An Assessment of Salicylic Acid-Induced Mucosal Damage *in Vivo* by Measuring the Metabolism of Salicylamide in Rabbit Intestine

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An assessment of salicylic acid-induced mucosal damage *in vivo* by measuring the metabolism of salicylamide (SAM) was investigated in rabbit intestine. The intestinal first-pass metabolism of SAM was studied using *in situ* intestinal sacs with complete mesenteric venous blood collection. The appearance of both SAM and its metabolites into the mesenteric venous blood was measured directly by cannulating the mesenteric vein of exposed intestine and collecting all venous blood draining from the absorbing region. Following oral pretreatment with salicylic acid, the appearance of SAM glucuronide (SAMG) in the mesenteric venous blood was significantly increased compared with the control. The increased blood concentration of SAMG following intraduodenal administration of SAM *in vivo* was observed in rabbits pretreated with salicylic acid orally. The blood concentration of SAMG after the intravenous administration of SAM was not increased compared with the control. We suggest that the change in the intestinal first-pass metabolism of SAM may be due to the intestinal mucosal damage induced by oral pretreatment with salicylic acid. The measurement of SAM metabolites may be of value in the assessment of intestinal mucosal damage *in vivo*.

Keywords mucosal damage; intestinal metabolism; salicylic acid; salicylamide; rabbit; intestinal absorption; screening test; membrane permeability; membrane transport; assessment

The ingestion of salicylic acid may result in epigastric distress, nausea and vomiting. Salicylic acid may also cause gastric ulceration, erosive gastritis and even hemorrhage. The gastrointestinal tract is an important site for adverse drug reaction. The gastrointestinal mucosal damage is usually assessed by macroscopical and microscopical examination. Measurement of gastrointestinal blood loss is also used extensively. The establishment of a convenient and noninvasive screening test for an assessment of gastrointestinal mucosal damage would considerably contribute to preformulation studies and preclinical stages of drug development, defining the potential for gastrointestinal mucosal damage in addition to biopharmaceutical, pharmacological and toxicological consideration. Studies using a scanning electron microscope have shown that the oral pretreatment of rabbits with salicylic acid (1 g/kg) before 24 h induced morphological changes in the gastrointestinal mucosa.[1] Other studies of gastrointestinal mucosal damage include use of the permeability of low molecular weight polyethylene glycols,[2-4] cellobiose and mannitol,[5-7] ^{51}chromium-labeled ethylenediaminetetraacetate[8] and phenolsulfonphthalein.[9-12] In the present study, an assessment of mucosal damage induced by oral pretreatment with salicylic acid was examined by measuring the metabolism of salicylamide (SAM) as a marker compound in rabbit intestine *in vivo*.

Experimental

Materials Salicylic acid and SAM were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and used as received. Carboxymethylcellulose sodium salt (CMC) was obtained from Hayashi Pure Chemicals Industries, Ltd. (Osaka, Japan), β-glucuronidase from Tokyo Zohki Kagaku Co., Ltd. (Tokyo, Japan), β-glucuronidase/arylsulfatase from Boehringer Mannheim GmbH (Mannheim, Germany) and heparin sodium salt from Novo Industries, Ltd. (Denmark). All other chemicals used in these experiments were of analytical or reagent grade.

Animal Experiments Male albino rabbits obtained from Kyudo Co., Ltd. (Kumamoto, Japan), 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). Salicylic acid (1 g/kg) suspended in a 1% CMC solution was administered by gastric intubation and animals were allowed free access to water only. Twenty four h later, *in situ* and *in vivo* absorption experiments were carried out.

