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A Novel Prodrug of Salicylic Acid, Salicylic Acid-glycylglycine Conjugate, Utilizing the Hydrolysis in Rabbit Intestinal Microorganisms

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Abstract—The hydrolysis of salicylic acid-glycylglycine conjugate (salicyl-glycylglycine) following oral, intravenous, intracaecal and rectal administration (434, 72, 36 and 36 μmol kg^{-1}, respectively: equivalent to salicylic acid) was examined in rabbits to develop a novel prodrug of salicylic acid. Salicylic acid was detected in the blood 2 h after oral administration of salicyl-glycylglycine and it reached a maximum level (55·6 μg mL^{-1}) at 15 h, whereas a small amount of salicyl-glycylglycine was found in the blood. In contrast, unchanged salicyl-glycylglycine was found mainly in the blood following its intravenous administration, suggesting the involvement of presystemic deconjugation in the hydrolysis of salicyl-glycylglycine. Immediate and very extensive salicyclic acid formation in the caecum was observed following intracaecal administration of salicyl-glycylglycine, suggesting that the intestinal microorganisms were responsible for the biotransformation of this compound. In-vitro incubation of salicyl-glycylglycine with caecal content showed that salicyl-glycylglycine was hydrolysed efficiently in the caecum. Consequently, the blood concentration of salicylic acid was prolonged extensively following rectal administration of salicyl-glycylglycine, indicating the usefulness of salicyl-glycylglycine as a prodrug of salicylic acid.

In our previous investigations, we demonstrated that the 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).

Recently, salicyluric acid-hydrolysing enzyme was purified from an intestinal bacterium in rabbits, and characterized by Ogushi et al (1988). They reported that this enzyme catalysed the hydrolysis of *N*-benzoyl amino acids and their derivatives. Taking this finding into consideration, we examined the behaviour of salicylic acid-L-alanine conjugate (salicyl-L-alanine) as an example of salicylic acid-amino acid conjugate in rabbits (Nakamura et al 1992). Although the bioavailability of salicylic acid following oral administration of salicyl-L-alanine was enhanced significantly compared with salicyluric acid, absorption of salicyl-L-alanine itself from the gastrointestinal tract was unknown. Absorption of the prodrug is a disadvantage when using the prodrug approach to control blood concentrations of the parent drug through utilization of the metabolic enzymes of intestinal microorganisms in the caecum and colon. Therefore, in the present study, we prepared a salicylic acid-glycylglycine conjugate (salicyl-glycylglycine) as a model prodrug of salicylic acid-dipeptide conjugate and examined its behaviour following oral, intravenous, intracaecal and rectal administration.

Materials and Methods

Chemicals

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

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Synthesis of salicyl-glycylglycine

Salicyl-glycylglycine was synthesized by coupling of glycylglycine methyl ester and acetylsalicylic acid. To a suspension of 10 g of glycylglycine in 200 mL of methanol, 8·3 mL of thionyl chloride was added slowly at 0°C with stirring and left overnight at room temperature (21°C). The reaction mixture was concentrated under reduced pressure. The product was recrystallized from methanol–ether to yield about 90% of glycylglycine methyl ester hydrochloride as white crystals (mp 128–130°C). *N*-Methylmorpholine (1 g) was added to a solution of 1·8 g of acetylsalicylic acid in 20 mL of anhydrous tetrahydrofuran. Isobutyl chlorocarbonate (1·4 g) was added to the stirred mixture at −15°C, resulting in formation of a gel. After about 5 min, glycylglycine methyl ester was dissolved in a mixture of 15 mL of *N*, *N*-dimethylformamide and 1 g of *N*-methylmorpholine was added to the former product with stirring. The reaction mixture was stirred overnight at room temperature, followed by stirring for 30 min below 0°C. It was dissolved in ethyl acetate and then washed with 5% $NaHCO_3$, followed by concentrating under reduced pressure. To the reaction product, dissolved in a small amount of methanol, 24 mL of 1 M NaOH was added and the mixture was stirred for 10 min at 0°C. After acidification with conc. HCl, the product was concentrated under reduced pressure and recrystallized from methanol to yield about 69% of salicyl-glycylglycine as white crystals: mp 238–240°C. The chemical structure of the product was confirmed by NMR, mass spectrum and elemental analyses. Analysis: calculated for $C_{11}H_{12}N_2O_5$: C, 52·38; H, 4·80; N, 11·11. Found: C, 52·26; H, 4·80; N, 10·96. EI/MS m/z: 252. 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 Micro-analysis, Nagasaki University. The analytical results obtained were within ±0·4% of the theoretical values.

