Effect of Small Intestinal Transit Time on Gastrointestinal Absorption of 2-[3-(3,5-Di-*tert*-butyl-4-hydroxyphenyl)-1*H*-pyrazolo[3,4-*b*]pyridin-1-yl]ethyl Acetate, a New Non-steroidal Anti-inflammatory Agent

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The gastrointestinal absorption of 2-[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)-1*H*-pyrazolo[3,4-*b*]pyridin-1-yl]ethyl acetate (1), a new non-steroidal anti-inflammatory agent was investigated in dogs. A method using acetaminophen and salicylazosulfapyridine as the markers (double-marker method) was applied to trace the gastrointestinal transit of orally dosed 1. The mean absorption time of acetaminophen in plasma was used as an indication of gastric emptying, and the first appearance time of sulfapyridine (a metabolite of salicylazosulfapyridine) in plasma was employed to detect the arrival of the marker to the colon. A remarkable inter-individual variation was observed in the absorption of 1. The extent of bioavailability was little affected by the gastric emptying time, but significantly influenced by the small intestinal transit time. Under a pretreatment with atropine, the transit time was prolonged to result in a significant enhancement of the bioavailability. Consequently, the absorption of 1 is confirmed to take place mainly in the small bowel.

Keywords oral absorption; dog; small intestinal transit time; gastric emptying time; salicylazosulfapyridine; acetaminophen; variation; atropine

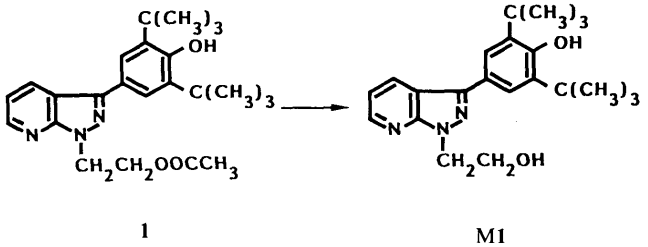
Introduction

The absorption of an orally administered drug is influenced by various physiological factors, such as gastric acidity[1] and bile flow.[2] Gastrointestinal transit is also considered to be one of the important determinants of drug absorption,[3] however, the oral bioavailability of a drug has been little discussed with the simultaneous monitoring of its gastrointestinal transit. In a previous paper,[4] we described a method to monitor the gastrointestinal transit of a drug in the dog, and discussed advantages of the method for use in a bioavailability study using chlorothiazide as a test compound. The method employed acetaminophen (AAP) and salicylazosulfapyridine (SASP) as respective marker compounds for the simultaneous determination of the gastric emptying time and small intestinal transit time of the drug.

2-[3-(3,5-Di-*tert*-butyl-4-hydroxyphenyl)-1*H*-pyrazolo-[3,4-*b*]pyridin-1-yl]ethyl acetate (**1**) is a new non-steroidal anti-inflammatory agent.[5] Compound **1** is poorly soluble in water over the physiological pH range, but is soluble in bile.[6] Therefore, we presumed this compound to be little affected by gastric acidity but influenced by the rate of transit through the gastrointestinal tract. During a preliminary formulation study for **1**, we evaluated its oral bioavailability in the dog while simultaneously watching the gastrointestinal transportation by the double-marker method.

Experimental

Material Compound **1** (mean particle size: 14.8 μm) and 2-[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)-1*H*-pyrazolo[3,4-*b*]pyridin-1-yl]ethanol (**M1**; an active metabolite of **1**)[5] were synthesized in our laboratories (Fig. 1). Sulfapyridine (SP), atropine sulfate and AAP were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). *p*-Hydroxyacetophenone (the internal standard; I.S.) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Salazopyrin tablets, each containing 500 mg of SASP were purchased from The Green Cross Co. (Osaka, Japan). All other reagents used were of analytical grade available from commercial suppliers.



