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Report

Pharmacokinetics of Sulfasalazine Metabolites in Rats Following Concomitant Oral Administration of Riboflavin

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Sulfasalazine, 60 mg/kg, was administered orally to groups of rats ($n = 4$) along with 1, 5, or 10 mg/kg of riboflavin. Plasma and urine were assayed for 5-aminosalicylic acid, acetyl-5-aminosalicylic acid, sulfapyridine, and acetyl-sulfapyridine using an HPLC method. The mean percent of dose recovered as total metabolites in urine was significantly greater ($\alpha = 0.01$) for the group receiving 10 mg/kg riboflavin compared to the controls or the group receiving 1 mg/kg riboflavin. Plasma AUC and C_{max} values were also significantly greater ($\alpha = 0.05$) for the 10 mg/kg riboflavin group. These results suggest that at higher doses, a significant fraction of riboflavin reaches the colon intact and stimulates more efficient reduction of the azo bond in sulfasalazine. Since the concentrations of 5-ASA achieved in the colon may be directly related to the efficacy of sulfasalazine in treating inflammatory bowel disease, concomitant administration of riboflavin may enhance sulfasalazine's efficacy in humans.

KEY WORDS: sulfasalazine; metabolites; riboflavin; azo-reduction; pharmacokinetics.

INTRODUCTION

Sulfasalazine is the most widely prescribed drug for the treatment of inflammatory bowel disease (1), the long term treatment of ulcerative colitis (2,3) and Crohn's disease (4). Sulfasalazine is a conjugate of 5-aminosalicylic acid (5-ASA) and sulfapyridine (SP) linked by an azo bond. Following oral administration, sulfasalazine is metabolized by the bacterial azoreductase enzymes in the colon (Fig. 1), reducing the azo bond and releasing these two components (5). Sulfasalazine itself may serve only as a prodrug to deliver the metabolic products, 5-ASA (a possible anti-inflammatory agent) and SP (an antibacterial agent), to the colon (6–9).

Riboflavin functions metabolically in the form of the two co-enzymes, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (10). The addition of flavins to *in vitro* tissue preparations enhanced the activities of the enzymes responsible for degrading azo compounds (11). Further hepatic carcinogenesis by azo dyes is potentiated by riboflavin deficiency (12–18), suggesting that the riboflavins, FAD and FMN affect azo reductase activity in the liver.

Khan *et al.* (19) reported that the cleavage of sulfasalazine at the azo bond in bacterial suspension and tissue homogenates *in vitro* was enhanced by NADP+, FAD+, and glucose 6-phosphate, suggesting that azoreduction is NADPH dependent and accelerated by flavins.

Although intestinal bacteria play an important role in the metabolism of sulfasalazine (20), no reports describing the effect of riboflavin *in vivo* on the reduction of the azo bond in sulfasalazine by the intestinal flora or in the liver have been published. This study reports the effect of oral riboflavin on the urinary excretion and pharmacokinetics of sulfasalazine metabolites in the rat.

MATERIALS AND METHODS

Materials

Sulfasalazine (lot 13F 0731), sulfapyridine (lot 54F 0635), 5-aminosalicylic acid (lot 15F 0803) and riboflavin (lot 16F 0216) were obtained from Sigma Chemical Company (St. Louis, MO). Acetyl-sulfapyridine (Ac-SP) and acetyl-5-aminosalicylic acid (Ac-5-ASA) were received as a gift from Pharmacia Laboratories (Sweden). All other chemicals were reagent grade, were purchased commercially, and were used as received.

Methods

Analytical. Analyses of urine and plasma samples for sulfasalazine metabolites were conducted using a reversed-phase HPLC method with uv detection at 254 mm (21). The assay was linear between 0.5 μg to 25 μg/mL for all sulfasalazine metabolite concentrations.

Animals. Male, CD albino strain rats (Charles River Laboratories, Wilmington, MA) weighing between 200–225 gm were used.

