ARTICLES

Tissue Residue Studies with Ronidazole: Effect of Label Site on Total Radioactivity Content of Rat Tissues

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The level of persistent tissue residues was determined in rats with ronidazole labeled with ^{14}C at four different sites. The time course of the residue disappearance and the total initial residue pool size were estimated following a single treatment using groups of rats killed at 2, 4, 7, and 14 days. As the observed tissue residue concentrations, the estimated rate of depletion, and the total pool size were different for each label site, the residues cannot consist only of substances containing an intact imidazole nucleus. In a dose–response study the tissue residue concentrations in all tissues examined were directly related to the dose over a range of 125-fold, 0.08–10 mg/kg. Simultaneous dosing with either methylamine or 1-methionine did not influence the tissue residue levels obtained with $^{14}CH_3$-labeled ronidazole. When ^{14}CO-labeled ronidazole was used, almost 60% of the label was eliminated as $^{14}CO_2$, indicative of extensive degradation of ronidazole. That fixation of $^{14}CO_2$ can only account for a small fraction of the residues from the ronidazole labeled at different sites is indicated by a comparison of the residue levels and the small amount, 0.5–2%, of the dose eliminated as $^{14}CO_2$. The 4,5 ring label gave the lowest overall tissue residue levels. In some tissues the other levels were as much as 6 times higher. That the 4,5 ring label also yields residues that contain substances that do not contain an intact imidazole nucleus was indicated by the observation that after 14 days the total residues in liver and kidney were lower in those animals dosed with methylene-labeled ronidazole.

Ronidazole is useful in animal husbandry for the prevention of swine dysentery and the treatment of turkey blackhead. Previously reported studies (Rosenblum et al., 1972) have shown that significant metabolic degradation of radioactive ronidazole occurs in the turkey, and studies in swine using [*methyl*-^{14}C]ronidazole (Figure 1) gave evidence for persistent residues due to incorporation of metabolites possibly derived from methylamine as well as residues that may contain an intact imidazole nucleus. (Wolf et al., 1983b).

When ^{14}C-labeled ronidazole is incubated anaerobically with rat microsomes in the presence of NADPH (West et al., 1982), the trichloroacetic acid (TCA) insoluble macromolecular fraction contains radiocarbon. In these studies there was no difference in the extent of incorporation radioactivity when ronidazole was labeled with $^{14}CH_3$, $^{14}CH_2$, or ring-4,5-^{14}C but ^{14}CO was poorly incorporated [Miwa et al., 1982].

With ^{14}C 4,5 ring labeled ronidazole, studies in germ-free and conventional rats (Wolf et al., 1983a) showed that the gut microflora contribute to the overall disposition of ronidazole. Levels of tissue residues 5 days after dosing are nearly the same for both types of animals. Thus the tissue residues appear to result primarily from metabolism by mammalian enzymes.

Persistent residues observed in vivo may be the result of activation and binding to cellular macromolecules to produce products similar to those observed in vitro and hence would contain the essential chemical features of the imidazole nucleus. In addition, persistent residues could result from metabolic degradation of the imidazole nucleus to one or two carbon fragments that could form endogenous substances capable of entering cellular macromolecules via normal protein synthesis reactions that do not occur in the in vitro system.

The studies were carried out to determine if the tissue residues in the rat are chemically similar to those produced in vitro or if the residues are due to incorporation of one and two carbon fragments of the drug. Fragmentation of the imidazole nucleus of metronidazole, a related 5-nitroimidazole, by rats (Koch et al., 1979) and humans (Koch et al., 1981) has been reported. Previous studies in turkey had shown that methylamine or a metabolite readily converted to methylamine is present in the tissues of these animals dosed with $^{14}CH_3$-labeled ronidazole. ^{14}C-labeled acetic acid was liberated by hydrolysis under weakly acidic conditions when the turkeys were dosed with ^{14}C 2 ring labeled ronidazole (Rosenblum et al., 1972).

The tissue distribution of radioactive residues following administration of $^{14}CH_3$-, $-^{14}CH_2-$, ^{14}C 4,5 ring, and ^{14}CO-labeled ronidazole is reported. In addition, the tissue distribution of ^{14}C-labeled methylamine was determined for comparison with $^{14}CH_3$-labeled ronidazole and a dose-dependency study was carried out with $^{14}CH_3$-labeled ronidazole.