1 M1

Fig. 1. Chemical Structures of **1** and **M1**

Preparation of Marker Suspension Salazopyrin tablets were crushed, added to AAP and suspended in distilled water so that the suspension contained 25 mg of SASP and 10 mg of AAP per 1 ml.

Analytical Method Since compound **1** is rapidly hydrolyzed *in vivo* and not detected in dog plasma, the concentration of **M1** was used to assess the bioavailability. The plasma **M1** concentration was determined by high performance liquid chromatography (HPLC). To 200 μl of plasma were added 1.25 μg of I.S. and 2 ml of hexane–ethyl acetate (1 : 1) solution. After shaking for 10 min and centrifuging at 3000 rpm for 5 min, 1.7 ml of the supernatant was transferred to a glass tube and evaporated to dryness under reduced pressure. The residue was dissolved in 200 μl of dichloromethane and 150 μl of this solution was injected into HPLC. The HPLC was carried out using a Shimadzu LC-6A apparatus equipped with a Nucleosil 50-5 (4.6 mm i.d. × 150 mm; M. Nagel) and a Shimadzu SPD-6A UV monitor (330 nm). The dichloromethane–methanol–28% ammonium water (99.2 : 0.76 : 0.04) mixture was employed as a mobile phase at a flow rate of 1 ml/min. The sensitivity was 10 ng/ml with less than 10% of the coefficient of variation of the assay. Typical chromatograms obtained from blank plasma and from plasma spiked with **M1** and I.S. are shown in Fig. 2.

Plasma concentrations of AAP and SP were measured simultaneously by HPLC under the conditions described previously.[7] The sensitivity was

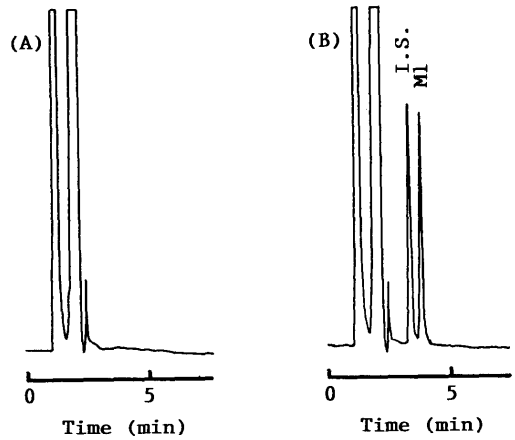


Fig. 2. HPLC Chromatograms of (A) Control Plasma and (B) Control Plasma Spiked with 200 ng/ml of **M1** and 2.5 μg/ml of I.S.

0.1 μg/ml for both compounds with less than 10% of the coefficient of variation of the assay.

Absorption Studies Six male beagle dogs weighing 11—13 kg were fasted from 20 h before to 24 h after the drug administration, but were allowed free access to water.

Each dog received drugs as follows: a 10 mg/kg intravenous dose of AAP as a 5% (w/v) solution in polyethylene glycol 400–saline (1 : 1) mixture; a 1 mg/kg intravenous dose of **1** as a 1% (w/v) solution in polyethylene glycol 400; a 3 mg/kg oral dose of **1** as a 0.3% (w/v) suspension in 0.5% (w/v) methyl cellulose solution (treatment A); the same treatment as treatment A, but immediately followed by 1 ml/kg oral dose of the marker suspension (treatment B); the same treatment as treatment B 10 min after a 0.1 mg/kg intravenous dose of atropine sulfate (treatment C). Treatment B was repeated twice on different days. Each dog received 30 ml (treatment A) or 20 ml (treatment B and treatment C) of water immediately after each treatment. Experiments were carried out at 1 to 2-week intervals. Blood samples (0.4—1 ml) were taken with heparinized syringes 0.25, 0.5, 1, 2, 3, 4 and 5 h after intravenous dosing with AAP, 0.5, 1, 2, 4, 6, 10 and 24 h after intravenous dosing with **1** and treatment A, and 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10 and 24 h after treatments B and C, respectively. The blood samples were centrifuged and the separated plasma samples were kept frozen until assay.

