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A Novel Prodrug of Salicylic Acid, Salicylic Acid–Glutamic Acid Conjugate Utilizing Hydrolysis in Rabbit Intestinal Microorganisms

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The fate of salicylic acid–glutamic acid conjugate (salicyl-glutamic acid) following oral, intravenous, intracecal and rectal administration (60, 10, 5 and 5 mg/kg, respectively: salicylic acid equivalent) was examined in rabbits. Salicylic acid was detected in the blood 2 h after oral administration of salicyl-glutamic acid and it reached the maximum level (69.4 μg/ml) at 18 h after the dose. A high blood concentration of salicylic acid (24.8 μg/ml) was observed up to 36 h. But only a small amount of salicyl-glutamic acid was detected in the blood (<2.5 μg/ml, as salicylic acid). In contrast, unchanged salicyl-glutamic acid was found mainly in the blood following intravenous administration of salicyl-glutamic acid, suggesting that presystemic de-conjugation of salicyl-glutamic acid predominantly occurred. The intestinal mucosal de-conjugation of salicyl-glutamic acid was negligible in the *in situ* intestinal sac preparation with complete mesenteric venous blood collection. Immediate and very extensive salicylic acid formation in the cecum was found following intracecal administration of salicyl-glutamic acid. After oral pretreatment of rabbits with kanamycin sulfate (6 × 400 mg), a significant inhibition of salicylic acid formation following intracecal administration of salicyl-glutamic acid was observed, indicating that the intestinal microorganisms were responsible for the biotransformation of salicyl-glutamic acid. Also, *in vitro* incubation of salicyl-glutamic acid with gut contents showed that the primary location of hydrolysis was the hind gut.

Keywords salicylic acid; salicylic acid–glutamic acid conjugate; prodrug; hydrolysis; presystemic de-conjugation; rabbit; intestinal microorganism

In our previous reports, we demonstrated that a glycine conjugate of salicylic acid (salicyluric acid) was metabolized to salicylic acid by intestinal microorganisms in rabbits,[1-3] rats[4] and dogs[5] and is a candidate as a prodrug to prolong the blood concentration of salicylic acid. However, a problem with the prodrug itself being absorbed from the stomach and/or small intestine to some degree remains to be improved.

There are two major considerations for enhancing the efficiency of a prodrug utilizing the metabolism of intestinal microorganisms: (1) orally administered prodrug reaches the large intestine, in which a large number of intestinal microorganisms exist, followed by passing through the stomach and the small intestinal tract; and (2) the parent drug is chemically modified to control metabolism by enzymes derived from intestinal microorganisms. Since the salicyluric acid-hydrolyzing enzyme purified from intestinal microorganisms was reported to catalyze the hydrolysis of *N*-benzoyl amino acids and their derivatives,[6] the metabolic activity to prodrug could be varied by changing the amino acid moiety of the salicylic acid conjugate. Furthermore, absorbability could be altered by substituting a side chain which could increase the hydrophilicity of the prodrug.

Based on these considerations, we prepared salicylic acid–glutamic acid conjugate (salicyl-glutamic acid) and examined its behavior in rabbits to develop a more potent prodrug of salicylic acid.

Materials and Methods

Chemicals Acetylsalicylic acid, L-glutamic acid, acetonitrile, acetic acid, methanol and *o*-ethoxybenzamide were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Kanamycin sulfate was obtained from Meiji Seika Kaisha, Ltd. (Tokyo, Japan). All other chemicals were of a reagent grade.