Urinary Excretion Studies. Rats were fasted overnight

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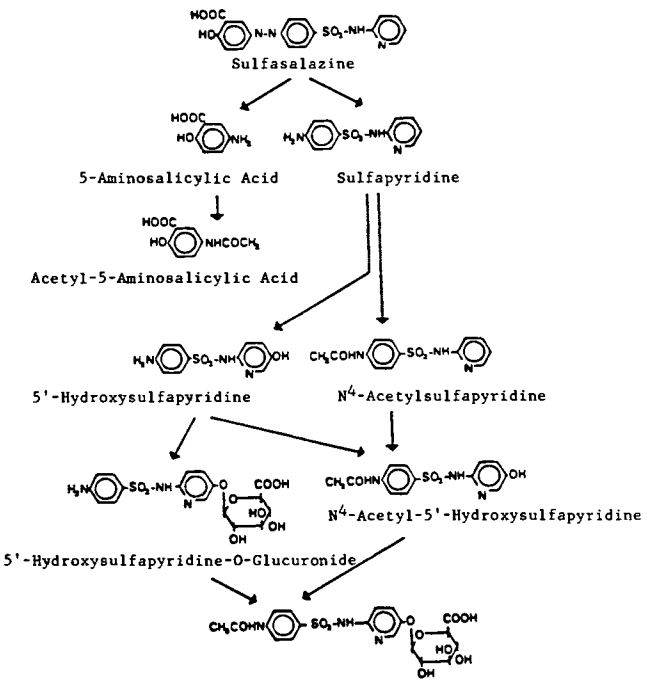


Fig. 1. Metabolism of sulfasalazine in man.

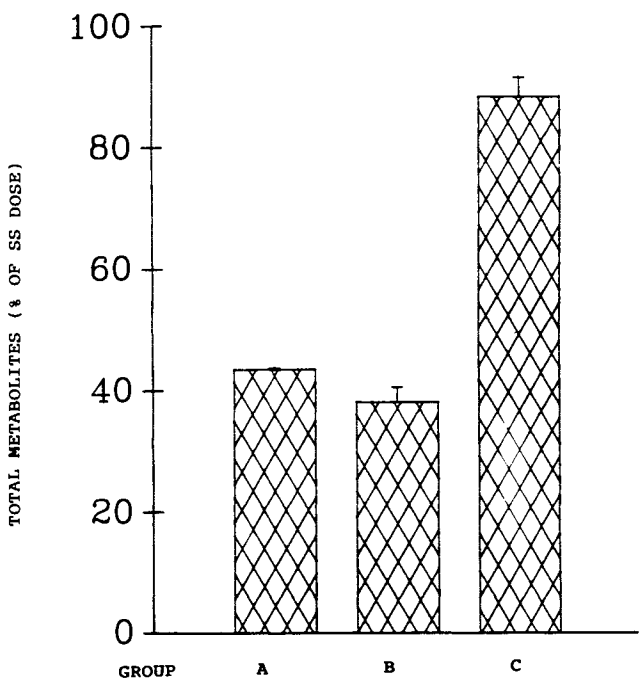


Fig. 2. Total metabolites as percent of sulfasalazine dose (60 mg/kg) excreted in urine over 48 hours. (A) control rats (43.3%); (B) riboflavin dose, 1 mg/kg (37.9%); and (C) riboflavin dose, 10 mg/kg (87.9%).

and water was allowed, *ad libitum*. A total of twelve rats were randomly assigned to three groups: A, B, and C (n = 4). Rats in Group A were administered 60 mg/kg of sulfasalazine suspended in aqueous media using 1% tragacanth gum by gastric gavage. Rats in Groups B and C were administered 60 mg/kg, respectively. All rats were housed in metabolism cages, and urine samples were collected at the end of 4, 8, 12, 24, and 48 hours, brought to 5 or 10 ml with distilled water and filtered. Diluted urine samples were frozen (−4°C) and stored in amber bottles in the dark until analyzed.

