J. Pharm. Pharmacol. 1992, 44: 295–299
Received May 11, 1991

Development of a Prodrug of Salicylic Acid, Salicylic Acid-L-alanine Conjugate, Utilizing Hydrolysis by Rabbit Intestinal Microorganisms

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Abstract—The hydrolysis of salicylic acid-L-alanine conjugate (salicyl-L-alanine) following oral, intravenous, intracaecal and rectal administration (60, 10, 5 and 5 mg kg^{-1}, respectively: salicylic acid equivalent) was examined in rabbits. Salicylic acid was detected in the blood 2 h after oral administration of salicyl-L-alanine and reached a maximum concentration at 10 h, whereas salicyl-L-alanine was rapidly eliminated. In contrast, unchanged salicyl-L-alanine only was found following intravenous administration of salicyl-L-alanine, suggesting that presystemic de-conjugation of salicyl-L-alanine was involved. The intestinal mucosal de-conjugation of salicyl-L-alanine was not recognized in the in-situ intestinal sac preparation with complete mesenteric venous blood collection. Immediate and very extensive salicylic acid formation in the caecum was found following intracaecal administration of salicyl-L-alanine. After oral pretreatment of rabbits with kanamycin sulphate, a significant inhibition of salicylic acid formation following intracaecal administration of salicyl-L-alanine was observed, indicating that the intestinal microorganisms were responsible for the biotransformation of salicyl-L-alanine. In-vitro incubation of salicyl-L-alanine with gut contents showed that the major source of its hydrolysis was the hind gut. Consequently, the blood concentration of salicylic acid was prolonged extensively following rectal administration of salicyl-L-alanine, suggesting the usefulness of salicyl-L-alanine as a prodrug of salicylic acid.

The significance of intestinal microorganisms to pharmacokinetics and pharmacology has been emphasized with respect to their ability to metabolize drugs and foreign compounds. The hydrolysis of glycine conjugates by intestinal microorganisms is well documented in various species (Norman & Grubb 1955; Hülsmann & Statius van Eps 1967; Boxenbaum et al 1974, 1979). In previous reports, we demonstrated that the glycine conjugate of salicylic acid (salicyluric acid) was metabolized to salicylic acid by intestinal microorganisms existing mainly in caecum and colon in rabbits (Shibasaki et al 1985; Nakamura et al 1986, 1988a), rats (Nakamura et al 1988b) and dogs (Nakamura et al 1989a). Furthermore, we examined the effects of fasting or pretreatment with antibiotics on the hydrolysis of salicyluric acid in rabbit intestinal microorganisms to obtain detailed information on the practical use of prodrugs utilizing metabolism by intestinal microorganisms (Nakamura et al 1989b, 1990).

Recently, a salicyluric acid-hydrolysing enzyme was purified from an intestinal bacterium in rabbits, and characterized by Ogushi et al (1988). They reported that this enzyme catalyzed the hydrolysis of *N*-benzoyl amino acids and their derivatives. Accordingly, the elucidation of the effect of amino acid moiety on the fate of salicylic acid was considered to offer a promising approach to developing an effective prodrug of salicylic acid. Thus, we examined the hydrolysis of salicylic acid-L-alanine conjugate (salicyl-L-alanine) following oral, intravenous, intracaecal and rectal administration in rabbits.

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Materials and Methods

Chemicals

Acetonitrile, acetic acid, methanol and *o*-anisic acid were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Kanamycin sulphate was obtained from Meiji Seika Kaisha Ltd (Tokyo, Japan). All other chemicals were of reagent grade.

