Physiologic Disposition and Metabolic Fate of Indomethacin Analogs II: 1-(*p*-Chlorobenzoyl)-5-dimethylamino-2-methylindole-3-acetic Acid

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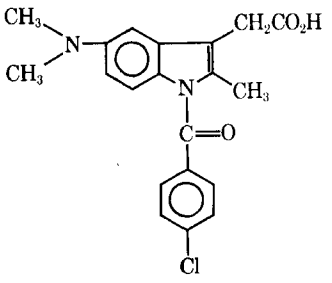
Abstract □ The physiologic disposition and metabolic fate of 1-(*p*-chlorobenzoyl)-5-dimethylamino-2-methylindole-3-acetic acid, an analog of indomethacin, were studied in several animal species and in man. This compound was well absorbed after oral administration and widely distributed in tissues. The drug was extensively metabolized in all species by hydrolysis of the *p*-chlorobenzoyl substituent, which was excreted in the urine as *p*-chlorobenzoylglycine. In this respect, the title compound differed from indomethacin in that the latter did not undergo hydrolysis in the dog. The acyl glucuronide was a metabolite of both drugs in man.

Keyphrases □ Indomethacin, indene analogs—physiologic disposition and metabolites, animals, man □ 1-(*p*-Chlorobenzoyl)-5-dimethylamino-2-methylindole-3-acetic acid—physiologic disposition and metabolites, animals, man □ Anti-inflammatory agents, potential—1-(*p*-chlorobenzoyl)-5-dimethylamino-2-methylindole-3-acetic acid, pharmacological evaluation □ GLC—determination

The anti-inflammatory activity of indomethacin, 1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid, was previously reported (1). The 5-dimethylamino analog (I) was one of several analogs synthesized (2) that retained pharmacologic activity comparable to that of indomethacin[1]. Replacement of the methoxy group by a dimethylamino substituent produced a more polar compound, with different ionization characteristics and a higher degree of water solubility. Insight as to the effect of this structural alteration on the physiologic disposition and metabolic fate of compounds in this series may be gained by comparison of such parameters for I and indomethacin. Results of such studies on I in several animal species and in man are described in the present report.



I

METHODS AND MATERIALS

I labeled with ^{14}C in the carbonyl carbon of the *p*-chlorobenzoyl group was prepared with a specific activity of 7.6 μc./mg.; it was >99% pure by TLC and paper chromatography. I labeled in the indole nucleus (2-position) was used in two experiments in rats. The specific activity was 6.8 μc./mg. *p*-Chlorobenzoic acid-7-^{14}C, with a specific activity of 37 μc./mg., was obtained[2]. *p*-Chlorobenzoylglycine was synthesized by reaction of *p*-chlorobenzoyl chloride with glycine as previously described (3). The crude product was washed with water and ether and recrystallized from hot water, m.p. 147–148°.

Anal.—Calc. for $C_9H_8ClNO_3$: C, 50.60; H, 3.78. Found: C, 50.90; H, 3.88.

Absorption and Excretion Studies—Adult male Sprague-Dawley rats, purebred beagle dogs of both sexes, male New Zealand white rabbits, male Carworth Farms mice, and male Hartley guinea pigs were used. Drug was administered as a solution of the hydrochloride in normal saline or water to animals and in gelatin capsules to normal human subjects. Doses were calculated in terms of free drug. Animals and human subjects were fasted overnight prior to oral administration of I. Urine was collected from animals housed individually in glass or stainless steel metabolism cages and was frozen as collected in dry ice-cooled containers. Urine was frozen after voiding in human experiments. Blood was collected in heparinized syringes, and the plasma was separated by centrifugation and frozen until assayed.

Tissue Distribution Studies—Tissues were obtained and prepared for analysis as previously described (4). Bile was collected from unanesthetized dogs by a technique previously described (4, 5).

Radioactivity Assay—Radioactive samples were assayed in a Tri-Carb liquid scintillation spectrometer[3] at 8° under conditions appropriate for measuring ^{14}C. All samples were counted in plastic vials containing 20 ml. of dioxane containing 8% naphthalene, 0.7% 2,5-diphenyloxazole, and 1,4-bis(5-phenyl-2-oxazolyl)-benzene. Plasma, urine, and aqueous extracts were counted directly; bile was diluted with water before counting. Feces were homogenized in a Waring blender with approximately 500 ml. of water and then diluted to 1 l. Aliquots of fecal and tissue homogenates were decolorized prior to counting by heating in alkaline peroxide (6). Counting was performed in the above medium containing 3% of a thixotropic gel[4]. Counting efficiency was determined by the internal standards method.

Measurement of I in Biological Samples—I was isolated from samples adjusted to pH 5 by extraction into heptane. The drug was back-extracted into 0.1 *N* NaOH, and the concentration was measured by spectrophotofluorometry. The procedure was as follows: One milliliter of plasma or urine was pipeted into a glass-stoppered centrifuge tube containing 2 ml. of 0.5 *M* citrate buffer, pH 5.0. Twenty-five milliliters of heptane, containing 3% isoamyl alcohol, was added, and the tube was shaken for 15 min. After centrifuging, 20 ml. of the organic phase was transferred into a second tube containing 5 ml. of 0.1 *N* NaOH. The tube was shaken for 5 min. and centrifuged. After removing the organic phase by aspiration, 2.5 ml. of the aqueous solution was transferred to a tube containing 0.5 ml. of 0.5 *M* citrate buffer, pH 5.0, and 0.2 ml. of 1 *N* HCl. The final pH was thereby brought to pH 5.0–5.5. The fluorescence of the solution was measured with an Aminco-Bowman spectrophotofluorometer (activation maximum, 295 nm.; fluorescence maximum, 385 nm., uncorrected).