MATERIALS AND METHODS

Animal Handling. Male Charles River CD rats, weight 180–220 g, were used. The animals were maintained on Purina chow. For all studies except the dose-dependence study with $^{14}CH_3$ label, the animals were dosed with 10 mg/kg, administered by gavage. Animals treated with methylamine hydrochloride received an equimolar dose, 3.35 mg/kg. Groups of three rats were killed and tissues

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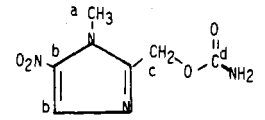

Figure 1. Ronidazole, (1-methyl-5-nitroimidazole-2-yl)methyl carbamate; label sites indicated as a, b, c, and d.

Table I. Elimination of Ronidazole Labeled at Different Sites with ^{14}C: Rats[a]

label site	dose, mg/kg	% eliminated[b] CO_2[c]	feces	uring	total
CH_3	10	1.39	42.1	44.2	87.7
CH_2	10	0.65	48.1	33.7	82.5
4,5 ring	10	0.17	50.8	39.0	90.0
C=O	10	57.5	5.6	21.8	84.9
CH_3[d]	10		43.2	30.4	73.6
CH_3[d]	2		48.0	33.0	91
CH_3[d]	0.4		52.0	32.6	84.6
CH_3[d]	0.08		52.2	28.5	80.7
CH_3[e]	10		44.9	39.8	84.7
CH_3[f]	10	0.6	50.6	32.5	83.7
CH_3NH_2[g]	3.4	23.8	2.6	16.2	42.6[h]

[a] All rats were male, Charles River C.D., weight 180–220 g, dosed orally by gavage with 1–2 mL of an aqueous solution of ronidazole. [b] Pooled samples from each group were collected daily for 4 days and the data from all groups in a single experiment combined. [c] Collected for 24 h after dosing; volatile bases from the CH_3 experiment amounted to 0.01%. [d] Average of six rats slaughtered 24 and 48 h after dose: GI tract contents included in feces. [e] As methylamine is a possible metabolic product of ronidazole that would lead to bound tissue residues, rats were maintained on a diet containing 0.7% methylamine hydrochloride beginning 3 days prior to treatment and continued throughout the experiment. [f] As N-demethylation produces formaldehyde, which in turn yields L-methionine, rats were maintained on a diet containing 2.5% L-methionine beginning 3 days before treatment and for 4 days after treatment. [g] Dosed as the hydrochloride salt. [h] The tissues contained about 28.4% of the dose; total accounted for was 71%.

analyzed at 2, 4, 7, and 11 or 14 days after dosing. Urine and feces were collected from each group for 4 days except the group killed at day 2. For collection of $^{14}CO_2$ the animals were placed in air-tight metabolism cages. Air flow was maintained by suction. The exit gases were passed through two consecutive gas scrubbing towers containing 100–125 mL of 1 N sodium hydroxide. One or two animals from the day 2 group were used.

Tissue Analysis. Rats were killed by heart puncture and exsanguination after being rendered unconscious with CO_2. The GI tract, liver, and kidney were excised intact. Fat samples were collected from the back. Muscle tissues were collected from the leg. Equal weight samples from all animals in a group were pooled for analysis.

Radioactivity Determination. Fecal and all tissue samples except kidney were homogenized separately in 3 parts by weight of water with a Virtis homogenizer. Kidney samples were homogenized as a 1:10 dilution. Duplicate samples of the homogenate (0.5–1 g) were weighed onto paper combustion cups and air-dried at room temperature for 24 h. Combustion of the dried samples was then carried out by means of a Packard Model 306 oxidizer. The $^{14}CO_2$ was absorbed in 8 mL of Carbosorb (Packard) and mixed with 13 mL of Permafluor V (Packard). Combustion of urine, plasma, and blood samples (0.3–0.5 mL) was carried out similarly. Samples were assayed for ^{14}C radioactivity with a Packard Model 3380 liquid scintillation spectrometer with external standardization. All determinations were carried out in duplicate.

Radioactive Synthesis. The radioactive compounds were synthesized in the Merck Sharp & Dohme Research Laboratories. All labeled compounds were tested for purity by TLC in two solvent systems. The purity was found to be as follows: $^{14}CH_3$, 99.4%; $-^{14}CH_2-$, 99.3%; ^{14}C 4,5 ring, 98.8%; ^{14}CO, 99.5%. No single impurity accounted for more than 0.5% of any lot.

Protein and Methylamine Determination. Protein was separated by a salt fractionation procedure. After 100 mg of sodium chloride/g of tissue was added, each homogenate was heated at 100 °C for 30 min in a boiling water bath. The suspension was centrifuged for 30 min at 10000 rpm, the solids were resuspended with 14 mL of 10% sodium chloride solution, and the heating and centrifuge steps were repeated. The solid product was suspended in 4 mL of acetone and filtered on a sintered glass funnel and air-dried. The dry product, amounting to 250–275 mg, was analyzed for total radioactivity by combustion as described above.

