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Hydrolysis of salicylic acid-tyrosine and salicylic acid-methionine prodrugs in the rabbit

Junzo Nakamura, Mitsuhiko Kido, Koyo Nishida and Hitoshi Sasaki

School of Pharmaceutical Sciences, Nagasaki University, Bunkyo-machi, Nagasaki 852 (Japan)

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Summary

We examined the hydrolysis of salicylic acid-tyrosine (salicyl-tyrosine) and salicylic acid-methionine (salicyl-methionine) conjugates following oral, intravenous, intracecal and rectal administration (434, 72, 36 and 36 μmol/kg, respectively: salicylic acid equivalent). Salicylic acid was detected in the blood following oral administration of salicyl-tyrosine and salicyl-methionine. In the case of salicyl-tyrosine, a relatively high blood concentration of salicylic acid was observed up to 36 h. Furthermore, the blood concentration of salicylic acid was sustained following rectal administration of salicyl-tyrosine and salicyl-methionine. A large difference in salicylic acid formation following oral administration was observed between salicyl-tyrosine and salicyl-methionine. Salicyl-tyrosine and salicyl-methionine were recovered from blood unchanged following intravenous administration, suggesting that presystemic de-conjugation was responsible for metabolism of the prodrugs following oral administration. Extensive salicylic acid formation in the cecum was found following intracecal administration of salicyl-tyrosine and salicyl-methionine. Also, in vitro incubation of salicyl-tyrosine and salicyl-methionine with gut contents showed that the primary location of hydrolysis was the hind gut. These results suggest that limited gastric and small intestinal absorbability of prodrug and metabolism by intestinal microorganisms were the major determinants of systemic bioavailability of parent drug.

Introduction

In our previous investigations, we demonstrated that glycine conjugate of salicylic acid (salicyluric acid) was metabolized to salicylic acid by intestinal microorganisms in rabbits (Shibasaki et al., 1985; Nakamura et al., 1986, 1988a, 1989a, 1990), rats (Nakamura et al., 1988b) and dogs (Nakamura et al., 1989b) and is a candidate for a prodrug to control the blood concentration of salicylic acid quantitatively.

Recently, the salicyluric acid-hydrolyzing enzyme purified from intestinal microorganisms was reported to catalyze the hydrolysis of *N*-benzoyl amino acids and their derivatives (Ogushi et al., 1988). Therefore, the metabolism of the prodrug could be varied by changing the amino acid moiety of the salicylic acid conjugate. Taking this finding into consideration, we examined the dis-

Correspondence to: J. Nakamura, School of Pharmaceutical Sciences, Nagasaki University, Bunkyo-machi, Nagasaki 852, Japan.

tribution and metabolism of salicylic acid-L-alanine conjugate (salicyl-L-alanine) in rabbits (Nakamura et al., 1992). Although extensive salicylic acid formation was observed following oral administration of prodrug, salicyl-L-alanine, the prodrug was absorbed considerably from the gastrointestinal tract.

In the present study, we prepared salicylic acid-tyrosine (salicyl-tyrosine) and salicylic acid-methionine (salicyl-methionine) conjugates, and examined their distribution and metabolism following oral, intravenous, intracecal and rectal administration to rabbits as part of our series of investigations on salicylic acid-amino acid conjugates. Since tyrosine and methionine, which respectively contain a benzene ring and a sulfur atom, are unique among amino acids, the pharmacokinetic characteristics of these compounds should provide useful information compared with other amino acid prodrugs. Finally, we aimed to develop an effective prodrug of salicylic acid, by exploring the general features of the pharmacokinetic characteristics of salicylic acid-amino acid conjugates.

Materials and Methods

Chemicals

Acetylsalicylic acid, L-tyrosine, L-methionine, acetonitrile, acetic acid, methanol and *o*-anisic acid were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals were of reagent grade.

