International Journal of Pharmaceutics, 99 (1993) 189–195

IJP 03232

Systemic and presystemic de-conjugation of salicylic acid-arginine conjugate as a prodrug of salicylic acid in rabbits

Junzo Nakamura, Mitsuhiko Kido, Chieko Tagami, Koyo Nishida and Hitoshi Sasaki

School of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852 (Japan)

(Received 19 May 1992)
(Modified version received 16 November 1992)
(Accepted 20 February 1993)

Key words: Salicylic acid-arginine conjugate; Prodrug; Hydrolysis; Systemic de-conjugation; Presystemic de-conjugation; Rabbit; Intestinal microorganism

Summary

The fate of salicylic acid-arginine conjugate (salicyl-arginine) following oral, intravenous, intracecal and rectal administration (434, 72, 36 and 36 μmol/kg, respectively) was examined in rabbits. A large amount of salicylic acid appeared in the blood following oral administration of salicyl-arginine with retention of the blood concentration of salicylic acid (above 12.0 μg/ml) up to 36 h, whereas salicyl-arginine was detected at low concentration (< 1.2 μg/ml, as salicylic acid). Both salicyl-arginine and salicylic acid could be found in the blood following intravenous administration of salicyl-arginine, suggesting that systemic de-conjugation of salicyl-arginine occurred. Extensive salicylic acid formation was observed following intracecal administration of salicyl-arginine. After oral pretreatment of rabbits with kanamycin sulfate (6 × 400 mg) and/or tinidazole (6 × 160 mg), significant inhibition of salicylic acid formation was observed following intracecal administration of salicyl-arginine, indicating that intestinal microorganisms were partly responsible for the biotransformation of salicyl-arginine.

Introduction

Prodrugs are used routinely in drug delivery systems. The bioavailability of the parent drug depends on the chemical or enzymatic converting activity of the prodrug at the target site. Although the liver is the major site for drug metabolism, the significance of intestinal microorganisms has been emphasized with respect to their ability to metabolize drugs. In our previous reports (Nakamura et al., 1992a,b,c,e), we investigated the behavior of acidic and neutral amino acid conjugates of salicylic acid in rabbits and established a fundamental principle for developing a potent prodrug utilizing metabolism in intestinal microorganisms.

Thus far, we have not examined the behavior of basic amino acid conjugates of salicylic acid. Among the amino acids, arginine is a characteristic basic one, of which the side chain is alkylguanidine. Accordingly, elucidation of the general features of the relationship between the physicochemical properties and disposition characteristics of these prodrugs was considered to offer a promising approach to developing an ef-

Correspondence to: J. Nakamura, School of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852, Japan.

fective prodrug of salicylic acid. In the present study, we prepared salicylic acid-arginine conjugate (salicyl-arginine) and examined its behavior following oral, intravenous, intracecal and rectal administration to rabbits.

Materials and Methods

Chemicals

Acetylsalicylic acid, L-arginine, acetonitrile and *o*-anisic acid were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Kanamycin sulfate and tinidazole were obtained from Meiji Seika Kaisha, Ltd (Tokyo, Japan) and Sigma Chemical Co. (St. Louis, MO, U.S.A.), respectively. All other chemicals were of reagent grade.