***In Situ* Absorption Experiments** *In situ* rabbit intestinal sacs with complete mesenteric venous blood collection were prepared as reported by Barr and Riegelman.[13] The technique of collecting all venous blood draining from the region of absorption was developed to provide an *in vivo* preparation with intact circulation. The usefulness of this preparation is that free drug and its metabolites which are absorbed into the capillary blood can be collected in the venous effluent without reaching the general circulation. Animals were anesthetized with sodium pentobarbital (25 mg/kg), given intravenously, *via* an ear vein. Additional sodium pentobarbital was administered as necessary during the experiments to maintain anesthesia. After complete anesthesia, a midline incision was made, and the mid-ileal portion of the intestine (5 cm) was cut. The intestinal lumen was washed with 0.9% NaCl, and both ends of the mid-ileal portion of intestine were ligated to prepare a closed sac. This portion was selected because of its accessibility and suitable vasculature to facilitate cannulation. The mesenteric vein was cannulated with polyethylene tubing (SP 45, i.d. 0.58 mm, o.d. 0.96 mm, Natsume Seisakusho Co., Ltd., Tokyo, Japan). The coagulation of blood was prevented by the intravenous administration of heparin sodium salt (1000 I.U./rabbit). All venous blood was collected in centrifuge tubes and assayed for SAM and its metabolites. The amounts of SAM and its metabolites appearing in the blood were estimated by multiplying the volume of blood collected by the concentration of SAM and its metabolites in the blood. A SAM solution (3 ml of 67 μg/ml) was administered by direct injection into the intestinal sac with a syringe. Leakage of SAM solution at the injection site was not found. SAM was dissolved in the pH 7.2 buffer solution (145 mM NaCl, 4.56 mM KCl, 1.25 mM $CaCl_2$, 1.33 mM Na_2HPO_4 and 0.33 mM NaH_2PO_4) reported by Schanker and Tocco.[14] The blood lost from the mesenteric vein was replaced continuously by an intravenous infusion of 0.9% NaCl *via* an ear vein. The temperature of isolated intestine was kept at 37 °C by a lamp and it was moistened by the frequent application of 0.9% NaCl to a paper covering the intestine. Determination of SAM and its metabolites in the intestinal luminal solution was carried out as follows. At the end of an absorption period, the intestinal closed sac was isolated by tearing off the mesentery and the serosal surface was blotted with paper. The intestinal luminal solution was withdrawn as completely as possible, and the intestinal lumen was washed with distilled water. The washings were combined with the intestinal luminal solution and made up to 100 ml with distilled water. This sample solution was assayed for SAM and its metabolites. Results were compared statistically using Student's *t*-test.

Intravenous Administration of SAM SAM solution (30 mg/kg) dissolved in 0.1 N NaOH was administered intravenously *via* an ear vein. The blood was collected from the vein of another ear with a heparinized syringe at appropriate time intervals.

Intraduodenal Administration of SAM Animals were anesthetized with sodium pentobarbital (25 mg/kg), given intravenously, *via* an ear vein. After complete anesthesia, a midline incision (5 cm) was made, and SAM solution (30 mg/kg) dissolved in 0.1 N NaOH was administered with a

syringe by direct injection into the duodenum. Leakage of SAM solution at the injection site was not observed. The blood was collected from an ear vein with a heparinized syringe at appropriate time intervals.

Analytical Methods SAM, SAM glucuronide (SAMG) and SAM sulfate (SAMS) were quantitated from venous blood and intestinal luminal solution by the spectrofluorometric assay method reported by Shibasaki *et al.*[15] A Shimadzu RF-510 spectrofluorometer (Shimadzu Co., Ltd., Kyoto, Japan) was used. SAMG and SAMS were analyzed as SAM after the hydrolysis of the sample with β-glucuronidase or β-glucuronidase/arylsulfatase at 37 °C for 24 h.

Results and Discussion

In the previous report,[1] we examined the intestinal mucosal damage following oral administration of salicylic acid (1 g/kg) before 24 h using a scanning electron microscope in rabbits. In control rabbits, the duodenal villi were broad and occasionally folded on the longitudinal axis. Individual cells could not be discriminated at this magnification (×200). By the oral pretreatment with salicylic acid, some changes of the mucosal surface in the duodenum were recognized. Broad and tongue-shaped villi were found in the jejunum of the control rabbits. Oral pretreatment with salicylic acid resulted in morphological change of the jejunal mucosa as well as mucus secretion. In control rabbits, the ileal villi were broad and tongue-shaped structures. Following oral pretreatment with salicylic acid, the villi of the ileal mucosa were broken down and the villus surface was covered with secreted mucus.

In the present study, SAM was used as a marker compound for an assessment of the mucosal damage in rabbit intestine. SAM, a mild analgesic and antipyretic agent, was reported to be subjected to first-pass metabolism during intestinal absorption in rabbits,[13,16-18] rats[15,19-23] and dogs.[24-27]

The effect of oral pretreatment with salicylic acid on the intestinal first-pass metabolism of SAM was examined in rabbits using *in situ* intestinal sacs with complete mesenteric venous blood collection. The appearance of both SAM and its metabolites into the mesenteric venous blood was measured directly by cannulating the mesenteric vein of exposed rabbit intestine and collecting all venous blood draining from the absorbing region. SAM is metabolized to SAMG and SAMS in the rabbit intestine. Minor metabolites of SAM were not determined in this study.