Synthesis of Salicyl-glutamic Acid Salicyl-glutamic acid was synthesized by coupling glutamic acid methyl ester and acetylsalicylic acid using a carbodiimide method. To a suspension of 41 g of glutamic acid in 200 ml of methanol, 30 ml of thionyl chloride was added slowly at 0 °C with stirring. The mixture was then left overnight at room temperature. The reaction mixture was concentrated under reduced pressure. It was recrystallized from methanol–ether to yield 89% of glutamic acid methyl ester hydrochloride as white crystals (mp 78 °C). To a solution of 10 g of glutamic acid methyl ester hydrochloride in methanol, 11 g of triethylamine was added slowly and stirred for 2 h at 0 °C. The reaction mixture was then filtered. An oily glutamic acid methyl ester was obtained, followed by concentrating the filtrate under reduced pressure. *N,N'*-Dicyclohexylcarbodiimide (11.4 g) was added to a solution of 10 g of acetylsalicylic acid in a small amount of tetrahydrofuran anhydride–methylene chloride. Subsequently, 8 g of glutamic acid methyl ester was added to the reaction mixture. It was stirred for 3 h at 0 °C and left overnight at room temperature, then filtered. The filtrate was concentrated under reduced pressure. The residue was washed with 5% $NaHCO_3$. To a solution of 8 g of acetylsalicylic acid–glutamic acid methyl ester in a small amount of methanol, 100 ml of 1 N NaOH solution was added and then stirred for 1 h. Next, methanol was evaporated under reduced pressure. The reaction mixture was washed with ether, followed by acidification with conc. HCl at 0 °C. It was extracted from ethyl acetate, and then washed with water and subsequently saturated NaCl solution. After being dried with Na_2SO_4 anhydride, the product was concentrated under reduced pressure and then recrystallized from ethyl acetate–*n*-hexane to yield 33% of salicyl-glutamic acid as white crystals: mp 162—164 °C; $[\alpha]_D^{21}$ −3.8° (1% (w/v) in MeOH). The chemical structure of the product was ascertained by nuclear magnetic resonance, mass spectrum and elemental analyses. Analysis: Calculated for $C_{12}H_{13}NO_6$: C, 53.93; H, 4.90; N, 5.24. Found; C, 53.87; H, 4.90; N, 5.17. EI-MS *m/z*: 267. Nuclear magnetic resonance 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 Micro-analysis, Nagasaki University.

Determination of Apparent Partition Coefficient To a 5 ml portion of salicylic acid or its derivatives (0.5 mM) dissolved in 0.1 N HCl (pH 1.2), 5 ml of chloroform saturated with distilled water was added. The mixture was strongly shaken for 30 s at 10-min intervals for 1-h periods, and then left for 4 h at 37 °C. Concentrations in the aqueous phase were determined by high performance liquid chromatography. The apparent partition coefficient was calculated employing the equation $(C_0 - C_w)/C_w$, where C_0 and C_w are the initial concentration and the concentration after shaking in the water phase, respectively. The apparent partition coefficients of salicylic acid, salicyl-glutamic acid and salicyluric acid were calculated to be 3.40, 0.03 and 0.10, respectively.

Animals Male albino rabbits (2—3 kg) 4—5 months of age 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-glutamic acid was dissolved in NaOH (2 eq of salicyl-glutamic acid) and administered as follows. Following oral, intravenous, intracecal and rectal administration of the drug (60, 10, 5 and 5 mg/kg, respectively: salicylic acid equivalent), blood was collected with a heparinized syringe at appropriate time intervals from an ear vein. The area under the blood concentration–time curve (*AUC*) was calculated by the trapezoidal method.[7]

Oral Administration: The drug solution was administered orally by gastric intubation.

Intravenous Administration: The drug solution was administered intravenously *via* an ear vein.

Intracecal Administration: Animals were anesthetized with sodium pentobarbital (25 mg/kg), given intravenously, *via* an ear vein. After complete anesthesia, a midline incision (2—3 cm) was made, and the drug solution was administered by direct injection into the cecum by syringe. No leakage of the drug solution at the injection site was observed. The abdomen was closed with operative stitching. Oral pretreatment of rabbits with kanamycin sulfate before intracecal administration of the drug was carried out as follows. The administration time of kanamycin sulfate was established by Gingell *et al.*[8] The animals received 6 × 400 mg/animal kanamycin sulfate which was dissolved in the water. Kanamycin sulfate was given orally twice daily for 2 d before salicyl-glutamic acid administration, and then 4 h before and 4 h after salicyl-glutamic acid administration.

Rectal Administration: The drug solution 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 prior to use for experiments but had free access to water. The surgical operation and other procedures were the same as reported previously.[9] In order to prepare the intestinal sac, midileal portions of the intestine (5—8 cm) were used. The intestinal lumen was washed with saline as completely as possible, and both sides of the intestine were ligated to prepare a closed sac. The mesenteric vein was cannulated with 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 3 ml of salicyl-glutamic acid solution (333 μg/ml: salicylic acid equivalent) into the intestinal lumen. The appearance of salicyl-glutamic acid and salicylic acid in the mesenteric venous blood was examined for 2 h. 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-glutamic Acid with Gut Contents** Fed 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. A 1 ml portion of salicyl-glutamic acid and salicyluric acid (100 μg/ml: salicylic acid equivalent) in saline was added to the gut content (0.1 g wet weight), and the mixture was incubated for 6 h at 37 °C. At appropriate time intervals, the mixture was centrifuged at 8000 × *g* for 10 min, and the supernatant (0.2 ml) was subjected to assay.