Synthesis of salicyl-arginine

Salicyl-arginine was synthesized by coupling of arginine methyl ester and acetylsalicylic acid-succinimide by means of the carbodiimide method as follows. To a suspension of 17 g of arginine in 100 ml of methanol, 11 ml of thionyl chloride was added slowly at 0°C with stirring and left overnight at room temperature. The reaction mixture was concentrated under reduced pressure. It was recrystallized from methanol-ether to yield arginine methyl ester dihydrochloride as white crystals. Acetylsalicylic acid (3.6 g) and *N*-hydroxysuccinimide (2.3 g) were dissolved in 20 ml of tetrahydrofuran. *N,N'*-Dicyclohexylcarbodiimide (4.2 g) in 20 ml of tetrahydrofuran was added to the former mixture below 0°C. The mixture was then stirred for 1 h at 0°C, left overnight at room temperature, and subsequently filtered. The filtrate was concentrated under reduced pressure and yielded acetylsalicylic acid-succinimide. To a solution of 5.2 g of arginine methyl ester dihydrochloride in 15 ml of dimethyl sulfoxide, 2 g of triethylamine was added. The mixture was added to the solution of acetylsalicylic acid-succinimide in 40 ml of ethylene glycol dimethyl ether and then stirred overnight at room temperature. The solvent was then evaporated under reduced pressure. The reaction mixture dissolved in water was washed with ethyl acetate. To a solution of acetylsalicylic acid-arginine methyl ester in 20 ml of methanol, 3 g of NaOH dissolved in 20 ml of distilled water was added and the mixture was stirred for 1 h. The methanol was subsequently evaporated under reduced pressure, followed by neutralization with 6 N HCl at 0°C. In addition, the water was evaporated at 60°C. The residue was dissolved in methanol. After drying with anhydrous Na_2SO_4, the methanol was evaporated under reduced pressure. Then, the product was purified by column chromatography. The product was applied to a silica gel column (Wakogel C-200, Wako Pure Chemical Industries, Ltd, Osaka, Japan; 16×3 cm i.d.). The column was eluted with methanol-ethyl acetate (3:2, v/v). The desired fraction was collected and evaporated under reduced pressure, followed by recrystallization from methanol-ethyl acetate to yield salicyl-arginine as white crystals. The chemical structure of the product was ascertained by NMR, mass spectral and elemental analyses. Analysis: Calc. for $C_{13}H_{18}N_4O_4$: C, 53.05; H, 6.16; N, 19.04. Found: C, 52.97; H, 6.11; N, 18.86. FAB-MS m/z: 295. 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.

Stability evaluation was carried out in blood or 0.1 M phosphate buffer solutions of pH 2.5, 6.0 and 7.5 at 37°C at a drug concentration of 100 μg/ml as salicylic acid. Salicyl-arginine was completely stable (100% remaining) in blood and phosphate buffer solutions at each pH after 24 h incubation.

Animals

Male albino rabbits (weight, 2–3 kg; age, 4–5 months) 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

Rabbits were starved for about 24 h prior to use for experiments but had free access to water.

Salicyl-arginine was dissolved in aqueous NaOH solution (equivalent to salicyl-arginine). Appropriate amounts of drug solution were administered as described previously (Nakamura et al., 1992a,d). Following oral, intravenous, intraduodenal, intracecal and rectal administration of drug (434, 72, 72, 36 and 36 μmol/kg, respectively), blood was collected with a heparinized syringe at appropriate time intervals from an ear vein and centrifuged at $8000 \times g$ for 5 min. Plasma samples (0.2 ml) were subjected to assay. The blood concentration of drug was calculated as salicylic acid from the calibration curve. The area under the blood concentration-time curve (AUC) was calculated according to the trapezoidal method (Yamaoka et al., 1978).

Oral pretreatment of rabbits with kanamycin sulfate and/or tinidazole before intracecal administration of drug was carried out as described previously (Nakamura et al., 1992d). Rabbits were divided into three groups (each $n = 4$), i.e., pretreatment with kanamycin sulfate, tinidazole and both kanamycin sulfate and tinidazole.

In vitro incubation of salicyl-arginine with gut contents

In vitro incubation of salicyl-arginine (100 μg/ml: salicylic acid equivalent) in saline with the contents (1 g wet weight) of five segments (jejunum, upper ileum, lower ileum, cecum and colon) of rabbit intestine was performed as described previously (Nakamura et al., 1992a).

Analytical method

Salicyl-arginine and salicylic acid in blood and supernatant fluid in the in vitro experiment were determined by high-performance liquid chromatography according to the method of Cham et al. (1979) with slight modifications. Details of the analytical method are described in our previous paper (Nakamura et al., 1992a). The chromatographic mobile phase consisted of a mixture of methanol-0.072% (v/v) of H_3PO_4 (55:45, v/v) and was filtered by passing 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-arginine, salicylic acid and the internal standard *o*-anisic acid were 2, 6.5 and 4 min, respectively.