The fluorescence reading was corrected for a "blank" value, obtained by analyzing control plasma or urine. The amount of drug present in the sample (assayed in duplicate) was calculated by comparison with the fluorescence obtained on assay of control plasma or urine to which known amounts of I were added. The fluorescence reading was proportional to the concentration from 0.1 to 10 mcg./ml. Recoveries of I added to plasma or urine in amounts

[1] Unpublished observations of Dr. C. A. Winter.

[2] New England Nuclear Corp.

[3] Model 314 EX or 3375, Packard Instrument Co.

[4] Cab-O-Sil.

Table I—TLC of Authentic I and Apparent Drug Isolated from Plasma and Urine

Solvent System	R_f Authentic I	R_f Apparent I
n-Butanol saturated I with 1 *N* ammonia	0.28	0.27
Ethyl acetate–isopropanol–10% ammonia (5:4:3)	0.48	0.46
Acetic acid–chloroform (5:95)	0.00	0.00
Isopropanol–7.5 N ammonia (4:1)	0.46	0.47
Acetic acid–isopropanol (5:95)	0.22	0.18

of 1–100 mcg. ranged from 75 to 93% for analyses performed over several months; excellent precision was obtained for any given day's analyses ($SD = \pm 2.3\%$).

Samples containing higher concentrations of I were assayed spectrophotometrically by a similar procedure, differing only in that the drug was back-extracted from the heptane into 5 ml. of 0.1 *N* HCl. The absorbance of the acidic solution at 265 nm. was measured, and the concentration was determined by comparison with readings from assay of known amounts of I. The absorbance (10-mm. lightpath) of a solution of 10 mcg./ml. of I in 0.1 *N* HCl was 0.55. Recoveries from urine were of adequate precision (85.3% ± 7.5 *SD*).

Possible interference in the assay by metabolites of the drug was studied by several techniques. Apparent I, isolated by the spectrophotometric procedure from plasma and urine obtained from various species, was reextracted from the dilute acid into ether after adjusting the pH to 5. The concentrated extract was subjected to TLC in several solvent systems. In all cases, only one spot was observed on the developed chromatoplate, corresponding in R_f value to that of authentic drug (Table I).

In addition, when heptane extracts of plasma from human subjects given I were assayed by GLC a major peak corresponding in retention time to authentic I methyl ester was observed.

Likewise, the excitation and fluorescence spectra of apparent drug extracted from plasma and urine were identical with those of authentic I.

GLC—GLC was performed on an F&M model 810 instrument, equipped with flame-ionization detectors and a column containing 3% QF-1 on Chromasorb G. The column, detector, and flash heater temperatures were 260, 280, and 280°, respectively. Helium was used as a carrier gas at a flow rate of 50 ml./min.

I was isolated from biological samples as was described for the spectrophotometric assay. The acid extract was shaken for 5 min. with heptane to remove isoamyl alcohol. The pH was then adjusted to 5 by addition of solid sodium acetate, and the solution was shaken with 10 ml. of chloroform for 10 min. The organic phase was evaporated to dryness *in vacuo*, and the residue was dissolved in 0.03 ml. of ether. About 0.05 ml. of an ethereal solution of diazomethane (prepared from 100 mg. of *N*-nitrosomethylurea) was added. After 5 min. the tube was placed in warm water in a hood, all solvent and unreacted reagent were allowed to evaporate, and the residue was dissolved in 0.01 ml. of ether. Approximately 3 μl. was injected into the chromatograph. The retention time (T_R) for authentic I methyl ester was 9.4 min.

TLC—Commercial silica gel-coated (250 μ) glass plates were used. I was visualized as a yellow spot after development by exposure of the plate to ammonia vapor. Radioactive compounds were located by scanning with a Packard model 7201 radiochromatogram scanner.

RESULTS

Oral Absorption and Plasma Levels—Plasma concentrations of I at various times after oral and intravenous administration of a single 5-mg./kg. dose of the ^{14}C-labeled drug were measured in rats (Table II). The plasma levels after either route of administration were comparable, indicating that oral absorption of drug was rapid and complete in this species.

Radiometric assay of plasma after extraction of I revealed that essentially all radioactivity had been extracted, indicating that only unchanged I was present. The half-life of I at this dose level in rat plasma was approximately 4 hr. A similar value was obtained after a lower oral dose of I (1 mg./kg.).

Plasma concentrations of I were also measured in dogs at various times after oral and intravenous administration of drug (Table II).

Plasma levels after oral administration were comparable to those after an intravenous dose, indicating that the drug was also well absorbed in the dog. Rapid drug absorption was indicated by the appearance of a peak plasma concentration 30 min. after oral administration. The plasma half-life was approximately 4 hr. in this species. The plasma contained only unchanged I, as shown by solvent extraction and TLC.