Analytical Method Salicyl-glutamic acid and salicylic acid in the blood and supernatant fluid in the *in vitro* experiment were analyzed by high performance liquid chromatography according to the method of Cham *et al.*[10] with slight modifications. Blood and supernatant fluid samples (0.2 ml) were added to acetonitrile (0.4 ml) containing the internal standard, *o*-ethoxybenzamide (30 μg/ml). The samples were mixed on a vortex-type mixer and centrifuged at 8000 × *g* for 10 min. The supernatant was filtered by passing it through a 0.45 μm pore size membrane filter (SJHVL04NS, Nihon Millipore Kogyo K. K., Yonezawa, Japan). Then, using a Hamilton syringe, 20 μl of the supernatant fluid was withdrawn and loaded onto the column. Calibration curves were constructed from data on the peak-area ratios of salicyl-glutamic acid and salicylic acid to the internal standard. We used an LC-6A pump, a RF-535 fluorescence detector, a Chromatopac C-R6A recorder (all from Shimadzu Co., Ltd., Kyoto, Japan) and a model 7125 sample injection valve (Rheodyne Inc., CA, U.S.A.). The stationary phase used was a Cosmosil $5C_{18}$ packed column (150 × 4.6 mm i.d., Nacalai Tesque, Inc.), which was used at room temperature. The peak area of fluorescence intensity was recorded at excitation and emission wavelengths 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 by passing it through a 0.5 μm pore size membrane filter (T050A047A, Toyo Roshi Co., Ltd., Tokyo, Japan) before use. The flow rate was 1.0 ml/min. The retention times of salicyl-glutamic acid, salicylic acid and the internal standard were 6, 12 and 10.5 min, respectively.

Results

Oral Administration of Salicyl-glutamic Acid The blood concentration of salicyl-glutamic acid and salicylic acid following oral administration of salicyl-glutamic acid was determined. As shown in Fig. 1, a small amount of salicyl-glutamic acid (< 2.5 μg/ml, as salicylic acid) was detected in the blood. The absorption ratio of salicyl-glutamic acid was calculated to be 26% by dividing the *AUC*/dose of salicyl-glutamic acid following oral administration by that following intravenous administration. On the other hand, salicylic acid was detected at 2 h after the dose and reached a peak blood concentration (69.4 μg/ml) at 18 h, indicating that the hydrolysis of salicyl-glutamic acid occurred. The blood concentration of salicylic acid remained above 24.8 μg/ml at even 36 h. The *AUC* value (0—12 h) for salicylic acid following oral administration of salicyl-glutamic acid was 3-fold larger than that of salicyluric acid[11] (20889.0 *vs.* 6785.4 μg/ml·min).

Intravenous Administration of Salicyl-glutamic Acid In order to examine the systemic de-conjugation of salicyl-glutamic acid, it was administered intravenously. The results are shown in Fig. 2. Salicyl-glutamic acid was detected in the blood although it was rapidly eliminated from the blood within 2 h. In contrast, a trace amount of salicylic acid (< 0.11 μg/ml) was detected, suggesting that presystemic de-conjugation of salicyl-glutamic acid was mainly involved in its hydrolysis.

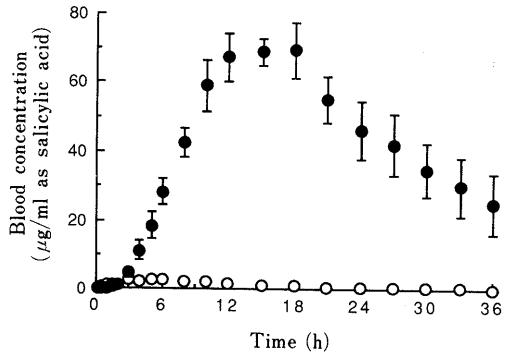

Fig. 1. Blood Concentration of Salicyl-glutamic Acid and Salicylic Acid Following Oral Administration of Salicyl-glutamic Acid (60 mg/kg: Salicylic Acid Equivalent) to Rabbits

○, salicyl-glutamic acid; ●, salicylic acid. Each point represents the mean ± S.E. of 5 experiments.