The stability experiment was carried out in 0·1 M phos-

phate buffer solutions of pH 2·5, 6·0 and 7·5 at 37°C at a drug concentration of 100 μg mL^{-1} as salicylic acid. Salicyl-glycylglycine was fairly stable after a 24 h incubation.

Determination of apparent partition coefficient

To a 5 mL portion of salicylic acid or salicyl-glycylglycine (0·5 mM) dissolved in 0·1 M HCl (pH 1·2), 5 mL of chloroform, saturated with distilled water, was added. The mixture was 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 HPLC. 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 and salicyl-glycylglycine were calculated to be 3·40 and 0·05, respectively.

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 24 h before experimentation but had free access to water. Salicyl-glycylglycine was dissolved in NaOH (equivalent to salicyl-glycylglycine). Appropriate amounts of drug solution were administered as follows. Following oral, intravenous, intracaecal and rectal administration of drug, 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 (Yamaoka et al 1978).

For oral administration of drug, the drug solution (434 μmol kg^{-1}: salicylic acid equivalent) was administered by gastric intubation.

For intravenous administration of drug, the drug solution (72 μmol kg^{-1}) was administered intravenously via an ear vein.

For intracaecal administration of drug, animals were first anaesthetized with sodium pentobarbitone (25 mg kg^{-1}), given intravenously, via an ear vein. After complete anaesthesia, a midline incision (2–3 cm) was made, and the drug solution (36 μmol kg^{-1}) 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.

For rectal administration of drug, the drug solution (36 μmol kg^{-1}) 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-glycylglycine with caecal contents

Fed rabbits were anaesthetized with intravenous injection of sodium pentobarbitone (25 mg kg^{-1}). After an incision into the caecum, the caecal contents were collected. A 1 mL portion of salicyl-glycylglycine (100 μg mL^{-1}) in saline was added to the caecal 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 HPLC assay. Salicyl-glycylglycine and salicylic acid were determined separately.

Analytical method

Salicyl-glycylglycine and salicylic acid in blood and supernatant fluid in the in-vitro experiment were analysed by HPLC according to the method of Cham et al (1979) with slight modifications. We used fluorescence intensity for detection instead of absorption measurement at 313 nm, which was employed by Cham et al (1979). Blood and supernatant fluid samples (0·2 mL) were added to acetonitrile (0·4 mL) containing 30 μg of the internal standard, *o*-anisic acid, in 1 mL. The samples were mixed on a vortex-type mixer and centrifuged at 8000 *g* for 10 min. The supernatant was filtered by passage through a 0·45 μm pore size membrane filter (SJHVL04NS, Nihon Millipore Kogyo K.K., Yonezawa, Japan). Twenty microlitres of the supernatant fluid was withdrawn using a Hamilton syringe and loaded onto the column. Calibration curves were constructed from data on the peak-area ratios of salicyl-glycylglycine and salicylic acid to 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, USA). The stationary phase used was a Cosmosil $5C_{18}$ packed column (150 × 4·6 mm i.d., Nacalai Tesque, Inc.). This column was used at room temperature (21°C). 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), which was filtered by passage 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^{-1}. The retention times of salicyl-glycylglycine, salicylic acid and the internal standard were 4, 12 and 7 min, respectively.

Results and Discussion

The blood concentration of salicyl-glycylglycine and salicylic acid following oral administration of salicyl-glycylglycine was determined in rabbits. As shown in Fig. 1, a large amount of salicylic acid was found in the blood, indicating that the hydrolysis of salicyl-glycylglycine occurred. A small amount of salicyl-glycylglycine was detected in the blood, suggesting that salicyl-glycylglycine itself was poorly absorbed from the stomach and small intestine. The AUC value (0–30 h) for salicyl-glycylglycine was about one-fourth of that for salicyl-L-alanine (850·0 vs 3720·5 μg mL^{-1} min) (Nakamura et al 1992). The apparent partition coefficient of salicyl-glycylglycine (0·05) was relatively low. This low partition coefficient may explain the limited penetration of salicyl-glycylglycine across the gastric and the intestinal mucosal membrane. On the other hand, salicylic acid was detected at 2 h after the dose and reached a peak blood concentration (55·6 μg mL^{-1}) at 15 h. The blood concentration of salicylic acid remained above 13·3 μg mL^{-1}, 36 h after the administration of the dose. The AUC value (0–30 h) for salicylic acid, calculated from the blood concentration profile following oral administration of salicyl-glycylglycine,