Results and Discussion

Oral administration of salicyl-arginine

The blood concentration profiles of salicyl-arginine and salicylic acid following oral administration of salicyl-arginine are shown in Fig. 1. A small amount of salicyl-arginine was detected in the blood (< 1.2 μg/ml, as salicylic acid), suggesting that salicyl-arginine was not absorbed and/or absorbed salicyl-arginine was rapidly metabolized by liver before reaching the systemic circulation. Also, by performing in vitro incubation of salicyl-arginine with blood, it was confirmed that hydrolysis of salicyl-arginine in the blood did not occur. On the other hand, salicylic acid appeared in the blood after the dose and reached a peak blood concentration (63.3 μg/ml) at 18 h, indicating that the hydrolysis of salicyl-arginine occurred extensively. In comparison with other amino acid conjugates of salicylic acid (Nakamura et al., 1992a,c,e), the lag time for appearance of salicylic acid in blood after oral administration of salicyl-arginine was short. The blood concentration of salicylic acid remained above 12.0 μg/ml up to 36 h with the AUC(0–36 h) value of salicylic acid amounting to 1230.1 μg/ml per h. The metabolism ratio of salicyl-arginine following oral administration was calcu-

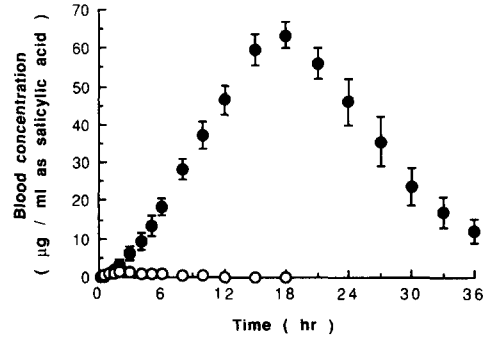

Fig. 1. Blood concentration of salicyl-arginine (○) and salicylic acid (●) following oral administration of salicyl-arginine (434 μmol/kg: salicylic acid equivalent) to rabbits. Each point represents the mean ± S.E. of six experiments.

lated to be 89%, by dividing the AUC/dose of salicylic acid appearing following oral administration of salicyl-arginine by that after intravenous administration of salicylic acid.

Intravenous administration of salicyl-arginine

Salicyl-arginine was administered intravenously to examine its systemic de-conjugation (Fig. 2). Salicyl-arginine was rapidly eliminated from the blood. On the other hand, salicylic acid appeared in the blood gradually and reached a peak blood concentration (7.8 μg/ml) at 30 min, suggesting that systemic de-conjugation of salicyl-arginine was involved in its hydrolysis. This result was not compatible with our previous data on the other amino acid conjugates of salicylic acid (Nakamura et al., 1992a,b,c,e). The metabolism ratio of salicyl-arginine in the systemic circulation was assumed to be 13%, by dividing the AUC/dose of salicylic acid appearing following intravenous administration of salicyl-arginine by that after intravenous administration of salicylic acid.

Intraduodenal administration of salicyl-arginine

In order to determine the involvement of metabolism by small intestinal mucosa in the presystemic de-conjugation of salicyl-arginine, it was intraduodenally administered to rabbits (data not shown). The absorption ratio of salicyl-arginine was calculated to be 15.9%, by dividing the AUC/dose of salicyl-arginine following intraduodenal administration of salicyl-arginine by that

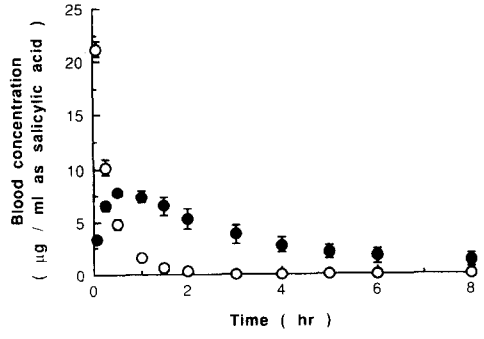

Fig. 2. Blood concentration of salicyl-arginine (○) and salicylic acid (●) following intravenous administration of salicyl-arginine (72 μmol/kg: salicylic acid equivalent) to rabbits. Each point represents the mean ± S.E. of five experiments.