Samples containing at least 10000 dpm of ^{14}C radioactivity were hydrolyzed with 6 N hydrochloric acid in a sealed tube heated at 120–130 °C for 16 h. Crystalline methylammonium *p*-toluenesulfonate was added as a carrier prior to the hydrolysis. The hydrolysis mixture was made strongly alkaline with sodium hydroxide and the methylamine distilled by using a nitrogen gas sweep. The gas stream was passed through a scrubber containing ethanol and *p*-toluenesulfonic acid. Methylammonium *p*-toluenesulfonate was isolated from the scrubbing solution by evaporating to dryness and crystallizing from acetonitrile. The specific activity was determined by using aliquots of the crystalline salt.

RESULTS

Excretion Routes. The data obtained for the elimination of the radiocarbon are summarized in Table I. The feces generally contain 40–50% of the dose and urine contains 30–40% for the methyl-, and methylene-, and 4,5 ring labeled drug. For these labels only a small fraction is converted to $^{14}CO_2$. When radioactive methylamine is dosed, a much higher fraction appears as CO_2. With carbonyl-labeled ronidazole very little of the dose appears in the feces, the feces/urine ratio is much lower, and almost 60% of the dose was recovered as $^{14}CO_2$.

Excretion in the urine and feces is quite rapid. Averaged daily excretion during the first four days are presented in

Table II. Daily Elimination of Radiocarbon from Rats Dosed with Ronidazole Labeled at Different Sites, Percent of Dose

label site	urine day 1	urine day 2	urine day 3	urine day 4	feces day 1	feces day 2	feces day 3	feces day 4
CH_3	41.6	2.2	0.36	0.16	30.3	11.1	0.88	0.18
$-CH_2-$	31.1	1.7	0.8	0.3	44.9	2.8	0.31	0.19
4,5 ring	36.5	2.0	0.44	0.3	45.8	4.7	0.3	0.22
av[a]	36.4	1.97	0.53	0.25	40.3	6.2	0.5	0.2
-CO	16.7	0.4	0.1	0.07	1.9	3.4	0.37	0.13
% total excreted[b]	93.0	5.0	1.4	0.6	85.4	13.1	1.1	0.4

[a] Average of CH_3, $-CH_2-$, and 4,5 ring label. [b] Fraction present in urine or feces based on overall average of ronidazole-dosed animals excluding the ^{14}CO label.

Table III. Tissue Residues of Ronidazole as a Function of Oral Dose Administered to Rats[a]

tissue dose, mg/kg	day	residue, ppm per mg/kg ronidazole dosed[b] liver	muscle	kidney	fat	plasma
10	1	0.182	0.136	0.229	0.248	0.05
	2	0.062	0.062	0.078	ND[c]	0.0093
2	1	0.115	0.135	0.175	0.17	0.0435
	2	0.055	0.05	0.055	0.07	0.0105
0.4	1	0.072	0.095	0.14	0.085	0.035
	2	0.032	ND	0.03	0.05	0.0075
0.08	1	0.09	0.1	0.11	0.09	0.025
	2	0.09	0.05	0.04	0.06	0.012

[a] Three male Charles River CD rats, average weight 180 g, were dosed with 1.8 mL of a solution containing sufficient ronidazole to give the indicated dose. The $^{14}CH_3$-labeled drug was adjusted to a specific activity of 0.06, 0.28, 1.4, and 7.1 μCi/mg for 10, 2, 0.4, and 0.08 mg/kg dose, respectively. [b] Total radioactivity expressed as ronidazole. [c] Detection limit was taken as 2σ above average background of five reagent blanks and is as follows: dose 10 mg/kg, tissues 0.18 ppm, plasma 0.045 ppm; dose 2 mg/kg, tissues 0.045 ppm, plasma 0.01 ppm; dose 0.4 mg/kg, tissues 0.01 ppm, plasma 0.0025 ppm; dose 0.08 mg/kg, tissues 0.002 ppm, plasma 0.0005 ppm.

Table II. For all label sites and for both urine and feces about 98% of the 4-day total is eliminated in the first 48 h. For urine alone 93% of the 4-day total is obtained in the first 24 h. Thus, most of the dose is eliminated rapidly. The total recovery averaged 84% for animals dosed with ronidazole and 71% for animals dosed with methylamine.

