experiment (using two birds) it can be seen that for most of the eggs examined a plateau level of radioactive products was established within 10 days following the administration of decoquinate-^{14}C at the rate of 3 mg daily. This level, approximately 0.9 ppm (wet weight), remained constant until 2 days after the last administration of the drug, then declined rapidly to a level of 0.0 to 0.1 ppm on the fifteenth day after the last administration of the drug. These results correspond closely with those reported by Filer *et al.* (1969) and Button *et al.* (1969) where a plateau of radioactivity in tissues of broiler chickens was obtained after administration of decoquinate through the feed. In both cases a rapid disappearance of residue occurred when medication ceased.

The tlc examination of the egg residue showed that decoquinate-^{14}C was metabolized to at least one other compound in the egg yolk. This compound possessed chromatographic characteristics similar to those reported to be present in chicken tissues (Craine *et al.*, 1971). Whether the nondecoquinate component was metabolized *in situ* or deposited at the time of yolk formation was not established.

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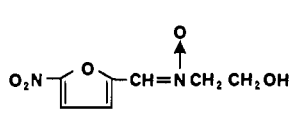
Urinary Excretion of Nifuratrone Metabolites by Swine (Gilts)

Elliott M. Craine,* Janice Carman, and Dale L. Hobson

Gilts were given single oral doses of nifuratrone at a level of 6 or 12 mg/kg body weight. Urine was collected and assayed by two procedures which measure the compound: by reaction with phenylhydrazine and by extraction with nitromethane. Compounds measured by each procedure appeared in the urine rapidly and were not detected after 8 hr. A total of 6.4% of the dose was measured in the urine by the phenylhydrazone method and 1.0% was measured by the nitromethane assay. Ion exchange chromatography detected seven urinary components induced by nifuratrone medication. Five of the seven had absorption spectra similar to the parent compound and three of these responded positively to the phenylhydrazone method, indicating they contained a nitrofurfural moiety. One of the components was nifuratrone and was responsible for the nitromethane assay of the original urine. Thus, only 1.0% of the dose administered was excreted intact in the urine. The components measured by the phenylhydrazone assay procedure had a urinary excretory half-life of less than an hour. All metabolites detected by the chromatography, including those measured by the phenylhydrazone method, were eliminated at similar rates.

Nifuratrone (1) [*N*-(2-hydroxyethyl)-α-(5-nitro-2-furyl)nitrone] has antibacterial activity in swine (Kim and Bambury, 1969; Dainippon Pharmaceutical Co., 1968). In the present work the compound was given to female pigs in single oral doses. Urine was collected and examined by four procedures to determine whether nifuratrone or its metabolites could be detected.



(1)

In general, the group of compounds known as nitrofurans is rapidly metabolized by avians or mammalians (Paul and Paul, 1964). The degradation pathways vary from one compound to another but some generalities do seem to exist. Metabolites closely related to the parent compound are excreted in the urine in many cases. Also, with some compounds, metabolism is so extensive that carbon atoms of the molecule appear in the normal body constituents (Buzard, 1962; Tennent and Ray, 1971; Herrett *et al.*, 1967). Thus, in metabolic experiments with ^{14}C as a tracer, many labeled metabolites appear, including normal body components, and confuse the studies.

Most body tissues have enzymes capable of degrading the nitrofurans (Paul *et al.*, 1960). It is thus not surprising that no evidence has been obtained to show deposition or accumulation of nitrofuran residues in tissues. From a public health standpoint it is important to know whether metabolites closely related to the parent drug pose residue problems, *i.e.*, those which retain the furan ring. Where the molecule is extensively degraded to normal body components or small molecules, the significance from a residue standpoint becomes less important.

In earlier work Paul *et al.* (1960) detected drug-related metabolites of nitrofurans in urine by examining the ultraviolet absorption spectra. Tennent and Ray (1971) separated these drug related metabolites by ion exchange chromatog-

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raphy. In the present work similar approaches were taken to determine the extent of metabolism of nifuratrone in pigs medicated with the compound.

