Effect of Fasting on the Hydrolysis of Salicyluric Acid in Rabbit Intestinal Microorganisms

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The effect of fasting on the hydrolysis of salicyluric acid in rabbit intestinal microorganisms was investigated. The blood concentration of salicyluric acid and salicylic acid following oral, intracecal and rectal administration of salicyluric acid was determined. In fasted rabbits (24 and 48 h), the blood concentration of salicylic acid after oral administration was changed compared to the control. However, a significant effect of fasting was not observed in the blood concentration of salicylic acid after rectal administration. Following intracecal administration, the blood concentration of salicylic acid was increased in fasted rabbits compared to the control. From these results, it seems that the slow rate of stomach emptying due to coprophagy during fasting is the principal reason for the change of blood concentration of salicylic acid following oral administration of salicyluric acid.

Keywords — salicyluric acid; salicylic acid; fasting; starvation; gut flora; intestinal microorganism; prodrug; rabbit; stomach emptying; glycine conjugate

Introduction

Intestinal microorganisms play an important role in the metabolism and toxicity of a number of drugs and other foreign compounds.¹⁻⁵⁾ In previous reports, we examined the blood concentration of salicyluric acid and salicylic acid following the intravenous, oral, intracecal and rectal administration of salicyluric acid in rabbits,^{6,7)} rats⁸⁾ and dogs.⁹⁾ In these species, salicyluric acid is metabolized to salicylic acid by intestinal microorganisms. Following rectal administration of salicyluric acid, prolonged blood concentration of salicylic acid was observed. However, species differences in the metabolic fate of salicyluric acid following oral and intravenous administration of salicyluric acid were recognized.

In order to avoid the possible interactions between various foods and drugs and the effect of food on stomach emptying time in the study of drug absorption from the gastrointestinal tract, it is a common practice to administer drugs to fasted human subjects or experimental animals. Rabbits have often been used as a tool in the evaluation of oral absorption characteristics of drugs because of their low cost and ease of handling. Chiou *et al.* demonstrated that the

stomach of rabbits fasted for 24 h was almost as full as that of unfasted rabbits, suggesting the complications and shortcomings of using rabbits for oral absorption studies of drugs.¹⁰⁾ Also, several investigators reported change of intestinal drug absorption by fasting.¹¹⁻¹⁷⁾

To provide more information on the physiological factors in the hydrolysis of salicyluric acid by intestinal microorganisms, we examined the oral, intracecal and rectal administration of salicyluric acid in fasted rabbits.

Materials and Methods

Materials — Salicyluric acid was obtained from Sigma Chemical Co. (St. Louis, U.S.A.). Acetonitrile, acetic acid, methanol and *o*-methoxybenzoic acid were purchased from Nakalai Tesque, Inc. (Kyoto, Japan). All other chemicals used in these experiments were of reagent grade.

Animal Experiments — Male albino rabbits weighing 1.8—2.8 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). The rabbits were starved for 24 and 48 h prior to use

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for experiments but had free access to water. The control rabbits were not starved. Salicyluric acid was dissolved in NaOH (equivalent to salicyluric acid). Appropriate amounts of drug solution were administered orally, intracecally and rectally.

Oral Administration of Drug: The drug solution (4 ml/kg) was administered by gastric intubation.

Intracecal Administration of Drug: Animals were anesthetized with sodium pentobarbital, given intravenously, *via* ear vein. After complete anesthesia, a midline incision (2–3 cm) was made, and the drug solution (6 ml/kg) 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 (2 ml/kg) was administered rectally, and the anus was closed with a plastic clip to prevent leakage of the rectal contents during the experiments. Following oral, intracecal and rectal administration of drug, blood was collected with a heparinized syringe at appropriate time intervals from an ear vein.