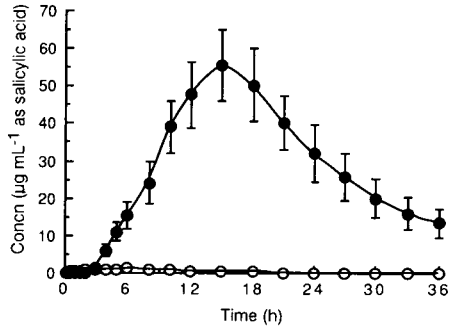

FIG. 1. Blood concentration of salicyl-glycylglycine (○) and salicylic acid (●) following oral administration of salicyl-glycylglycine (434 μmol kg^{-1}: salicylic acid equivalent) to rabbits. Each point represents the mean ± s.e. of 6 experiments.

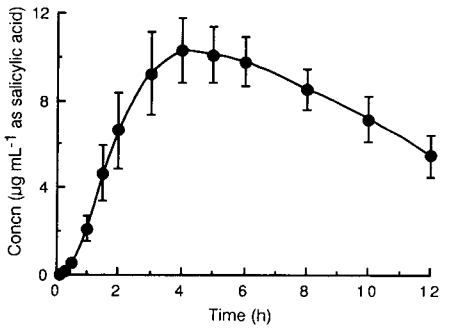

FIG. 3. Blood concentration of salicylic acid following intracaecal administration of salicyl-glycylglycine (36 μmol kg^{-1}: salicylic acid equivalent) to rabbits. Each point represents the mean ± s.e. of 6 experiments.

was about 3-fold that of salicyl-L-alanine (55720·6 vs 19933·0 μg mL^{-1} min) (Nakamura et al 1992).

In order to examine the systemic de-conjugation of salicyl-glycylglycine, it was administered intravenously to rabbits. The results are shown in Fig. 2. Salicyl-glycylglycine was detected in the blood although it was rapidly eliminated from the blood within 2 h. In contrast, negligible amounts of salicylic acid were detected in the blood at any time. These observations suggest the existence of presystemic de-conjugation of salicyl-glycylglycine.

Salicyl-glycylglycine was administered intracaecally to examine the mechanism of salicyl-glycylglycine hydrolysis in rabbits. Fig. 3 shows the blood concentration of salicylic acid following intracaecal administration of salicyl-glycylglycine. Salicylic acid reached a peak blood concentration (10·3 μg mL^{-1}) at 4 h after the dose, after which its blood concentration declined slowly. Salicyl-glycylglycine was not detected over the course of this experiment. The data presented in Fig. 3 indicate rapid and extensive formation of salicylic acid from salicyl-glycylglycine in the caecum. Salicylic acid was detected in the blood at 2 h and reached a maximum level at 15 h after oral administration of salicyl-glycylglycine (Fig. 1). From these results, we assume that the delay in detection and the maximum level of salicylic acid in blood following oral administration of salicyl-glycylglycine were mainly due to the time required for the gastrointestinal transit, and partially due to the hydrolysis to salicylic acid.

Salicyl-glycylglycine-hydrolysing activity of the caecal contents was examined by an in-vitro incubation experiment. The formation of salicylic acid from salicyl-glycylglycine increased with time in the caecal content. After a 60 min incubation period, 24·8% of applied dose was hydrolysed. Since many studies indicate that intestinal microorganisms exist mainly in the caecum and colon, data collected support the hypothesis that salicyl-glycylglycine was hydrolysed by intestinal microorganisms primarily in the caecum and large intestine of rabbits. Activities of pancreatic amylase and total protease reportedly decrease distally from the small bowel to the sigmoid/rectum region of the large intestine in man (Macfarlane et al 1989), suggesting that salicyl-glycylglycine is not hydrolysed by digestive enzymes, but by intestinal microorganisms.