Plasma concentrations of I were determined in six human subjects given the drug orally (Table II). Levels were maximal 1 hr. after drug administration in five of the six subjects; the remaining subject had a peak concentration 2 hr. after drug administration. These results indicate that the drug was rapidly absorbed in man. The half-life was approximately 1 hr. Low, but measurable, concentrations of drug were found in plasma 25 hr. following drug administration. Similar results were obtained in four subjects after oral administration of I-^{14}C (300 mg. of I, including 25 μc. of I-^{14}C). Only I was present in plasma during the first 2–4 hr. after drug administration. At 6–8 hr., however, about 50% of the plasma radioactivity was not extractable into heptane and presumably represented a metabolite(s) of the drug.

Tissue Distribution—Binding of I to nondialyzable constituents of plasma was measured, using ^{14}C-labeled drug, by equilibrium dialysis at 22° for 18 hr. Under these conditions, approximately 97% of the compound was bound in human plasma when added in concentrations ranging from 1 to 25 mcg./ml. Extensive binding was also noted in similar experiments with plasma from rats (96%), dogs (92%), rabbits (98%), and guinea pigs (93%).

Distribution of radioactivity in rat tissues after intraperitoneal administration of I labeled in either the chlorobenzoyl carbonyl-^{14}C or the 2-indole-^{14}C position was determined after 1 and 24 hr. (Table III). After 1 hr., radioactivity was widely distributed among the various tissues, with the possible exception of the brain. Radioactivity was, however, not concentrated, relative to plasma, in any organ. It was localized, to some extent, in the liver, kidney, small intestine, and red blood cells. After 24 hr., radioactivity levels in the liver, kidney, and large intestine had increased relative to plasma, whereas levels in the other tissues had declined at nearly the same rate as in plasma.

Table II—Plasma Levels of I in Various Species following a Single Oral or Intravenous Dose[a]

Hours	Plasma Concentration, mcg./ml. Rat Intravenous	Rat Oral	Dog Intravenous	Dog Oral	Man Oral
0.25	25.0 ± 3.6	23.4 ± 3.4	8.5 ± 0.8	—	—
0.5	19.3 ± 1.2	24.0 ± 0.4	3.6 ± 0.6	2.3 ± 0.2	—
1.0	16.4 ± 0.2	19.9 ± 0.1	1.7 ± 0.2	2.2 ± 0.9	13.6 ± 1.2
2.0	11.1 ± 0.4	19.4 ± 0.8	1.6 ± 0.7	1.5 ± 0.5	8.2 ± 1.3
3.0	—	—	—	—	2.9 ± 0.5
4.0	11.4 ± 1.6	12.0 ± 1.0	1.0 ± 0.1	1.3 ± 0.6	1.4 ± 0.4
5.0	—	—	—	—	1.0 ± 0.4
6.0	7.6 ± 0.8	9.9 ± 0.3	1.1 ± 0.1	1.1 ± 0.4	0.9 ± 0.3
8.0	—	—	—	—	0.9 ± 0.2
24.0	0.5 ± 0.0	0.1 ± 0.0	—	0.1 ± 0.1	0.6 ± 0.3

[a] Values represent the mean ± *SE* for three rats, four dogs, and six human subjects by each route; animals received 5 mg./kg. of I-^{14}C, and human subjects received 300 mg.

Table III—Tissue Distribution of Radioactivity in Rats after a Single Intraperitoneal Dose of I-^{14}C (3 mg./kg.)[a]

Tissue	Tissue Radioactivity			
	1 hr.		24 hr.	
	Group A	Group B	Group A	Group B
Liver	0.60 (0.55–0.64)	0.58 (0.48–0.67)	1.06 (0.86–1.26)	0.98 (0.90–1.07)
Testes	0.15 (0.13–0.16)	0.08 (0.07–0.08)	0.12 (0.10–0.14)	0.17 (0.15–0.19)
Fat	0.17 (0.17–0.17)	0.12 (0.11–0.13)	0.13 (0.09–0.16)	0.16 (0.14–0.18)
Kidney	0.41 (0.39–0.43)	0.28 (0.25–0.31)	1.26 (1.12–1.40)	1.18 (1.12–1.25)
Spleen	0.10 (0.09–0.10)	0.14 (0.13–0.14)	0.08 (0.08–0.08)	0.16 (0.13–0.19)
Small intestine	0.39 (0.29–0.48)	0.62 (0.41–0.92)	0.39 (0.26–0.48)	1.64 (0.68–2.59)
Brain	0.02 (0.02–0.03)	0.02 (0.02–0.02)	0.02 (0.00–0.03)	0.05 (0.03–0.08)
Lung	0.28 (0.24–0.31)	0.29 (0.27–0.31)	0.24 (0.23–0.24)	0.19 (0.17–0.22)
Muscle	0.10 (0.09–0.11)	0.04 (0.04–0.04)	0.06 (0.05–0.06)	0.10 (0.10–0.11)
Heart	0.19 (0.18–0.20)	0.17 (0.13–0.21)	0.19 (0.15–0.22)	0.34 (0.34–0.35)
Red cells	0.63 (0.57–0.69)	—	0.72 (0.63–0.80)	—
Stomach	0.16 (0.09–0.23)	0.15 (0.10–0.20)	0.13 (0.12–0.14)	0.43 (0.25–0.60)
Large intestine	0.17 (0.12–0.21)	—	0.45 (0.29–0.57)	—
Plasma	13.2 (12.3–14.2)	17.3 (17.1–17.4)	0.7 (0.3–1.1)	1.50 (1.23–1.77)

[a] Group A represents four rats given I-chlorobenzoyl carbonyl-^{14}C; Group B represents four rats given I-indole-2-^{14}C. Two rats of each group were sacrificed at each time. Plasma levels are given as micrograms per milliliter; tissue levels are expressed as the ratio of tissue concentration to the corresponding plasma concentration. Average values are shown with individual figures in parentheses.