Synthesis of salicyl-L-alanine

Salicyl-L-alanine was synthesized by coupling L-alanine methyl ester and acetylsalicylic acid by means of the carbodiimide method as follows. To a solution of 25 g of L-alanine in 200 mL of methanol, 30 mL of thionyl chloride was added slowly at 0°C and stirred overnight at room temperature (21°C). The reaction mixture was concentrated under reduced pressure. Recrystallization from methanol and ether yielded about 90% of L-alanine methyl ester hydrochloride as white crystals (mp 154–155°C). To a solution of L-alanine methyl ester hydrochloride in 50 mL of methanol, 50 g of triethylamine was added slowly and stirred for 2 h at 0°C. The reaction mixture was then filtered. L-Alanine methyl ester was obtained as an oil by concentrating the filtrate under reduced pressure. To a solution of 150 mmol of acetylsalicylic acid in methylene chloride and a small amount of tetrahydrofuran, 150 mmol of *N,N'*-dicyclohexylcarbodiimide was added at under 0°C. After 30 min, 150 mmol of L-alanine methyl ester was added. The reaction mixture was left overnight at room temperature, and then filtered. The filtrate was washed with 5% $NaHCO_3$ and concentrated under reduced pressure to obtain acetylsalicylic acid-L-alanine methyl ester as an oil. To a solution of acetylsalicylic acid-L-alanine methyl ester in a small amount of methanol, 300 mL of 1 M NaOH was added and stirred for

1 h. The methanol was evaporated under reduced pressure. The reaction mixture was acidified with conc. HCl, washed with ether and extracted with ethyl acetate, which was washed with water and saturated NaCl solution. After drying with Na_2SO_4, the product was concentrated under reduced pressure and then recrystallized from ethyl acetate to yield about 50% of salicyl-L-alanine as white crystals: mp 162–164°C; $[\alpha]_D^{20} = +24{\cdot}2°$ (c = 0·91, EtOH). Analysis: Calculated for $C_{10}H_{10}NO_4$: C, 57·42; H, 5·26; N, 6·70. Found: C, 57·92; H, 5·30; N, 6·66. EI-MS m/z: 209. The chemical structure was confirmed by NMR, mass spectral and elemental analyses. NMR and mass spectra were taken on a JEOL FX90Q Fourier transform spectrometer (JEOL Ltd, Tokyo, Japan) and a JEOL JMS-DX303 mass spectrometer (JEOL Ltd), respectively. Elemental analyses were performed by the Center for Organic Elemental Microanalysis, Nagasaki University. The analytical results obtained were within ±0·4% of the theoretical values.

Animals

Male albino rabbits, 2–3 kg, were individually housed in cages in an air-conditioned room and maintained on a standard laboratory diet (ORC4, Oriental Yeast Co. Ltd, Tokyo, Japan).

In-vivo experiments

The rabbits were starved for about 24 h before use but had free access to water. Salicyl-L-alanine was dissolved in NaOH (equivalent to salicyl-L-alanine). Appropriate amounts of drug solution were administered as described below. Following oral, intravenous, intracaecal and rectal administration of drug, blood was collected with a heparinized syringe at appropriate time intervals from an ear vein.

Oral administration. The drug solution (60 mg kg^{-1}: salicylic acid equivalent) was administered orally by gastric intubation.

Intravenous administration. The drug solution (10 mg kg^{-1}: salicylic acid equivalent) was administered intravenously via an ear vein.

Intracaecal administration. Animals were anaesthetized with sodium pentobarbitone, given intravenously, via an ear vein. After complete anaesthesia, a midline incision (2–3 cm) was made, and the drug solution (5 mg kg^{-1}: salicylic acid equivalent) was administered by direct injection into the caecum by syringe. Leakage of drug solution at the injection site was not observed. The abdomen was closed with operative stitching. Oral treatment of rabbits with kanamycin sulphate before intracaecal administration of drug was carried out as follows, using the administration time of kanamycin sulphate established by Gingell et al (1971). The animals each received 6 × 400 mg kanamycin sulphate in aqueous solution. Kanamycin sulphate was given orally twice daily for 2 days before salicyl-L-alanine administration and then 4 h before and 4 h after salicyl-L-alanine administration.

Rectal administration. The drug solution (5 mg kg^{-1}: salicylic acid equivalent) was administered rectally, and the anus was closed with a plastic clip to prevent leakage of the rectal contents during the experiment.