Tissue Residues. *Dosage Response.* The tissue analysis data are contained in Table III. Comparison of the concentration of tissue residues on day 1 indicates a higher level in those rats receiving the highest dose. Although the difference is significant at the 1% level, the difference on day 2 is not significant at the 5% level. This observation can be explained if the rate of absorption for the highest dose is lower during the first few hours after dosing. Thus, the total tissue exposure would be proportional to the dose, producing equal residues on day 2 when most of the ronidazole and low molecular weight metabolites have been eliminated. Total radioactivity residues on day 1 would therefore contain a higher fraction of low molecular weight substances than the residues at lower doses.

Effect of Label Site. Analysis results of the pooled tissues are contained in Table IV. Persistent residues are present in all tissues. At the last time point muscle contains the most radioactivity in all cases except that of the $-CH_2-$ label. For this label fat contained a greater quantity than muscle. At 2 days the largest concentration of radioactivity was found in the kidney with the exception of the CO label in which the liver contained the highest quantity of radioactivity. The markedly different level of radioactivity in tissues with different labels excludes the possibility that the tissue residues contain only substances with an intact *N*-methylimidazole or imidazole nucleus. This is even more clear when the concentration of residues relative to the 4,5 label (Table V) and pool size and half-life (Table VI) are examined. Added methylamine or L-methionine had little effect on the overall residue formation, although tissue residue ratios appear more consistent with time (Table V).

Tissue Fractionation. The fraction of the total residue obtained in the protein fraction of muscle and liver was higher for animals dosed with $^{14}CH_3$-labeled ronidazole than for those dosed with ^{14}C-labeled methylamine although the total level was much lower (Table VII). Residues that liberated methylamine account for a higher

Table IV. Total Radioactivity Content of Rat Tissues from Animals Dosed with Ronidazole Labeled at Different Sites[a]

label site	time after dose, days	tissue, ppm[b] liver	muscle	kidney	fat	plasma
$-CH_3$	2	0.33	0.31	0.48	0.14	0.08
	4	0.27	0.26	0.41	0.13	0.05
	7	0.16	0.23	0.26	0.09	0.02
	11	0.11	0.18	0.17	0.07	0.01
$-CH_2-$	2	0.22	0.18	0.35	0.23	0.089
	4	0.10	0.13	0.26	0.14	0.033
	7	0.07	0.095	0.11	0.079	0.020
	14	0.02	0.051	0.042	0.061	0.002
4,5 ring	2	0.18	0.17	0.26	0.04	0.06
	4	0.10	0.11	0.14	0.03	0.03
	7	0.06	0.07	0.07	0.02	0.02
	14	0.03	0.06	0.04	0.02	0.004
CO	2	0.40	0.12	0.19	0.07	0.12
	4	0.25	0.11	0.17	0.11	0.24
	7	0.12	0.07	0.08	0.07	0.09
	14	0.04	0.06	0.04	0.05	0.02
$-CH_3$[c]	2	0.43	0.49	0.58	0.11	0.06
	4	0.36	0.33	0.50	0.15	0.04
	7	0.16	0.15	0.16	0.06	0.02
	14	0.05	0.13	0.08	0.03	0.005
$-CH_3$[d]	2	0.40	0.26	0.47	0.17	0.10
	4	0.21	0.20	0.24	0.07	0.05
	7	0.15	0.17	0.18	0.07	0.03
	14	0.08	0.12	0.09	0.04	0.01
CH_3NH_2	2	1.2	1.6	2.5	1.3	0.2
	4	1.1	1.8	2.2	0.70	0.12
	7	0.58	1.2	0.94	0.43	0.06
	14	0.28	0.74	0.44	0.23	0.033

[a] Male Charles River CD rats, weight 180–220 g, were used. The dose, 10 mg/kg, was administered orally by gavage. Tissues from three rats per group were combined for analysis. [b] Average of two or four replicates per sample, 10 dpm–0.001 ppm in plasma or 0.65 ppm in tissue, expressed in terms of ronidazole. [c] With added CH_3NH_2; see Table I, footnote *e*, for details. [d] With added L-methionine; see Table I, footnote *f*, for details.

