Biological Disposition of 2-(4-Phenyl-1-piperazinylmethyl)cyclohexanone-1-C^{14} (C^{14}-MA1050)

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MA1050 is distributed in tissues as a typical organic base and excreted primarily in urine. Repeated treatment of rats does not result in marked alteration of tissue distribution, although excretion is prolonged, and the relative importance of the urinary and fecal routes is altered. The reduced (cyclohexanol) derivative of MA1050 and a metabolite presumed to be its *O*-glucuronide are found in urine, along with an additional unidentified metabolite. The major urinary metabolites may be the *p*- and/or *o*-phenolic derivatives of the Mannich base. Mannich hydrolysis was not shown to occur to a significant extent, and the suggestion that products of Mannich hydrolysis might be responsible for the ocular toxicity elicited by MA1050 was not supported.

MA1050 [2-(4-phenyl-1-piperazinylmethyl)-cyclohexanone] is a Mannich base (Fig. 1) possessing analgetic activity in mice and anti-inflammatory activity in rats (1). Disposition of the compound was of interest since there have been no reports regarding the metabolism of *N*-aromatic derivatives of piperazine or Mannich bases. In addition, chronic administration of MA1050 to dogs[1] leads to development of a mucopurulent ocular discharge and, eventually, lens opacity. The ocular discharge has also been observed during chronic administration of a number of related Mannich bases in which the size of the cycloalkanone ring is altered (all of which can yield a methylene cycloalkanone upon Mannich hydrolysis[2]), but not during the chronic administration of relevant doses of phenylpiperazine. These toxicity data suggested that methylenecyclohexanone and/or its dimer or other product(s) resulting from Mannich hydrolysis might account for the ocular toxicity observed. Hence, it was of interest to determine the importance of Mannich hydrolysis as a metabolic route.

EXPERIMENTAL

Male Sprague-Dawley rats from 150 to 250 Gm. body weight and Beagle dogs of both sexes from 6 to 10 Kg. body weight were employed in these studies.

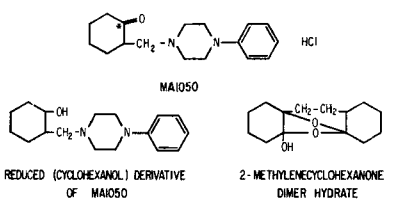

Fig. 1.—Structure of MA1050, its reduced derivative, and a product of Mannich hydrolysis *in vitro*.

All animals were allowed food and water *ad libitum* up to the time of drug administration; animals employed in excretion studies had access to food and water throughout the study. Repeatedly treated rats received single intraperitoneal daily doses of 25 mg./Kg. of unlabeled MA1050 (as the monohydrochloride) for 13 days and were utilized on the fourteenth day.

C^{14}-MA1050[3] was administered to rats orally or intraperitoneally as a 0.6% aqueous solution at a dose of 25 mg./Kg.; dogs received 10 mg./Kg. orally. Rats received 23.53 μc. of C^{14}/Kg. of body weight; dogs received 1.88 μc./Kg. All animals were dosed from freshly prepared solutions of drug.

Four separate studies were conducted: excretion studies in orally dosed rats and dogs, and acute and subchronic distribution and excretion studies in intraperitoneally dosed rats.

Distribution of C^{14} was investigated in two groups of 21 rats, each divided into seven subgroups of three rats. In each group, animals were sacrificed 0.5, 1, 2, 4, 8, 16, and 24 hr. after drug administration. Samples of blood, brain, lung, liver, kidney, adrenal, spleen, fat, heart, and muscle were obtained from each rat. Tissues were prepared for liquid scintillation counting as described previously (2).

Urinary and fecal excretion of C^{14} was investigated in three groups of rats containing six animals each and one group of three dogs. Following administration of labeled drug, the animals were placed in stainless steel metabolism cages.[4] Feces and urine were collected (urine in containers packed in dry ice) at intervals for 72 hr. after drug administration. Techniques described previously were employed in the preparation of urine (3) and feces (2) samples for liquid scintillation counting.

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[1] Industrial Bio-Test Laboratories, Inc., Northbrook, Ill.

[2] Mannich hydrolysis in this paper refers to the typical hydrolysis of Mannich bases to yield an amine and an α-methylene ketone.

[3] Synthesized by Gust Nichols of this laboratory.

[4] Acme Metal Products, Inc., Chicago, Ill.

