Table VIII—Binding of I and 5-Chlorosalicylic Acid to Plasma

Species	Percent Bound[a]	
	I[b]	5-Chlorosalicylic Acid[c]
Human	80.5	99.4
Sprague—Dawley rat	54.6	71.1
Rhesus monkey	83.2	97.2
Beagle dog	78.9	97.5
Control, buffer only	−1.7	0.3

[a] Average of duplicates. [b] Initially there was 0.4 mg of I to be partitioned between 5 ml of plasma and 5 ml of buffer. [c] Initially there was 0.5 mg of 5-chlorosalicylic acid to be distributed between 5 ml of plasma and 5 ml of buffer.

In all three species, rat, dog, and human, cleavage of the oxazine ring of I yielded 5-chlorosalicylic acid, which was excreted into the urine in the free form and as conjugates with glucuronic acid and glycine.

The radioactivity from 3-labeled I appeared as respiratory carbon dioxide in exhaled breath and as β-hydroxybutyric acid and its metabolites fumaric, citric, α-ketoglutaric, succinic, and malic acids in the urine.

The metabolic fate of I is summarized in Scheme I.

CONCLUSIONS

The present study showed that I was converted to 5-chlorosalicylic acid by rats, beagle hounds, and humans and that the chemical entity present in the systemic circulation after the oral administration of I was not the intact drug but was the metabolite, 5-chlorosalicylic acid. The latter compound was excreted through the kidneys either free or conjugated with glucuronic acid and glycine. Elements of the isoxazole ring were converted to β-hydroxybutyric acid, which was metabolized by enzymes of the citric acid pathway.

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ACKNOWLEDGMENTS AND ADDRESSES

Received April 5, 1974, from *Wallace Laboratories, Division of Carter-Wallace, Inc., Cranbury, NJ 08512*

Accepted for publication August 1, 1974.

Presented in part at the meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N.J., April 1973.

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Comparative Anti-Inflammatory, Analgesic, and Antipyretic Activities of 7-Chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo-(3,2-*b*)(1,3)-benzoxazin-9-one and 5-Chlorosalicylic Acid in Rats

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Abstract □ Evidence is presented which indicates that 7-chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo-(3,2-*b*)(1,3)-benzoxazin-9-one (I) and 5-chlorosalicylic acid, its major metabolic end-product, are equally effective as anti-inflammatory and antipyretic agents, while the former is a somewhat more effective analgesic than its metabolite in the rat. However, at the equimolar doses used in this study, I is not ulcerogenic, while 5-chlorosalicylic acid does possess this untoward effect in the fasted rat. Moreover, the LD_{50} for 5-chlorosalicylic acid (261.0 mg/kg) is approximately 6.5 times less than that of I (1710.0 mg/kg) in the nonfasted rat. These results support the postulation that 5-chlorosalicylic acid is most likely responsible for the pharmacological activity displayed by I; *i.e.*, the latter acts as a carrier or delivery system, allowing attenuation of the toxic properties of its active metabolite.

Keyphrases □ 7-Chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo-(3,2-*b*)(1,3)-benzoxazin-9-one—anti-inflammatory, analgesic, and antipyretic activities compared to 5-chlorosalicylic acid, rats □ 5-Chlorosalicylic acid—anti-inflammatory, analgesic, and antipyretic activities compared to 7-chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo-(3,2-*b*)(1,3)-benzoxazin-9-one, rats □ Anti-inflammatory activity—7-chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo-(3,2-*b*)-(1,3)-benzoxazin-9-one and 5-chlorosalicylic acid compared in rats □ Analgesic activity—7-chloro-3,3a-dihydro-2-methyl-2*H*,-9*H*-isoxazolo-(3,2-*b*)(1,3)-benzoxazin-9-one and 5-chlorosalicylic acid compared in rats □ Antipyretic activity—7-chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo-(3,2-*b*)(1,3) - benzoxazin-9-one and 5-chlorosalicylic acid compared in rats

7-Chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo-(3,2-*b*)(1,3)-benzoxazin-9-one[1] (I) has been reported to be an orally effective nonsteroidal anti-inflammatory, analgesic, and antipyretic agent in rats (1). Unlike most other clinically useful nonsteroidal anti-inflammatory drugs, I is not ulcerogenic at markedly effective doses nor does it promote GI blood loss. In addition, I has a mild diuretic action, which does not contribute to its anti-inflammatory action.