Figure 1 shows the appearance of SAM (Fig. 1A), SAMG (Fig. 1B) and SAMS (Fig. 1C) in the mesenteric venous blood after an injection of SAM into the intestinal lumen. Following oral pretreatment with salicylic acid, a significant effect was not found in the appearance of SAM compared with the control (Fig. 1A). The cumulative amounts of SAM in the mesenteric venous blood tended to reach a plateau within 30 min, suggesting the rapid absorption of SAM in the intestine. Levels of SAMG increased from 30 to 120 min compared with the control (Fig. 1B). However, no effect was observed in the appearance of SAMS in the mesenteric venous blood (Fig. 1C). These results indicate the alteration of SAM metabolism by the intestinal mucosal damage.

To provide more information on the intestinal first-pass metabolism of SAM, SAM and its metabolites in the intestinal luminal solution after an injection of SAM were determined. The results are shown in Table I. In control rabbits, 1.1 μg of SAM (0.5% of dose) remained in the intestinal luminal solution. On the other hand, 1.7 μg of SAM (0.8% of dose) remained in the intestinal luminal solution in rabbits pretreated with salicylic acid orally. These results show almost complete absorption of SAM both in control and in salicylic acid-pretreated rabbits. In contrast with the result of an increased appearance of SAMG into the mesenteric venous blood, a significant decrease of SAMG appearance in the intestinal luminal solution was recognized compared with the control. In pretreated rabbits, the appearance of SAMS in the intestinal luminal solution tended to decrease compared with the control.

In order to examine the effect of oral pretreatment with salicylic acid on the distribution and elimination patterns of SAM, the blood concentration of SAM and its metabolites following the intravenous administration of SAM was determined. The results are shown in Fig. 2. In

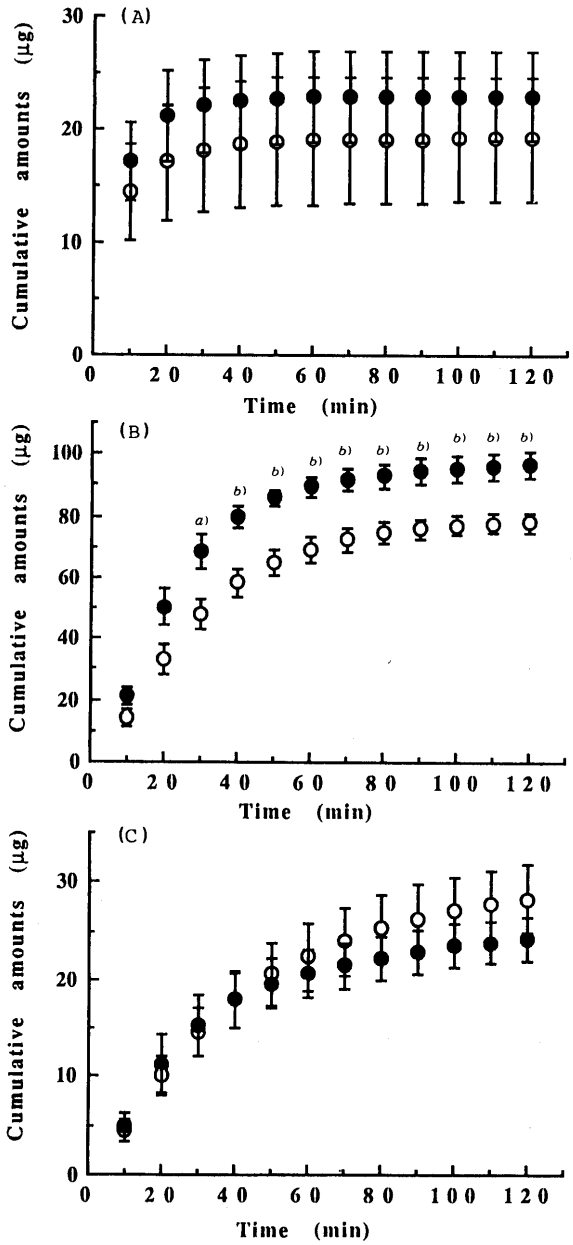

Fig. 1. Appearance of SAM and Its Metabolites in Mesenteric Venous Blood after an Injection of SAM in the Intestinal Lumen

(A): SAM, (B): SAMG, (C): SAMS. Key: (○), control (5); (●) salicylic acid pretreatment orally (5). Dose: 3 ml of 67 μg/ml solution of SAM. The amounts of SAMG and SAMS were calculated as SAM. Results are expressed as the mean ± S.E. Numbers in parentheses represent number of experiments. Statistical significance: *a*) $p<0.05$, *b*) $p<0.01$. The results of control were reported previously.[18]

Fig. 2A, the blood concentration of SAM in rabbits pretreated with salicylic acid orally did not change compared with the control, except for the decreased blood concentration of SAM in 5 min. Both the control and the salicylic acid-pretreated rabbits orally showed an almost identical blood concentration of SAMG and SAMS in Fig. 2B and Fig. 2C, respectively.