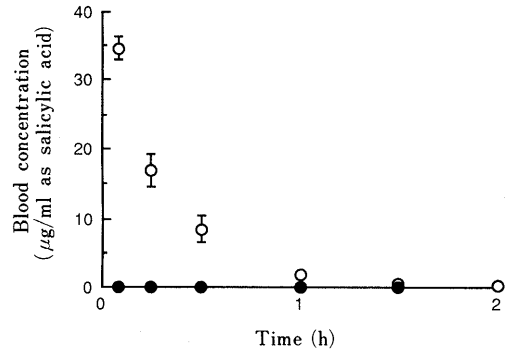

Fig. 2. Blood Concentration of Salicyl-glutamic Acid and Salicylic Acid Following Intravenous Administration of Salicyl-glutamic Acid (10 mg/kg: Salicylic Acid Equivalent) to Rabbits

○, salicyl-glutamic acid; ●, salicylic acid. Each point represents the mean ± S.E. of 4 experiments.

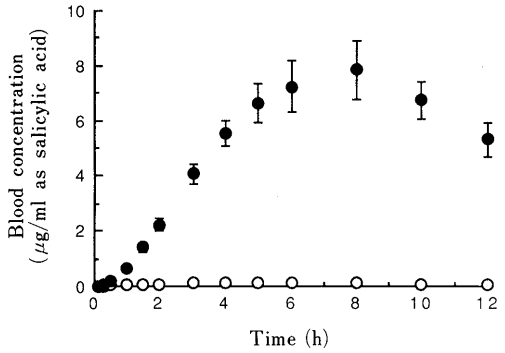

Fig. 3. Blood Concentration of Salicyl-glutamic Acid and Salicylic Acid Following Intracecal Administration of Salicyl-glutamic Acid (5 mg/kg: Salicylic Acid Equivalent) to Rabbits

○, salicyl-glutamic acid; ●, salicylic acid. Each point represents the mean ± S.E. of 8 experiments.

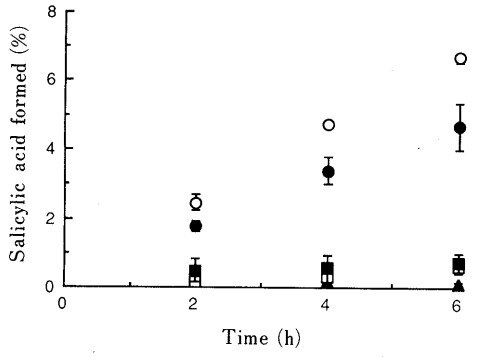

Fig. 5. Time Courses of Salicylic Acid Appearing in the Medium during Incubation of Salicyl-glutamic Acid (500 μg: Salicylic Acid Equivalent) with Rabbit Gut Contents (0.5 g)

▲, jejunum; □, upper ileum; ■, lower ileum; ○, cecum; ●, colon. Each point represents the mean ± S.E. of 6 experiments.

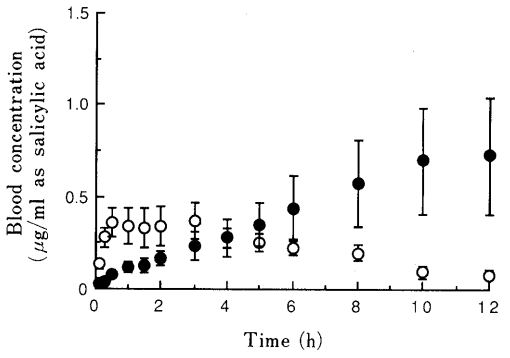

Fig. 4. Effect of Oral Pretreatment with Kanamycin Sulfate on Blood Concentration of Salicyl-glutamic Acid and Salicylic Acid Following Intracecal Administration of Salicyl-glutamic Acid (5 mg/kg: Salicylic Acid Equivalent) to Rabbits

○, salicyl-glutamic acid; ●, salicylic acid. Each point represents the mean ± S.E. of 5 experiments.