Table V. Ratio of Concentration of Total Radioactivity in Tissues of Rats Dosed with ^{14}C-Labeled Ronidazole Relative to That Observed with 4,5 Ring Label[a]

label site	time, days postdose	liver	muscle	kidney	fat	plasma
$-CH_3$	2	1.83	1.82	1.85	3.5	1.33
	4	2.7	2.36	2.93	4.33	1.67
	7	2.67	3.29	3.71	4.5	1.0
	11	3.17	3.0	4.25	3.5	2.5
	14 (est)[b]	(4.7)	(5.0)	(6.0)	(5.0)	(2.5)
$-CH_2-$	2	1.22	1.06	1.35	5.75	1.48
	4	1.0	1.18	1.86	4.67	1.1
	7	1.17	1.36	1.57	3.95	1.0
	14	0.67	0.85	1.05	3.05	0.5
CO	2	2.22	0.71	0.73	1.75	2.0
	4	2.5	1.0	1.21	3.67	8.0
	7	2.0	1.0	1.14	3.5	4.5
	14	1.33	1.0	1.0	2.5	5.0
$-CH_3$[c]	2	2.39	2.88	2.23	2.75	1.0
	4	3.6	3.0	3.57	5.0	1.3
	7	2.67	2.14	8.57	3.0	1.0
	14	1.67	2.17	2.0	1.5	1.25
$-CH_3$[d]	2	2.22	1.53	1.81	4.25	1.67
	4	2.1	1.82	1.71	2.33	1.67
	7	2.5	2.43	2.57	3.5	1.5
	0.4	2.67	2.0	2.25	2.0	2.5
CH_3NH_2	2	6.67	9.41	9.62	32.5	3.53
	4	11.0	16.4	15.7	23.3	4.0
	7	9.7	17.1	13.4	21.5	3.0
	14	9.33	12.3	11.0	11.5	8.25

[a] Based on data in Table IV. [b] Extrapolated from previous time points assuming first-order depletion. [c] With added CH_3NH_2; see Table I, footnote *e*, for details. [d] With added L-methionine; see Table I, footnote *f*, for details.

fraction of the total in muscle compared to that in liver. For both tissues the fraction of the residue that liberated methylamine declines with time. No radioactive methylamine was liberated from the tissues of animals dosed with ^{14}C-labeled methylamine.

DISCUSSION

Excretion. The comparative fecal excretion data indicate no difference in the extent of fecal excretion except for the CO-labeled drug. As it is likely that the same fraction of the *N*-methylimidazole nucleus is present in the feces regardless of label site, it appears that the feces do not contain metabolites retaining the CO group. When ronidazole was dosed to germ-free rats, the fraction excreted in the feces was much lower than that excreted by normal animals. This suggests that the high fraction of radiocarbon in the feces is not due to unabsorbed drug or biliary excretion of the absorbed drug (Wolf et al., 1983a) and that metabolism by the microflora is involved in the fecal excretion. As the covalently bound material produced by microsomal reaction with ronidazole also does not contain the CO label, it is likely that most of the radioactivity in the feces is covalently bound to the fecal solids. As the fraction in the feces is not influenced by the label site, the intact *N*-methylimidazole nucleus is probably present.

For all labels the total recovery is lower than that usually observed in our laboratory with other compounds. Recently, it has been observed in our laboratory that 2-carboxyl-1-methyl-5-nitroimidazole, a potential metabolite, is readily decarboxylated to yield 1-methyl-5-nitroimidazole. The latter substance volatilizes readily and, if present, would have been partially lost when samples were dried prior to combustion.

All one-carbon compounds produce $^{14}CO_2$ via known metabolic pathways. Similarly, most two-carbon compounds are at least partially converted to $^{14}CO_2$. Hence, the yield of $^{14}CO_2$ is an indication of one- and two-carbon metabolism which could yield intermediary metabolites. On the basis of this observation, tissue residues from the 4,5 ring would contain the lowest fraction of endogenous substances.

The excretion of the drug and most metabolites is rapid. Thus, 93% of that excreted in the urine is contained in the first 24-h collection and 97.4% of that excreted in the first 4 days was obtained in the first 48 h. As the cages were not washed daily, much of that obtained in the day 3 and 4 collections may actually have been excreted earlier. Similary, about 98% of the fecal excretion occurs in the first 2 days. These data indicate that most of the drug and metabolites are rapidly excreted.

Tissue Residues. *Dose–Response.* Several conditions are necessary for a measured response to remain proportional to the dose. The most important of these are (1) the drug must not influence gastric mobility, blood flow, respiration, and the like by direct action at the cellular level or indirectly via the central nervous system, (2) the effective K_m of metabolic reactions important in drug disposition is at least an order of magnitude greater than the effective cellular concentration of the drug at the highest dose level employed, and (3) cofactors and substances that combine with the drug metabolites must not be depleted at high concentrations of the drug. In this study the dose was varied from 0.08 to 10 mg/kg (125-fold) and the concentration of total radioactivity in tissues determined in 1 and 2 days after a single dose. From the excretion rate studies it appears that most of the dose has been eliminated by 24 h; even so the tissue residue determinations at this time could contain sufficient quantities of readily eliminated substances to influence the tissue level as only a small fraction of the dose remains in the tissues. The tissue residue concentrations taken at day 1 indicate a dose dependency, especially for liver, kidney, and fat. At the second day there is no significant difference in the tissue residue level related to dose. Thus, the rate of formation of more persistent residues does not exhibit dose dependency over the dosage range explored. Depletion of cofactors or substances reacting with the drug did not occur.

