Unequal Hydrolysis of Salicylic Acid–D-Alanine and Salicylic Acid–L-Alanine Conjugate in Rabbit Intestinal Microorganisms

Junzo NAKAMURA,* Chieko TAGAMI, Koyo NISHIDA and Hitoshi SASAKI

School of Pharmaceutical Sciences, Nagasaki University, Bunkyo-machi, Nagasaki 852, Japan. *Received June 11, 1991*

The behavior of salicylic acid-D-alanine conjugate (salicyl-D-alanine) following intravenous, oral and intracecal administration was examined in rabbits, then compared with that of salicylic acid-L-alanine conjugate (salicyl-L-alanine) as reported previously. Following intravenous administration, salicyl-D-alanine eliminated rapidly from the blood, and its blood concentration was almost identical with that of salicyl-L-alanine. In both cases, salicylic acid could not be detected in the blood, indicating that systemic de-conjugation of D-alanine might not occur. Unchanged salicyl-D-alanine was found in the blood mainly following oral and intracecal administration of salicyl-D-alanine. On the other hand, salicylic acid formed extensively following oral and intracecal administration of salicyl-L-alanine, suggesting that the presystemic de-conjugation of D-alanine and L-alanine was unequal. Furthermore, *in vitro* incubation of salicyl-D-alanine with cecal content, in which the major source of salicyl-L-alanine hydrolysis is found, showed that the hydrolysis of salicyl-D-alanine was negligible in rabbit intestinal microorganisms.

Keywords salicylic acid; salicylic acid-D-alanine conjugate; salicylic acid-L-alanine conjugate; prodrug; rabbit; intestinal microorganism; presystemic de-conjugation; hydrolysis; enantioselectivity

Differences in pharmacodynamic and pharmacokinetic properties of drugs existing as enantiomers have been widely recognized.[1-4] The therapeutic importance of pharmacokinetic studies of enantiomers was thus emphasized. Most previous studies have focused on the enantioselectivity at hepatic metabolism. Yet, it has become apparent that intestinal microorganisms are capable of metabolizing a variety of drugs, thus potentially influencing and/or altering drug activity and toxicity.[5-8] However, so far, there is no available information concerning enantioselective metabolism in the intestinal microorganisms. In the previous study,[9] we demonstrated that salicylic acid-L-alanine conjugate (salicyl-L-alanine) was metabolized to salicylic acid by rabbit intestinal microorganisms and was a potent prodrug by reason of extensive formation and prolonged blood concentration of salicylic acid. Accordingly, we examined the behavior of salicylic acid-D-alanine conjugate (salicyl-D-alanine), an enantiomer of salicyl-L-alanine, following intravenous, oral and intracecal administration to rabbits, and we compared it with that of salicyl-L-alanine, aiming to advance the practical use of a prodrug utilizing the metabolism in intestinal microorganisms.

Experimental

Chemicals Chemicals were of reagent grade and obtained commercially from Nacalai Tesque, Inc. (Kyoto, Japan). Salicyl-D-alanine and salicyl-L-alanine were synthesized by the coupling of D-alanine methyl ester or L-alanine methyl ester and acetylsalicylic acid by means of the carbodiimide method as described previously.[9] Salicyl-D-alanine was yielded as white crystals: mp 162—164 °C; $[\alpha]_D^{20}$ −23.3° (c=0.91, EtOH). The chemical structure of salicyl-D-alanine was ascertained by nuclear magnetic resonance, mass spectrum and elemental analyses.

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* Experiment** The rabbits were starved for about 24 h prior to use for the experiments but had free access to water. Salicyl-D-alanine was dissolved in NaOH (equivalent to salicyl-D-alanine). Following intravenous, oral and intracecal administration of appropriate amounts of drug solution, blood was collected with a heparinized syringe at appropriate time intervals from an ear vein and subjected to assay. The area under the blood concentration–time curve (AUC) was calculated by the trapezoidal method.[10]

Intravenous Administration of Drug: The drug solution (10 mg/kg: salicylic acid equivalent) was administered intravenously *via* an ear vein. The half-life of blood elimination ($t_{1/2}$) was calculated from the blood elimination rate constant which is estimated by applying mono-exponential regression analysis to the blood concentration–time curve.

Oral Administration of Drug: The drug solution (60 mg/kg: salicylic acid equivalent) was administered orally by gastric intubation.

Intracecal Administration of Drug: 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 (5 mg/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.

Statistical analysis of the results was carried out using the Student's t-test.