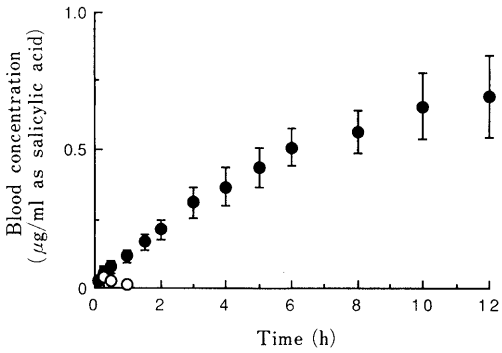

Fig. 6. Blood Concentration of Salicyl-glutamic Acid and Salicylic Acid Following Rectal Administration of Salicyl-glutamic Acid (5 mg/kg: Salicylic Acid Equivalent) to Rabbits

○, salicyl-glutamic acid; ●, salicylic acid. Each point represents the mean ± S.E. of 5 experiments.

Intracecal Administration of Salicyl-glutamic Acid Salicyl-glutamic acid was administered directly into the cecum to examine the mechanism of salicyl-glutamic acid hydrolysis in rabbits. The results are shown in Fig. 3. Salicyl-glutamic acid was detected at a very low concentration (<0.13 μg/ml, as salicylic acid). The blood concentration profile in Fig. 3 indicated immediate and very extensive salicylic acid formation from salicyl-glutamic acid in the cecum.

The effect of oral pretreatment with kanamycin sulfate (6 × 400 mg) on the blood concentration of salicyl-glutamic acid and salicylic acid following intracecal administration of salicyl-glutamic acid was examined. The oral pretreatment of rabbits with kanamycin sulfate resulted in a 92% reduction in the *AUC* value (0—12 h) of salicylic acid calculated from its blood concentration profile shown in Figs. 3 and 4 (3862.0 *vs.* 305.1 μg/ml·min).

Hydrolysis of Salicyl-glutamic Acid by Intestinal Mucosa The hydrolysis of salicyl-glutamic acid by the intestinal mucosa was examined by employing an *in situ* intestinal sac preparation into which the drug solution was injected. Only a very small percent of salicyl-glutamic acid and salicylic acid appeared in the mesenteric venous blood (4.7 and 0.3% of applied dose, respectively) and the greater part of applied salicyl-glutamic acid remained in the intestinal lumen (95.0 % of applied dose) at 2 h, indicating that the hydrolysis of salicyl-glutamic acid by the intestinal mucosa was negligible and salicyl-glutamic acid was poorly absorbed from the intestine.

***In Vitro* Incubation of Salicyl-glutamic Acid with Gut Contents** Salicyl-glutamic acid-hydrolyzing activities of the contents from different regions of the intestinal tract were determined. The results are shown in Fig. 5. The hydrolyzing activity in the contents from the jejunum, upper ileum and lower ileum were almost negligible. In contrast, the formation of salicylic acid from salicyl-glutamic acid increased with time in the contents from the cecum and colon, indicating that the contents from the hind gut were the major source of hydrolysis of salicyl-glutamic acid.

Rectal Administration of Salicyl-glutamic Acid Figure 6 shows the blood concentration of salicyl-glutamic acid and salicylic acid following rectal administration of salicyl-glutamic acid. Salicyl-glutamic acid was poorly absorbed in an intact form. A part of salicyl-glutamic acid was hydrolyzed to salicylic acid, which was subsequently absorbed. The blood concentration of salicylic acid increased gradually with time up to 12 h after the dose. It is suggested that salicyl-glutamic acid following the rectal administration was metabolized by intestinal microorganisms, judging from the results of *in vitro* incubation experiments.

Discussion

Prodrugs have been commonly used in drug delivery systems. A prodrug is inactive as administered and, then, should be converted to the parent drug chemically or by specific enzymes at the target site.

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.[12-16] Because drug glycosides are hydrophilic, they are poorly absorbed from the small intestine. Once such a glycoside reaches the colon it can be hydrolyzed by bacterial glycosidases, releasing the parent drug to be absorbed by the colonic mucosa. Furthermore, the significance of intestinal microorganisms to colon-specific delivery of other drugs, *i.e.*, insulin,[17] vasopressin,[18] naproxen[19-21] and narcotic antagonists[22] has been emphasized.