Effect of Atropine on Systemic Clearance of M1 Four male beagle dogs weighing 11—12 kg were used. Each dog received a 1 mg/kg intravenous dose of **1** as a 1% (w/v) solution in polyethylene glycol 400 (treatment 1); the same treatment as treatment 1 10 min after a 0.1 mg/kg intravenous dose of atropine sulfate (treatment 2). A 2-week interval separated the two treatments. The other experimental conditions were the same as those in the absorption studies described above.

Pharmacokinetic Analysis The maximum plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were determined from individual plasma concentration curves of **M1**. The area under the plasma **M1** concentration–time curve (AUC_{0-24h}) was calculated by the linear trapezoidal method and the mean residence time (MRT) was computed by moment analysis.[8] The extent of bioavailability was calculated as follows: $AUC_{0-24h,\,p.o.} \times dose_{i.v.}/AUC_{0-24h,\,i.v.} \times dose_{p.o.}$.

Gastrointestinal Transit Time The mean absorption time (MAT) of AAP was used as an index of the rate of gastric emptying of **1**. The value was calculated according to the following equation: $MAT = MRT_{p.o.} - MRT_{i.v.}$, where $MRT_{p.o.}$ and $MRT_{i.v.}$ represent the values of oral and intravenous AAP, respectively. Small intestinal transit time of **1** was assessed by the time for first appearance of SP in plasma.[4,7,9]

Statistical Analysis Pharmacokinetic parameters and transit times obtained from 3 treatments were subjected to analysis of variance according to the randomized block design, and subsequently to Tukey's multiple-range test. Differences between 2 mean values for paired treatments were evaluated by paired t-test. The correlation coefficients (r) between gastric emptying time and small intestinal transit time, gastric emptying time and the extent of bioavailability, and small intestinal transit time and the extent of bioavailability were respectively calculated by linear regression analysis.

Results

Bioavailability of Compound 1 A remarkable inter-individual variation in the plasma **M1** level was recognized from treatment A, as shown in Fig. 3. The pharmacokinetic parameters (mean ± S.D.) obtained were as follows: C_{max} (ng/ml), 179.1 ± 71.6; T_{max} (h), 1.7 ± 1.2; AUC_{0-24h} (ng·h/ml), 2048 ± 1234; MRT (h), 10.1 ± 0.6.

The bioavailability parameters of treatment B are listed in Table I. The MRT (h) from this treatment were 10.1 ±