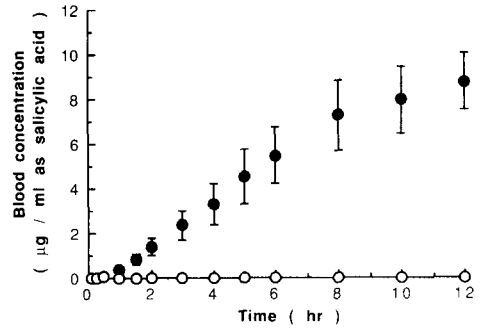

Fig. 3. Blood concentration of salicyl-arginine (○) and salicylic acid (●) following intracecal administration of salicyl-arginine (36 μmol/kg: salicylic acid equivalent) to rabbits. Each point represents the mean ± S.E. of four experiments.

after intravenous administration of salicyl-arginine. Although salicyl-arginine was poorly absorbed from the small intestine, the estimated ratio of salicylic acid appearing in the blood (15.7%), which was calculated by dividing the AUC/dose of salicylic acid following intraduodenal administration of salicyl-arginine by that after intravenous administration of salicylic acid, was relatively high. Therefore, this result suggests the involvement of metabolism by small intestinal mucosa during absorption in the presystemic metabolism of salicyl-arginine.

Intracecal administration of salicyl-arginine

Salicyl-arginine was administered directly into the cecum, in which intestinal microorganisms are mainly distributed, to examine the mechanism of salicyl-arginine hydrolysis in rabbits. Fig. 3 shows the blood concentration of salicyl-arginine and salicylic acid following intracecal administration of salicyl-arginine. Unchanged salicyl-arginine was detected at very low concentration (< 0.09 μg/ml, as salicylic acid), whereas extensive salicylic acid formation from salicyl-arginine in the cecum was observed, suggesting the possibility of salicyl-arginine hydrolysis by intestinal microorganisms.

The effect of oral pretreatment with kanamycin sulfate (6 × 400 mg) or tinidazole (6 × 160 mg) on the blood concentration of salicylic acid following intracecal administration of salicyl-arginine was examined. Kanamycin sulfate and tinidazole are known to inhibit selectively the growth of aerobic

and anaerobic bacteria, respectively (Maeda et al., 1989). The oral pretreatment of rabbits with kanamycin sulfate or tinidazole resulted in a significant reduction in the formation of salicylic acid as shown in Fig. 4A and B, as judged from the decreased blood concentration of salicylic acid up to 12 h compared with the control (Fig. 3). Furthermore, the simultaneous oral pretreatment of rabbits with both kanamycin sulfate and tinidazole resulted in almost complete inhibition of salicylic acid formation from salicyl-arginine (Fig. 4C). These results support the hypothesis that salicyl-arginine was hydrolyzed to salicylic acid by intestinal microorganisms in rabbits. The inhibitory effect of kanamycin sulfate on salicylic acid formation from salicyl-arginine was greater than that of tinidazole.

In vitro incubation of salicyl-arginine with gut contents

The salicyl-arginine-hydrolyzing activities of the contents from different regions of the intestinal tract were determined. The amount of salicylic acid appearing in the medium increased linearly with time up to 6 h in the contents from every region, similarly to other prodrugs (Nakamura et al., 1992a,c,e), and their values after a 6 h incubation are listed in Table 1. In contrast with the other amino acid conjugates of salicylic acid (Nakamura et al., 1992a,c,e), salicyl-