Syntheses of salicyl-tyrosine and salicyl-methionine

Salicyl-tyrosine and salicyl-methionine were synthesized by coupling of tyrosine or methionine methyl ester, and acetylsalicylic acid, respectively, using a carbodiimide as described previously (Nakamura et al., 1992). Finally, 17.5% of salicyl-tyrosine or 38.5% of salicyl-methionine was obtained as white crystals: m.p. 172 and 95°C; $[\alpha]_D^{20} = -50.0$ and $-19.3°$ (1% (w/v) in acetone), respectively. The chemical structure of the products were ascertained by NMR, mass spectrum and elemental analyses. Analysis of salicyl-tyrosine: Calculated for $C_{16}H_{15}NO_5$: C, 63.78; H, 5.02; N, 4.65. Found: C, 63.43; H, 5.08; N, 4.63. EI-MS m/z: 301. Analysis of salicyl-methionine: Calculated for $C_{12}H_{15}NO_4S$: C, 53.52; H, 5.61; N, 5.20. Found: C, 53.31; H, 5.48; N, 5.21. EI-MS m/z: 269. 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.

Stability analysis was carried out in 0.1 M phosphate buffer solutions of pH 2.5, 6.0 and 7.5 at 37°C, employing a drug concentration of 100 μg/ml as salicylic acid. The pH of the buffer solution was adjusted to the desired value by using a buffer system consisting of 0.1 M H_3PO_4 and 0.1 M $Na_2HPO_4 \cdot 12H_2O$. Salicyl-tyrosine and salicyl-methionine were completely stable (100% remaining) at each pH after a 24 h incubation.

Animals

Male albino rabbits weighing 2–3 kg were used throughout the study. The animals 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 prior to use for experiments but had free access to water. Salicyl-tyrosine and salicyl-methionine were dissolved in NaOH (equivalent to salicyl-tyrosine or salicyl-methionine). Following oral, intravenous, intracecal and rectal administration of drug, blood was collected with a heparinized syringe at appropriate time intervals from an ear vein and centrifuged at $8000 \times g$ for 5 min. Blood concentration of drug was calculated as salicylic acid from the calibration curve. The area under the blood concentration-time curve (AUC) was calculated by the trapezoidal method (Yamaoka et al., 1978).

Oral administration of drug: The drug solution (434 μmol/kg: salicylic acid equivalent) was administered orally by gastric intubation.

Intravenous administration of drug: The drug solution (72 μmol/kg: salicylic acid equivalent) was administered intravenously via an ear vein.

Intracecal administration of drug: Animals were anesthetized with sodium pentobarbital (25 mg/kg), given intravenously, via an ear vein. After complete anesthesia, midline incision (2–3 cm) was made, and the drug solution (36 μmol/kg: salicylic acid equivalent) was administered by direct injection into the cecum by syringe. Leakage of drug solution at the injection site was not observed. The abdomen was closed with operative stitching.

Rectal administration of drug: The drug solution (36 μmol/kg: 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 vitro incubation of salicyl-tyrosine and salicyl-methionine with gut contents

Non-starved rabbits were anesthetized with an intravenous injection of sodium pentobarbital (25 mg/kg). After the five segments (jejunum, upper ileum, lower ileum, cecum and colon) of the intestine were cut open, their contents were collected separately. 10 ml portion of salicyl-tyrosine or salicyl-methionine (100 μg/ml: salicylic acid equivalent) in saline was added to a gut content (1 g wet weight) and the mixture was incubated for 6 h at 37°C. At appropriate time intervals, a 1 ml portion of the mixture was withdrawn and centrifuged at $8000 \times g$ for 5 min. The supernatant (0.2 ml) was subjected to assay.

Analytical method

Salicyl-tyrosine, salicyl-methionine and salicylic acid in blood and supernatant fluid in the in vitro experiment were analyzed by HPLC according to the method of Cham et al. (1979) with slight modifications. Details of the analytical method have been described in our previous paper (Nakamura et al., 1992). The chromatographic mobile phase consisted of a mixture of acetic acid-methanol-water (4 : 35 : 65 and 4 : 45 : 55 v/v, for detection of salicyl-tyrosine and salicyl-methionine, respectively). In the case of detection of salicyl-tyrosine, the retention times of salicyl-tyrosine, salicylic acid and the internal standard were 14.7, 12, and 6.8 min, respectively. For salicyl-methionine, the retention times of salicyl-methionine, salicylic acid and the internal standard were 9.7, 7.4, and 4.3 min, respectively.