Effect of Label Site. If the tissue residue contained only substances with an intact imidazole nucleus, each label site should produce the same level of residues in each tissue at each analysis time, and such parameters as half-life of the residue and tissue/plasma ratios would be identical. On the other hand, if the tissue residues contain only fragments of ronidazole, the different label sites would be expected to produce differing levels of residues in various tissues as both the efficiency of fragmentation and incorporation into macromolecular constituents would vary.

The half-life and total pool size of the persistent residues are contained in Table VI. It is evident from examination of this table and the observed tissue residue values in Table IV that the tissue residues cannot contain exclusively an intact imidazole nucleus. Residues due to $-CH_3$ and $-CH_2-$ labels are both greater than those due to the 4,5 ring. Residues due to the CO label appear greater than those due to the 4,5 ring and $-CH_2-$ labels in liver and plasma, while being similar in muscle. Thus the residues cannot contain the 4,5 ring, $-CH_2-$, and CO labels in the same proportion as the parent molecule. For all tissues except fat, values for the 4,5 ring and $-CH_2-$ labels are similar and the residues could contain a fragment containing both portions of the imidazole nucleus. The high value for the $-CH_2-$ label in fat indicates that this is also unlikely. The fat residues are probably due to the incorporation of labeled acetyl into fatty acids by de novo synthesis, and it is likely that much of the residue in the other tissues is due to acetyl incorporation into other endogenous metabolites.

As about 60% of the CO label appeared as $^{14}CO_2$, it is likely that most of the residues observed with this label are due to incorporation of $^{14}CO_2$ into endogenous substances. The relatively higher initial residue levels in liver, kidney, and plasma relative to muscle at the early times but higher levels in muscle at later times for the CO label are similar to that observed when mice are dosed with $^{14}CO_2$ (Skipper et al., 1949 a,b; Buchanan, 1951). The fact that the $-CH_3$ label is generally higher than all of the other labels proves that the fragmentation of the imidazole nucleus occurred *prior* to incorporation into the persistent products. The in vitro studies show that the intact imidazole nucleus forms macromolecular complexes (West et al., 1982). If the in vivo residues were generated in this manner, the residues due to $-CH_3$ label could not be higher than either the 4,5 ring or $-CH_2-$ labels.

Nature of the Residues. The fact that the in vitro experiments clearly show that the intact imidazole nucleus can be reductively activated to a reactive intermediate that binds to microsomal proteins and the in vivo experiments show equally clearly that the residue cannot contain an intact imidazole nucleus exclusively is readily explained by the fact that microsomal system cannot synthesize macromolecules and probably has limited capability for the synthesis of endogenous metabolites such as methionine. It is possible that residues containing the intact imidazole nucleus are also formed in vivo but are elimi-

Table VI. Half-Life and Pool Size of Persistent Residues in Tissues of Rats Dosed with Ronidazole Labeled with ^{14}C at Different Sites[a]

	liver		muscle		kidney		fat		plasma	
label site	C_0, ppm	$t_{1/2}$, days	C_0, ppm	$t_{1/2}$, days	C_0, ppm	$t_{1/2}$, days	C_0, ppm	$t_{1/2}$, days	C_0, ppm	$t_{1/2}$, days
$-CH_3$	0.43	5.5	0.33	13.1	0.66	5.6	0.18	8.0	0.11	3.1
$-CH_2-$	0.20	4.2	0.19	7.5	0.46	4.0	0.16	9.3[b]	0.12	2.4
4,5 ring	0.15	5.9	0.12	12.9[c]	0.19	5.9	0.03	21[c]	0.08	3.4
CO	0.47	3.9	0.12	12.9[c]	0.26	5.1	0.13	9.4	0.57	2.9
CH_3NH_2	1.7	5.3	2.4	8.1[c]	3.4	4.6[b]	1.35	5.6	0.17	5.7
$-CH_3$[d]	0.70	3.6	0.36	8.6	0.76	4.1	0.23	4.6	0.09	3.4
$-CH_3$[e]	0.30	7.3	0.24	13.6	0.36	7.1	0.1	11.5	0.09	4.3

[a] Linear regression analysis of data presented in Table IV omitting the 2-day result in all cases, based on first-order kinetics using the relation $C = C_0e^{-kt}$; ppm expressed in terms of ronidazole. [b] Correlation coefficient less than 0.95. [c] Correlation coefficient less than 0.90. [d] With added CH_3NH_2; see Table I, footnote *e*, for details. [e] With added L-methionine; see Table I, footnote *f*, for details.