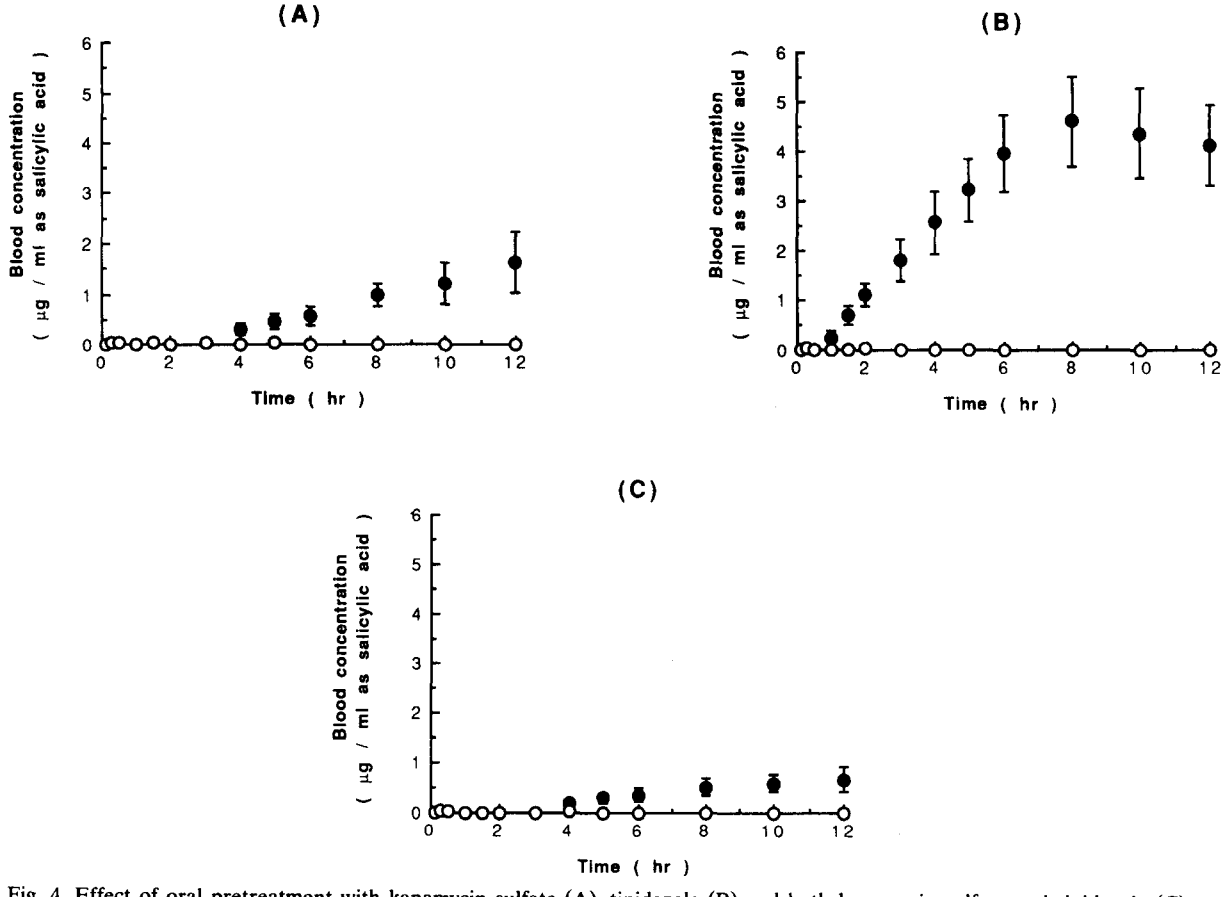

Fig. 4. Effect of oral pretreatment with kanamycin sulfate (A), tinidazole (B) and both kanamycin sulfate and tinidazole (C) on blood concentration of salicyl-arginine (○) and salicylic acid (●) following intracecal administration of salicyl-arginine (36 μmol/kg: salicylic acid equivalent) to rabbits. Each point represents the mean ± S.E. of four experiments.

arginine-hydrolyzing activities of the contents from the jejunum, upper ileum and lower ileum were recognized. Aspirin-L-arginine conjugate was reported to be hydrolyzed by trypsin and carboxypeptidase (Tsunematsu et al., 1991). Therefore, it is suggested that salicyl-arginine might be partly hydrolyzed by digestive enzyme in the small intestinal contents. The short lag time for the appearance of salicylic acid in blood following oral administration of salicyl-arginine might be explained by this result. On the other hand, the formation of salicylic acid from salicyl-arginine was relatively extensive in the contents from the cecum and colon, indicating that the contents from the hind gut were the major source of hydrolysis of salicyl-arginine.

Rectal administration of salicyl-arginine

Fig. 5 shows the blood concentration of salicyl-arginine and salicylic acid following rectal administration of salicyl-arginine. Salicyl-arginine was poorly detectable as the intact form due to low absorbability and/or rapid systemic de-conjugation of absorbed salicyl-arginine. Part of the salicyl-arginine was hydrolyzed to salicylic acid, followed by its absorption. The blood concentration of salicylic acid increased gradually with time and reached its maximum level at 12 h after the dose. From the result of in vitro incubation of salicyl-arginine with the contents from colon (Table 1), it was suggested that intestinal microorganisms in the rectum hydrolyzed salicyl-arginine partly.