Analyses of plasma, liver, kidney, and small intestine for I after administration of I labeled in the chlorobenzoyl carbonyl group revealed that virtually all of the radioactivity in these tissues after 1 hr. was present as unchanged drug.

Tissue levels after administration of drug labeled in different positions were generally comparable after 1 hr. This also suggested that tissue radioactivity at that time was mainly present as I, since greater discrepancies presumably would have appeared if the *p*-chlorobenzoyl moiety of I were cleaved and distributed in tissues. After 24 hr., the only marked difference between the two labeled forms was in the small intestine, suggesting that a metabolite(s) was present.

Urinary and Fecal Excretion—Urinary and fecal excretion of I was measured in rats and dogs after single oral and intravenous doses of labeled drug (Table IV). A total of 45.7% of an oral dose to rats was recovered in the urine, and approximately 26% of the dose was recovered in the feces. Intravenous administration to rats resulted in an almost identical excretion pattern, 49.5% being found in the urine and 28.4% in feces after 4 days. The results demonstrated that the drug was well absorbed in the rat.

Approximately 32% of the dose of I-^{14}C to dogs was excreted in the urine in 24 hr. after either route of administration; excretion in the feces amounted to 52.6 and 40.7% of the dose after intravenous and oral administration, respectively.

Urinary and fecal excretion of radioactivity was also measured in several other species after a single oral dose of labeled drug (Table V). Mice given I orally excreted 66% of the dose in the urine and 9.0% in feces. Guinea pigs given labeled I intracardially excreted 76% of the dose in urine and 12% in the feces. Rabbits given labeled I intravenously excreted 88% of the dose in the urine

Excretion of total radioactivity in the urine and feces of four human subjects was measured after oral administration of a single dose of 300 mg. of I (including 25 μc. of ^{14}C-labeled I) (Table V). The drug was excreted primarily (62–75% of the dose) in the urine, chiefly in the first 24 hr. These findings indicate that I was extensively absorbed after oral administration to man and that it was excreted relatively rapidly and completely, mainly in urine. The excretion pattern found in man resembled that found in mice, rabbits, and guinea pigs more closely than the pattern found in rats and dogs.

Biliary Excretion—Biliary excretion of radioactivity and of free I was determined in a dog given a single 5-mg./kg. dose of I (including about 14 μc. of ^{14}C drug) intravenously. The results (Table VI) indicated that the drug was extensively excreted in bile (94.7% of the dose in 6 hr.). However, only 1.3% of the dose was present in the bile as apparent free drug. Plasma levels of ^{14}C declined very rapidly after intravenous administration to a dog with a bile duct fistula. The estimated half-life was <30 min. Almost all of the radioactive material (>90%) in plasma was apparent free drug.

A second experiment, in which the same dose was given orally to this dog, showed extensive biliary excretion as well, but somewhat less than after the intravenous dose. Approximately 10% of the total

Table IV—Urinary and Fecal Excretion of Radioactivity in Rats and Dogs after Administration of I-^{14}C[a]

Day	Dose Recovered, %							
	Rat				Dog			
	Oral		Intravenous		Oral		Intravenous	
	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces
1	39.5 ± 2.0	14.9 ± 0.4	38.1 ± 1.6	6.9 ± 4.8	24.6 ± 1.1	26.1 ± 9.0	26.8 ± 1.4	36.8 ± 4.6
2	5.4 ± 0.9	13.1 ± 0.9	6.5 ± 1.9	14.3 ± 1.4	4.9 ± 0.6	12.9 ± 4.3	4.6 ± 1.6	10.2 ± 1.2
3	2.6 ± 0.2	0.3 ± 0.0	1.1 ± 0.4	4.5 ± 2.2	2.1 ± 0.6	1.7 ± 1.0	1.6 ± 0.6	4.2 ± 1.4
4	2.0 ± 0.6	0.1 ± 0.1	—	—	—	—	—	—
Total	49.5 ± 3.1	28.4 ± 0.6	45.7 ± 3.1	25.7 ± 1.8	31.6 ± 1.3	40.7 ± 4.3	33.0 ± 3.2	51.2 ± 4.0

[a] Values represent the mean ± *SE* for three rats and four dogs given 5 mg./kg. of I-^{14}C.

Table V—Urinary and Fecal Excretion of Radioactivity in Mouse, Rabbit, Guinea Pig, and Man after Administration of I-^{14}C[a]

Day	Dose Recovered, %							
	Mouse		Rabbit		Guinea Pig		Man	
	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces
1	60.0	4.0	51.9 ± 18.4	0.0	68.0 ± 3.2	11.0 ± 2.2	58.6 ± 6.4	2.2 ± 2.0
2	6.3	5.0	28.6 ± 13.5		6.0 ± 0.7	0.6 ± 0.1	11.4 ± 3.8	1.9 ± 1.2
3	—	—	7.0 ± 6.8		1.7 ± 0.2	—	—	—
Total	66.3	9.0	87.5 ± 8.5	0.0	75.7 ± 3.8	11.6 ± 1.0	70.0 ± 5.2	4.1 ± 3.0

[a] Values represent the mean ± *SE* for three rabbits, three guinea pigs, and four human subjects and pooled excreta from eight mice. I was administered orally to mice (5 mg./kg.), intravenously to rabbits and guinea pigs (5 mg./kg.), and orally to man (300 mg., equivalent to 273 mg. of free I).

