alously with the glucosyl bromide to give N-alkylation or phenyl alkylation was eliminated, for either possibility would result in a product containing a phenolic group. The absence of an aromatic hydroxyl was demonstrated by determining the uv spectra in neutral and in basic solution. Whereas the absorption maxima of **1** and **2** were shifted in the presence of sodium hydroxide, no shift occurred in the uv maxima of **5**, **6**, or indeed chlorpropham itself.

The Michael synthesis is known to yield β- rather than α-glucosides. Confirmation of the β configuration of compounds **3**, **4**, **5**, and **6** was obtained by determining their specific optical rotations. These rotations, ranging from $-39°$ to $-69°$, were in the range of values of the β isomers of known substituted phenyl glucosides and their tetraacetates ($-103°$ to $+45°$) rather than in the range of the corresponding α isomers ($+137°$ to $+212°$) (Bonner *et al.*, 1952). The susceptibility of **5** and **6** to hydrolysis by the enzyme, β-glucosidase, also indicates β configuration. Efforts to confirm the configuration of **3**, **4**, **5**, and **6** by their nmr spectrum were unsuccessful. The H-1′ doublet could not be observed because of signal overlap and difficulties with radiofrequency saturation.

The mass spectrum of **3** showed mass peaks corresponding to those reported by Still and Mansager (1972) for a sample of acetylated chlorpropham conjugate from soybeans.

In the final step of the reaction sequence, the carbamate group appeared to be quite stable. Deacylation occurred without any major tendency for replacement of isopropoxy by methoxy, as was evident by the 86% yield of both **5** and **6**.

The alternate route analogous to the preparation of glucuronides used by Paulson *et al.* (1972) was not considered, as the metabolites **1** and **2** were independently needed. His procedure involved the reduction of commercially available *p*-nitrophenyl-β-glucuronide, followed by reaction with isopropyl chloroformate.

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The author is indebted to Derek Horton of Ohio State University for many helpful suggestions on these syntheses and to the following PPG Industries staff for their part in this work: Kenneth Dress (uv determinations), James Gregory (synthesis of **1**), Robert Pfost (mass spectra interpretation), Robert Shupe (ir and nmr determinations), Albert Strong (synthesis of **2**), and Jerome Wiedmann (enzymatic hydrolysis studies).

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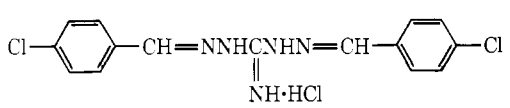
Absorption, Excretion, and Metabolism of Robenz, Robenidine Hydrochloride [1,3-Bis(*p*-Chlorobenzylideneamino)guanidine Hydrochloride], in the Rat

Jack Zulalian* and Paul E. Gatterdam

The results of the absorption, excretion, and metabolism in the rat of a new anticoccidial agent, 1,3-bis(*p*-chlorobenzylideneamino)guanidine hydrochloride [Robenz, robenidine hydrochloride], are presented. Rats given a single oral dose of robenidine hydrochloride, labeled with ^{14}C in the α-carbon atom of the *p*-chlorobenzylidene moiety, excreted approximately 58% of the radioactive dose in the feces and 20% in the urine within 24 hr. Tissue retention was low and accounted for 0.4% of the dose 96 hr after oral treatment. Two urinary metabolites were identified as *p*-chlorohippuric acid and *p*-chlorobenzoic acid and these accounted for 88 and 2%, respectively, of the total urinary radioactivity. Unmetabolized robenidine accounted for the major portion of the fecal radioactivity. Qualitatively, the metabolites isolated from the liver, kidney, and muscle were the same as those found in urine. *p*-Chlorohippuric acid appeared as the predominant metabolite in liver and kidney, while robenidine was predominant in skin and fat.

1,3-Bis(*p*-chlorobenzylideneamino)guanidine hydrochloride, the active ingredient in American Cyanamid's Robenz, robenidine hydrochloride medicated premix coccidiostat, is a new and highly effective product for preventing coccidiosis (Kantor *et al.*, 1970) and has been developed for use in broiler chickens. The absorption, excretion, and metabolism of ^{14}C-labeled robenidine hydrochloride (I) was studied in a mammal, the rat, as part of the program to aid toxicologists in evaluating robenidine data and in evaluating the safety to the consumer associated with the commercial use of this compound in poultry.