Analytical Method — Salicyluric acid and salicylic acid in blood were analyzed by high-performance liquid chromatography after modifying the method described by Cham *et al.*¹⁸⁾ We used fluorescence intensity for detection instead of absorption measurement at 313 nm, which was employed by Cham *et al.* Blood samples (0.4 ml) were added to an equal volume of acetonitrile containing 30 μ g of the internal standard, *o*-methoxybenzoic acid, in 1 ml. The samples were mixed on a vortex-type mixer and centrifuged at 10000 rpm for 10 min. Then 20 μ l 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 salicyluric acid and salicylic acid to the internal standard. We used a LC-6A pump, a RF-530 fluorescence detector, a Chromatopac C-R3A recorder (all from Shimadzu Co., Ltd., Kyoto, Japan) and a model 7125 sample injection valve (Rheodyne Inc., CA, U.S.A.). The prepared column was a bonded octadecylsilane-silica gel type (Fine SIL

C₁₈, Japan Spectroscopic Co., Ltd., Tokyo, Japan), average particle size 10 μ m and 250 $\times$ 4.6 mm i.d. 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

Fig. 1. Blood Concentration of Salicyluric Acid and Salicylic Acid Following Oral Administration of Salicyluric Acid

(a), control (6); (b), fasting for 24 h (4); (c), fasting for 48 h (4). ○, salicyluric acid; ●, salicylic acid. Blood concentration and dose (60 mg/kg) of salicyluric acid: salicylic acid equivalent. Each point represents the mean $\pm$ S.E. Numbers in parentheses represent number of experiments. The control rabbits were not starved.

chromatographic mobile phase consisted of a mixture of acetic acid-methanol-water (4:40:60, v/v/v) and was filtered by passing through a 0.5 μm pore membrane filter (Toyo Roshi Co., Ltd., Tokyo, Japan) before use. The flow rate was 1.5 ml/min. The retention times of salicyluric acid, salicylic acid and the internal standard were 4.9, 9.5 and 7.5 min, respectively.

Results and Discussion

The blood concentration of salicyluric acid and salicylic acid following oral administration of salicyluric acid was determined in rabbits. In control rabbits (Fig. 1a), salicyluric acid reached a peak blood concentration (11.4 $\mu\text{g/ml}$) at 1.5 h after the dose and then decreased. Salicylic acid reached a peak blood concentration (10.2 $\mu\text{g/ml}$) in about 5 h and then decreased. The results in fasted rabbits are shown in Fig. 1b (24 h) and Fig. 1c (48 h). In fasted rabbits, the time taken for salicyluric acid to reach the peak level is shorter. The blood concentration of salicylic acid in fasted rabbits (24 h) was maintained at 10.8–12.4 $\mu\text{g/ml}$ from 4–12 h. However, salicylic acid was detected at low concentration for 12 h in fasted rabbits (48 h). In the previous reports,⁶⁾ only unchanged salicyluric acid was detected in the blood following intravenous administration of salicyluric acid in rabbits, suggesting that presystemic deconjugation of glycine was involved. After oral pretreatment of rabbits with kanamycin sulfate, complete inhibition of the formation of salicylic acid following oral administration of salicyluric acid was demonstrated, indicating that the intestinal microorganisms were responsible for the biotransformation. Furthermore, *in vitro* incubation of salicyluric acid with gut contents showed that the major location of the hydrolysis was the hind gut.

In order to examine the mechanism of change of salicylic acid concentration in the blood following the oral administration of salicyluric acid in fasted rabbits, salicyluric acid was administered intracecally. The results are shown in Fig. 2. In control (Fig. 2a) and fasted rabbits (Fig. 2b and 2c), salicyluric acid was detected at low concentration for 5 to 6 h. The

blood concentration of salicylic acid was increased in fasted rabbits compared to the control. The metabolic activity of intestinal microorganisms in the cecum may be changed by fasting. Further studies will be required to clarify the mechanism of increase in the blood concentration of salicylic acid in fasted rabbits. As shown in Fig. 1c, the blood concentration of salicylic acid following oral administration of salicyluric

Fig. 2. Blood Concentration of Salicyluric Acid and Salicylic Acid Following Intracecal Administration of Salicyluric Acid

(a), control (5); (b), fasting for 24 h (5); (c), fasting for 48 h (5). $\circ$, salicyluric acid; $\bullet$, salicylic acid. Blood concentration and dose (5 mg/kg) of salicyluric acid: salicylic acid equivalent. Each point represents the mean $\pm$ S.E. Numbers in parentheses represent number of experiments. The control rabbits were not starved.