Table VI—Biliary Excretion of Radioactivity in Dogs Given I-^{14}C[a]

Hours	Dose Excreted in Bile, %—Dog 1	Dose Excreted in Bile, %—Dog 2	^{14}C Plasma Concentration, mcg./ml.—Dog 1
0.25	—	—	15.0
0.50	—	—	9.1
1.0	52.4	13.7	2.9
2.0	32.3	22.4	0.8
3.0	6.5	11.5	—
4.0	1.8	3.9	0.1
5.0	1.2	0.9	—
6.0	0.5	1.8	<0.1
24.0	—	—	<0.1
Total	94.7	54.2	—

[a] Dose = 5 mg./kg., including approximately 10 μc. of I-^{14}C. Dog 1 received the drug intravenously, and Dog 2 received the drug orally.

radioactivity excreted after oral administration was apparent free drug.

These results are consistent with the appearance of a significant fraction of an intravenous dose of I-^{14}C in feces (Table IV). Whether or not labeled material excreted in the bile was reabsorbed and reexcreted in the bile is not known. These results also suggest that the excretion average of 40.7% of an oral dose of I-^{14}C in feces did not indicate incomplete absorption. The more likely explanation is that the drug was extensively excreted in the bile and then eliminated in the feces.

Urinary Metabolites of I in Various Species—Only about 5–15% of the administered dose of radioactive I (Tables IV and V) was excreted in the urine of all species as free drug, indicating that the drug was extensively metabolized. No labeled carbon dioxide was detected in the expired air of rats given labeled I, indicating that the administered radioactivity was not lost during these experiments. The following metabolites of I were isolated and identified in the excreta of various species.

p-*Chlorobenzoic Acid*—This metabolite was isolated, identified, and determined in urine of various species given labeled I. A suitable aliquot was adjusted to pH 5 with 1 *N* HCl and extracted with heptane–3% isoamyl alcohol (5 volumes). The heptane extract was back-extracted with 0.1 *N* HCl ($^1/_5$ volume) to remove I and then shaken with 0.1 *N* NaOH. Aliquots of the base were counted to determine the *p*-chlorobenzoic acid content. Recovery of added *p*-chlorobenzoic acid was quantitative. For chromatographic studies, the 0.1 *N* NaOH extract was acidified and extracted with ether and the concentrated ether extract was used.

Apparent *p*-chlorobenzoic acid was identical with authentic compound by TLC as shown in Table VII. Cochromatography gave only a single radioactive peak. This metabolite represented about 2–6% of the urinary radioactivity in all species examined.

p-*Chlorohippuric Acid*—This metabolite was isolated from urine by the following procedure. Urine from animals and human subjects given I-^{14}C was adjusted to pH 5 and extracted with heptane to remove I and *p*-chlorobenzoic acid-^{14}C. After adjusting to pH 3, the residue was extracted three times with equal volumes of ether. About 45% of the urinary radioactivity was found in the ether extract.

The ether extract was subjected to TLC, with the results shown in Table VIII. As indicated, the metabolite was identical with *p*-chlorohippuric acid. The R_f value of the metabolite was unaltered after

Table VII—TLC of Authentic *p*-Chlorobenzoic Acid and Metabolite Isolated from Urine of Human Subjects Given I-^{14}C

Solvent System	R_f—Authentic *p*-Chlorobenzoic Acid	R_f—Metabolite
Ethanol–water–ammonia (100:12:16)	0.52	0.52
Ethyl acetate–methanol–acetic acid (7:4:1)	0.72	0.72
Benzene–dioxane–ammonia (10:80:10)	0.06	9.06

Table VIII—TLC of Authentic *p*-Chlorohippuric Acid and Metabolite Isolated from Urine of Human Subjects Given I-^{14}C[a]

Solvent System	R_f—Authentic *p*-Chlorohippuric Acid	R_f—Metabolite
Ethyl acetate–methanol–acetic acid (7:4:1)	0.79	0.81
Butanol–acetic acid–water (4:1:1)	0.73	0.68
Acetic acid–isopropanol (5:95)	0.73	0.74
Ether (saturated with water)–methanol–88% formic acid (95:1:4)	0.77	0.77

[a] Spots were located by radiometric scanning and by spraying with 4% *p*-dimethylaminobenzaldehyde in acetic anhydride (7).

treatment with 0.1 *N* NaOH, also indicating that the *p*-chlorobenzoylindole group was not present in this metabolite since I was deacylated by dilute alkali.

The metabolite was also identified by reverse-isotope dilution in the following experiment. Authentic *p*-chlorohippuric acid (200 mg.) was dissolved in 150 ml. of I-^{14}C human urine. The urine was extracted twice with equal volumes of ether, the solvent was evaporated, and the residue was crystallized from hot water to constant melting point and specific activity. The results shown in Table IX indicate that the metabolite was identical to *p*-chlorohippuric acid.