The preceding study (2) showed that, in both the

[1] Wallace Laboratories W-2395.

Table I—Oral Activity and Toxicity of Compound I and 5-Chlorosalicylic Acid in the Carrageenan-Induced Edema Test in Rats

Drug	Carrageenan		Acute Toxicity[a]		Therapeutic Index, LD_{50}/ED_{50}
	ED_{50} (95% C.L.), mg/kg	mM[b]	LD_{50} (95% C.L.), mg/kg	mM[a]	
I	34.5 (23.3–51.1)	0.144	1710.0 (1335.0–2190.0)	7.16	49.6
5-Chlorosalicylic acid	25.8 (18.8–38.3)	0.150	261.0 (193.0–352.0)	1.52	10.0

[a] LD_{50} values for both the 24-hr and 7-day observation periods following single-dose administration were identical. [b] ED_{50} or LD_{50} values are expressed as millimolar.

Table II—Prophylactic Effect of Compound I and 5-Chlorosalicylic Acid on Adjuvant-Induced Polyarthritis in Rats

Treatment Group[a]	[Drug] in Diet, %	Actual Mean Dosage Consumed, mg/kg/day (mM)	Day 21 Measurements, Mean ± SE				
			Foot Volume, ml		Body Weight Net Gain, g, Mean ± SE	Erythrocyte Sedimentation Rate, mm/hr	Serum Albumin/ Globulin
			Injected	Uninjected			
Nonarthritic controls	—	— —	2.50 ± 0.08[b]	2.49 ± 0.08[b]	139.0 ± 3.1	2.8 ± 0.7	1.40 ± 0.09[b]
Arthritic controls	—	— —	4.79 ± 0.38	3.68 ± 0.25	61.0 ± 2.7[c]	6.0 ± 0.9	0.37 ± 0.05
I	0.1	76.5 (0.32)	3.66 ± 0.19[b]	2.70 ± 0.19[b]	91.5 ± 12.1[c]	4.0 ± 0.3	0.65 ± 0.10[b]
	0.2	150.4 (0.63)	2.93 ± 0.18[b]	2.40 ± 0.17[b]	73.5 ± 8.4[c]	2.6 ± 0.4[b]	0.86 ± 0.16[b]
5-Chlorosalicylic acid	0.1	74.3 (0.43)	3.03 ± 0.10[b]	2.44 ± 0.12[b]	82.5 ± 9.0[c]	2.6 ± 0.8[b]	1.26 ± 0.20[b]
	0.2	150.0 (0.87)	2.53 ± 0.07[b]	1.96 ± 0.06[b]	39.6 ± 6.1[c]	2.2 ± 0.8[b]	1.97 ± 0.19[b]

[a] n = eight rats per treatment group. [b] $p \leq 0.05$ when compared with arthritic control group. [c] $p \leq 0.05$ when compared with nonarthritic control group.

rat and dog, orally administered I was converted to its metabolite 5-chlorosalicylic acid by the intestinal wall during absorption. In addition, Edelson *et al.* (2) revealed that very little intact I could be detected in the systemic circulation. Moreover, these investigations (2) showed that absorption of I from the GI tract is not rate limiting. Since 5-chlorosalicylic acid appears to be the major metabolite of I, it was of interest to determine to what extent it might be contributing to the pharmacological properties of I. This report describes the results of a direct comparative study of I and 5-chlorosalicylic acid in various test systems.

EXPERIMENTAL

Animals—Male rats of the Sprague–Dawley strain[2] were used. They were allowed to acclimate to the laboratory environment for at least 5 days prior to use, with food and water allowed *ad libitum*.

Carrageenan-Induced Edema—A modification of the method of Winter *et al.* (3) was used. At least six rats, 100–120 g, were used at each dose level tested. All doses of I and 5-chlorosalicylic acid were prepared as suspensions in a 1% acacia vehicle for this and subsequent experiments unless otherwise stated.