TABLE I.—DISTRIBUTION OF C^{14} FOLLOWING THE INTRAPERITONEAL ADMINISTRATION OF 25 mg./Kg. C^{14}-MA1050 TO MALE RATS

Tissue	Mean[a] Concn. of C^{14}-mcg./Gm. Wet Wt. MA1050	Acute I[b]	Acute II[c]	Acute Total	Repeated[d] Total	Biological Half-Life of C^{14}, hr. Acute	Repeated
Cerebrum	0.29	0.31	1.46	2.06	2.25	6.40	6.45
Fat	0.78	2.05	2.72	5.55	8.88	3.91	3.28
Muscle	0.30	0.43	1.43	2.16	2.35	8.47	6.00[e]
Eye	0.19	0.37	0.53	1.09	1.19	7.22	7.22
Blood	0.31	0.42	1.20	1.93	2.08	7.98	9.35
Lung	...	...	...	4.63	3.92	6.05	5.42
Heart	0.44	0.52	1.03	1.99	2.08	6.87	6.77
Spleen	1.67	1.13	1.72	4.52	4.71	7.14	7.37
Kidney	3.80	2.47	3.24	9.51	10.14	7.04	5.46
Liver	2.77	7.23	5.38	15.38	18.09	7.82	9.68
Adrenal	...	...	...	5.31	6.47	8.67	6.96

[a] Over-all time intervals investigated, from 0.5 to 24 hr. [b] The reduced derivative of MA1050. [c] Unidentified radioactive material(s). [d] Each rat received 25 mg./Kg. of MA1050 intraperitoneally once daily for 13 days prior to receiving C^{14}-MA1050 on day 14. [e] Significantly (at the 0.025 level) shorter than in acutely treated rats.

TABLE II.—URINARY, FECAL, AND RESPIRATORY EXCRETION OF C^{14} FOLLOWING THE ORAL OR INTRAPERITONEAL ADMINISTRATION OF C^{14}-MA1050 TO RATS AND DOGS

hr.	Mean Cumulative Dose Excreted, %: Acute Oral Rat, $n = 6$	Acute i.p. Rat, $n = 6$[c]	Repeated i.p. Rat, $n = 6$[c]	Acute Oral Dog[a]
Urine				
2	5.00	14.78	3.14	
4	23.54	36.75	12.93	
6				5.55
8	48.32	53.52	30.91	
12	58.51	59.73	40.42	17.29
24	71.85	67.84	50.70	29.60
36	75.05	70.15	54.02	31.83
48	76.62	71.34	55.87	38.76
60	76.74	71.56	56.48	40.90
72	77.87 ± 4.73	73.53 ± 2.31 (I)	58.26 ± 1.60 (II)	41.51 ± 4.79
Feces				
24	8.40	8.65	12.69	0.51
48	15.42	14.86	18.11	1.08
72	15.76 ± 1.34	15.18 ± 0.70 (III)	19.93 ± 0.98 (IV)	6.54 ± 1.32
Total	93.63 ± 3.81	88.71 ± 2.00 (V)	78.19 ± 1.54 (VI)	47.91 ± 5.81
Respiratory $C^{14}O_2$				
20		0.12 ± 0.02[b]		

[a] $n = 3$; two males, one female. [b] $n = 3$. [c] I *versus* II, III *versus* IV, and V *versus* VI, all significantly (at the 0.01 level) different.

TABLE III.—EXTRACTION OF C^{14} FROM RAT AND DOG URINES WITH CHLOROFORM AND BUTANOL

Urine	Total C^{14} Extracted, %: With Chloroform[a]	With Butanol[b]	Total	Dose Chloroform Soluble, %
Rat[c], untreated	12.8 ± 0.46	61.0 ± 0.79	73.8 ± 0.65	9.4
Rat[c], β-glucuronidase hydrolyzed	25.3 ± 2.36	37.4 ± 1.71	62.7 ± 1.66	18.6
Dog, untreated	33.6 ± 4.77	...	...	14.0

[a] Urine adjusted to pH 11. [b] Urine adjusted to pH 7, buffered with an equal volume of 0.1 *M* pH 7 phosphate buffer, and extracted three times with *n*-butanol saturated with water. [c] From acutely intraperitoneally dosed rats.

Samples prepared as above were cooled to 0° and counted in a liquid scintillation spectrometer[5] for a period sufficient to give less than 2% counting error. Count rates obtained were corrected to absolute efficiency by the internal standard technique.

Recovery of metabolites from urine was accomplished by extraction of the total volume excreted, after adjustment to pH 11 with aqueous sodium hydroxide, with 1 vol. of chloroform by high speed mechanical shaking for 15 min. After one extraction with chloroform, the urines were adjusted to pH 7, buffered with an equal volume of 0.1 *M* pH 7 phosphate buffer, and extracted three times with 1.5 vol. of *n*-butanol saturated with water by high speed mechanical shaking for 1 hr. Urines were hydrolyzed with β-glucuronidase as described previously (2). Analogous tissues obtained at all time intervals were pooled, lyophilized, and metabolites were recovered by extraction three times with methanol. The amount of C^{14} removed by each extraction was determined by liquid scintillation

[5] Packard Tri-Carb model 314-EX, Packard Instrument Co., Inc., LaGrange, Ill.