I

Robenz, robenidine hydrochloride

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Table I. Cumulative Excretion of Carbon-14 in Feces and Urine from the Rat after a Single Oral Dose of *p*-Chlorobenzylidene-^{14}C-Robenidine Hydrochloride

Time, hr	% of dose	
	Feces	Urine
6	0.03	2.8
12	0.1	9.9
24	58.2	19.6
48	68.6	21.8
96	70.4	22.2
	Overall recovery, 92.6%	

Table II. Carbon-14 in Tissues from the Rat 96 Hr after a Single Oral Dose of *p*-Chlorobenzylidene-^{14}C-Robenidine Hydrochloride

Tissues	% of dose
Liver	0.057
Kidney	0.006
Blood	0.014
Muscle	0.001
Brain	0.001
Gastrointestinal tract	0.093
Fat[a]	0.001
Skin[a]	0.003
Carcass	0.182
Total recovery tissues,	0.36
Respiratory $^{14}CO_2$,	0.07
Urine,	22.2
Feces,	70.4
Overall recovery,	93.0

[a] Includes extractable and unextractable residual radioactivity.

MATERIALS AND METHODS

Radiolabeled Robenidine Hydrochloride. *p*-Chlorobenzaldehyde (carbonyl-^{14}C) obtained from Mallinckrodt Nuclear was used for the synthesis of radiolabeled robenidine. The radiotracer (321 mg, 3.28 mCi/m*M*) was dissolved in 8 ml of ethanol and added dropwise to a solution of 1,3-diaminoguanidine hydrochloride (144 mg, 1.14 m*M*) dissolved in 8 ml of boiling ethanol. The solution was stirred, heated 1 hr in a hot water bath, and then cooled. The precipitate that formed was collected by suction filtration, washed with hot benzene, and allowed to dry. The yield was 75.3% (319 mg) and the final product had a specific activity of 12.7 μCi/mg. By two-dimensional thin-layer chromatography in chloroform–methanol–formic acid (75:22.5:2.5, v/v/v) *vs.* chloroform–acetone–acetic acid (60:30:10, v/v/v), the product had a radiochemical purity of 99.2%.

***p*-Chlorohippuric Acid.** *p*-Chlorohippuric acid (II) prepared from *p*-chlorobenzoyl chloride and glycine (Novello *et al.*, 1926) had mp 142–144.5°.

Treatment of Rats. Fifteen male Charles River rats were given single oral doses of robenidine hydrochloride by stomach tube. The dose contained 21 mg of the drug/kg of body weight (56 μCi of carbon-14) and was selected to provide sufficient amounts of radioactivity in the excreta so as to facilitate two-dimensional thin-layer chromatography, with minimum cleanup of urine and the extracts of feces and tissues. Extrapolating from published dietary intake data for rats that the animals used in this study consumed about 15 g of food per day, it was calculated that the dosage rate used in this study corresponded to a concentration in the diet of 280 ppm of robenidine hydrochloride.

Each rat was placed in a metabolism cage that allowed for the separate collection of urine and feces. Food and water were provided *ad libitum*. The rats were divided into five groups of three rats each. The rats were sacrificed 6, 12, 24, 48, and 96 hr after treatment, as indicated in Table I. The urine was collected in glass containers surrounded by Dry Ice. Feces were collected and frozen at 6 and 12 hr after dosing and daily thereafter throughout the study.

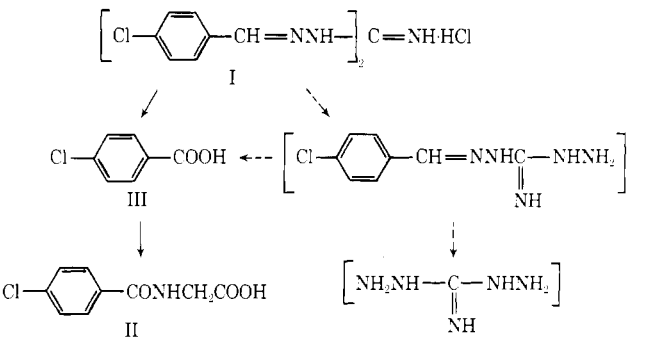


Figure 1. Metabolic route for Robenz, robenidine hydrochloride, metabolism in the rat.

At the same time intervals, one group of three rats was sacrificed and liver, kidney, blood, muscle, brain, fat, and skin were removed (Table II). The same tissues within each group were pooled, homogenized with 5–10 ml of water/g of sample, and lyophilized. In addition, the digestive tract, a portion of skin, and the carcass of one rat from each group were taken for the determination of their radioactive residues.