Drugs were administered orally by gavage in a volume of 0.5 ml/100 g of body weight. One hour after dosing, 0.05 ml of a 1% solution of calcium carrageenan was injected subcutaneously into the plantar surface of the right hindpaw of each rat. The volume of the injected foot was measured by water displacement to the lateral malleolus immediately before drug administration and again 3 hr after carrageenan injection. The difference between the two was called the edema volume.

The mean edema volume was determined for each experimental group. An ED_{50} value, defined as that dose at which the edema volume was decreased by 25% or greater in 50% of the rats studied, was determined by the method of Litchfield and Wilcoxon (4).

Adjuvant-Induced Polyarthritis—Polyarthritis was induced in rats, 150–170 g at the start of the experiment, by injection of heat-killed *Mycobacterium tuberculosis* organisms (5). Each animal received 0.1 ml of adjuvant suspension (0.5% in heavy mineral oil) injected subcutaneously into the plantar surface of the right hindfoot. At least eight individually caged rats were used in each test group.

Appropriate concentrations of I and 5-chlorosalicylic acid were blended into a commercial diet, and the resulting mixtures were fed continuously to the test animals beginning immediately after injection of *M. tuberculosis*. The animals were maintained on this drug–diet admixture for the duration of the experiment, *i.e.*, 21 days. Nonarthritic (normal) and arthritic control animals received only the commercial diet. Beginning on Day 0 and subsequently on Days 7, 14, and 21 of the experiment, the following parameters were measured: drug–diet consumption, injected and uninjected hindpaw volumes, and body weight.

Group mean drug dosage was calculated from food consumption and body weight data. Following all measurements on Day 21, each rat was sacrificed by decapitation and a 2- or 3-ml blood sample was collected in heparinized vials. Erythrocyte sedimentation rate (6) and serum albumin/globulin ratios (7) were determined.

Analgesic Activity—The paw pressure method described by Randall and Selitto (8) was used to measure the analgesic effectiveness of I and 5-chlorosalicylic acid in rats (100–120 g). Inflammation was produced in the right hindpaw of each rat by injection of 0.1 ml of a 20% suspension of brewer's yeast in saline. One hour later, all rats (eight per group) received the test drug or vehicle orally (0.5 ml/100 g of body weight).

Pain threshold was measured[3] 1 hr following drug administration by applying pressure to the inflamed paw at a steadily increasing rate of 14 g/sec. The end-point, or pain threshold, was defined as that pressure necessary to cause the animals to struggle and/or vocalize. An analgesic effect was denoted if the individual reaction thresholds were 2 SD or greater than the mean threshold for the vehicle-treated control group (9). An ED_{50} value was determined (4) based on this all-or-none response.

Antipyretic Activity—Eighteen hours before oral drug administration, rats (~200 g) were rendered pyretic by subcutaneously injecting a 15% suspension of brewer's yeast (1.0 ml/100 g of body weight) according to the method of Smith and Hambourger (10). At least eight rats were used for each dose of drug and for fevered and nonfevered control groups.

Rectal temperatures were determined before the yeast injection and immediately before (designated the 0-hr reading) and at 2 and

[2] Charles River, Inc.

[3] Analgesy-Meter, Ugo Basile, Milan, Italy.

Table III—Oral Analgesic Effectiveness of Compound I and 5-Chlorosalicylic Acid in Rats as Measured by the Randall–Selitto Paw Pressure Test

Drug	Analgesia		Acute Toxicity		Therapeutic Index, LD_{50}/ED_{50}
	ED_{50} (95% C.L.), mg/kg	m*M*[a]	LD_{50} (95% C.L.), mg/kg	m*M*[a]	
I	84.0 (46.4—152.0)	0.35	1710.0 (1335.0—2190.0)	7.16	20.4
5-Chlorosalicylic acid	182.0 (92.0—360.0)	1.06	261.0 (193.0—352.0)	1.52	1.4

[a] ED_{50} or LD_{50} values are expressed as millimolar.

4 hr after oral drug administration. These measurements were recorded on a telethermometer[4] from a thermistor probe inserted a constant depth of 5.0 cm.

Drug-Induced Gastric Lesions—Ulcerogenic activity was studied by a method similar to that described by Domenjoz (11). Rats (~200 g), in groups of at least six, received the test drug or vehicle orally at 9 am and 4 pm on the test day. These animals were fasted for 18 hr prior to dosing, but they had access to water throughout the experiment beginning 0.5 hr prior to drug administration.