To assess the intestinal mucosal damage induced by oral pretreatment with salicylic acid *in vivo*, the blood concentration of SAM and its metabolites was determined after the intraduodenal administration of SAM. The results are presented in Fig. 3. In Fig. 3A, no effect was found in the blood concentration of SAM in rabbits pretreated with salicylic acid orally compared with the control. As shown in Fig. 3B, however, the blood concentration of SAMG in rabbits orally pretreated with salicylic acid was significantly increased from 15 min to 2 h, suggesting it was due to the alteration of SAM metabolism, but not due to the increased permeability of SAM in the intestine. In Fig. 3C, the blood concentration of SAMS in rabbits orally pretreated with salicylic acid did not change compared with the control,

TABLE I. SAM and Its Metabolites in the Intestinal Luminal Solution in 120 min

Pretreatment	SAM	SAMG	SAMS	Total
		(μg)		
Control (5)	1.1±0.6	23.3±1.8	16.9±1.8	41.3±3.9
Salicylic acid (5)	1.7±0.9	11.7±3.9[a)]	10.9±2.7	24.2±4.7[a)]

Dose: 3 ml of 67 μg/ml solution of SAM. Each value is expressed as the mean ± S.E. Numbers in parentheses represent number of experiments. The amounts of SAMG and SAMS were calculated as SAM. Statistical significance: *a*) $p<0.05$. The results of control were reported previously.[18)]

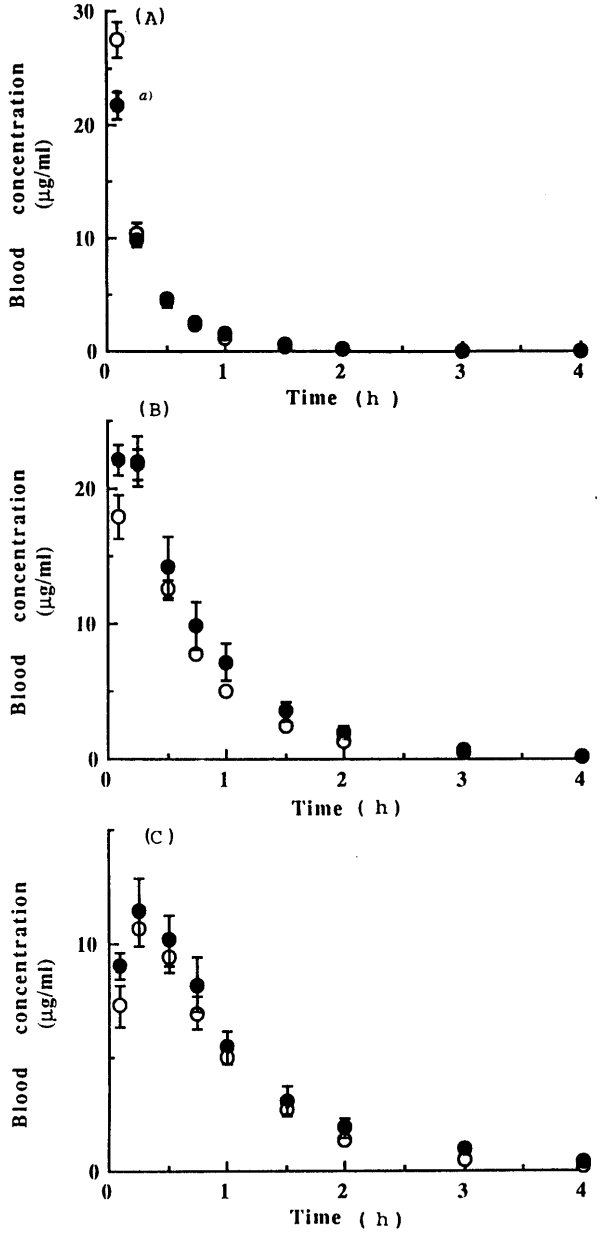

Fig. 2. Blood Concentration of SAM and Its Metabolites Following Intravenous Administration of SAM in Rabbits

(A): SAM, (B): SAMG, (C): SAMS. Key: (○), control (6); (●), salicylic acid pretreatment orally (5). Dose: 30 mg/kg of SAM. The blood concentration of SAMG and SAMS was calculated as SAM. Results are expressed as the mean ± S.E. Numbers in parentheses represent number of experiments. Statistical significance: *a*) $p<0.05$.