In addition, the metabolite was also subjected to GLC after conversion to its methyl ester by means of diazomethane (Table X). Under the conditions used, *p*-chlorohippuric acid and hippuric acid were well separated. The metabolite had a retention time identical with that of authentic *p*-chlorohippuric acid. Cochromatography of the metabolite and authentic *p*-chlorohippuric acid gave no additional peaks but enlarged the metabolite peak. This metabolite represented 35–48% of the urinary radioactivity in four human subjects.

p-Chlorohippuric acid was also identified in guinea pig, rabbit, dog, and rat urine by TLC, GLC, and reverse-isotope dilution analyses. This metabolite accounted for approximately 59, 75, 80, and 78%, respectively, of the urinary radioactivity in these species.

I-Glucuronide—Incubation of human urine with β-glucuronidase (6 mg./ml.; pH 7.0, 37° for 18 hr.) released additional quantities of I. The amount of I liberated in this manner was proportional to the enzyme concentration over a range of 25–1000 mcg./ml. Addition of saccharolactone (10 mg.) to the incubation mixture resulted in almost complete inhibition (90%) of I release by 100 mcg. of β-glucuronidase.

Extraction of urine with ethyl acetate at pH 3, following removal of I and *p*-chlorohippuric acid with heptane and ether, removed about 35% of the urinary radioactivity. TLC of the extract in isopropanol–acetic acid (95:5) revealed three peaks on scanning: R_f 0.03, 0.28, and 0.61. Similarly, paper chromatography in methanol–

Table IX—Reverse-Isotope Dilution Analysis of Apparent *p*-Chlorohippuric Acid Isolated from Human Urine

Crystallization Number	Melting Point	Specific Activity, d.p.m./mg.
1	147.5–148.5°	1833
2	147.5–148.5°	1818
3	147.5–148.0°	1820

Table X—GLC of Authentic *p*-Chlorohippuric Acid and Metabolite Isolated from Urine of Human Subjects Given I[a]

Compound	T_R, min.
Hippuric acid, methyl ester	2.6
p-Chlorohippuric acid, methyl ester	4.3
Metabolite, methyl ester	4.3

[a] Column was packed with 1% QF-1 on Chromasorb G and operated at 170°. All compounds were esterified with diazomethane prior to chromatography.

benzene–water–butanol (2:1:1:1) revealed three peaks, suggesting that the extract contained three metabolites.

The ethyl acetate extract was incubated with β-glucuronidase, and the incubation mixture was extracted sequentially with heptane, ether, and ethyl acetate. TLC of the ethyl acetate fraction now yielded only two spots: R_f 0.03 and 0.61. Evidently, the spot at R_f 0.28 was labile to β-glucuronidase.

On the basis of this evidence, this metabolite was tentatively identified as the acyl glucuronide of I. No synthetic sample was available as a reference. Urine from four human subjects given labeled I was assayed for this glucuronide by analysis of I present before and after enzymatic hydrolysis. The results indicated that 20–32% of the urinary radioactivity was present as the acyl glucuronide of I.

Analysis of urine of dogs, guinea pigs, and rabbits for I-glucuronide showed that the metabolite was not present in dog or rabbit urine. Approximately 20% of the radioactivity present in guinea pig urine was present as apparent I-glucuronide.

Other Metabolites—The remaining metabolites in the ethyl acetate extract of human urine apparently retained the *p*-chlorobenzoylindole structure, since treatment with 0.1 *N* NaOH altered their solvent partitioning behavior as well as TLC pattern. GLC of the base-treated metabolites revealed five peaks, two of which corresponded to *p*-chlorobenzoic acid and 4-chlorosalicylic acid. *p*-Chlorobenzoic acid was identified in the base-treated material by reverse-isotope dilution analysis. However, 4-chlorosalicylic acid was shown not to be present.

The unknown metabolites in the ethyl acetate extract were treated with acetic anhydride, since, if they were *N*-demethylated derivatives of I (5-position), acetylation should influence their solvent partitioning and TLC patterns. Marked changes were noted in these criteria after acetylation, suggesting that the metabolites may be *N*-demethylated structures.

Larger quantities of the metabolites were prepared by TLC in isopropanol–acetic acid (95:5). Major radioactive peaks at R_f 0.02 and 0.56 were eluted and rechromatographed in *n*-propanol–1 *N* acetic acid (3:1) and isopropanol–acetic acid (95:5), respectively. The 0.02 peak was run a second time in this system and then in ethyl acetate–methanol–acetic acid (7:4:1) on a cellulose plate. The original 0.56 peak was eluted and run twice in ether (saturated with water)–methanol–88% formic acid, followed by elution. Eluates of these TLC plates contained approximately 40 mcg. of the 0.02 peak present and 200 mcg. of the 0.56 peak.

An aliquot of each fraction was subjected to GLC following treatment with diazomethane. The 0.82 R_f metabolite gave a peak at 3.7 min. (170°, 1% QF-1 on Chromasorb Q); the 0.56 R_f metabolite gave a peak at 2.6 min. No further conclusions as to the structure of these metabolites were possible.

Since I was shown to be extensively debenzoylated in the rat, a preliminary experiment was performed in which rats were given I labeled in the 2-carbon of the indole nucleus. Excretion of label in this case would reflect primarily the physiologic disposition of the indole nucleus of I. In this experiment, 23% of the dose (5 mg./kg. p.o.) was excreted in the urine and 52% in feces (average of four rats) compared to 49.5 and 28.4%, respectively, when I labeled in the chlorobenzoyl-carbonyl carbon was given (Table IV).