Results and Discussion

The use of metabolism in intestinal microorganisms has been attracting attention in the field of prodrug development. However, little is known about the relationship between the physicochemical properties and disposition characteristics of

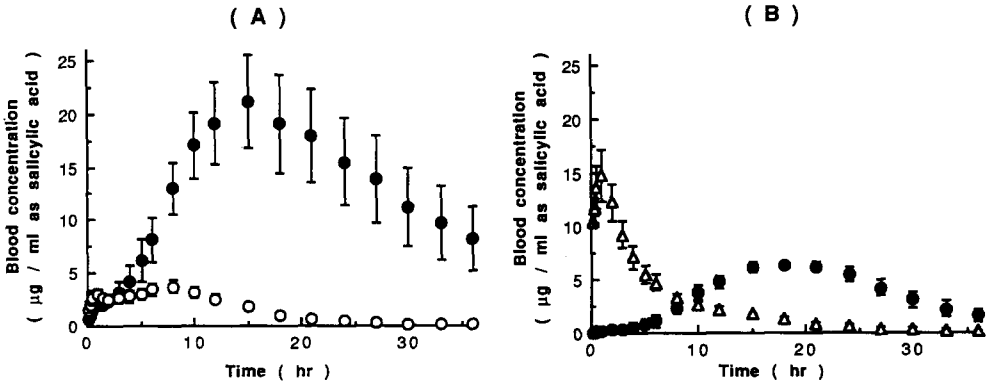

Fig. 1. Blood concentration of salicyl-tyrosine or salicyl-methionine, and salicylic acid following oral administration of salicyl-tyrosine (A) and salicyl-methionine (B) (434 μmol/kg: salicylic acid equivalent) to rabbits. (A) (○) Salicyl-tyrosine, (●) salicylic acid (6); (B) (△) salicyl-methionine, (●) salicylic acid (5). Numbers in parentheses represent number of experiments. Each point represents the mean ± S.E.

prodrugs. The overall goal of this research is the accumulation of systematic information on the pharmacokinetic characteristics of several amino acid-salicylic acid conjugates having various physicochemical properties.

Oral administration of salicyl-tyrosine and salicyl-methionine

The blood concentrations of salicyl-tyrosine or salicyl-methionine, and salicylic acid following oral administration of these prodrugs were determined in rabbits. As shown in Fig. 1A, a small amount of salicyl-tyrosine (< 3.7 μg/ml, as salicylic acid) was detected in the blood. Salicylic acid appeared in the blood gradually and reached a peak blood concentration (21.1 μg/ml) at 15 h, indicating hydrolysis of salicyl-tyrosine. The blood concentration of salicylic acid remained above 8.2 μg/ml up to 36 h. Similarly to salicyl-tyrosine, salicylic acid was also detected in the blood following oral administration of salicyl-methionine and reached a maximum level (6.4 μg/ml) at 18 h after dosing (Fig. 1B). The AUC(0–36 h) value for salicylic acid following oral administration of salicyl-tyrosine was greater than that of salicyl-methionine (28586.2 vs 7915.9 μg ml^{-1} min). In contrast, the AUC values for salicyl-tyrosine and salicyl-methionine were reversed (3153.8 vs 5536.9 μg ml^{-1} min). The difference in the blood concentration of salicylic acid following oral administration of salicyl-tyrosine and salicyl-methionine may be due to variation in the absorbability of prodrug from the stomach and intestines. In order to deliver salicylic acid as proposed, the prodrugs must reach the cecum/colon in large amounts in order to utilize the metabolic activity of the gut microflora to slowly release salicylic acid, followed by its absorption across the colonic mucosa. Also, the difference in hydrolyzing activity of enzyme was considered to be one of the causes of differences in the systemic bioavailability of salicylic acid.

Recently, it was reported that steroid glycosides and the unique glycosidase activity of the colonic microflora form the basis of a colon-specific drug delivery system (Friend and Chang, 1984, 1985; Friend et al., 1991a,b; Tozer et al., 1991). Drug glycosides are hydrophilic and, thus, are poorly absorbed from the stomach and small intestine. Once such a glycoside reaches the colon it can be cleaved by bacterial glycosidases, releasing the parent drug to be absorbed by the colonic mucosa. Based on the data obtained, salicyl-tyrosine was apparently transported to the cecum and colon after passing through the stomach and small intestine without appreciable loss due to absorption.