acid was detected at low concentration for 12 h in fasted rabbits (48 h). However, the blood concentration profile of salicylic acid in Fig. 2c indicates immediate and very extensive salicylic acid formation from salicyluric acid in the cecum. From these results, it seems that salicyluric acid could not pass down to the cecum following oral administration and the slow rate of stomach emptying due to coprophagy during fasting is the principal reason for the change of blood concentration of salicylic acid following oral administration of salicyluric acid. It is well known that rabbits take feces directly from the anus as they are being excreted. Chiou *et al.* reported that the stomachs of rabbits after fasting for 24 h were almost as full as those of unfasted animals, although the rabbits were maintained on a wire mesh support which allowed feces to drop to the bottom of the cage, thus

reducing coprophagy.¹⁰⁾ Also, Maeda *et al.* showed that the retardation and variation of oral drug absorption seen in the conventionally fasted rabbits are presumably due to the decrease and difference in the rate in which gastric contents transferred to the intestine, because their stomachs should have been full of solid material as a result of coprophagy.¹⁹⁾ Furthermore, they reported that the stomach-emptying-controlled rabbit can be a satisfactory model for evaluation of gastrointestinal absorption characteristics of these drugs with limited or regional solubility.²⁰⁻²³⁾

Figure 3 shows the blood concentration of salicyluric acid and salicylic acid following rectal administration of salicyluric acid. In control (Fig. 3a) and fasted rabbits (Fig. 3b and 3c), a small amount of salicyluric acid was absorbed in intact form. The rest was hydrolyzed to salicylic acid, which was subsequently absorbed. The blood concentration of salicylic acid was maintained for several hours. From these results, a significant effect of fasting was not observed in the blood concentration of salicylic acid following rectal administration of salicyluric acid. Therefore, it is suggested the benefit of rectal administration of the prodrug, which is converted to the parent drug by intestinal microorganisms.

Starvation has a profound influence not only on the growth and development of the body as a whole, but appears to affect different organs to varying degrees. The small intestine has been shown to be susceptible to the influences of prolonged fasting. Ju and Nasset demonstrated a disproportionately greater and more rapid loss of weight from the small intestine than the body as a whole during starvation in rats.²⁴⁾ In addition, Steiner *et al.* examined the effect of starvation for 6 d on the major tissue constituents of the rat small intestine.²⁵⁾ The data indicate that during starvation the weight loss of the small intestine is proportionally greater than that of the whole body, the total intestinal cell population is diminished, and there is a decrease in the RNA, protein and water content of the individual cells.

Doluisio *et al.* examined the effect of fasting on the intestinal absorption profiles of salicylic acid, barbital, haloperidol and chlorpromazine using *in situ* technique in rats.¹¹⁾ No apparent

Fig. 3. Blood Concentration of Salicyluric Acid and Salicylic Acid Following Rectal Administration of Salicyluric Acid (a), control (5); (b), fasting for 24 h (5); (c), fasting for 48 h (5). ○, salicyluric acid; ●, salicylic acid: Blood concentration and dose (5 mg/kg) of salicyluric acid: salicylic acid equivalent. Each point represents the mean $\pm$ S.E. Numbers in parentheses represent number of experiments. The control rabbits were not starved.

deviation in absorption patterns occurred when fasting periods were less than 20 h. However, when the period of starvation exceeded 20 h, absorption rates were found to decrease significantly and the decrease was dependent on the duration of the fasting period. Therefore, it appears that some physiologic and/or biochemical change that occurs during periods of starvation might be responsible for slowing the absorption rate of drugs. Diamond *et al.* reported that prolonged fasting may inhibit the intestinal drug absorption process by virtue of an induced diminution in intestinal blood perfusion in dogs.¹²⁾ In addition, Komuro *et al.* demonstrated that a reduction in the absorptive surface of the small intestine and the diminished volume of distribution induced by fasting is one of the main causes of the decrease of drug absorption in rats.^{14,15)} On the other hand, it is known that environmental and dietary stress may alter the gastrointestinal microorganisms.²⁶⁻²⁸⁾ Therefore, additional studies are needed to clarify the physiological factors affecting the formation of parent drug from prodrug by intestinal microorganisms before prodrug can be applied in humans.

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