EXPERIMENTAL

Chemicals and Materials. Nifuratrone was prepared by R. E. Bambury; phenylhydrazine hydrochloride, and reagent grade hydrochloric acid, isopropyl alcohol, sodium chloride, potassium phosphate, monobasic and potassium phosphate, dibasic, J. T. Baker Co.; spectrophotometric grade toluene, Matheson Coleman and Bell; reagent grade aluminum oxide, Merck & Co.; reagent nitromethane, Aldrich Chemical Co.; enzyme grade ammonium sulfate, Mann Research; 95% ethanol, U.S. Industrials Chemical Co.; practical grade 2,6-lutidine (redistilled), Eastman Kodak Co.; 5-nitro-2-furoic acid, Norwich Pharmacal Co.

Phenylhydrazone Assay. Nifuratrone and related nitrofurans react in acid with phenylhydrazine to form 5-nitro-2-furfuraldehydephenylhydrazone which can be transferred to a solvent and measured spectrophotometrically (Buzard *et al.*, 1956). An aliquot of solution to be assayed was diluted to 10 ml in a centrifuge tube. One milliliter of a 1.5% solution of phenylhydrazine (prepared daily) was added with vigorous mixing, followed by addition of 1 ml of concentrated hydrochloric acid with vigorous mixing. After 40 min at room temperature, the reaction mixture was extracted with 5.0 ml of toluene. Emulsions which developed with samples such as urine were broken by slow speed centrifugation. For purification, a 4.0-ml portion of the toluene phase was mixed with 1 g of aluminum oxide in a centrifuge tube. The 5-nitro-2-furfuraldehydephenylhydrazone adsorbed to the alumina, showing a red color which was a qualitative test for that specific hydrazone. To elute the hydrazone, isopropyl alcohol (0.4 ml) was added to the mixture, which was then centrifuged. The absorbance of the solution was determined at 437 nm in a Beckman DU spectrophotometer modified with a Gilford photometer. A linear relation between absorbance and concentration was obtained in a range of 0.02 to 0.75 μmol of nifuratrone. Values are expressed as μmol equivalents of nifuratrone.

Nitromethane Assay. The method was similar to that of Leeson *et al.* (1970) for nifuratrone. A 1.0-ml portion of aqueous solution to be assayed was mixed with 1.0 ml of saturated ammonium sulfate solution and 10.0 ml of nitromethane. The mixture was centrifuged at low speed to separate the phases. The absorbance of the nitromethane or upper phase was measured at 380 nm. A linear relation was obtained between absorbance and concentration of nifuratrone in the range of 0.05 to 2.0 μmol.

Absorption Spectra. All spectra were run in a Beckman DK-1a Spectrophotometer. Urine samples were diluted with pH 7 phosphate buffer. Fractions from the chromatography were diluted with the buffer used in elution.

Chromatography. Urine samples were chromatographed on columns (11 × 300 mm) of Dowex 1-X2 in the chloride form. Elution was accomplished with a three-chamber gradient system similar to that of Peterson and Sober (1959) to raise sodium chloride concentration to 2.0 *M*. The columns were operated at room temperature in a darkened area with a flow of about 0.6–1.0 ml/min. The effluent was collected in 5-ml fractions with a siphon device. The absorbance of each fraction was measured at 320 and 375 nm in the Beckman DU spectrophotometer modified with a Gilford photometer. Selected fractions or combinations were assayed by the phenylhydrazone and the nitromethane procedures and absorption spectra were determined.

Animal Treatment. The swine were Yorkshire gilts from the Hess & Clark herd. Routinely, sows of the herd consumed a ration containing 100 g of chlortetracycline per ton during gestation and 150 g of furazolidone per ton during lactation. Baby pigs had access to a creep feed containing ASP-250 until they were weaned. Shortly after weaning, pigs weighing about 8 kg were placed in metabolism units, and were maintained on a nonmedicated grower ration.