Fat and skin were extracted with benzene. The benzene fractions were refrigerated and the residual fractions dried and stored. Rat feces, liver, and kidney were extracted with ethanol.

$^{14}CO_2$ Determination. In order to estimate the extent of breakdown of robenidine hydrochloride to $^{14}CO_2$, a rat was given a single oral dose containing 25.2 mg of the drug/kg of body weight (60 μCi of carbon-14). The rat was placed in an all-glass metabolism chamber (Delmar Scientific Laboratories, Model DS 7005) and the respiratory radioactivity measured by passage of respired CO_2 through 150 ml of 3 *N* sodium hydroxide solution contained in a scrubbing bottle. The rat was kept in the apparatus for 24 hr, food and water being provided *ad libitum* during the experiment. Recovery of $^{14}CO_2$ from several samples of standard sodium carbonate-^{14}C (Nuclear Chicago Corp., Model RS14A) treated with hydrochloric acid, using the same metabolism chamber used in the animal experiment, was approximately 95%.

TOXICITY OF ROBENIDINE HYDROCHLORIDE AND ITS METABOLITES

Oral LD_{50} values were determined in female albino rats. Robenidine hydrochloride and metabolites II and III (Figure 1) were fed *via* stomach tube as aqueous suspensions in volumes of 1 ml/rat. In the LD_{50} region, five replicates were used. The mortality counts were taken 24 hr after treatment and the LD_{50} values are given in Table III.

Determination of Radioactivity. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. Appropriate aliquots of urine and other liquid samples were counted directly after the addition of DAM-611 solution containing 5% Cab-O-Sil thixotropic gel. The total radioactivity in respired air was determined by counting 0.5- and 1.0-ml aliquots of the absorber solution (3 *N* sodium hydroxide) in 20 ml of DAM-611 scintillator solution (Davidson and Feigelson, 1957) containing 5% Cab-O-Sil thixotropic gel.

Radioactive spots on thin-layer chromatograms were located by means of radioautography using Kodak Royal Pan professional grade photographic film. For purposes of quantitation, each radioactive zone was scraped from the

Table III. Comparative Toxicities to Female Albino Mice of the Identified Metabolites and Robenidine Hydrochloride

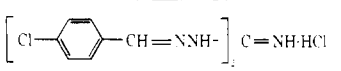
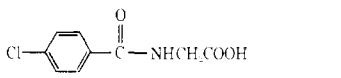
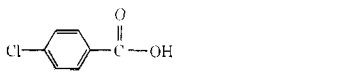
Compd no.	Structure	LD_{50}, mg/kg
I		390
II		2230
III		1170

plate and placed into scintillation vials for direct counting.

Radioactivity in tissues, excreta, intestine, and carcass was determined by modification of the oxygen flask combustion method (Kelly *et al.*, 1961).

Chromatography. Commercially available glass plates (20 cm × 20 cm) precoated with silica gel were used for the two-dimensional tlc analysis. System I [chloroform–methanol–diethylamine (75:20:5, v/v/v) *vs.* chloroform–ethyl acetate–formic acid (60:30:10, v/v/v)] and system II [ethyl acetate–1-propanol–water–formic acid (30:50:15:5, v/v/v/v) *vs.* ethyl acetate–1-propanol–water–ammonium hydroxide (30:50:15:5, v/v/v/v)] were the solvent systems of choice in studying the radioactive metabolic patterns in urine, feces, and tissue extracts.

Metabolite Identification. As an aid to the identification of metabolites, authentic standards prepared as potential metabolites were chromatographed with the radiometabolite mixture. Coincident spots, detected by radioautography followed by visualization under ultraviolet light (254 nm) of the standards, provided tentative identification. Isotope dilution *via* cocrystallization of the standards, tentatively identified as metabolites, with the isolated radioactive metabolites to achieve a constant specific activity was used to confirm the identification.

RESULTS AND DISCUSSION

Results. Rats excreted approximately 58% of the radioactive dose in the feces and 20% in the urine within 24 hr, as shown in Table I. Tissue retention accounted for 0.36% of the dose after 96 hr, as shown in Table II. Metabolism to respiratory $^{14}CO_2$ was 0.07% of the dose during the first 24 hr and it is unlikely that significant additional $^{14}CO_2$ was respired after this time, since only a small fraction of the dose remained in the body after 24 hr. The overall recovery of the administered dose was 93%.