In-situ intestinal experiment

The rabbits were starved for about 24 h before use but had free access to water. The surgical operation and other procedures were the same as reported previously (Podder et al 1986). In order to prepare the intestinal sac, midportions of the intestine (5–8 cm) were used. The intestinal lumen was washed with 0·9% NaCl (saline) as completely as possible, and both sides of the intestine were ligated to prepare a closed sac. The mesenteric vein was cannulated with a polyethylene tubing SP 45 (0·96 mm o.d., Natsume Seisakusho Co. Ltd, Tokyo, Japan), through which all venous blood was collected in heparinized tubes at successive intervals after injecting salicyl-L-alanine solution (3 mL) into the intestinal lumen. The appearance of salicyl-L-alanine and salicylic acid in the mesenteric venous blood was examined. The blood lost from the mesenteric vein was continuously replaced by an intravenous infusion of saline via an ear vein.

In-vitro incubation of salicyl-L-alanine with gut contents

Non-fasted rabbits were anaesthetized by intravenous injection of sodium pentobarbitone. After the five segments (jejunum, upper ileum, lower ileum, caecum and colon) of intestine were cut open, the content of each segment (1 g wet weight) was collected separately. A ten mL portion of salicyl-L-alanine (100 μg mL^{-1}: salicylic acid equivalent) in saline was added to the gut content (1 g wet weight) and the mixture was incubated for 6 h at 37°C. One mL portions of the mixture were collected at appropriate time intervals and centrifuged at 12000 rev min^{-1} for 10 min, and the supernatant (0·4 mL) was subjected to assay.

Similarly, in-vitro incubation of salicyl-L-alanine with caecum content was carried out. To a sample of caecum content (2 g wet weight), 5 mL saline was added and then mixed. The mixture was separated into supernatant and residue by centrifugation at 3500 rev min^{-1} for 10 min. A 5 mL portion of salicyl-L-alanine (200 μg mL^{-1}: salicylic acid equivalent) in saline was added to the supernatant and the residue suspended in 5 mL saline. The mixture was incubated for 4 h at 37°C, and then centrifuged at 12000 rev min^{-1} for 10 min. The supernatant (0·4 mL) was subjected to assay.

Analytical method

Salicyl-L-alanine and salicylic acid in blood, and in supernatant fluid in the in-vitro experiment, were analysed by HPLC according to the method of Cham et al (1979) with fluorescence for detection instead of absorption at 313 nm. Blood and supernatant fluid samples (0·4 mL) were added to an equal volume of acetonitrile containing 30 μg of the internal standard, *o*-anisic acid, in 1 mL distilled water. The samples were mixed on a vortex mixer and centrifuged at 12000 rev min^{-1} for 10 min. The supernatant was filtered through a 0·45 μm pore size membrane (SJHVL04NS, Nihon Millipore Kogyo K.K., Yonezawa, Japan). Twenty μL of the supernatant fluid was withdrawn using a Hamilton syringe and loaded onto the column. Calibration curves were constructed from the peak-area ratios of salicyl-L-alanine and salicylic acid to internal standard. We used an LC-6A pump, an RF-530 fluorescence detector, a Chromatopac C-R3A recorder (all from Shimadzu Co. Ltd, Kyoto, Japan) and a model 7125 sample injector valve (Rheodyne Inc., CA, USA). The stationary phase used was a Cosmosil 5C18

packed column (150 × 4·6 mm i.d., Nacalai Tesque, Inc.). This column was used at room temperature. The peak area of fluorescence intensity was recorded at excitation and emission wave-lengths of 300 and 410 nm, respectively. The chromatographic mobile phase consisted of a mixture of acetic acid-methanol-water (4:35:65, v/v/v) and was filtered through a 0·5 μm pore size membrane (T050A047A, Toyo Roshi Co. Ltd, Tokyo, Japan) before use. The flow rate was 1·0 mL min^{-1}. The retention times of salicyl-L-alanine, salicylic acid and the internal standard were 8·4, 12·5 and 6·8 min, respectively.

Results and Discussion

The blood concentrations of salicyl-L-alanine and salicylic acid following oral administration of salicyl-L-alanine were determined in rabbits. As shown in Fig. 1a, both salicyl-L-alanine and salicylic acid were detected, indicating that the hydrolysis of salicyl-L-alanine occurred. Salicyl-L-alanine reached a peak blood concentration (24·9 μg mL^{-1}, as salicylic acid) at 30 min after the dose and then decreased to below 1 μg mL^{-1} (as salicylic acid) at 10 h. On the other hand, salicylic acid was detected at 2 h and reached a peak blood concentration (20·2 μg mL^{-1}) at 10 h. The blood concentration of salicylic acid remained above 5 μg mL^{-1} even at 30 h, indicating the extensive prolongation of blood concentration of salicylic acid. The blood concentration of salicylic acid increased significantly following oral administration of salicyl-L-alanine compared with salicyluric acid (Shibasaki et al 1985).