Each rat was sacrificed by chloroform inhalation 24 hr after the first dosing; its stomach was removed, opened along the greater curvature, and examined with a dissecting microscope for the presence of gastric ulcers. Animals with stomachs exhibiting at least one ulcer were considered positive responders. Statistical comparison among groups was carried out using the χ-square test with the Yates correction.

Toxicity—Single-dose oral LD_{50} values for I and 5-chlorosalicylic acid were determined in nonfasted rats (150–170 g) 24 hr and 7 days following drug administration. Ten rats per treatment group and at least three doses were used in this study. The number of animals dead in each group at each time interval was tallied and LD_{50} values were calculated (4).

RESULTS

Carrageenan-Induced Edema—Compound I and 5-chlorosalicylic acid were orally effective inhibitors of carrageenan-induced hindpaw edema in rats (Table I). When a comparison of their ED_{50} values was made, both compounds were equally potent anti-inflammatory drugs. The acute oral LD_{50} values for these compounds and their corresponding therapeutic index (LD_{50}/ED_{50}) were also calculated. Lethalities following a single oral administration of various doses of I or 5-chlorosalicylic acid occurred within the first 24 hr; hence, LD_{50} values for this period and for the 7-day observation period were identical. Although not depicted graphically, this comparison is valid since the slopes of the dose–response curves to determine the ED_{50} and LD_{50} of each drug are parallel. Table I reveals that although both compounds did not differ in their anti-inflammatory potency, I possessed a therapeutic index nearly five times greater than that observed for the metabolite.

Assuming that I (mol. wt. 239) was completely biotransformed to 5-chlorosalicylic acid (mol. wt. 172), justification for transformation of ED_{50} and LD_{50} values to millimolar units was warranted. Although the ED_{50} for I was somewhat, but not statistically significantly, greater than 5-chlorosalicylic acid, its corresponding millimolar value (0.144) was identical to that of its major metabolite (0.150).

Adjuvant-Induced Polyarthritis—Results of a comparative study of the prophylactic effect of I and 5-chlorosalicylic acid on adjuvant-induced polyarthritis in rats are shown in Table II. Dietary concentrations of 0.1 and 0.2% (approximately 75.0 and 150.0 mg/kg/day) of each drug significantly inhibited the development of adjuvant-induced polyarthritis. Enlargement of the injected foot (primary lesion) was markedly inhibited by each drug in a dose-related manner. However, the metabolite was significantly more effective than identical doses of I, with the highest concentration (0.2% or 150.0 mg/kg/day) of the former completely suppressing edema formation.

Similarly, a dose-dependent reduction of swelling in the contralateral, uninjected hindpaw (secondary lesion) was observed for each drug. The high concentration of I (0.2% or 150.4 mg/kg/day), as well as the low concentration of 5-chlorosalicylic acid (0.1% or 74.3 mg/kg/day), completely abolished this secondary involvement associated with adjuvant disease. On the other hand, the volume of the uninjected hindpaw of rats receiving the 0.2% drug–diet admixture of 5-chlorosalicylic acid was markedly less than the nonarthritic control group.

When the milligram per kilogram doses of I and 5-chlorosalicylic acid are converted to millimolar values (Table II), it is apparent that the millimolar doses of the latter compound are approximately 35% greater. This fact most likely accounts for the slightly greater activity of 5-chlorosalicylic acid against adjuvant-induced polyarthritis.

Another prominent manifestation of adjuvant-induced polyarthritis was a significant attenuation of body weight gain over the 21-day period, *e.g.*, 61.0 g for the arthritic controls compared to 139.0 g for the nonarthritic controls or approximately a 56.2% reduction (Table II). Suppression of body weight gain in arthritic animals treated with I or 5-chlorosalicylic acid was not restored to the rate of gain displayed by the nonarthritic control group. However, a trend toward normal body weight gain was noticed in the 0.1% I-treated (91.5 g) and 5-chlorosalicylic acid-treated (82.5 g) groups, since they gained significantly more weight than the arthritic control rats. But 0.2% of 5-chlorosalicylic acid further retarded body weight gain.