Oral Absorption Studies. One day prior to drug administration, rats were prepared surgically for jugular cannulation as described earlier (22). Food was withdrawn 12 to 16 hours prior to dosing. Sulfasalazine (60 mg/kg) and riboflavin were administered as described above, except that riboflavin doses of 5 mg/kg and 10 mg/kg were used for Groups B and C, respectively. Blood (0.25 mL) was drawn from the jugular vein at 0, 1, 2, 3, 4, 6, 8, 12, and 24 hours in heparinized tubes, and the plasma was harvested following centrifugation. The plasma samples were stored frozen (−4°C) and in the dark until analyzed.

RESULTS AND DISCUSSION

Table I shows the percent recovery of the metabolites of sulfasalazine (SS) excreted in the urine over 48 hours in rats. The data presented show that when 10 mg/kg of riboflavin (RF) is concomitantly administered with 60 mg/kg of SS, the recoveries of the metabolites (% dose) were significantly higher ($\alpha = 0.01$; Scheffe's test [23]) than either the control group (Group A) or the group receiving 1 mg/kg RF (Group B).

Levy and Jusko (24) have shown that RF absorption takes place mainly from the proximal region of the intestinal tract in man, and its absorption is site specific and saturable. The upper limit of intestinal absorption of RF appears to be about 25 mg in normal subjects (25). The RF dose of 10

Table I. Recovery of Sulfasalazine Metabolites Excreted in Urine[a]

Compound	Group A (60 mg/kg sulfasalazine) (n = 4)	Group B (60 mg/kg sulfasalazine + 1 mg/kg riboflavin) (n = 4)	Group C (60 mg/kg sulfasalazine + 10 mg/kg riboflavin) (n = 4)	ANOVA ($\alpha = 0.01$)
5-Aminosalicylic acid	4.6 ± 0.19	4.9 ± 0.61	8.2 ± 0.8	C > B & A
Sulfapyridine	9.7 ± 0.16	7.4 ± 0.67	13.9 ± 1.1	C > B & A
Acetylsulfapyridine	13.7 ± 0.18	11.9 ± 1.0	30.3 ± 1.9	C > B & A
Acetylaminosalicylic acid	15.3 ± 0.33	13.7 ± 0.63	35.7 ± 4.5	C > B & A

[a] Each value represents mean 48 hour recovery (% dose ± S.D.) following concomitant oral administration of sulfasalazine and riboflavin in fasting rats.