In order to examine the systemic de-conjugation of salicyl-L-alanine, the conjugate was administered intravenously.

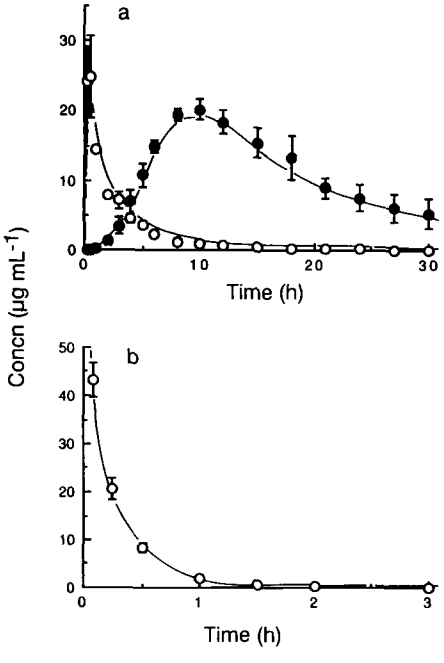

FIG. 1. Blood concentrations of salicyl-L-alanine measured as salicylic acid equivalent (○) and salicylic acid (●) following oral (a) and intravenous (b) administration of salicyl-L-alanine (60 and 10 mg kg^{-1}, respectively: salicylic acid equivalent) to rabbits. Each point represents the mean ± s.e. of 4 experiments.

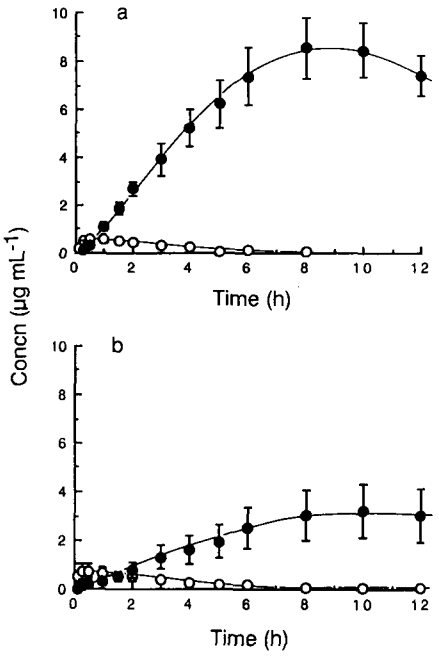

FIG. 2. Blood concentrations of salicyl-L-alanine measured as salicylic acid equivalent (○) and salicylic acid (●) following intracaecal administration of salicyl-L-alanine (5 mg kg^{-1}: salicylic acid equivalent) to control rabbits (a) and rabbits orally pretreated with kanamycin sulphate (b). Each point represents the mean ± s.e. of 6 experiments.

The results are shown in Fig. 1b. Salicyl-L-alanine was detected in the blood, but was rapidly eliminated. In contrast, salicylic acid could not be detected, suggesting that presystemic de-conjugation of salicyl-L-alanine was needed.

Salicyl-L-alanine was administered intracaecally to examine the mechanism of salicyl-L-alanine hydrolysis in rabbits. Fig. 2a shows the blood concentration of salicyl-L-alanine and salicylic acid following intracaecal administration of salicyl-L-alanine. Salicylic acid reached a peak blood concentration (8 μg mL^{-1}) at 8 h. Salicyl-L-alanine was detected at low concentrations (< 0·6 μg mL^{-1}, as salicylic acid) for 8 h. The blood concentration profile in Fig. 2a indicated immediate and extensive salicylic acid formation from salicyl-L-alanine in the caecum. Salicylic acid was detected in the blood at 2 h and reached a maximum level at 10 h after oral administration of salicyl-L-alanine (Fig. 1a). Thus, it seemed that the delays in detection and the maximum level of salicylic acid following oral administration of salicyl-L-alanine were mainly due to the time required for the gastrointestinal transit and partially due to the hydrolysis to salicylic acid.