Table II also reveals that the erythrocyte sedimentation rate was markedly increased while the serum albumin/globulin ratio was severely lowered in adjuvant-induced polyarthritis. Compound I, as a 0.2% drug–diet admixture, significantly reversed the elevated erythrocyte sedimentation rate to the level of the nonarthritic control group. This effect was dose related, since the lower concentration (0.1%) of I did not significantly affect this measurement, although a trend was apparent.

Both concentrations of 5-chlorosalicylic acid were equally effective in completely reversing this elevated erythrocyte sedimentation rate. Likewise, the altered serum albumin/globulin ratio was partially restored by each concentration of I, whereas the 0.1% drug–diet admixture of the metabolite completely protected rats from this phenomenon. When the concentration of 5-chlorosalicylic acid in the diet was raised to 0.2%, serum albumin/globulin ratios were significantly elevated beyond the value for nonarthritic control rats.

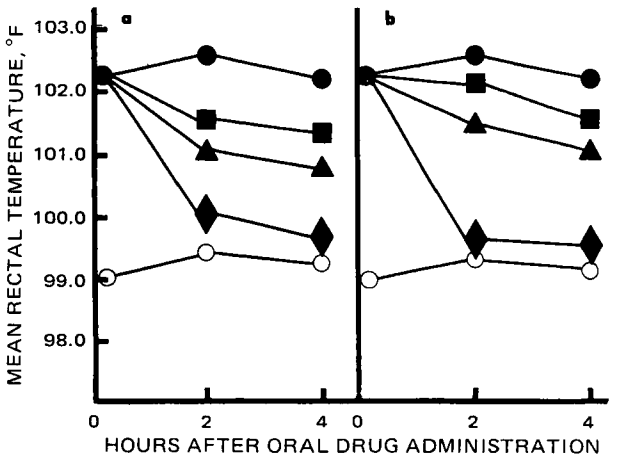

Figure 1—*Antipyretic activity of Compound I (a) and 5-chlorosalicylic acid (b) in rats with yeast-induced fever. Key: ●, fevered controls; ○, nonfevered controls; ■, 25.0 mg/kg; ▲, 50.0 mg/kg; and ♦, 100.0 mg/kg.*

[4] Yellow Springs Instrument Co., Yellow Springs, Ohio.

Table IV—Comparative Ulcerogenic Activity of Compound I, 5-Chlorosalicylic Acid, and Phenylbutazone in Rats[a]

Drug	Oral Dose, mg/kg (m*M*) bid	Number of Rats with Stomach Ulcers: Number of Animals Studied	Mortality
1% Acacia	—	0/44	0/44
I	139.0 (0.58)	0/15	0.15
	208.5 (0.87)	0/15	0/15
5-Chloro-	100.0 (0.58)	1/38	0/38
salicylic acid	150.0 (0.87)	11/28**	10/28*
Phenylbutazone	150.0 —	16/18***,†	0/18‡

[a] *$p < 0.05$, **$p < 0.02$, ***$p < 0.001$ when compared to the group receiving the high dose of I (208.5 mg/kg bid). †$p < 0.01$, ‡$p < 0.02$ when compared to the group receiving the high dose of 5-chlorosalicylic acid (150.0 mg/kg bid).

Analgesic Activity—Compound I and 5-chlorosalicylic acid were both orally effective analgesic agents when evaluated in rats using the Randall–Selitto paw pressure test (Table III). However, I was approximately 2.2 and 3.9 times more potent than its metabolite on a milligram per kilogram and millimolar basis, respectively. When the acute lethality of these compounds is considered, I has a substantially higher therapeutic index (20.4) than 5-chlorosalicylic acid (1.4).

Antipyretic Activity—When administered at oral doses (25.0, 50.0, and 100.0 mg/kg) which did not affect rectal temperature in nonpyretic rats, I and 5-chlorosalicylic acid significantly reduced fever 2 and 4 hr after dosing (Fig. 1). This activity was dose related for each compound, with the 100.0-mg/kg dose returning rectal temperature back to normal (nonfevered control). Moreover, the overall antipyretic effectiveness of I and 5-chlorosalicylic acid was nearly identical.