Table VII. Chemical Analysis of Muscle and Liver Protein from Rats Dosed with [*methyl*-^{14}C]Ronidazole and $^{14}CH_3NH_2$, Percent and Concentration of Total Radioactivity in Protein and Methylamine Produced on Hydrolysis

	time postdose, days			
	2	4	7	11
	[*methyl*-^{14}C]Ronidazole[a]			
liver, protein, %[b]	54	59	68	75
ppb[c]	178	159	107	83
$^{14}CH_3NH_2$, %[d]	16	15	13	11
$^{14}CH_3NH_2$, ppb	29	24	14	9
muscle, protein, %[b]	61	61	69	59
ppb[c]	189	159	159	106
$^{14}CH_3NH_2$, %[d]	69	40	28	27
$^{14}CH_3NH_2$, ppb	130	63	44	29
	$^{14}CH_3NH_2$[a]			
liver, protein, %[b]	55	66	65	67[e]
ppb[c]	660	726	377	188
CH_3NH_2, %[c]	0.8	0.4	ND[f]	ND
muscle, protein, %[b]	37	44	47	41
ppb[c]	593	792	441	180
CH_3NH_2, %[d]	ND	ND	0.03	0.13

[a] Tissue samples from animals in Table IV. [b] Protein separated as described under Materials and Methods. [c] Expressed in terms of ronidazole. [d] $^{14}CH_3NH_2$ analysis as described under Materials and Methods. [e] 14 days postdose. [f] ND = not detectable.

nated relatively rapidly so that the persistent residues are indeed due largely to naturally occurring metabolites. However, some portion of the residue could contain an intact imidazole nucleus.

Treatment of *N*-methyl-5-nitroimidazoles and the 5-acetylamino compound derived from ronidazole with 6 N hydrochloric acid at 120 °C degrades the imidazole nucleus and liberates *N*-methylamine (Wolf et al., 1983b). It seems likely that all metabolites containing an *N*-methylimidazole nucleus would respond similarly. When $^{14}CH_3$-labeled ronidazole was dosed to swine, a portion of the tissue residue was found to liberate $^{14}CH_3NH_2$ on strong acid hydrolysis (Wolf et al., 1983b). Consequently, the protein fraction of rat muscle and liver was examined for $^{14}CH_3NH_2$ liberation. Tissues from rats dosed with $^{14}CH_3NH_2$ served as a control. The data contained in Table VII show that a substantial fraction of the radioactivity is contained in the "protein" fraction for animals dosed with either ronidazole or methylamine. Little, if any, radioactive methylamine is liberated from tissues of rats dosed with methylamine, but the residues from tissues of the animals dosed with $^{14}CH_3$-labeled ronidazole contain substances that liberate methylamine. As the fraction of the protein that liberates methylamine declines with time, these residues, which may contain an intact imidazole nucleus, deplete at a faster rate than the residues that do not liberate methylamine. These drug-related residues appear to comprise about 30% of the total radioactivity in muscle at 7 or 11 days and about 12% of the liver residues. Thus, a fraction of the residues may be produced by the same reactions as those observed in vitro.

Residues due to degradation of ronidazole to methylamine could be produced by formation of both [^{14}C]formaldehyde and $^{14}CO_2$. Indeed, if the formation of $^{14}CO_2$ from [*methyl*-^{14}C]ronidazole proceeds via $^{14}CH_3NH_2$ only, the residues due to this pathway should be related to the $^{14}CO_2$ formed. At equimolar dose the [*methyl*-^{14}C]ronidazole-dosed rats produced 5.8% as much $^{14}CO_2$ as the $^{14}CH_2NH_2$-dosed rats. From this ratio the residues due to this metabolic pathway in liver and muscle tissue is estimated to account for 20–30% of the total residues. Thus, it would appear that the genesis of residues due to the $-CH_3$ label is not primarily via the methylamine pathway. This is confirmed by the relatively slight effect on the total residues observed in the rats loaded with cold methylamine or L-methionine prior to dosing with $^{14}CH_3$-labeled ronidazole.

These studies are also useful to determine which label site is the most desirable for the determination of tissue residues in the target animal. As the residue levels in rats dosed with 4,5 ring label are generally lower than those obtained with the other labels, this would most nearly approximate the residues of concern. Examination of the level of "drug related" residues as indicated by the methylamine generation test indicates that even for this label most of the residues present in liver and muscle are probably of the endogenous type. Hence, there is no way of labeling ronidazole so that a radioactive depletion study detecting only drug-related residues can be carried out.