Metabolites in Bile—Bile from a dog given I-^{14}C containing 95% of the administered radioactivity was assayed for I. The results showed that only approximately 1% of the biliary radioactivity was present as free I. The bile was incubated with β-glucuronidase and again analyzed for I. The results indicated that approximately 90% of the biliary radioactivity was now accounted for by I. The amount released was proportional to the enzyme concentration, and release was inhibited by addition of saccharolactone.

The apparent I released after incubation was identified as I by TLC and GLC of the heptane extract. Only one spot was seen after TLC in the systems described in Table I, and only one peak after GLC (after methylation) as described in the *Methods* section. In each case, the metabolite was identical with authentic I.

In a separate experiment, bile was extracted with ethyl acetate at pH 5. The extract, containing 92% of the biliary label, was subjected to TLC in two solvent systems. In isopropanol–acetic acid (95:5), only one radioactive spot was observed: R_f 0.08. No free drug (R_f 0.20) or glucuronic acid (R_f 0.0) was present. The spot was eluted with 0.1 *N* NaOH, and the eluate gave a positive test for glucuronic acid (8).

In ethyl acetate–isopropanol–10% ammonia (5:4:3), two radioactive components were observed: R_f 0.51 and 0.88. The lower R_f spot corresponded with I; free glucuronic acid was detected at R_f 0.05.

DISCUSSION

Comparison of I with the closely related structure of indomethacin offers an opportunity to observe the effect of replacing the 5-methoxy substituent by a dimethylamino group on the physiologic disposition and metabolic fate of compounds in the *N*-benzoylindole acetic acid series. These parameters were previously described for indomethacin (4, 9). Both I and indomethacin were well absorbed after oral administration. The rate of metabolism of I, as defined by the plasma half-life, appeared to be more rapid than indomethacin in man but slower in the dog. Rates were equivalent in the rat.

Both compounds were widely distributed in rat tissues but highest concentrations of drug remained in plasma, presumably a reflection of the extensive binding of both drugs to plasma protein. I was excreted more rapidly and more extensively in rat feces than was indomethacin, possibly due to more extensive biliary excretion of I. I labeled with ^{14}C in the *p*-chlorobenzoyl carbonyl carbon was used in most of these experiments. A different excretion pattern was obtained in rats given I labeled in the indole-2 carbon, which suggested that hydrolysis of the amide bond was an important metabolic route.

A major metabolite of I in human urine was *p*-chlorohippuric acid. This metabolite accounted for approximately 40% of the urinary radioactivity and resulted from hydrolysis of the *p*-chlorobenzoyl substituent of I and subsequent conjugation with glycine. Although it was originally reported (9) that only free indomethacin and its acyl glucuronide were excreted by man, more recent data (10) show that this drug is also extensively metabolized in man by *O*-demethylation and *N*-debenzoylation. In the dog, however, a marked difference in the metabolic fate of I and indomethacin existed. The latter compound was rapidly excreted in dog bile as the acyl glucuronide and excreted almost completely in feces (4), whereas I in dogs was extensively *N*-debenzoylated and excreted in urine, *p*-chlorohippuric acid accounting for about 80% of the urinary radioactivity.

The acyl glucuronide of I was also identified as a metabolite of I in man and accounted for about 25% of the urinary radioactivity. Unknown metabolites, possibly *N*-dealkylated derivatives of I, represented about 25% of the urinary radioactivity in man. Minor amounts of unchanged I and *p*-chlorobenzoic acid were also identified in human and animal urine.

McChesney *et al.* (11) reported that *p*-chlorobenzoic acid, formed as a metabolite of chlormezanone in the dog and man, was also excreted in the urine as *p*-chlorohippuric acid. Benzoic acid, however, is excreted largely as benzoylglucuronic acid in the dog (12) but as hippurate in man, suggesting that the *p*-chloro substituent, in view of the results with I, caused increased conjugation with glycine in the dog. Quick (13) reported that a *p*-chloro substituent had little effect on the extent of glycine conjugation in the benzoic acid series. The dose administered is, however, important in selection of the means of conjugation, but it is possible that *p*-chlorobenzoic acid formed *in situ* is conjugated differently than when administered *per se*.

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ACKNOWLEDGMENTS AND ADDRESSES

Received October 9, 1970, from the *Merck Institute for Therapeutic Research, West Point, PA 19486*

Accepted for publication March 9, 1971.

A preliminary report of this work appeared in *Fed. Proc.*, **27**, 238(1968).

The authors thank Dr. N. H. R. Cantwell and Dr. R. N. Pierson for providing the material from human studies performed at St. Lukes' Hospital, New York, N. Y.; and Dr. R. L. Ellsworth and Dr. C. Rosenblum for supplies of radioactive drug.