The metabolism cages were constructed of lucite and stainless steel. The floor of expanded galvanized iron with $^5/_8$-in. openings trapped feces, while urine flowed down a V-shaped galvanized trough to bottles. Nifuratrone is unstable in light, and under acidic or basic conditions it hydrolyzes readily. To prevent degradation of the compound and its metabolites urine was collected in dark brown bottles containing 40 ml of pH 7.0 phosphate buffer and 1 ml of toluene. Aliquots of urine were analyzed by each procedure as soon as possible after the collection period was complete. Where necessary, urine was stored for 3 to 12 hr at 5°C. It was not always possible to carry out chromatographic analysis immediately with equipment available. Several portions of urine were frozen individually and stored at −25°C. Repeated freeze–thaw steps were avoided.

Nifuratrone was administered in water at a concentration of about 5 mg/ml. A piece of Tygon tubing was worked down the pig's esophagus to the stomach and the dose was forced down the tube with a syringe.

Experiment Schedule. Basically all experiments involved an oral medication of female pigs. Urine was collected after the medication and examined by four procedures. Each pig was used as a source of control urine and values obtained with control urine were used as blanks in the assays.

EXPERIMENT 1. Three weeks after weaning, pig 1 was given nifuratrone in her drinking water at 300 mg/l. for a 3-day period. Medication was withheld for 48 hr and control urine was collected. One day later the pig was medicated with 200 mg of nifuratrone or approximately 12 mg/kg.

EXPERIMENT 2. Pigs 2 and 3 had mild diarrhea shortly after weaning and were given 300 mg of nifuratrone/l. in their drinking water for 2 days. Fifteen days later, at the beginning of Experiment 2, pig 2 weighed 11.4 kg and pig 3 weighed 13.6 kg. Control urine was collected and the pigs were given doses of nifuratrone at a level of 6 mg/kg.

EXPERIMENT 3. Pigs 2 and 3 were held in the metabolism cages for 7 days after Experiment 2. Nifuratrone was then placed in their drinking water for a period of 10 days at a level of 300 mg/l. The medicated water was removed and control urine was collected after 8 hr. Pigs 2 and 3 weighed 20.2 and 22.1 kg, respectively. Each pig was given a single dose of nifuratrone at a level of 6 mg/kg.

RESULTS

In three separate experiments, three pigs (gilts) were given single oral doses of nifuratrone dissolved in water. The urine was collected from each pig in succeeding time periods for 10–12 hr. The urine was examined by four procedures: (1) phenylhydrazone assay; (2) nitromethane assay; (3) direct spectrophotometric spectra; and (4) column chromatography on anion exchange resin.

Phenylhydrazone Assay. Each pig excreted into the urine measurable amounts of components which contained the nitrofurfural moiety, *i.e.*, there was a positive response to the phenylhydrazone assay. The amounts excreted by individual

Table I. The Measure of Compounds[a] in the Urine of Pigs after Single Oral Doses of Nifuratrone

Experiment	Pig	Length of collection periods, time	μmol equivalents[a] excreted in collection period A	B	C	D	E
1	1	3 hr	*b*	48.5	11.0	0.0	0.0
2	2	2 hr	*b*	11.8	5.5	0.6	0.2
	3		*b*	16.1	14.3	0.8	0.0
3	2[c]	2 hr	8.0	*b*	15.5	0.5	0.0
	3		24.0	*b*	20.0	0.5	0.0

[a] Measured by the phenylhydrazone procedure using nifuratrone as a standard. [b] No urine voided in the period. [c] Pig 2 regurgitated a portion of the dose.

Table II. The Excretion of Compounds in the Urine of Pigs after Single Oral Doses of Nifuratrone Expressed as a Percent of the Dose Administered

Experiment	Pig	Nifuratrone administered, mg/kg	Amount of dose excreted: Phenylhydrazone Assay, %	Nitromethane assay, %
1	1	12	6.0	1.3
2	2	6	5.3	1.0
	3	6	7.7	0.8
3[a]	2[b]	6	4.1	0.5
	3	6	6.7	1.1
Average			6.4	1.0

[a] In Experiment 3 the pigs were medicated with nifuratrone in the drinking water at a level of 300 mg/l. for 10 days before the experiment. [b] Pig 2 regurgitated a portion of the dose; therefore, the values are not included in the average.