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. A previous report (Shibasaki et al 1985) showed that the luminal contents of the colon and faeces were responsible for hydrolysis of salicyluric acid in rabbits. A further study (Nakamura et al 1988a) indicated that microbial metabolism

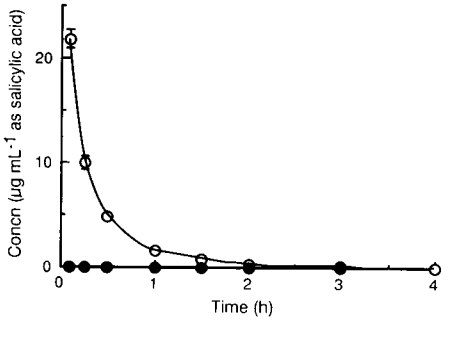

FIG. 2. Blood concentration of salicyl-glycylglycine (○) and salicylic acid (●) following intravenous administration of salicyl-glycylglycine (72 μmol kg^{-1}: salicylic acid equivalent) to rabbits. Each point represents the mean ± s.e. of 5 experiments.

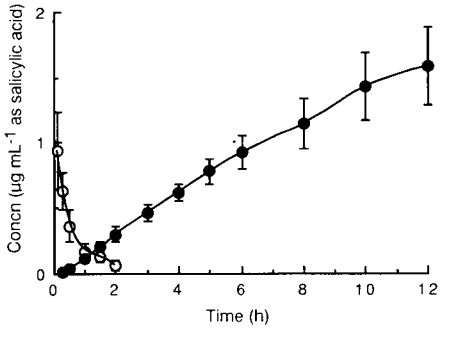

FIG. 4. Blood concentration of salicyl-glycylglycine (○) and salicylic acid (●) following rectal administration of salicyl-glycylglycine (36 μmol kg^{-1}: salicylic acid equivalent) to rabbits. Each point represents the mean ± s.e. of 6 experiments.

of salicyluric acid might be responsible for sustained blood levels of salicylic acid following rectal administration in rabbits. These results suggested that salicyluric acid was hydrolysed by microorganisms within the rectum and that liberated salicylic acid was subsequently absorbed. Fig. 4 shows the blood concentration of salicyl-glycylglycine and salicylic acid following rectal administration of salicyl-glycylglycine to rabbits. Salicyl-glycylglycine was rapidly absorbed from the rectum. Salicyl-glycylglycine was apparently hydrolysed to salicylic acid, which was subsequently absorbed. The blood concentration of salicylic acid increased gradually with time over the 12 h experiment. These results suggest the involvement of microbial metabolism in the hydrolysis of salicyl-glycylglycine to salicylic acid after rectal administration.

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 & 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. In a similar pattern, salicyl-glycylglycine was apparently transported to the caecum and colon following passage through the stomach and small intestine. Furthermore, the significance of intestinal microorganisms to colon-specific delivery of other drugs, including insulin (Saffran et al 1986), vasopressin (Saffran et al 1988), naproxen (Harboe et al 1989a, b; Larsen et al 1989) and narcotic antagonists (Simpkins et al 1988) has been emphasized.

In conclusion, blood concentration profiles of salicylic acid following oral and rectal administration of salicyl-glycylglycine suggest the potential use of intestinal microorganisms in releasing salicylic acid from the prodrug.

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References

Cham, B. E., Johns, D., Bochner, F., Imhoff, D. M., Rowland, M. (1979) Simultaneous liquid-chromatographic quantitation of salicylic acid, salicyluric acid, and gentisic acid in plasma. Clin. Chem. 25: 1420–1425

Friend, D. R., Chang, G. W. (1984) A colon-specific drug-delivery system based on drug glycosides and the glycosidases of colonic bacteria. J. Med. Chem. 27: 261–266

Friend, D. R., Chang, G. W. (1985) Drug glycosides: potential prodrugs for colon-specific drug delivery. Ibid. 28: 51–57

Friend, D. R., Phillips, S., McLeod, A., Tozer, T. N. (1991a) Relative anti-inflammatory effect of oral dexamethasone-β-D-glucoside and dexamethasone in experimental inflammatory bowel disease in guinea-pigs. J. Pharm. Pharmacol. 43: 353–355

Friend, D. R., Phillips, S., Tozer, T. N. (1991b) Colon-specific drug delivery from a glucoside prodrug in the guinea-pig. Efficacy study. J. Contr. Rel. 15: 47–54

Harboe, E., Larsen, C., Johansen, M., Olesen, H. P. (1989a) Macromolecular prodrugs. XIV. Absorption characteristics of naproxen after oral administration of a dextran T-70-naproxen ester prodrug in pigs. Int. J. Pharm. 53: 157–165