The effect of oral pretreatment with kanamycin sulphate on the blood concentrations of salicyl-L-alanine and salicylic acid following intracaecal administration of salicyl-L-alanine was examined. As shown in Fig. 2b, the oral pretreatment of rabbits with kanamycin sulphate caused a significant decrease in the formation of salicylic acid, suggesting that salicyl-L-alanine was hydrolysed to salicylic acid by the intestinal microorganisms in rabbits. In control and kanamycin sulphate-pretreated rabbits, the areas under the blood concentration-time curves of salicyl-L-alanine were

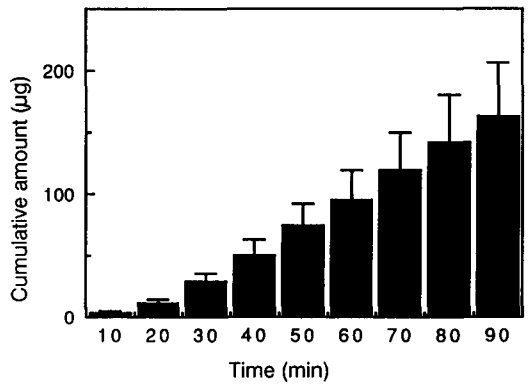

FIG. 3. Cumulative amount of salicyl-L-alanine measured as salicylic acid equivalent appearing in the mesenteric venous blood after injection into the intestinal lumen. In this case, salicylic acid could not be detected. Animals received 3 mL of salicyl-L-alanine solution (333 $\mu g\ mL^{-1}$: salicylic acid equivalent). Values are mean ± s.e. of 6 experiments.

133·7 ± 64·8 and 177·7 ± 131·4 $\mu g\ mL^{-1}$ min, respectively, indicating no significant difference. In the present study, we did not examine the effect of oral pretreatment with kanamycin sulphate on the disposition of salicyl-L-alanine administered intravenously, because of poor absorption of kanamycin sulphate from the gastrointestinal tract.

The intestinal epithelial cells contain many enzymes including peptidases. Prodrugs based on this consideration were reported by Koike et al (1986a, b) and Persico et al (1988). Therefore, the hydrolysis of salicyl-L-alanine by the intestinal mucosa was examined employing an in-situ intestinal sac preparation, into which drug solution was injected. The appearances of salicyl-L-alanine and salicylic acid into the mesenteric venous blood was measured directly by cannulating the mesenteric vein of exposed rabbit intestine and collecting all venous blood draining from the absorbing region. Fig. 3 shows the appearance of salicyl-L-alanine into the mesenteric venous blood after injection in the intestinal lumen, while salicylic acid could not be detected, indicating that salicyl-L-alanine was not metabolized by the intestinal mucosa.

Salicyl-L-alanine-hydrolysing activities of the contents from different regions of the intestinal tract were examined (Fig. 4). Salicyl-L-alanine-hydrolysing activities of the contents from the jejunum and upper ileum were negligible. The formation of salicylic acid from salicyl-L-alanine increased with time in the experiments with caecum and colon contents, indicating that the contents from the hind gut were the major sources of metabolism of salicyl-L-alanine. These findings were consistent with the results indicating the *N*-benzoyl amino acid derivatives are hydrolysed by salicyluric acid-hydrolysing enzyme (Ogushi et al 1988). Activities of pancreatic amylase and total protease were reported to decrease distally from the small bowel to the sigmoid/rectum region of the large intestine in man (Macfarlane et al 1989), suggesting that salicyl-L-alanine is not hydrolysed by digestive enzymes, but by intestinal microorganisms.

In order to investigate the distribution of salicyl-L-alanine-hydrolysing enzyme in the caecum content, we separated the caecum content by centrifugation and examined the hydrolysing activities in the supernatant and the residual fractions of caecum content. After incubation of salicyl-L-alanine with the supernatant and the residue fractions for 4 h at 37°C, 10 μg (1%) and 180 μg (18%) of salicylic acid was formed in the incubation media, respectively. Hydrolysing activity in the supernatant is considered to be due to the enzymes derived from the intestinal microorganisms, while the residual fraction contains intracellular and surface-bound enzymes of the intestinal microorganisms. Therefore, the intracellular and surface-bound enzymes seem to play a major role on the hydrolysis of salicyl-L-alanine.