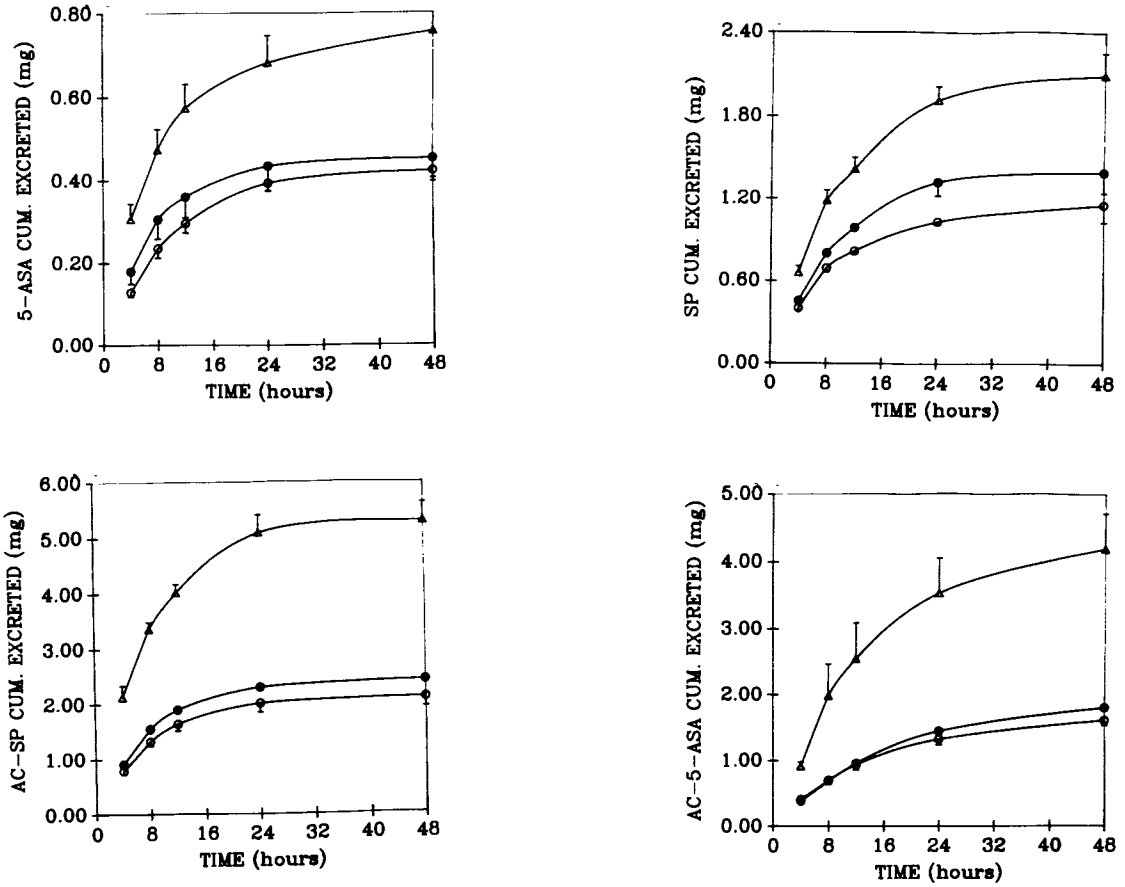


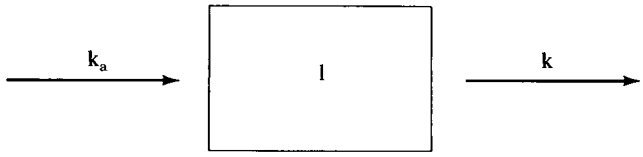
Fig. 3. Cumulative amounts of sulfasalazine metabolites excreted in urine following 60 mg/kg sulfasalazine: control group (○); riboflavin dose, 1 mg/kg (●); and riboflavin dose, 10 mg/kg (△).

mg/kg in the present study appears to exceed the saturable concentration in rats.

In a study of the effect of oral neomycin on the oral administration of SS to rats, Peppercorn and Goldman (20) found that none of the SS was recovered intact in urine, feces or cecum when the rats received SS alone. However, when the rats were pretreated with oral neomycin, about 50% of the administered SS was recovered intact in the cecum and feces. In addition, orally administered SS is known to reach the colon of humans intact where it is split by the bacteria into SP and 5-ASA (26,27). Results of the present study suggest that at a 10 mg/kg oral dose, significant amounts of RF reach the colon of rats where it stimulates the intestinal bacteria to efficiently cleave the azo bond of sulfasalazine. Figure 2 shows the total metabolites recovered from rat urine over the 48 hour period following oral administration of SS alone and with two dose levels of RF. The data presented show that about 88% of the dose is recovered as metabolites in rats when 10 mg/kg of RF is administered with SS.

The cumulative amounts of 5-ASA, SP, Ac-SP and Ac-5-ASA excreted in urine following the three treatments are shown in Fig. 3. These data also demonstrate that consistently higher amounts of each of the metabolites were excreted in urine in the group treated with 10 mg/kg riboflavin.

Mean plasma concentrations of 5-ASA, SP, Ac-SP and Ac-5-ASA following oral administration of SS (60 mg/kg) alone and with 5 and 10 mg/kg of RF are shown in Fig. 4. The plasma data were fitted by a one compartment pharmacokinetic model (Scheme I) using the PC NONLIN program (28):where k_a is the apparent first-order rate constant for



Scheme I

appearance of the metabolites in the central compartment (1) and k is the apparent first-order elimination rate constant for disappearance of the metabolites from the central compartment.