Harboe, E., Larsen, C., Johansen, M., Olesen, H. P. (1989b) Macromolecular prodrugs. XV. Colon-targeted delivery—bioavailability of naproxen from orally administered dextran-naproxen ester prodrugs varying in molecular size in the pig. Pharm. Res. 6: 919–923

Larsen, C., Harboe, E., Johansen, M., Olesen, H. P. (1989) Macromolecular prodrugs. XVI. Colon-targeted delivery—comparison of the rate of release of naproxen from dextran ester prodrugs in homogenates of various segments of the pig gastrointestinal (GI) tract. Ibid. 6: 995–999

Macfarlane, G. T., Cummings, J. H., Macfarlane, S., Gibson, G. R. (1989) Influence of retention time on degradation of pancreatic enzymes by human colonic bacteria grown in a 3-stage continuous culture system. J. Appl. Bacteriol. 67: 521–527

Nakamura, J., Inoue, Y., Sasaki, H., Shibasaki, J. (1986) Prolonged blood concentration of salicylic acid following the simultaneous oral administration of salicylic acid and salicyluric acid in rabbits. Chem. Pharm. Bull. 34: 2624–2627

Nakamura, J., Shiota, H., Haraguchi, Y., Sasaki, H., Shibasaki, J. (1988a) Further studies on the hydrolysis of salicyluric acid in intestinal microorganisms and prolonged blood concentration of salicylic acid following rectal administration of salicyluric acid in rabbits. J. Pharmacobiodyn. 11: 53–57

Nakamura, J., Shiota, H., Sasaki, H., Shibasaki, J. (1988b) Hydrolysis of salicyluric acid in intestinal microorganisms and prolonged blood concentration of salicylic acid following rectal administration of salicyluric acid in rats. Ibid. 11: 625–629

Nakamura, J., Haraguchi, Y., Sasaki, H., Shibasaki, J. (1989a) Effect of fasting on the hydrolysis of salicyluric acid in rabbit intestinal microorganisms. Ibid. 12: 602–607

Nakamura, J., Shiota, H., Sasaki, H., Shibasaki, J. (1989b) Sustained blood concentration of salicylic acid following rectal administration of salicyluric acid in dogs. Chem. Pharm. Bull. 37: 2537–2538

Nakamura, J., Haraguchi, Y., Asai, K., Sasaki, H., Shibasaki, J. (1990) Effect of pretreatment with antibiotics on the hydrolysis of salicyluric acid in rabbit intestinal microorganisms. J. Pharmacobiodyn. 13: 461–467

Nakamura, J., Tagami, C., Nishida, K., Sasaki, H. (1992) Development of a prodrug of salicylic acid, salicylic acid-L-alanine conjugate, utilizing hydrolysis by rabbit intestinal microorganisms. J. Pharm. Pharmacol. 44: 295–299

Ogushi, S., Watanabe, H., Nakayama, M., Tsuru, D. (1988) Salicylurate-hydrolyzing enzyme from intestinal bacterium in rabbit: purification and characterization. Chem. Pharm. Bull. 36: 4539–4546

Saffran, M., Kumar, G. S., Savariar, C., Burnham, J. C., Williams, F., Neckers, D. C. (1986) A new approach to the oral administration of insulin and other peptide drugs. Science 233: 1081–1084

Saffran, M., Bedra, C., Kumar, G. S., Neckers, D. C. (1988) Vasopressin: a model for the study of effects of additives on the oral and rectal administration of peptide drugs. J. Pharm. Sci. 77: 33–38

Shibasaki, J., Inoue, Y., Kadosaki, K., Sasaki, H., Nakamura, J. (1985) Hydrolysis of salicyluric acid in rabbit intestinal microorganisms. J. Pharmacobiodyn. 8: 989–995

Simpkins, J. W., Smulkowski, M., Dixon, R., Tuttle, R. (1988) Evidence for the delivery of narcotic antagonists to the colon as their glucuronide conjugates. J. Pharmacol. Exp. Ther. 244: 195–205

Tozer, T.N., Rigod, J., McLeod, A. D., Gungon, R., Hoag, M. K., Friend, D. R. (1991) Colon-specific delivery of dexamethasone from a glucoside prodrug in the guinea pig. Pharm. Res. 8: 445–454

Yamaoka, K., Nakagawa, T., Uno, T. (1978) Statistical moments in pharmacokinetics. J. Pharmacokinet. Biopharm. 6: 547–558