***In Vitro* Incubation of Salicyl-D-alanine and Salicyl-L-alanine with Cecal Content** Rabbits without starvation were anesthetized with an intravenous injection of sodium pentobarbital (25 mg/kg). After the intestine was excised from the animals the cecal content was collected. A 10 ml portion of salicyl-D-alanine or salicyl-L-alanine (10 μg/ml: salicylic acid equivalent) in saline was added to the cecal content (1 g wet weight) and the mixture was incubated for 6 h at 37 °C. A 1 ml portion of the mixture was centrifuged at 12000 rpm for 10 min, and the supernatant (0.4 ml) was subjected to assay.

Analytical Method Salicyl-D-alanine, salicyl-L-alanine and salicylic acid in blood and in the supernatant fluid in the *in vitro* experiment were analyzed by high performance liquid chromatography according to the method of Cham *et al.*[11] with slight modifications. We used fluorescence intensity for detection instead of absorption measurement at 313 nm, which was employed by Cham *et al.*[11] 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. The samples were mixed on a vortex-type mixer and centrifuged at 12000 rpm for 10 min. The supernatant was filtered by passing through a 0.45 μm pore size membrane filter (SJHVL04NS, Nihon Millipore Kogyo K.K., Yonezawa, Japan). Then, 20 μl of the supernatant fluid was withdrawn using a Hamilton syringe and loaded onto the column. Calibration curves were constructed from comparing data on the peak-area ratios of salicyl-D-alanine, salicyl-L-alanine and salicylic acid to an 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, U.S.A.). 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. 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-D-alanine,

salicyl-L-alanine, salicylic acid and the internal standard were 8.4, 8.4, 12.5 and 6.8 min, respectively.

Results and Discussion

The aims of the present study are to establish whether enantioselectivity is involved in drug metabolism by the intestinal microorganisms and to obtain more useful information on the development of a prodrug utilizing the metabolism in the intestinal microorganisms. Therefore, we compared the behavior of salicyl-D-alanine and salicyl-L-alanine, as model enantiomers, following intravenous, oral and intracecal administration to rabbits.

In the first instance, salicyl-D-alanine was administered intravenously. The blood concentration–time curve of salicyl-D-alanine is shown in Fig. 1 together with that of salicyl-L-alanine for comparison.[9] Both salicyl-D-alanine and salicyl-L-alanine were rapidly eliminated from the blood, with $t_{1/2}$ of 20.5 ± 0.7 and 17.6 ± 2.7 min (mean $\pm$ S.E., $N=4$) (not significant), respectively. The shapes of their blood concentration profiles were almost the same, and no significant difference was seen in the AUC value between salicyl-D-alanine ($1102.5 \pm 288.7\ \mu g \cdot ml^{-1} \cdot min$) (mean $\pm$ S.E., $N=4$) and salicyl-L-alanine ($863.8 \pm 72.1\ \mu g \cdot ml^{-1} \cdot min$) (mean $\pm$ S.E., $N=4$). It was therefore conceivable that the *in vivo* elimination processes were almost identical with both enantiomers. Furthermore, salicylic acid could not be detected with either enantiomer, indicating that systemic de-conjugation of D-alanine and L-alanine might not occur.

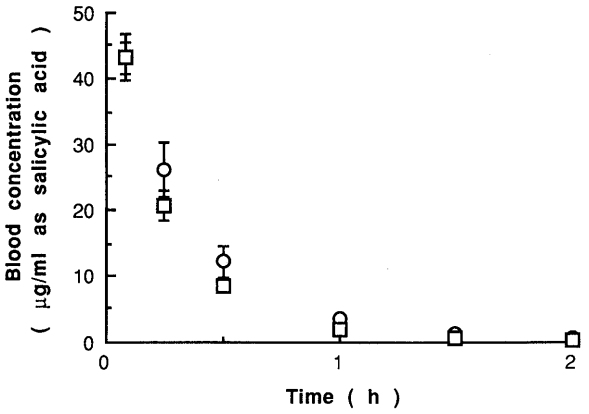

Fig. 1. Blood Concentration of Salicyl-D-alanine and Salicyl-L-alanine Following Intravenous Administration of Salicyl-D-alanine and Salicyl-L-alanine (10 mg/kg: Salicylic Acid Equivalent) to Rabbits, Respectively

○, salicyl-D-alanine; □, salicyl-L-alanine. Each point represents the mean $\pm$ S.E. of 4 experiments.