The mean values for the absorption half-life (k_a), elimination half-life (k), time of maximum metabolite concentration (T_{max}), maximum plasma metabolite concentration (C_{max}) and area under the plasma metabolite concentration-time curve (AUC, 0–24 hrs) of 5-ASA, SP, Ac-SP, and Ac-5-ASA are shown in Tables II, III, IV, and V, respectively.

The areas under the plasma concentration versus time curves for each of the metabolites for Group C; i.e., 10 mg/kg riboflavin dose, were consistently higher than for the con-

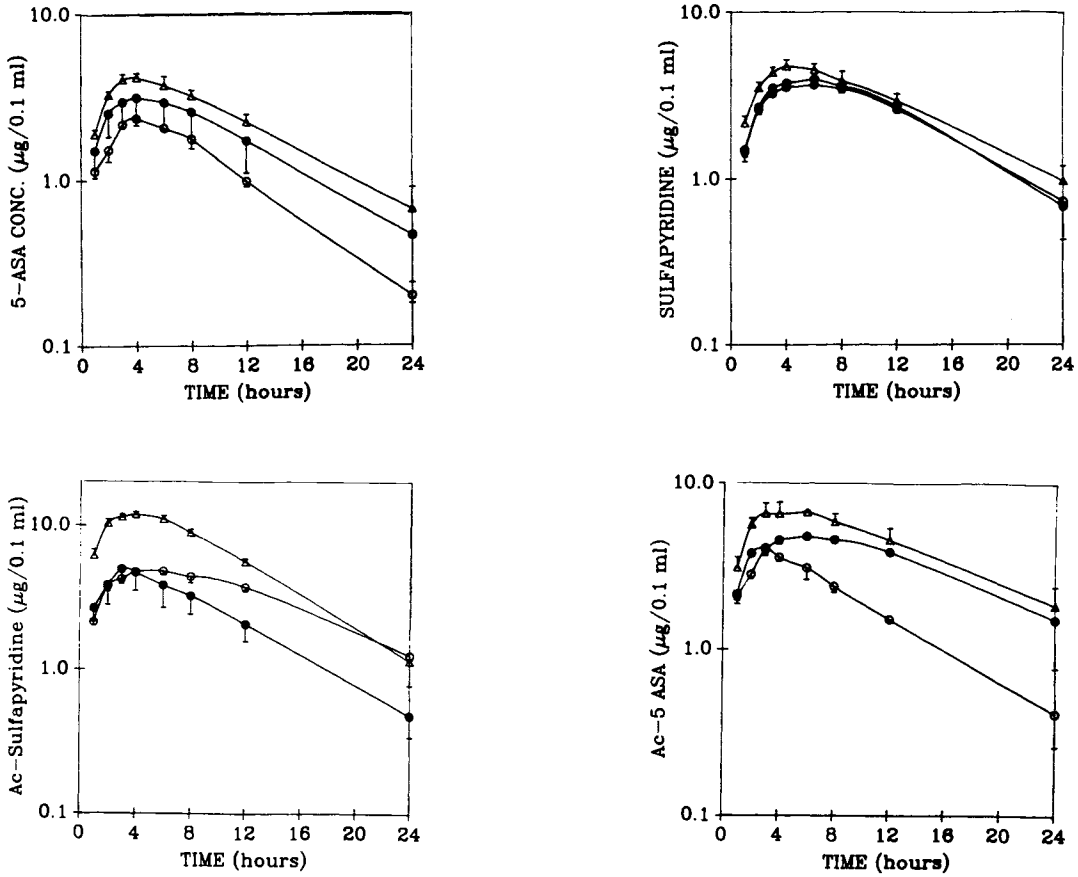


Fig. 4. Semi-logarithmic plots of sulfasalazine metabolite plasma concentrations (±S.D.) versus time following sulfasalazine (60 mg/kg) administered orally: control group (○); riboflavin dose, 5 mg/kg (●); and riboflavin dose, 10 mg/kg (△).