TABLE 1

Amount of salicylic acid appearing in the medium after 6 h incubation of salicyl-arginine (1000 μg: salicylic acid equivalent) with rabbit gut contents (1 g wet weight)

Site	Salicylic acid appearing (μg)
Jejunum	3.53 ± 1.67
Upper ileum	6.77 ± 2.45
Lower ileum	4.29 ± 2.45
Cecum	25.86 ± 0.75
Colon	16.62 ± 2.64

Values are mean ± S.E. of at least six experiments.

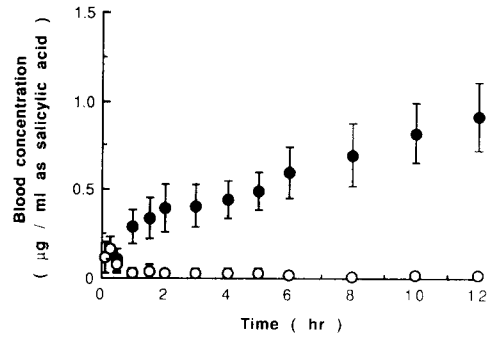

Fig. 5. Blood concentration of salicyl-arginine (○) and salicylic acid (●) following rectal administration of salicyl-arginine (36 μmol/kg: salicylic acid equivalent) to rabbits. Each point represents the mean ± S.E. of four experiments.

In conclusion, an immediate and sustained blood concentration of salicylic acid following oral administration of salicyl-arginine was observed, suggesting its usefulness as a prodrug utilizing metabolism probably in the intestinal mucosa, liver and intestinal microorganisms. However, the underlying mechanism by which salicyl-arginine is metabolized to salicylic acid systemically and presystemically remains to be clarified in the future.

Acknowledgments

We thank Professor Sunao Furukawa, School of Pharmaceutical Sciences, Nagasaki University, for helpful discussion on the synthesis of salicyl-arginine. We wish to thank Toshiko Ezoe, Haruki Kishikawa and Yukiko Nakamura for skillful 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, by a Grant-in-Aid from the Sankyo Foundation of Life Science, and by a Grant-in-Aid from the Nakatomi Foundation.

References

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

Gingell, R., Bridges, J.W. and Williams, R.T., The role of the gut flora in the metabolism of prontosil and neoprontosil in the rat. *Xenobiotica*, 1 (1971) 143–156.

Maeda, Y., Takahashi, M., Tashiro, H. and Akazawa, F., The rapid evaluation of intestinal bacterial growth using a conjugate of ursodeoxycholic acid with *para*-aminobenzoic acid. *J. Pharmacobio-Dyn.*, 12 (1989) 272–280.

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

Nakamura, J., Tagami, C., Nishida, K. and Sasaki, H., Unequal hydrolysis of salicylic acid-D-alanine and salicylic acid-L-alanine conjugate in rabbit intestinal microorganisms. *Chem. Pharm. Bull.*, 40 (1992b) 547–549.

Nakamura, J., Asai, K., Nishida, K. and Sasaki, H., A novel prodrug of salicylic acid, salicylic acid-glutamic acid conjugate utilizing hydrolysis in rabbit intestinal microorganisms. *Chem. Pharm. Bull.*, 40 (1992c) 2164–2168.

Nakamura, J., Kido, M., Nishida, K. and Sasaki, H., Effect of oral pretreatment with antibiotics on the hydrolysis of salicylic acid-tyrosine and salicylic acid-methionine prodrugs in rabbit intestinal microorganisms. *Chem. Pharm. Bull.*, 40 (1992d) 2572–2575.

Nakamura, J., Kido, M., Nishida, K. and Sasaki, H., Hydrolysis of salicylic acid-tyrosine and salicylic acid-methionine prodrugs in the rabbit. *Int. J. Pharm.*, 87 (1992e) 59–66.

Tsunematsu, H., Ishida, E., Yoshida, S. and Yamamoto, M., Synthesis and enzymatic hydrolysis of aspirin-basic amino acid ethyl esters. *Int. J. Pharm.*, 68 (1991) 77–86.

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