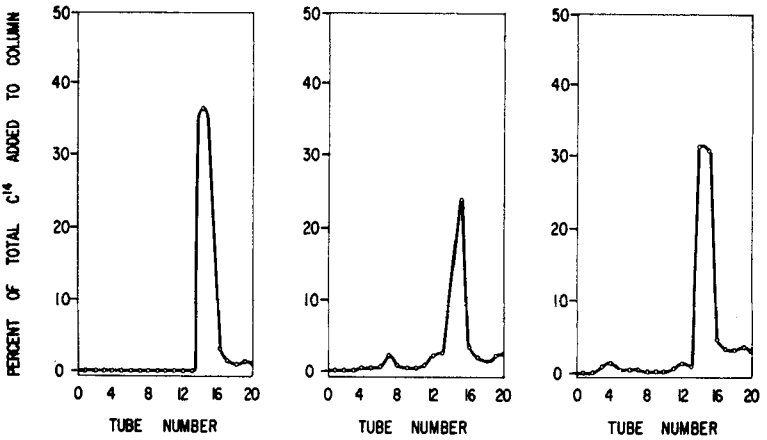

Fig. 2.—Column chromatography of chloroform extracts of urines from rats and dogs treated with C^{14}-MA1050. Key: left, untreated rat urine; middle, β-glucuronidase hydrolyzed rat urine; right, untreated dog urine.

counting of appropriate samples. Phenylpiperazine, a potential nonradioactive metabolite, was determined by a modification of the spectrophotometric method of Umbreit (4).

Chloroform and butanol extracts of urine were chromatographed on Whatman No. 1 paper in one of four solvent systems: (*a*) *n*-butanol saturated with water, (*b*) *tert*-amyl alcohol–acetic acid–water (60:15:25), (*c*) pyridine–ammonium hydroxide–water (35:15:50), and (*d*) butanol–ethanol–water (25:25:50). The distribution of C^{14} along these chromatograms was determined using a chromatogram scanning technique described previously (3). In addition, MA1050 and its metabolites were visualized with the iodoplatinate reagent described by Smith (5).

Chloroform extracts of urine and methanol extracts of tissue were also separated by a column chromatographic technique. A 110 × 13 mm. column was prepared from a slurry of neutral alumina[6] in ether. The extract and/or authentic C^{14}-MA1050 and its reduced derivative were added to the column and eluted successively with 4-ml. fractions of ether, ether–chloroform (1:1), chloroform, chloroform–methanol (1:1), and methanol. Liquid scintillation counting of an aliquot of each fraction was employed to determine the amount of C^{14} eluted.

The biological half-life of C^{14} in the various tissues was calculated using first-order rate equations (6). All differences of interest were tested by the *t* test.

RESULTS

Distribution.—Data representing the average levels of radioactivity obtained, over all time intervals examined, in the various tissues of acutely and repeatedly dosed rats are summarized in Table I. The results reveal no marked alterations in the tissue distribution of C^{14} following repeated treatment with MA1050, compared with acute treatment. There were no significant differences in levels of C^{14} reached in corresponding tissues of the two groups of rats at any of the seven time intervals examined. With the exception of muscle, no significant differences were observed in the rates of disappearance of C^{14} from corresponding tissues of the two groups of rats. It is of interest that the level of C^{14} reached in the eye is the lowest of any tissue examined and that the biological half-life of C^{14} in this tissue is intermediate in the spectrum of half-lives observed. Metabolite studies revealed that, while in nearly every tissue an unidentified material accounts for 35–70% of the radioactivity, the reduced derivative of MA1050 reaches greater tissue levels than MA1050 itself.

C^{14} Excretion.—The excretion of C^{14} following the oral or intraperitoneal administration of C^{14}-MA1050 to rats and dogs is summarized in Table II. Respiratory excretion of C^{14} as $C^{14}O_2$ was shown to be an unimportant route. The pattern of urinary and fecal excretion of C^{14} following acute intraperitoneal or oral administration of C^{14}-MA1050 to rats is virtually identical. However, a marked difference in the excretion of C^{14} by acutely and repeatedly intraperitoneally treated rats is evident. Acutely treated rats excreted 26% more C^{14} in urine in 72 hr. than repeatedly treated rats. The major difference in rates of urinary excretion occurred during the initial 8 hr., during which time repeatedly treated rats excreted 42% less C^{14} than acutely treated animals. This trend toward decreased urinary excretion was partly compensated for by increased fecal excretion; however, the net result of the alterations in excretory routes is that repeatedly treated rats excrete about 12% less C^{14} in 72 hr. than acutely treated rats. The results indicate that C^{14} is excreted much more slowly by dogs following oral administration of C^{14}-MA1050 than by rats.