We selected salicylic acid, an analgesic belonging to aromatic acids, as a model drug. Orally ingested salicylic acid is absorbed rapidly in humans. Appreciable concentrations are found in plasma in less than 30 min; after a single dose, a peak value is reached in about 2 h and then gradually declines. The biotransformation of salicylic acid takes place in many tissues, but particularly in the liver. The three chief metabolic products are salicyluric acid, the ether, or phenolic glucuronide, and the ester, or acyl glucuronide. Salicyluric acid has been known to be metabolized by intestinal microorganisms in rabbits,[1-3] rats[4] and dogs.[5] Therefore, we prepared salicyl-glutamic acid and examined its behavior in rabbits, aiming to design a potent prodrug which is transported to the hind gut without being absorbed by the stomach and small intestine, then metabolized efficiently to a parent drug by intestinal microorganisms.

Following oral administration of salicyl-glutamic acid, only a small amount of salicyl-glutamic acid appeared in the blood, whereas salicylic acid was found extensively in the blood. In comparison with salicyluric acid,[1] the blood concentration of salicylic acid after oral administration of salicyl-glutamic acid was extremely large. From these results, it seemed that salicyl-glutamic acid was transported to the hind gut and metabolized to salicylic acid without being absorbed by the stomach and small intestine. The observation that the apparent partition coefficient of salicyl-glutamic acid (0.03) was smaller than that of salicyluric acid (0.10) may explain the reduction in the gastric and intestinal absorption of salicyl-glutamic acid.

Salicylic acid formation was negligible in the blood following intravenous administration of salicyl-glutamic acid, indicating that presystemic de-conjugation of salicyl-glutamic acid in the intestinal mucosa and/or by intestinal microorganisms might be related to its hydrolysis. Because we observed that the intestinal mucosal de-conjugation of salicyl-glutamic acid was negligible in the *in situ* intestinal sac preparation with complete mesenteric venous blood collection, the metabolism in the intestinal microorganisms is considered to be the major determinant of the presystemic metabolism of salicyl-glutamic acid. Furthermore, from this experiment, it was ascertained that the intestinal absorbability of salicyl-glutamic acid was very low.

Following intracecal administration of salicyl-glutamic acid, a small amount of salicyl-glutamic acid was detected in the blood, while immediate and very extensive salicylic acid formation was observed. Scheline 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 is seen at the ileocecal valve in humans.[23,24] It was thus suggested that salicyl-glutamic acid was hydrolyzed by intestinal microorganisms. In the case of oral administration, a lag time of about 2 h was observed before the appearance of salicylic acid in the blood. From these results, it seemed that the delays in detection and the maximum level of salicylic acid following oral administration of salicyl-glutamic acid were due mainly to the time required for gastrointestinal transit and partially to the hydrolysis to salicylic acid.

Furthermore, oral pretreatment of rabbits with kanamycin sulfate caused a significant inhibition in salicylic acid formation. These results provide support for the hypothesis that salicyl-glutamic acid was hydrolyzed to salicylic acid by the intestinal microorganisms in rabbits.

Further study was carried out to investigate the distribution of salicyl-glutamic acid-hydrolyzing activity in the gut contents. From the results of *in vitro* experimentation of incubation with gut contents, it was proved that the cecum and colon area was the major region of salicyl-glutamic acid hydrolysis, similar to salicyluric acid.[1] In addition, the hydrolyzing activity of salicyl-glutamic acid in the cecal contents (6.7% salicylic acid formed in 6 h) was low compared with that of salicyluric acid (38.1% salicylic acid formed in 6 h). On the other hand, the absorption ratio of salicyl-glutamic acid (26%) following oral administration was lower than that of salicyluric acid (66%).[11] Therefore, an increased extent of salicylic acid bioavailability following oral administration of salicyl-glutamic acid is not related to the increase of hydrolyzing activity of enzymes, but to the decrease of gastric and/or intestinal absorbability of salicyl-glutamic acid compared to salicyluric acid.

In conclusion, an extended and prolonged blood concentration of salicylic acid following oral administration of salicyl-glutamic acid was observed, suggesting the usefulness of salicyl-glutamic acid as a prodrug utilizing the metabolism of intestinal microorganisms. Furthermore, accumulation of systematic information on the pharmacokinetic properties of various amino acids and/or peptide conjugates of salicylic acid would improve the design of a prodrug which can control the blood concentration of salicylic acid.

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