Intravenous administration of salicyl-tyrosine and salicyl-methionine

Salicyl-tyrosine and salicyl-methionine were administered intravenously to examine the sys-

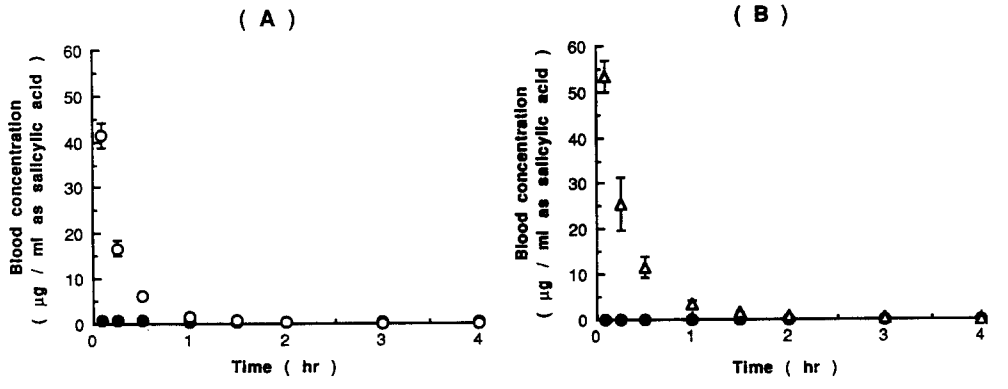

Fig. 2. Blood concentration of salicyl-tyrosine or salicyl-methionine, and salicylic acid following intravenous administration of salicyl-tyrosine (A) and salicyl-methionine (B) (72 μmol/kg: salicylic acid equivalent) to rabbits. (A) (○) Salicyl-tyrosine, (●) salicylic acid (4); (B) (△) salicyl-methionine, (●) salicylic acid (5). Numbers in parentheses represent number of experiments. Each point represents the mean ± S.E.

temic de-conjugation. Both salicyl-tyrosine and salicyl-methionine were rapidly eliminated from the blood (Fig. 2A and B). The blood concentration profiles of the two prodrugs were similar and no significant difference was seen in the AUC value between salicyl-tyrosine (749.2 ± 149.9 μg ml^{-1} min) (mean ± S.D., $N = 4$) and salicyl-methionine (1185.6 ± 406.8 μg ml^{-1} min) (mean ± S.D., $N = 5$). At the same time, a trace amount of salicylic acid was detected with salicyl-tyrosine and salicyl-methionine. The location of metabolism is probably the intestinal mucosa and/or in the lumen of the small and/or large intestine. In the latter case, intestinal microorganisms might be responsible for presystemic de-conjugation of the prodrugs. Since we previously showed that the intestinal mucosal de-conjugation of salicyl-L-alanine did not occur in the in situ intestinal sac preparation with complete mesenteric venous blood collection (Nakamura et al., 1992), it appears that metabolism by intestinal microorganisms is mainly responsible for the presystemic de-conjugation of salicyl-tyrosine and salicyl-methionine.

Intracecal administration of salicyl-tyrosine and salicyl-methionine

Scheline (1968, 1973) reviewed much of the literature on the distribution of microorganisms in the gastrointestinal tract and indicated that the stomach, duodenum, jejunum and upper ileum are only sparsely populated. Increasing numbers of organisms exist in the distal ileum, and a significant increase in number is seen at the ileocecal valve in humans. In addition, in the review of Williams (1972), it was reported that the location of microorganisms along the gastrointestinal tract was similar in rabbits and humans. Therefore, salicyl-tyrosine and salicyl-methionine were administered directly into the cecum followed by measurement of salicylic acid and its prodrugs in blood. Fig. 3A and B shows the blood concentration of salicyl-tyrosine or salicyl-methionine, and salicylic acid following intracecal administration of the prodrugs. Both salicyl-tyrosine and salicyl-methionine were detected at relatively low concentrations. The low blood concentration profiles of these prodrugs indicate the extensive formation of salicylic acid from them in the cecum. It was thus demonstrated that salicyl-tyrosine and salicyl-methionine were hydrolyzed by the intestinal microorganisms. Since there is a possibility that salicylic acid produced by microorganisms and these prodrugs has antibacterial activity, it might affect the hydrolytic activity of intestinal microorganisms. Therefore, further studies are needed to examine the effect of salicylic acid and these prodrugs on the intestinal microorganisms. No significant difference in the AUC(0–12 h) for salicylic acid was observed between salicyl-tyro-