Table III. Chromatographic Components Appearing in the Urine of Swine Medicated with Single Doses of Nifuratrone

Component	Band peak,[a] tube	Phenylhydrazone reaction	Control urine	Maximum in absorption spectrum, nm	Color
1	5	No	No	365	None
2	7	No	Yes	328	None
3	9	No	No	365	None
4	13	Yes	No	365	Yellow
5	29–30	No	Yes	None	None
6	35	Yes	No	365	None
7	43	No	Yes	325	None
8	47	Yes	No	365	Yellow
9	57	No	Yes	*b*	None
10	63	No	Yes	*c*	None
11	67	No	Yes	320	None
12	79	No	Yes	None	Yellow
13	95	No	Yes	325	None

[a] The position of elution for each component is designated by band peak which is the tube with the highest concentration. [b] Shoulder at 320 nm. [c] Absorption 300–350 nm, but not discrete.

pigs in each collection period expressed as μmol equivalents of nifuratrone are presented in Table I. In Experiment 1 the excretion was complete within 9 hr. In the other two experiments it was complete within 8 hr. In all cases there were metabolites present in the first urine sample obtained. Except for pig 2 in Experiment 3, the first collection period contained a higher concentration of metabolites than succeeding periods. This difference with pig 2 may be due to the regurgitation of a portion of the dose.

In Table II the compounds measured by the phenylhydrazone procedure excreted in the urine are expressed for each pig as a percent of the dose administered. The amounts were consistent, ranging from 5.3 to 7.7%; the value for pig 2, Experiment 3 was not considered quantitative because of the loss due to regurgitation after dosing. The average from four individual medications was 6.4%. There apparently was no drastic effect obtained by increasing the amount of nifuratrone in the dose from 6 to 12 mg/kg. Also, exposure of the pigs to nifuratrone for 10 days did not change the quantitative excretion of compounds measured by the phenylhydrazone assay.

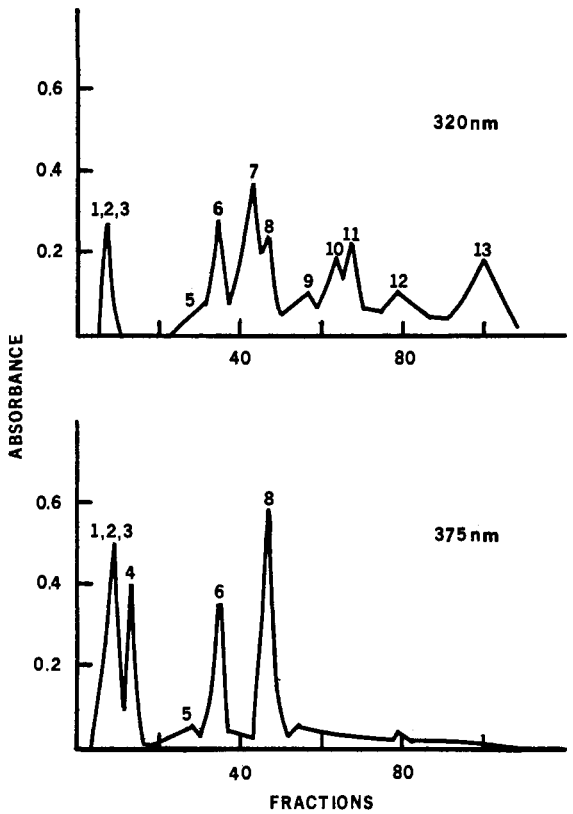

Figure 1. Ion exchange chromatography of urine from a pig medicated with nifuratrone

Nitromethane Assay. Table II also contains the excretion of metabolites measured by the nitromethane procedure expressed as a percent of the dose administered. Again, the amounts were consistent, ranging from 0.8 to 1.3%, with an average of 1.0%. In each case, the phenylhydrazone response was 5 to 10 times higher than the nitromethane response. As with the phenylhydrazone assay, the amount of metabolites excreted was not affected by the 10-day medication nor by varying the dose level.