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Tissue Residues Due to Ronidazole: Bioavailability of Residues in Swine Muscle on Ingestion by the Rat

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A substantial fraction of the radioactive residues present in muscle tissue from swine dosed with [*methyl*-^{14}C]ronidazole was absorbed and retained by the rat. This is characteristic of endogenous substances. On the other hand, drug-related residues characterized by methylamine liberation were poorly absorbed, apparently not metabolized and not covalently bound to rat tissue.

Ronidazole, (1-methyl-5-nitroimidazol-2-yl)methyl carbamate, is used in food animal production for controlling swine dysentery and for treatment of turkey blackhead. Although ronidazole disappears rapidly from edible tissues of the pig, radiocarbon from labeled ronidazole persists for prolonged periods (Wolf et al., 1983). Experiments in rats showed that most of the persistent tissue residues were derived from one- or two-carbon fragments of the drug (Wolf et al., 1983). However, a fraction of the residues may contain a drug-related moiety based on liberation of labeled methylamine when tissues of animals dosed with [*methyl*-^{14}C]ronidazole are subjected to strong acid hydrolysis.

When tissues containing radioactive endogenous substances are ingested, the radioactivity should be mostly absorbed and retained by the tissues of the dosed animal. The disposition of residues containing drug-related moieties is uncertain. This study was undertaken to determine the disposition of the radioactive residues in pig muscle on ingestion by the rat.

MATERIALS AND METHODS

Preparation of Swine Muscle Tissue. Muscle tissue from swine dosed with [*methyl*-^{14}C]ronidazole was obtained as described (Wolf et al., 1983). The animals were dosed once daily for 3 days. Seven days after the last dose, the pigs were slaughtered, and the muscle tissue was collected and homogenized by passing through a meat grinder several times. Convenient aliquots were packaged in polyethylene bags, frozen, and stored at −30 °C. The samples were thawed. Equal weights of tissue from three pigs were homogenized with 4 volumes of water, and the homogenate was freeze-dried.

Preparation of Rat Diets. The fluffy freeze-dried muscle was mixed with Purina Rat Chow at a ratio of 8 g of muscle tissue, which contained radioactive residues equivalent to 16 μg of ronidazole, and 10 g of Chow (by weight). A control diet was prepared with freeze-dried muscle from unmedicated pigs. After blending 16 μg of [*methyl*-^{14}C]ronidazole in 0.1 mL of ethanol was added to 18-g aliquots and each aliquot thoroughly blended. The specific activity of the ronidazole spike was 7.1 μCi/mg.

Animal Handling. Normal rats, male Charles River CD, weight approximately 250 g, were housed in individual metabolism cages equipped to collect separated excreta. However, fecal pellets may be washed with urine during collection. The cages were fitted with a specially designed feeding tunnel attached externally to the cage. The design is such that any spilled diet is readily collected without contamination with excreta.

Each animal was dosed with 18 g of the Chow–muscle mixture late in the day for 2 days. The total radioactivity ingested was determined by analysis of diet recovered daily from the feed compartment. On the second day each animal received 1 mL of an aqueous solution containing 2 μCi of ^{51}Cr EDTA complex to measure emptying of the GI tract. After the 2-day dosing period, each animal received 18 g of Purina Rat Chow daily for 2 days.

On the fifth day the animals were asphixiated with CO_2. After removal of the entire GI tract, the animals were skinned and the carcass homogenized by several passages through a meat grinder. Tissues samples were frozen and stored at −30 °C.

For collection of expired radioactivity, the metabolism cages were placed in plastic bags and the exiting air passed sequentially through absorption towers containing 1 N sulfuric acid and 2 N sodium hydroxide. The animals were dosed similarly except dosing with ^{51}Cr was omitted. In a control experiment urine, feces, and carcass were analyzed for methylamine liberation 48 h after dosing with 16 μg of [*methyl*-^{14}C]ronidazole.

Radiochemical Procedures. *Radioactive Ronidazole.* ^{14}C-Labeled methanol was converted to the methanesulfonate ester by treatment with methanesulfonic anhydride. Alkylation of 4-nitroimidazole yielded 1-[^{14}C]-methyl-5-nitroimidazole, which was converted to ronidazole by reaction with formaldehyde and transesterification with methyl carbamate. The product was found to be 99.4% pure by thin-layer chromatography using benzene–methanol (4:1) and benzene–dioxane–concentrated ammonium hydroxide (25:70:5). No single impurity accounted for

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