CNS Activities of Lactam Derivatives

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Abstract □ Data presented show that lactams, which have the resonating structure $\rangle\ddot{N}-C(=A)- \leftrightarrow \rangle\overset{+}{N}=C(-\bar{A})-$ commonly associated with CNS depressants and CNS stimulants, have significant CNS activities. Results of preliminary testing show that lactams of medium ring size and lacking *N*-substitution are fairly potent CNS stimulants. The acute lethal toxicity (log $1/C$) of four CNS stimulants of the lactam type is found to be highly correlated with log P, where P is the 1-octanol–water partition coefficient for the compound. The rate of respiration in mice depressed with sodium pentobarbital is stimulated by 2-azacyclononanone, and the rate and depth of respiration were increased by 2-azacyclooctanone in dogs anesthetized with sodium pentothal. Both 2-azacyclooctanone and 2-azacyclononanone are capable of reducing considerably the sodium pentobarbital sleeping time in mice. At higher doses, the CNS stimulant effects of these drugs are manifested by convulsions. For 2-azacyclooctanone, the convulsant ED_{50} is about 37 mg./kg. and the LD_{50} is about 300 mg./kg. Direct evidence for CNS stimulation by 2-azacyclooctanone was obtained by recording changes in EEG patterns in artificially ventilated dogs paralyzed with *d*-tubocurarine. Diphenylhydantoin is much less effective as an antagonist to the convulsant actions of 2-azacyclooctanone than sodium pentobarbital. This finding, taken together with the tonic-clonic convulsions produced by this drug, strongly suggests that, like nikethamide and pentylenetetrazole, the site of action for its CNS effects is subcortical and supraspinal. The conclusion is drawn that the resonating moiety shown here confers upon many compounds CNS-stimulant activities.

Keyphrases □ Lactams, medium ring derivatives—CNS activities, mice, dogs □ CNS depressants, stimulants—medium ring lactams, mice, dogs □ Tonic-clonic convulsions—lactam derivatives, subcortical, supraspinal CNS effects □ Resonating structure, lactams—CNS activities, mice, dogs

Lien and Kumler (1) pointed out that many CNS depressants and CNS stimulants have a common resonating structure: $\rangle\ddot{N}-C(=A)- \leftrightarrow \rangle\overset{+}{N}=C(-\bar{A})-$, where A = O or S. Since lactams are simple compounds possessing this resonating structure, and since very few studies on the biological activities of these compounds have been reported (2), it was decided to study the physicochemical properties and pharmacological activities of these compounds in the CNS. The purpose of this report is to present results of the preliminary screening of some compounds containing the lactam moiety. γ-Thiobutyrolactone, which has the same resonance structure as lactams, is also included in this study. Also, as general representatives of these drugs, 2-azacyclooctanone and 2-azacyclononanone were subjected to pharmacological investigation.

MATERIALS AND METHODS

γ-Thiobutyrolactone was provided by Kharasch and Langford (3) and was purified before use by distillation under atmospheric pressure (b.p. 198°). δ-Valerolactam (m.p. 35–39°), ε-caprolactam (m.p. 69–70°), 2-azacyclooctanone (m.p. 36°), 2-azacyclononanone (m.p. 77–79°), 1-methyl-2-piperidone (n_D^{20} = 1.4823), and 1-butyl-2-pyrrolidinone (n_D^{20} = 1.4640) were purchased[1] and used without further purification.

N-Ethyl-ε-caprolactam was prepared by alkylation of ε-caprolactam using sodium hydride and ethyl iodide, according to the previously reported procedure for the alkylation of cyclic thiourea (1). The procedure was slightly modified by using a reflux period of 3 rather than 5 hr. after the addition of ethyl iodide. The product was purified by vacuum distillation (b.p. 77–78°/1.3 mm.). Elemental analysis[2] gave the following results.

Anal.—Calc. for C, H, N: C, 68.20; H, 10.63; N, 9.995. Found: C, 68.50; H, 10.82; N, 10.36. IR, 1640 $cm.^{-1}$ (C=O stretching).

Preliminary Test for LD_{50}—Groups of six mice, three males and three females weighing 17–27 g., were injected intraperitoneally with a series of logarithmic doses (30, 100, 300, and 1000 mg./kg., *etc.*). Signs of CNS stimulant activity were observed continuously for 2 hr. and then at regular intervals for 2 days. All drugs were administered in aqueous solutions in a volume of 1 ml. or less. Control animals received saline injections. From the number of animals that did not survive, the LD_{50} was estimated for each drug according to the method of Miller and Tainter (4).

Partition Coefficient—The *n*-octanol–water partition coefficients of γ-thiobutyrolactone and *N*-ethyl-ε-caprolactam were measured using a Carey-14 spectrophotometer. γ-Thiobutyrolactone was found to have an absorption maximum ($\lambda_{max.}$) of 2340 Å and a molar absorptivity ($\epsilon_{max.}$) of 4.358×10^3. The partition coefficients of 2-azacyclononanone and *N*-ethyl-ε-caprolactam were measured by GLC[3]. The logarithms of the partition coefficients (log P) are summarized in Table I.

Analeptic Effect of 2-Azacyclononanone in Mice against Sodium Pentobarbital—2-Azacyclononanone (30 mg./kg.) was injected intraperitoneally into a group of 12 mice, which 10 min. earlier had been pretreated with 60 mg./kg. of sodium pentobarbital. Changes in the respiratory rate of control and experimental animals, as well as the mean sleeping times, were compared.

Estimation of ED_{50} and LD_{50} of 2-Azacyclooctanone—Male Swiss albino mice, weighing 20–30 g., were used. The experiments were carried out in a room constantly maintained at 26°. Graded doses of 2-azacyclooctanone dissolved in physiological saline were administered intraperitoneally. For a given experiment,

[1] Aldrich Chemical Co., Inc.
[2] Performed by C. F. Geiger, Ontario, Calif.
[3] Hydrogen-flame detector, column 3% OV-17 on Gas Chrom Q, Applied Science Laboratories.