Metabolite Excretion.—The data in Table III summarize the extraction of C^{14} from untreated and β-glucuronidase-hydrolyzed urines with chloroform and butanol. Chloroform soluble material in urine accounts for a relatively minor portion of the dose in both the rat and the dog.

Paper chromatography of chloroform extracts of urine demonstrated that none of the C^{14}-labeled material was present as the dimer of 2-methylenecyclohexanone. In addition, less than 1.0% of the dose could be detected in urine as phenylpiperazine following oral or intraperitoneal administration of MA1050 to rats.

Subsequently, aliquots of these chloroform extracts were chromatographed on top of authentic C^{14}-MA1050; in each solvent system the C^{14}-labeled material moved to the R_f value of authentic MA1050. However, chromatogram scans indicated

[6] Fisher reagent grade, 80–200 mesh, Brockman activity one.

TABLE IV.—PAPER (WHATMAN NO. 1) CHROMATOGRAPHY OF REFERENCE PHENOLIC AMINES AND BUTANOL EXTRACTS OF RAT URINES

Solvent System	R_f, *o*-Hydroxy	R_f, *p*-Hydroxy	R_f, Butanol Extract
(*a*) Amyl alcohol–acetic acid–water (60:15:25)	0.71	0.72	0.71
(*b*) Pyridine–ammonium hydroxide–water (35:15:50)	0.91	0.93	0.92
(*c*) Butanol–ethanol–water (25:25:50)	0.91	0.88	0.89

slight peak separation in each case. Additional chromatograms were prepared to compare the behavior of the radioactive material in the chloroform extracts, authentic MA1050, and the reduced (cyclohexanol) derivative of MA1050. However, it was not possible to obtain adequate resolution of even the two reference compounds.

At this point, chloroform extracts of rat and dog urines were subjected to column chromatography; the results of these studies are illustrated in Fig. 2. These results indicate that 11.2% of an acute intraperitoneal dose of C^{14}-MA1050 is excreted in urine by rats as the reduced derivative of MA1050, 8.9% as the free compound, and an additional 2.3% as a material extracted following hydrolysis with β-glucuronidase. No C^{14}-MA1050 was detected. Elution of dog urines indicated that 9.2% of the administered dose is excreted as the reduced derivative, 0.2% as an unidentified material analogous to a metabolite observed in the chloroform extract of enzymatically hydrolyzed rat urine, as well as 0.3% as unchanged MA1050. These findings demonstrate that MA1050 is handled in a qualitatively similar manner in the rat and the dog.

The chromatographic behavior of the radioactive material in butanol extracts of urine and reference (*o*- and *p*-hydroxy) phenolic derivatives of MA1050 is summarized in Table IV. The results were inconclusive and suggested that either or both phenolic amines might be present in urines.

DISCUSSION

These findings illustrate a pattern of distribution of C^{14} following the administration of C^{14}-MA1050 which is typical of weak organic bases in general—relatively high levels in reticuloendothelial tissues and relatively low levels in blood, brain, and muscle. The results suggested that the C^{14} is distributed as an organic base rather than as a neutral product(s) of Mannich hydrolysis. This was supported by the identification of MA1050 and its reduced derivative in tissues. The reduced derivative of MA1050 has been investigated pharmacologically and was found to possess no analgetic activity and 80% of the anti-inflammatory activity of MA1050.

MA1050 is unstable in acid, undergoing Mannich hydrolysis to give, *in vitro*, phenylpiperazine and the dimer of 2-methylenecyclohexanone. The failure to find this dimer or significant amounts of phenylpiperazine in urine suggested that Mannich hydrolysis did not occur *in vivo*. Hence, the studies offer no support for a mechanism explaining lens opacity which would require Mannich hydrolysis to yield a methylene cycloalkanone.

Calculations from rate constant values obtained in a study of the stability of MA1050 (7) indicate that 18.8% of the Mannich base present in a urine sample subjected to the conditions employed for β-glucuronidase hydrolysis would undergo Mannich hydrolysis. This partially accounts for the additional radioactive material extractable from enzymatically hydrolyzed urine with chloroform, as a derivative of 2-methylenecyclohexanone, and suggests that the additional material is such a derivative rather than an additional *O*-glucuronide conjugate(s).

Chromatographic evidence suggesting the presence of both *o*- and *p*-phenolic amines in urine is consistent with the findings of Smith and Williams (8) and Parke and Williams (9) that both *o*- and *p*-hydroxy metabolites of aniline are formed by rats. Due to the similarity of R_f values of MA1050 and its reduced derivative, it is possible that urine contains a mixture of the phenols of both the Mannich base and its reduced derivative.

Although butanol extracts of dog urines were not prepared, it is evident from the amount of C^{14} excreted in urine and the portion of this which is chloroform soluble that metabolism to phenolic amines is a much less important route in the dog.

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