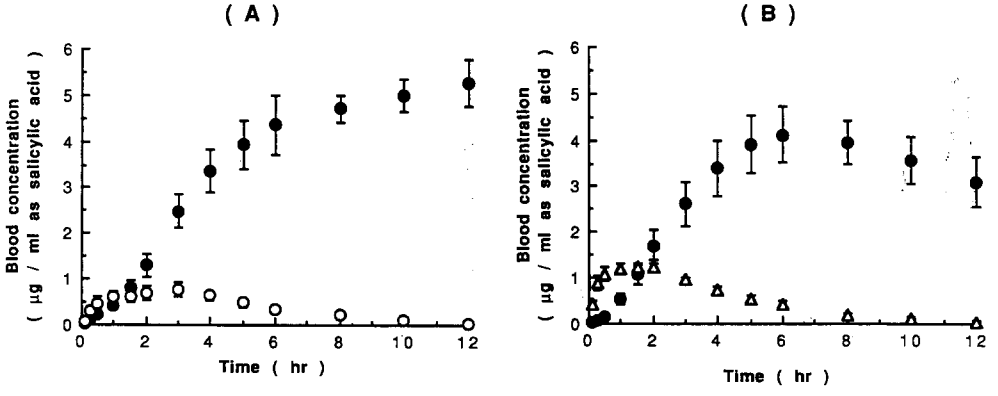

Fig. 3. Blood concentration of salicyl-tyrosine or salicyl-methionine, and salicylic acid following intracecal administration of salicyl-tyrosine (A) and salicyl-methionine (B) (36 μmol/kg: salicylic acid equivalent) to rabbits. (A) (○) Salicyl-tyrosine, (●) salicylic acid (5); (B) (△) salicyl-methionine, (●) salicylic acid (7). Numbers in parentheses represent number of experiments. Each point represents the mean ± S.E.

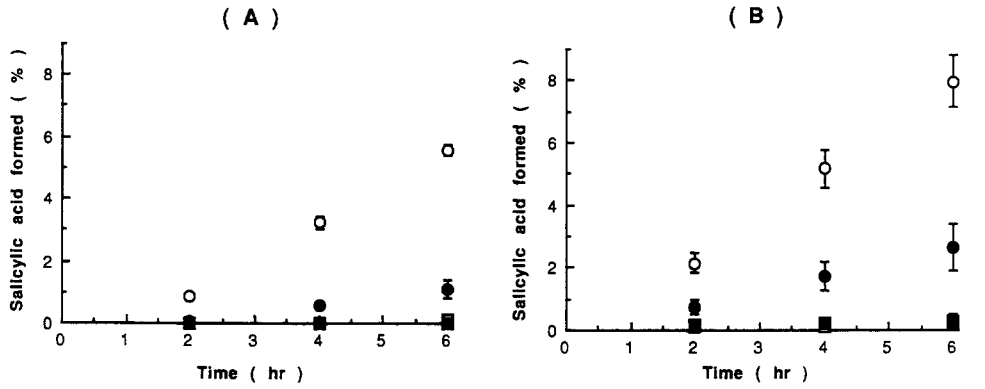

Fig. 4. Time courses of salicylic acid appearing in the medium during incubation of salicyl-tyrosine (A) and salicyl-methionine (B) (1000 μg salicylic acid equivalent) with rabbit gut contents (1 g wet weight). Each point represents the mean $\pm$ S.E. of eight experiments. (▲) jejunum, (□) upper ileum, (■) lower ileum, (○) cecum, (●) colon.

sine (2562.6 $\pm$ 458.5 μg ml^{-1} min) (mean $\pm$ S.D., $N = 5$) and salicyl-methionine (2187.1 $\pm$ 762.9 μg ml^{-1} min) (mean $\pm$ S.D., $N = 7$).