Table II. Mean Values of Pharmacokinetic Parameters for 5-Aminosalicylic Acid Following Oral Administration of Sulfasalazine and Riboflavin

Parameter	Group A (60 mg/kg sulfasalazine) (n = 4)	Group B (60 mg/kg sulfasalazine + 5 mg/kg riboflavin) (n = 4)	Group C (60 mg/kg sulfasalazine + 10 mg/kg riboflavin) (n = 4)	ANOVA (α = 0.05)
k_a half-life (hours)	3.87 ± 0.94	6.1 ± 0.87	6.85 ± 1.13	C & B > A
k half-life (hours)	2.34 ± 0.39	1.5 ± 0.11	1.18 ± 1.13	A > B & C
T_{max} (hours)	4.26 ± 0.05	4.18 ± 0.26	3.94 ± 0.28	N.S.[a]
C_{max} (μg/0.1 mL)	2.3 ± 0.27	3.14 ± 0.84	4.11 ± 0.29	C > B > A
AUC (0–24 hr) (μg • hr/0.1 mL)	26.4 ± 1.8	44.5 ± 15.4	58.59 ± 9.2	C & B > A

[a] Not significant.

Table III. Mean Values of Pharmacokinetic Parameters of Sulfapyridine Following Oral Administration of Sulfasalazine and Riboflavin

Parameter	Group A (60 mg/kg sulfasalazine) (n = 4)	Group B (60 mg/kg sulfasalazine + 5 mg/kg riboflavin) (n = 4)	Group C (60 mg/kg sulfasalazine + 10 mg/kg riboflavin) (n = 4)	ANOVA (α = 0.05)
k_a half-life (hours)	2.47 ± 0.12	3.01 ± 0.89	7.47 ± 0.63	C > B & A
k half-life (hours)	5.95 ± 0.28	4.81 ± 2.3	1.52 ± 0.08	C & B > A
T_{max} (hours)	5.36 ± 0.19	5.28 ± 0.28	4.54 ± 0.13	A & B > C
C_{max} (μg/0.1 mL)	3.76 ± 0.14	4.03 ± 0.38	4.67 ± 0.42	C > A & B
AUC (0–24 hr) (μg • hr/0.1 mL)	60.5 ± 2.9	60.74 ± 16.2	75.97 ± 11.4	N.S.[a]

[a] Not significant.

Table IV. Mean Values of Pharmacokinetic Parameters for Acetylsulfapyridine Following Oral Administration of Sulfasalazine and Riboflavin

Parameter	Group A (60 mg/kg sulfasalazine) (n = 4)	Group B (60 mg/kg sulfasalazine + 5 mg/kg riboflavin) (n = 4)	Group C (60 mg/kg sulfasalazine + 10 mg/kg riboflavin) (n = 4)	ANOVA (α = 0.05)
k_a half-life (hours)	2.38 ± 1.37	1.11 ± 0.18	5.2 ± 0.16	C > B & A
k half-life (hours)	7.87 ± 3.4	5.65 ± 0.14	1.48 ± 0.16	A & B > C
T_{max} (hours)	5.27 ± 0.59	3.43 ± 0.26	3.87 ± 0.06	A > B & C
C_{max} (μg/0.1 mL)	4.91 ± 0.16	4.67 ± 1.1	11.99 ± 0.54	C > B & A
AUC (0–24 hr) (μg • hr/0.1 mL)	56.75 ± 14.2	91.1 ± 13.1	148.77 ± 6.63	C > B > A

trol group (Group A) or the 5 mg/kg riboflavin dose (Group B). Analysis of variance (ANOVA) using Duncan's multiple range test (29) showed that the AUC values for 5-ASA for rats given 5 or 10 mg/kg of riboflavin (Groups C and B) were significantly higher (α = 0.05) compared to the controls (Group A). SP showed no statistically significant differences among the AUC values, but a trend toward higher AUC values was observed for Group C (Table III). The AUC's for the acetylated metabolites, Ac-5-ASA and Ac-SP, were, significantly higher (α = 0.05 and 0.,01, respectively) for Group C compared to Groups B and A.