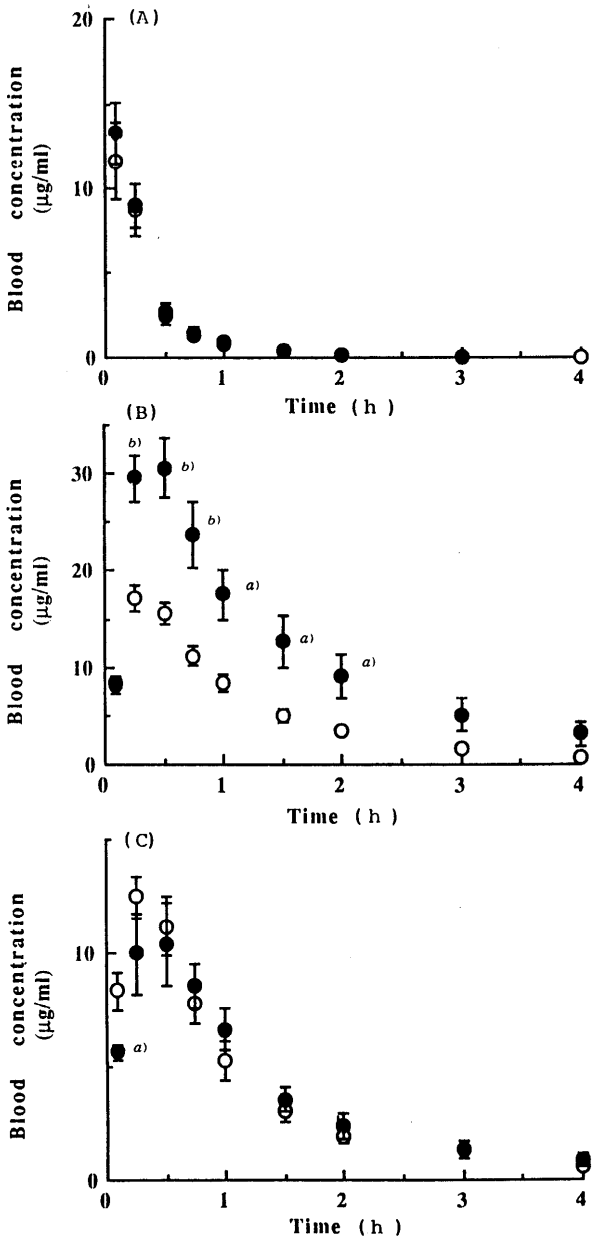

Fig. 3. Blood Concentration of SAM and Its Metabolites Following Intraduodenal Administration of SAM in Rabbits

(A): SAM, (B): SAMG, (C): SAMS. Key: (○), control (5); (●), salicylic acid pretreatment orally (5). Dose: 30 mg/kg of SAM. The blood concentration of SAMG and SAMS was calculated as SAM. Results are expressed as the mean ± S.E. Numbers in parentheses represent number of experiments. Statistical significance: *a*) $p<0.05$, *b*) $p<0.01$.

except for a decreased blood concentration of SAMS in 5 min.

In the previous report,[18] we demonstrated the change in the intestinal first-pass metabolism of SAM in rabbits orally pretreated with indomethacin using *in situ* intestinal sacs with complete mesenteric venous blood collection. By oral pretreatment with indomethacin, the total amounts of SAM absorbed in 20 and 120 min were significantly increased compared with the control. These results indicated the alteration of the permeability in the intestinal mucosa. In 20 min, indomethacin pretreatment resulted in an increased appearance of SAM and SAMG in the mesenteric venous blood. In 120 min, an increased appearance of SAM and a decreased appearance of SAMS were observed, compared with the control. On the basis of these considerations, it is suggested that the change in the intestinal first-pass metabolism of SAM is probably due to the mucosal damage in the intestine by oral pretreatment with indomethacin or salicylic acid. The mechanism by which the mucosal damage causes the change of SAM metabolism in the intestine is poorly understood. Further studies are needed to investigate the blood concentration and the urinary recovery of SAM and its metabolites after an oral administration of SAM. The alteration of the intestinal first-pass metabolism of a marker compound may be utilized as a convenient and noninvasive screening test for the quantitative assessment of intestinal mucosal damage *in vivo*.

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