The blood concentration of salicyl-D-alanine and salicylic acid following oral administration of salicyl-D-alanine was determined in rabbits. As shown in Fig. 2, salicyl-D-alanine reached a peak blood concentration (32.0 μg/ml, as salicylic acid) at 90 min after the dose and then decreased, while a trace amount ($<0.2\ \mu g/ml$) of salicylic acid was detected, indicating that the presystemic de-conjugation of salicyl-D-alanine was extremely minimal. In the case of salicyl-L-alanine reported previously,[9] salicylic acid was detected at 2 h after the dose and its blood concentration remained above 5.3 μg/ml at even 30 h, indicating extensive presystemic de-conjugation of L-alanine. The AUC value for salicylic acid following oral administration of salicyl-L-alanine ($19933.0\ \mu g \cdot ml^{-1} \cdot min$) was about 200 times as large as that of salicyl-D-alanine ($99.4\ \mu g \cdot ml^{-1} \cdot min$). The enantioselectivity at the presystemic metabolism of salicyl-D-alanine and salicyl-L-alanine can be caused by differences in metabolic activity in intestinal mucosa and/or in the intestinal microorganisms. Since we previously observed that 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,[9] metabolism in the intestinal microorganisms is considered to be the major determinant of the difference in the presystemic metabolism of salicyl-D-alanine and salicyl-L-alanine.

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.[6] Also, Williams reviewed findings that the location of microorganisms along the gastrointestinal tract was similar in rabbits and humans.[12] Therefore, salicyl-D-alanine was administered intracecally to study the enantioselective metabolism of salicyl-D-alanine and salicyl-L-alanine in intestinal microorganisms. Figure 3 shows the blood concentration of salicyl-D-alanine following intracecal administration of salicyl-D-alanine. A small amount of salicyl-D-alanine

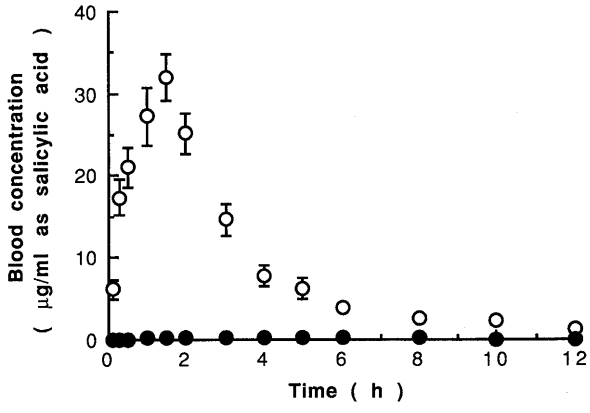

Fig. 2. Blood Concentration of Salicyl-D-alanine and Salicylic Acid Following Oral Administration of Salicyl-D-alanine (60 mg/kg: Salicylic Acid Equivalent) to Rabbits

○, salicyl-D-alanine; ●, salicylic acid. Each point represents the mean $\pm$ S.E. of 6 experiments.

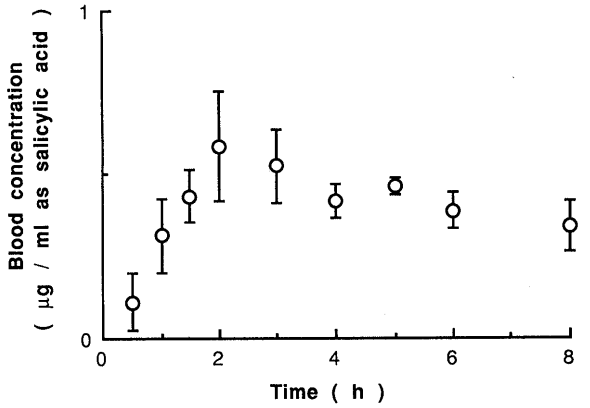

Fig. 3. Blood Concentration of Salicyl-D-alanine Following Intracecal Administration of Salicyl-D-alanine (5 mg/kg: Salicylic Acid Equivalent) to Rabbits

○, salicyl-D-alanine. Each point represents the mean $\pm$ S.E. of 3 experiments.