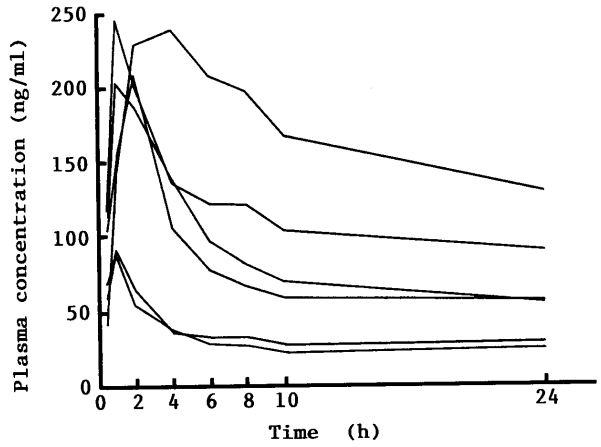


Fig. 3. Plasma Concentrations of **M1** in Individual Dogs Fllowing Treatment A

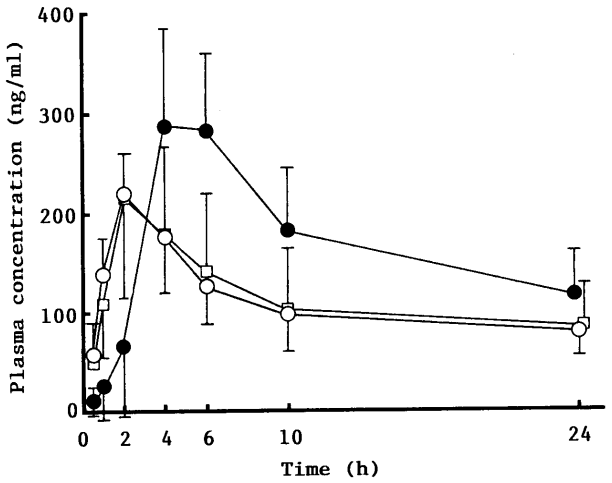


Fig. 4. Mean Plasma Concentrations of **M1** Following Treatment B (○, 1st Trial; □, 2nd Trial) and Treatment C (●)

Each point represents the mean ± S.D. ($n = 6$).

TABLE I. Pharmacokinetic Parameters of **M1** Following Treatment B and Treatment C

Dog No.	C_{max} (ng/ml)			T_{max} (h)			AUC_{0-24h} (μg·h/ml)			Bioavailability[a] (%)		
	Treatment B		Treatment C	Treatment B		Treatment C	Treatment B		Treatment C	Treatment B		Treatment C
	1st	2nd		1st	2nd		1st	2nd		1st	2nd	
1	261.4	236.4	261.0	2.0	2.0	6.0	2.38	2.41	3.50	33.8	34.3	49.8
2	193.6	270.4	393.5	2.0	4.0	4.0	2.15	3.69	4.81	20.3	34.7	45.3
3	256.8	342.5	341.6	2.0	2.0	6.0	3.90	4.87	5.54	32.6	40.7	46.3
4	153.7	32.5	144.8	2.0	4.0	6.0	2.14	0.60	1.98	32.5	9.1	30.1
5	256.8	263.6	279.6	4.0	2.0	4.0	2.98	2.57	3.12	49.5	42.7	51.7
6	193.4	218.8	373.7	2.0	2.0	6.0	2.05	2.00	4.88	23.9	23.3	57.0
Mean	219.3	227.4	299.0	2.3	2.7	5.3[b]	2.60	2.69	3.97[c]	32.1	30.8	46.7[c]
S.D.	45.2	104.5	91.6	0.8	1.0	1.0	0.72	1.46	1.34	10.1	12.6	9.1

a) The extent of bioavailability calculated as follows: $AUC_{0-24h,\,p.o.} \times dose_{i.v.}/AUC_{0-24h,\,i.v.} \times dose_{p.o.}$ *b*) Significantly different from treatment B ($p < 0.01$). *c*) Significantly different from treatment B ($p < 0.05$).

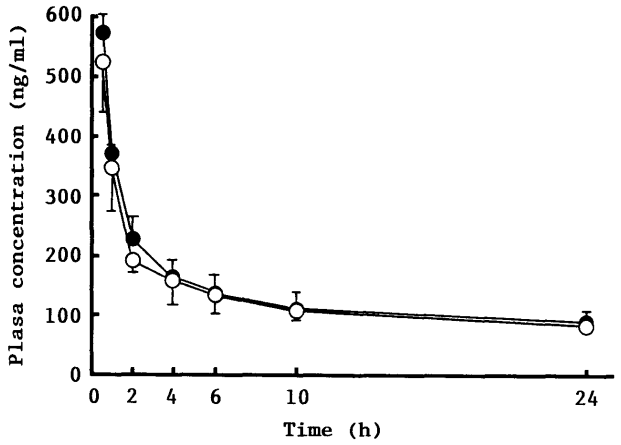


Fig. 5. Mean Plasma Concentrations of **M1** Following Treatment 1 (○) and Treatment 2 (●)

Each point represents the mean ± S.D. ($n=4$).