Since all the significant potential repositories of the radioactivity were analyzed, the failure to achieve 100% recovery of the radioactive dose may be attributed to the accumulation of experimental errors rather than to the actual loss of radioactivity in some unknown fashion, perhaps on the surfaces of the cages. Therefore, the expression of the radioactivity of the various samples as percent of the recovered radioactivity may be more valid than expressed in terms of the administered dose. When recovery is calculated on this basis, 99% of the radioactivity is accounted for by the combined urinary and fecal elimination and this shows that very little radioactivity is retained by the tissues.

Table IV shows the residual radioactivity levels in the selected tissues, expressed as ppm equivalents of robenidine hydrochloride at the various times after treatment. All the tissues except skin, which showed a maximum level of residual radioactivity at 12 hr, showed maximum levels of robenidine-derived radioactivity at 6 hr. After 96 hr the radioactivity levels in each of the tissues had declined to less than 0.3 ppm.

Table IV. Residual Radioactivity in Tissues of Rats Expressed as ppm Equivalents of Robenidine Hydrochloride

Tissue	Withdrawal time, hr →	Group A, 6	Group B, 12	Group C, 24	Group D, 48	Group E, 96
Liver		8.7	5.6	1.6	0.5	0.3
Kidney		3.6	3.0	0.9	0.3	0.2
Blood		2.4	1.6	0.75	0.2	0.1
Muscle		0.6	0.5	0.1	0.04	0.02
Brain		0.75	0.5	0.1	0.03	0.02
Gastrointestinal tract		158	155	11.4	0.5	0.16
Carcass		1.5	0.7	0.2	0.06	0.05
Fat[a]		3.6	3.0	0.95	0.14	0.08
Skin[a]		0.65	1.38	0.33	0.10	0.08

[a] Figures include the sum of extractable and unextractable residual radioactivity.

The residual radioactivity was higher in liver, kidney, blood, and fat than in muscle or brain, with the liver being the highest at all time intervals compared with these other tissues. The higher levels in the kidney and liver presumably result from their effectiveness in removing robenidine and its closely related metabolites from the blood.

The study was intended to elucidate the nature of the metabolites in the tissues resulting from a single oral dose of robenidine to mammals but was not intended to define quantitatively their residues in tissues. In this type of study the measurement of the residual radioactivity in tissues indicates whether any tissues serve as repositories for residues. This information can then be useful for designing residual analytical studies for the parent drug and its significant metabolites under realistic field conditions.

NATURE AND IDENTITY OF RADIOACTIVE COMPOUNDS

Urine. The radioactive urine samples were chromatographed using the solvent systems I and II. System I resolved the radioactivity in the urine into a minimum of six urinary metabolites. The major urinary metabolite of robenidine was identified as *p*-chlorohippuric acid by cochromatography with the synthetic standard. This metabolite accounted for 88% of the urinary radioactivity and final confirmation was achieved by successive recrystallization of authentic *p*-chlorohippuric acid with the urinary radiometabolite to a constant specific activity. A minor metabolite, identified as *p*-chlorobenzoic acid, accounted for 2% of the urinary radioactivity. About 6% of the radioactivity in the urine that was not identified remained at the origin of the thin-layer chromatogram. By chromatography in solvent system II, the urinary radioactivity was resolved into 13 radioactive spots with one predominating metabolite, *p*-chlorohippuric acid. Since there was no origin-bound radioactivity using system II, this suggests that the origin-bound radioactivity found in chromatograms with solvent system I is probably composed of several polar metabolites.

Feces. The 12–24-hr fecal sample, representing the peak excretion of radioactivity by rats treated with robenidine, was lyophilized and the powder extracted with boiling ethanol to recover 69% of the fecal radioactivity. Extraction of the fecal residue with boiling ethanol acidified with hydrochloric acid recovered another 28% of the radioactivity from feces.