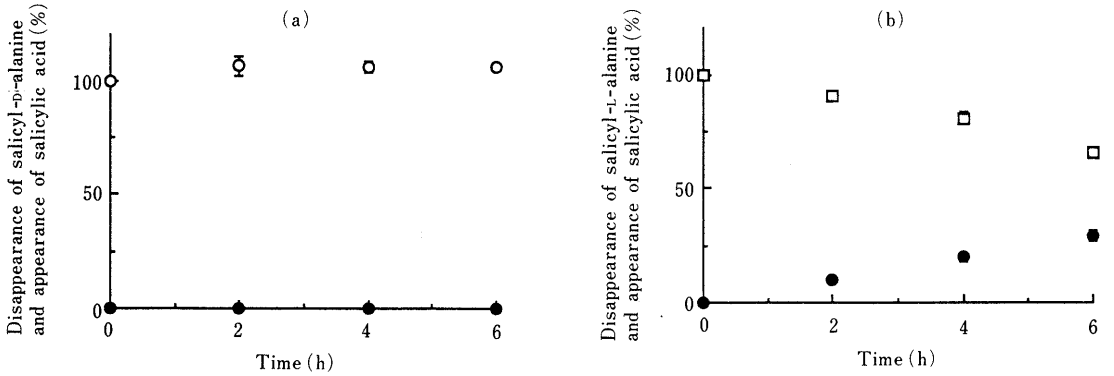

Fig. 4. Time Courses of Salicyl-D-alanine or Salicyl-L-alanine Disappeared and Salicylic Acid Appeared during Incubation of Salicyl-D-alanine (a) and Salicyl-L-alanine (b) (100 μg: Salicylic Acid Equivalent) with Rabbit Cecal Content (1 g)

○, salicyl-D-alanine; □, salicyl-L-alanine; ●, salicylic acid. Each point represents the mean ± S.E. of 6 experiments.

(< 0.6 μg/ml, as salicylic acid) was detected in the blood. Similar to the data on oral administration, salicylic acid could not be detected in the blood. Thus, this result supported the assumption that salicyl-D-alanine was not metabolized by intestinal microorganisms.

Salicyl-D-alanine-hydrolyzing activity of the cecal content, in which the major source of salicyl-L-alanine metabolism is found,[9] was examined to confirm the deficiency of salicyl-D-alanine-hydrolyzing activity in the intestinal microorganisms. The results are shown in Fig. 4a, together with those of salicyl-L-alanine (Fig. 4b) for comparison. The formation of salicylic acid from salicyl-L-alanine increased with time, and the amount of salicylic acid formed was 29% of applied dose at 6 h. This value corresponded to the amount of salicyl-L-alanine (34%) which disappeared, indicating that salicylic acid was the major metabolite in the metabolism by intestinal microorganisms. On the other hand, salicyl-D-alanine-hydrolyzing activity of the cecal content was so low that the amount of salicylic acid formed after incubation of salicyl-D-alanine with cecal content was negligible (< 0.3% of applied dose). It seemed that salicyl-D-alanine could not be metabolized by the hydrolyzing enzymes in rabbit intestinal microorganisms. However, the precise mechanism by which salicyl-L-alanine is hydrolyzed selectively in rabbit intestinal microorganisms remains to be elucidated.

In conclusion, a large difference in the presystemic hydrolysis between salicyl-D-alanine and salicyl-L-alanine by rabbit intestinal microorganisms was seen, though no significant difference was observed in the *in vivo* elimination processes. This finding may provide important implications for the practical use of a prodrug utilizing the metabolism in intestinal microorganisms. Furthermore, additional studies are needed to clarify whether none of the other D-amino acids conjugates of salicylic acid can be metabolized by the intestinal microorganisms.

Acknowledgments We thank Professor S. Furukawa, School of Pharmaceutical Sciences, Nagasaki University, for helpful discussion on the synthesis of salicyl-D-alanine. We wish to thank K. Asai, Y. Haraguchi, M. Kido, M. Matsui and Y. Maeda for skilled technical assistance. This work was supported in part 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.

References

1) E. J. Ariens, *Eur. J. Clin. Pharmacol.*, **26**, 663 (1984).
2) D. E. Drayer, *Clin. Pharmacol. Ther.*, **40**, 125 (1986).
3) B. Testa, *Trends Pharmacol. Sci.*, **1986**, 60.
4) T. Walle and U. K. Walle, *Trends Pharmacol. Sci.*, **1986**, 155.
5) R. R. Scheline, *J. Pharm. Sci.*, **57**, 2021 (1968).
6) R. R. Scheline, *Pharmacol. Rev.*, **25**, 451 (1973).
7) P. Goldman, *Annu. Rev. Pharmacol. Toxicol.*, **18**, 523 (1978).
8) H. G. Boxenbaum, I. Bekersky, M. L. Jack and S. A. Kaplan, *Drug Metab. Rev.*, **9**, 259 (1979).
9) J. Nakamura, C. Tagami, K. Nishida and H. Sasaki, *J. Pharm. Pharmacol.*, **44** (1992) in press.
10) K. Yamaoka, T. Nakagawa and T. Uno, *J. Pharmacokinet. Biopharm.*, **6**, 547 (1978).
11) B. E. Cham, D. Johns, F. Bochner, D. M. Imhoff and M. Rowland, *Clin. Chem.*, **25**, 1420 (1979).
12) R. T. Williams, *Toxicol. Appl. Pharmacol.*, **23**, 769 (1972).