C_{max} values were compared using Duncan's multiple range test (Tables II to V). Higher C_{max} values were associated with higher doses of riboflavin. The higher AUC and C_{max} values due to RF pretreatment are supported by the metabolites excretion data. Thus, a higher dose of riboflavin apparently reaches the colon and stimulates the azoreductase enzyme activity of the intestinal microflora to more efficiently reduce sulfasalazine. Alternatively, the gastrointestinal transit time may be delayed due to higher doses of riboflavin, allowing more time for the flora to metabolize the same dose of sulfasalazine.

The values for apparent absorption and elimination half-lives shown in Tables II, III, IV, and V were obtained by curve fitting and probably do not represent the true drug absorption and elimination half-lives (30).

Studies by Houston and Cassidy (31) suggested that the kinetics of 5-ASA and Ac-5-ASA are absorption rate limited and may be slower than the excretion rate following intraduodenal dosing in rats. After oral administration of sulfasalazine, sulfapyridine appears in the blood with a lag time of about 3 to 6 hours (32,33,34). The long lag time may be caused by the transit time for intestinal passage of sulfasalazine to its site of bacterial cleavage in the colon (7,35). Once sulfapyridine is formed, absorption occurs slowly with an apparent half-life of 2 to 5 hours (31,32). Thus, absorption and elimination processes tend to flip-flop and the terminal slope of the curve may represent either the absorption or the elimination or a hybrid of both processes.

The rate of disappearance of sulfasalazine metabolites from the gut of rats depends on (i) azoreduction of sulfasalazine in the colon and (ii) the absorption characteristics of the metabolites themselves. Estimation of the rate and extent of absorption of the primary metabolites is difficult because, in addition to the absorption process, conjugate of the primary metabolites may occur in the colon and liver. Therefore, the values of k_a and k presented in Tables II, III, IV, and V may not be true estimates of absorption and elimination rate constants for the metabolites. A further complicating factor is that the k (elimination rate constant) represents the sum of the rate constants for urinary excretion rate constant of SP or 5-ASA, the biotransformation rate constant for acetylation of the primary metabolites and "loss" due to other processes.

Since plasma concentration data following intravenous and oral administration of each individual metabolite were not available, estimates of true absorption and elimination rate constants could not be obtained.

The results of the present study demonstrate that rats given sulfasalazine and higher doses of riboflavin, increased the concentrations of 5-ASA and SP in the blood. These results, therefore, suggest that riboflavin stimulated the azoreduction of sulfasalazine and consequently increased the bioavailability of these metabolites. These results, in turn, suggest that higher concentrations of riboflavin at or above the saturable limit in the gastrointestinal tract pass down to the colon and stimulate the bacterial azoreductase enzyme system.

Table V. Mean Values of Pharmacokinetic Parameters for Acetyl-5-Aminosalicylic Acid Following Oral Administration of Sulfasalazine and Riboflavin

Parameter	Group A (60 mg/kg sulfasalazine) (n = 4)	Group B (60 mg/kg sulfasalazine + 5 mg/kg riboflavin) (n = 4)	Group C (60 mg/kg sulfasalazine + 10 mg/kg riboflavin) (n = 4)	ANOVA (α = 0.05)
k_a half-life (hours)	6.67 ± 1.73	9.25 ± 4.7	9.75 ± 2.6	N.S.[a]
k half-life (hours)	0.98 ± 0.26	2.44 ± 1.2	1.18 ± 0.44	N.S.
T_{max} (hours)	3.32 ± 0.25	5.55 ± 0.12	4.19 ± 0.37	B > C > A
C_{max} (μg/0.1 mL)	3.5 ± 0.33	4.88 ± 0.08	6.89 ± 0.63	C > B > A
AUC (0–24 hr) (μg • hr/0.1 mL)	47.17 ± 4.19	102.34 ± 23.8	128.91 ± 24.5	C & B > A

[a] Not significant.

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