In vitro incubation of salicyl-tyrosine and salicyl-methionine with gut contents

The salicyl-tyrosine- and salicyl-methionine-hydrolyzing activities in the contents from different regions of the intestinal tract were determined. The results are shown in Fig. 4A and B. The hydrolyzing activities in the contents from the jejunum, upper ileum and lower ileum were almost negligible. In contrast, the formation of salicylic acid from salicyl-tyrosine and salicyl-methionine increased with time in the contents from the cecum and colon, indicating that the contents from the hind gut were the major source of metabolism of salicyl-tyrosine and salicyl-methionine. In addition, the cecal content displayed greater hydrolysis activity than the colonic content in both prodrugs. This is probably due to the difference in population and class of microorganism in cecum and colon.

Since there are many indications that intestinal microorganisms exist mainly in the cecum and colon, such observations provide support for the hypothesis that salicyl-tyrosine and salicyl-methionine are hydrolyzed by intestinal microor-

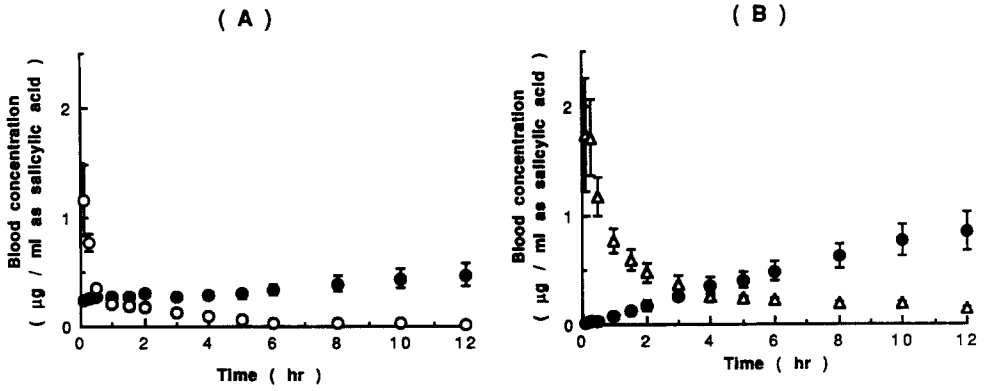

Fig. 5. Blood concentration of salicyl-tyrosine or salicyl-methionine, and salicylic acid following rectal administration of salicyl-tyrosine (A) and salicyl-methionine (B) (36 μmol/kg: salicylic acid equivalent) to rabbits. (A) (○) Salicyl-tyrosine, (●) salicylic acid (5); (B) (△) salicyl-methionine, (●) salicylic acid (5). Numbers in parentheses represent number of experiments. Each point represents the mean $\pm$ S.E.

ganisms in rabbits. 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 humans (Macfarlane et al., 1989). While this finding suggests that salicyl-tyrosine and salicyl-methionine are not hydrolyzed by digestive enzymes, further studies are needed to clarify the precise mechanism by which salicyl-tyrosine and salicyl-methionine are hydrolyzed in rabbit gastrointestinal tract.

The hydrolyzing activity of salicyl-methionine in the contents from the cecum and colon was higher than that of salicyl-tyrosine, but not significant. This finding suggests that the large difference in the systemic bioavailability of salicylic acid following oral administration of salicyl-tyrosine and salicyl-methionine was caused by differences in gastric and/or intestinal rate of absorption.

Rectal administration of salicyl-tyrosine and salicyl-methionine

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.

Both salicyl-tyrosine and salicyl-methionine were absorbed intact to some extent following rectal administration (Fig. 5A and B). Portions of salicyl-tyrosine and salicyl-methionine doses were hydrolyzed to salicylic acid, which was subsequently absorbed. The blood concentration of salicylic acid increased gradually with time up to 12 h following prodrug dosing. These results suggest that microbial metabolism of salicyl-tyrosine and salicyl-methionine was responsible for the prolonged blood concentration of salicylic acid after rectal administration. In contrast with oral administration, the AUC(0–12 h) value for salicylic acid following rectal administration of salicyl-tyrosine (252.8 μg ml^{-1} min) was similar to that of salicyl-methionine (340.4 μg ml^{-1} min).

In conclusion, extended blood concentration of salicylic acid following oral administration of salicyl-tyrosine was observed compared with salicyl-methionine. It appears that poor absorbability is required so that substantial amounts of prodrug reach the primary site utilizing the metabolism of the intestinal microorganisms. We are currently performing experiments to obtain more prolonged and constant blood concentrations of salicylic acid through the use of other prodrugs.

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