The rectal route has a definite advantage over the oral route for drugs that are destroyed by gastric acidity or by enzymes in the intestinal wall. Potentially, the rectal route may also partially reduce the first-pass hepatic loss. Our previous report (Shibasaki et al 1985) showed that the luminal contents of the colon and faeces were responsible for salicyluric acid hydrolysis in rabbits. Further study (Nakamura et al 1988a) indicated that microbial metabolism of salicyluric acid might be responsible for the prolonged retention of salicylic acid in the blood following rectal administration in rabbits. These results suggested that

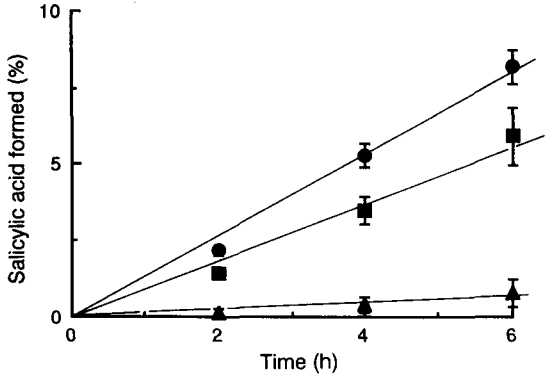

FIG. 4. Time courses of salicylic acid appearing in the medium during incubation of salicyl-L-alanine (500 μg: salicylic acid equivalent) with rabbit gut contents (0·5 g). Each point represents the mean ± s.e. of 6 experiments. ▲, Lower ileum; ●, caecum; ■, colon.

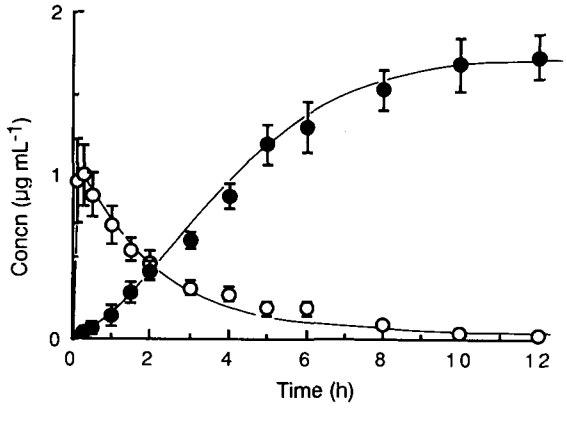

FIG. 5. Blood concentrations of salicyl-L-alanine measured as salicylic acid equivalent (○) and salicylic acid (●) following rectal administration of salicyl-L-alanine (5 mg kg^{-1}: salicylic acid equivalent) to rabbits. Each point represents the mean ± s.e. of 6 experiments.

salicyluric acid is hydrolysed by microorganisms within the rectum and that liberated salicylic acid is subsequently absorbed. Fig. 5 shows the blood concentrations of salicyl-L-alanine and salicylic acid following rectal administration of salicyl-L-alanine. Salicyl-L-alanine was absorbed intact or was hydrolysed to salicylic acid and subsequently absorbed. The blood concentration of salicylic acid increased gradually with time and reached a maximum at 12 h after the dose. These results suggest that microbial metabolism of salicyl-L-alanine may be responsible for the prolonged blood concentration of salicylic acid, and that salicyl-L-alanine is a useful prodrug which is converted to parent drug by intestinal microorganisms.

Acknowledgements

We thank Professor Sunao Furukawa, School of Pharmaceutical Sciences, Nagasaki University, for helpful discussion on the synthesis of salicyl-L-alanine. We wish to thank Kumiko Asai, Yoko Haraguchi, Mitsuhiko Kido, Michiyo Matsui and Yoshitake Maeda for skilled technical assistance. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, and by a Grant-in-Aid from the Mochida Memorial Foundation for Medical and Pharmaceutical Research.

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