Chromatography in solvent system I revealed that unchanged robenidine accounted for 90% of the ethanol-soluble radioactivity and 10% was distributed among nine minor metabolites. However, a comparison to the extract of control feces fortified with robenidine-^{14}C, wherein all the radioactivity was recovered by ethanol extraction,

suggested that five of the minor fecal metabolites were a result of nonmetabolic breakdown of the robenidine which occurred during the extraction process. Chromatography in solvent system I of the radioactivity extracted from the feces with acidified ethanol revealed that all of the radioactivity remained near the origin, while chromatography in solvent system II resolved the radioactivity into ten radioactive spots, none of which was identified. Since more than 60% of the radioactivity found in feces was identified as unmetabolized robenidine, this suggests that robenidine was incompletely absorbed from the digestive tract and does not undergo extensive breakdown in the body of the animal.

Tissues. In order to establish the relevance of the urinary metabolites to those occurring in tissues, the radioactivity in selected tissues was isolated by extraction and chromatographed in solvent system I. Benzene extracts of fat and skin, containing 93–95% of the total radioactivity in these tissues, revealed that robenidine was the only compound present. Ethanol extracts of liver, kidney, and muscle containing 87–97% of the total radioactivity in these tissues revealed the presence of *p*-chlorohippuric acid, *p*-chlorobenzoic acid, and robenidine as tissue residues.

CONCLUSION

Since *p*-chlorobenzoic acid is excreted as *p*-chlorohippuric acid in dogs (Novello *et al.*, 1926) and rabbits (Bray *et al.*, 1952) and these compounds have been identified as urinary metabolites of robenidine, this evidence indicates that the scheme shown in Figure 1. represents a major route for the metabolism of robenidine in the rat. No 1-amino-3-(*p*-chlorobenzylidineamino)guanidine, postulated to be a potential metabolite, was found.

It is speculated that this aminoguanidine fragment may be a transient metabolite. It could be split by the rat to yield *p*-chlorobenzoic acid and 1,3-diaminoguanidine, as suggested from the mammalian metabolism of the related aldehyde derivatives of thiosemicarbazide (Williams, 1959). Since there is little literature on the known fate of guanidine in mammalian metabolism, no proposals can be made to suggest the possible fate of the aminoguanidine moiety.

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Metabolism of the Herbicide Methazole in Cotton and Beans, and Fate of Certain of Its Polar Metabolites in Rats

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Methazole, 2-(3,4-dichlorophenyl)-4-methyl-1,2,4-oxadiazolidine-3,5-dione, was metabolized by cotton and beans to 3-(3,4-dichlorophenyl)-1-methylurea and 3-(3,4-dichlorophenyl)urea. These metabolites occurred in the free form and as polar products which were converted to the free form by acid treatment. ^{14}C residues in the solids after acetone extraction were removed by heating in 1 *N* HCl and extracting with ethyl acetate. The methylurea and urea derivatives of methazole were the major components of the ethyl acetate extract. When polar acetone-extractable metabolites from cotton and beans were administered orally to rats, 70 to 80% of the radioactivity was excreted within 24 hr. Residues were low, 0.13 ppm maximum, in tissues of animals sacrificed 12 hr after treatment orally for 14 days with polar methazole metabolites at a rate of approximately 1 μg of methazole-^{14}C equivalents/animal/day.

The general metabolic fate of methazole, 2-(3,4-dichlorophenyl)-4-methyl-1,2,4-oxadiazolidine-3,5-dione, in cotton has been reported (Jones and Foy, 1972). It was demonstrated that the herbicide was metabolized to 1-(3,4-dichlorophenyl)-3-methylurea, 1-(3,4-dichlorophenyl)urea, and to significant quantities of more polar materials which remained unidentified. In addition, large quantities of methazole-^{14}C equivalents, over 30% in leaves, could not be extracted from the treated plants with methanol.

Similar pathways have been described for monuron, 3-(4-chlorophenyl)-1,1-dimethylurea, and diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (Frear and Swanson, 1972; Onley *et al.*, 1968; Smith and Sheets, 1967; Swanson and Swanson, 1968). Frear and Swanson (1972) found that after 24 hr, 20 to 25% of the methanol-extractable monuron equivalents from excised cotton leaves treated with monuron was as β-D-glucosides of 3-(4-chlorophenyl)-1-hydroxymethyl-1-methylurea and 3-(4-chlorophenyl)-1-hydroxymethylurea. Subsequent studies showed that the hydroxymethyl intermediate was unstable but would react with methanol to form 3-(4-chlorophenyl)-1-methoxymethylurea, which was stable (Tanaka *et al.*, 1972).

The methoxymethylurea derivative of methazole was detected in methanol solutions of the herbicide after exposure to ultraviolet light (Ivie *et al.*, 1973). It was not a

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