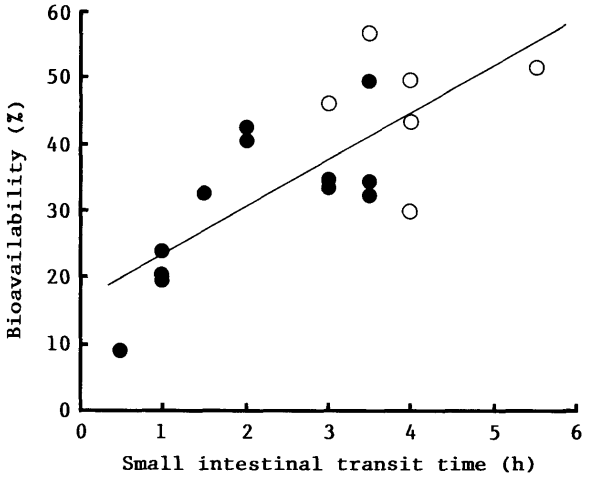


Fig. 6. Correlation between Small Intestinal Transit Time and the Extent of Bioavailability Following Treatment B (●) and Treatment C (○)

$r=0.73$, $p<0.01$.

TABLE II. Gastrointestinal Transit Time Following Treatment B and Treatment C

Dog No.	Gastric emptying time[a] (h)			Small intestinal transit time[b] (h)		
	Treatment B		Treatment C	Treatment B		Treatment C
	1st	2nd		1st	2nd	
1	0.12	0.10	1.41	3.0	3.5	4.0
2	0.04	0.50	1.33	1.0	3.0	4.0
3	0.23	0.29	1.24	3.5	2.0	3.0
4	0.39	0.17	2.18	1.5	0.5	4.0
5	0.32	0.46	1.42	3.5	2.0	5.5
6	0.17	0.17	1.03	1.0	1.0	3.5
Mean	0.21	0.28	1.44[c]	2.3	2.0	4.0[d]
S.D.	0.13	0.17	0.39	1.2	1.1	0.8

a) The mean absorption time of acetaminophen. *b*) The time for first appearance of sulfapyridine in plasma. *c*) Significantly different from treatment B ($p<0.01$). *d*) Significantly different from treatment B ($p<0.05$).

0.7 and 10.3 ± 0.8 (mean ± S.D.) for the 1st and 2nd trials, respectively. No significant differences ($p>0.05$) in corresponding parameters existed between treatment A and treatment B. Figure 4 shows the mean plasma **M1** levels resulting from treatment B and treatment C. The C_{max} from treatment C tended to be higher than that from treatment B, while the T_{max} from treatment C was significantly longer than that from treatment B. The values of AUC_{0-24h} and the extent of bioavailability for treatment C were both significantly larger than those for treatment B (Table I).

Effect of Atropine on Systemic Clearance of M1 The plasma **M1** concentrations declined similarly after treatment 1 and treatment 2 (Fig. 5). There was no significant difference ($p>0.05$) in the value of AUC_{0-24h} (ng·h/ml) between the two treatments: 3146 ± 534 for treatment 1 and 3343 ± 531 for treatment 2 (mean ± S.D.).

Gastrointestinal Transit Time Following treatments B and C, AAP was detected at the initial point (0.25 h) in all cases. Appreciable inter-individual variations were observed in the gastric emptying time and the small intestinal transit time for treatment B. Both transit times in treatment C were significantly longer than in treatment B (Table II).

There was no significant correlation between gastric emptying time and small intestinal transit time in treatment B ($r=0.22$, $p>0.05$), nor was an significant correlation observed between gastric emptying time and the extent of bioavailability ($r=0.55$, $p>0.05$); a significant correlation was recognized, however, between small intestinal transit time and the extent of bioavailability in treatment B ($r=0.73$, $p<0.01$), as shown in Fig. 6. The corresponding data for treatment C are also plotted in Fig. 6. These values were situated around the regression line resulting from treatment B.