Drug-Induced Gastric Lesions—Following oral administration of markedly effective anti-inflammatory doses (total doses of 278.0 and 417.0), I did not produce gastric ulcers nor was it fatal to rats (Table IV). 5-Chlorosalicylic acid produced ulcers in 39% and killed 36% of the rats receiving a total dose of 300.0 mg/kg, which was equimolar to the high dose of I. Likewise, a 300.0-mg/kg total dose of phenylbutazone, a nonsteroidal anti-inflammatory agent with a propensity for ulcerogenesis, produced ulcers in 89% of the rats dosed but was not lethal. These data indicate that phenylbutazone is significantly more ulcerogenic than 5-chlorosalicylic acid and that each of these compounds is excessively ulcerogenic compared to I.

DISCUSSION

The results of the present investigation clearly demonstrate that both I and 5-chlorosalicylic acid, its major biotransformation product, were effective anti-inflammatory, analgesic, and antipyretic agents in the rat. On a milligram per kilogram basis, I and its metabolite were equally effective inhibitors of carrageenan-induced edema and brewer's yeast-induced pyresis. However, I was slightly, but significantly, less effective than 5-chlorosalicylic acid in the adjuvant-induced polyarthritis test when both were studied as 0.1 and 0.2% drug–diet admixtures. On the other hand, I was approximately 2.2 times as potent as 5-chlorosalicylic acid in its analgesic effectiveness.

A toxic manifestation shared by most clinically useful nonsteroidal antiarthritic compounds, including the salicylates, administered acutely or for long periods to laboratory animals (12) and humans (13) is a propensity to induce GI disturbances such as ulceration. Results obtained with I (Table IV) reveal that total doses as high as 417.0 (or 208.5 mg/kg bid) given orally did not induce ulcer formation in fasted rats. However, an equimolar dose of 5-chlorosalicylic acid (total dose of 300.0 or 150.0 mg/kg bid) not only caused ulcers to occur but proved fatal to a substantial number of the rats dosed. Likewise, phenylbutazone was markedly ulcerogenic but not lethal at 300.0 mg/kg. In a previous study (1), ulcer formation as well as lethality did occur following oral administration of a higher total daily dose of 600.0 mg/kg (or 300.0 mg/kg bid) of I.

Similarly, inspection of the LD_{50} values obtained following a single oral dose of I or its metabolite (Tables I and III) to nonfasted rats points out another striking difference in the toxicity of these two compounds. 5-Chlorosalicylic acid (261.0 mg/kg) was approximately 6.5 times as toxic as I (1710.0 mg/kg). The calculated LD_{50} for I in the present investigation closely correlates with the value of 1660.0 mg/kg for the nonfasted rat obtained in earlier experiments (1). Moreover, all deaths following oral administration of these compounds occurred within the first 24 hr.

The LD_{50} data reported for the nonfasted rat in this study, along with lethality data obtained previously (1) for nonfasted and fasted rats, clearly suggest that I is more toxic to fasted rats. This finding is not unreasonable since Zbinden (14) pointed out that many compounds are more toxic in fasted as compared to nonfasted rats. Therefore, this point may explain the discrepancy observed between the doses that were lethal and induced ulcer formation in the previous study (1) and the findings obtained in this study.

Based on the results of this investigation, it appears reasonable to speculate that 5-chlorosalicylic acid, the major metabolic product of I (2), may be largely responsible for the anti-inflammatory and antipyretic properties of this compound and, to a lesser degree, for its analgesic effectiveness. However, the profound ulcerogenic and lethal activity of 5-chlorosalicylic acid is avoided when this compound is metabolically generated following administration of I. As pointed out previously (2), orally administered I was converted to 5-chlorosalicylic acid as it passed through the intestinal wall. Therefore, I most likely functions as a carrier or unique delivery system for 5-chlorosalicylic acid *in vivo*, permitting manifestation of its beneficial pharmacological properties with complete avoidance of its deleterious GI effects.

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ACKNOWLEDGMENTS AND ADDRESSES

Received April 5, 1974, from *Wallace Laboratories, Division of Carter-Wallace, Inc., Cranbury, NJ 08512*

Accepted for publication December 26, 1974.

The authors acknowledge the technical assistance of Heidi Vassar, Linda Knobloch, Caroline Galbreath, Rose Mary Terzi, Elizabeth Kelton, James Harrison, Chin Chuen Ma, and John Melton.

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