’s method. N.D.: not detected. Mean± S.E. are given (n=3). β-d-Glucosidase and β-d-fucosidase activities were determined using p-nitrophenyl β-d-glucoside and p-nitrophenyl β-d-fucose as substrates, respectively.

than 20% of the administered dose was absorbed, but the AUC0−10h value was 2.5 times larger than that in the case of the conventional and gnotobiote rats. This finding revealed that I was gradually absorbed during a long period of time (10h) in the germ-free rats, relative to the conventional rats (4h). A similar finding was reported for paoniflorin administered orally to germ-free and conventional rats. Accordingly, we suggest that the intestinal bacteria also play a major role in the elimination of I as well as with paoniflorin.

We previously isolated two enzymes, saikosaponin-hydrolyzing β-d-glucosidase and prosaikogenin-hydrolyzing β-d-fucosidase, from Eubacterium sp. A-44, responsible for the hydrolysis of saikosaponins. In the present study, both enzymatic activities could be detected in the feces and cecal contents of the gnotobiote rats, but not in those of the germ-free rats. Similar activities were detected in the feces and cecal contents of conventional rats. However, the hydrolyzing activities in the feces and cecal contents of the gnotobiote rats were higher than those of the conventional rats, compatible with the finding that the AUC0−10h values of both metabolites (2 and 3) in the plasma of the gnotobiote rats were larger than those of the conventional rats. This result indicates that the saikosaponin b2- and prosaikogenin A-hydrolyzing activities of intestinal bacteria, such as Eubacterium sp. A-44, are essential for the production of 2 and 3.

3. the main metabolite of I, was reported to show anti-inflammatory, analgesic, sedative, antitussive, and hypothermic effects as well as anti-tumor action. Cheng and Tsai reported that the anti-inflammatory effects of I were due to the increase in corticosterone level caused by the release of adrenocorticotrophic hormone (ACTH) into rat plasma. In the case of other saikosaponins, it has been reported that prosaikogenin F and saikogenin F are detected in the blood stream and cumulative feces of conventional rats after the oral administration of saikosaponin a. Likewise, the metabolites of saikosaponin b2 were detected in the feces of conventional rats. When compared with the present results, the saikosaponin a- or b2-hydrolyzing activity of intestinal bacteria also seems to be responsible for the production of their respective metabolites. Since various pharmacological effects, such as anti-inflammatory and anti-hyperchlolesteremic actions, have been reported for the metabolites from saikosaponins, the present findings led us to speculate that these metabolites formed by intestinal bacteria play an important role in the clinical effects of orally administered prescriptions containing Bupleuri Radix in traditional Chinese medicine. In addition, since the transformation of saikosaponins by a mixture of bacteria from human feces was much faster than by bacteria of rat feces when the saponins were incubated in vitro under the same conditions, it is suggested that higher concentrations of their metabolites appear in human plasma than in rat plasma.

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