Discussion

The rate of gastric emptying was assessed using AAP as a marker. Although AAP is poorly absorbed from the stomach, it is rapidly absorbed from the small bowel,[10] and hence the mean absorption time corresponds to the mean gastric emptying time. The rate of transit through the small bowel was assessed using SASP as a marker. Upon oral administration, SASP is partially absorbed during its transit through the small bowel and the unabsorbed part of the dose is metabolized to form SP in the colon.[11] Timing of the first appearance of SP in plasma corresponds to the arrival of the head of SASP in the colon.[12] Since a portion of SASP seems to leave the stomach immediately after administration, the time for first appearance of SP should indicate the time taken for the head of SASP to pass through the samll bowel. It has been pointed out that once liquid and pellets have left the stomach there is little additional spreading within the small bowel.[13] In fact, Bond and Levitt[14] described that the time to the inception of H_2 rise gave an good indication of the small intestinal transit time of the bulk of the lactulose orally administered. Since the SASP method has been proven to correlate well with the lactulose/breath hydrogen method,[12] the time for first appearance of SP seems to represent not only the small intestinal transit time of the head but also that of the whole bulk of SASP.

Compound **1** was not detected in the plasma after either oral or intravenous administration. In a preliminary study using rats, it was noted that plasma **M1** concentration after oral **1** was approximately 2.5-fold higher than that after an

oral equivalent dose of **M1**. This suggests that most of **1** is absorbed from the intestinal tract in an intact form. Compound **1** can be assumed to penetrate quickly into the intestinal wall after dissolution owing to its high lipophilicity.[6]

It has already been confirmed that the absorption of compound **1** from the gastrointestinal tract is dependent on the bile flow.[6] The bile salts are not absorbed in the proximal small bowel, bur are almost completely reabsorbed in the ileum.[15] Consequently, compound **1** apparently does not dissolve after arrival at the colon, and the absorption site is supposed to be restricted to the small bowel.

The extent of bioavailability of **1** was little affected by the rate of gastric emptying, but significantly correlated with the rate of transit through the small bowel. These results reveal that the absorption of **1** primarily occurs in the small bowel, *viz.* over a relatively wide range of the tract. The longer the residence time of **1** in the small intestinal tract is, the more of the compound may dissolve into the intestinal fluid resulting in an increased absorption.

The marker compounds, that is, AAP and SASP did not alter the absorption and elimination characteristics of **1**, since no significant differences in each corresponding pharmacokinetic parameter of **M1** were observed between treatment A and treatment B. Pretreatment with atropine, a potent inhibitor of gastrointestinal motility, enhanced the absorption of **1** and prolonged the small intestinal trasit time. The degree of increased absorption in treatment C was that predicted from the regression line for small intestinal transit time *vs.* the extent of bioavailability for treatment B. It is apparent that the atropine pretreatment had no effect on the systemic clearance of **M1**, since no difference in the values of AUC_{0-24h} existed between treatment 1 and treatment 2. Therefore, the prolonged transit time through the small bowel seems to cause the increased absorption of **1**. Moreover, the delayed T_{max} by atropine suggests that little of the compound is absorbed from the stomach. This seems to suggest that the compound does not dissolve in the stomach owing to its extremely poor water-solubility.

The bioavailability of many drugs with poor water-solubility has been reported to be variable.[16] The absorption efficiency of a drug is frequently affected by other drugs which depress or accelerate the gastrointestinal motility.[17] These phenomena were often interpreted to be caused by alteration in gastrointestinal transit, however, such discussions were made without any actual data on the transit. The transit through the small bowel is considered to be one of the important physiological fractors determining drug absorption,[3] nevertheless, there have been only limited reports which demonstrated this with the simultaneous measurement of the transit.

In the present study, the relationship between the absorption of lipophilic compound (**1**) and its transit in the gastrointestinal tract was investigated using AAP and SASP as markers for the monitoring of the transit. As mentioned above, we clarified that variations in the absorption of **1** arose mainly from the difference in the rate of transit through the small bowel. In addition, from *in vivo* absorption experiments we clarified that the main site of the absorption of **1** is the small bowel. This double-marker method is thus shown to be a useful tool to investigate the effect of the gastrointestinal transit time on drug absorption in the dog.

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