Absorption Spectra. The urine samples of Experiment 1 were diluted at 1.0 to 50 ml with phosphate buffer and scanned in the recording spectrophotometer. With control urine there was absorption in the region from 280 to 360 nm but it was nonspecific without significant maxima. The absorption spectrum of the parent compound, nifuratrone, has a maximum at 365 nm. This is typical of nitrofurans which have strong absorption bands in the general region of 350–400 nm. When nifuratrone was added to control urine, its presence was readily detected because of low absorbance of urine at 365 nm. The urine obtained after medication had an increased absorption in the region of 310–380 nm, although no significant maxima appeared.

Column Chromatography. Portions of the various urine samples collected were chromatographed on anion exchange resin columns. The absorption spectra of the urine obtained indicated that spectrophotometric monitoring of the column effluent was a reasonable approach to detect metabolites. With the system used, 13 urinary components were found, as

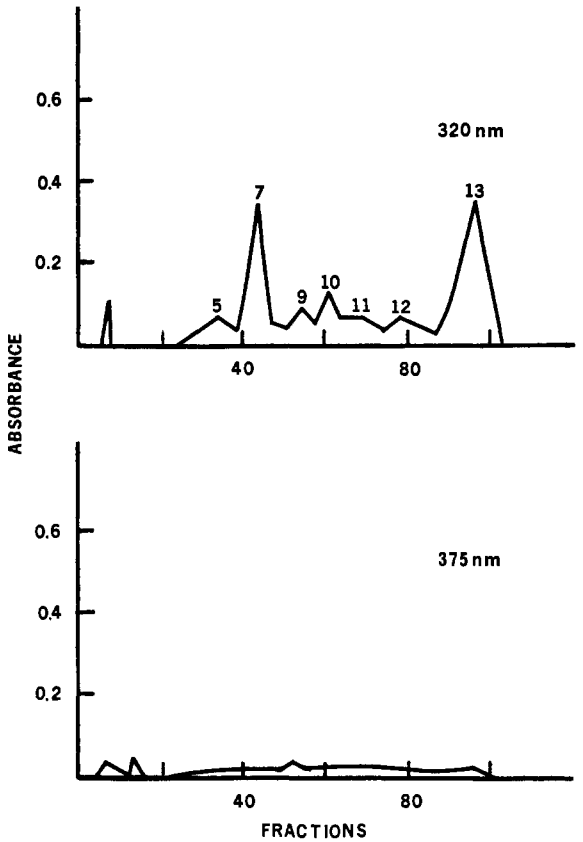

Figure 2. Ion exchange chromatography of control pig urine

listed in Table III. The absorbance of the effluent of the chromatography of 5 ml of urine from period B of Experiment 1 at 320 and 375 nm is shown in Figure 1. The pattern was typical of all urine samples where metabolites were detected.

Control urine contained peaks corresponding to seven of the thirteen components (Figure 2). In a 20-ml sample of control urine there were peaks corresponding to components 2, 5, 7, 9, 10, 11, 12, and 13. After medication component 12 contained yellow color, which was not detected in the control urine. If metabolites were eluted in positions of peaks 9, 11, or 12, they were relatively minor. At present it is concluded that seven metabolites containing a furan ring were detected by the chromatography at the position of components 1, 3, 4, 6, 8, 10, and 12. Component 10 was present at low concentrations in control urine but increased after medications. The increase appeared to be due to the presence of 5-nitro-2-furoic acid. Component 10 eluted in the same position as an authentic sample of 5-nitro-2-furoic acid. The absorption spectra of component 10 had an absorption band in the region of 320 to 350 nm, while authentic 5-nitro-2-furoic acid has a maximum at 315 nm.

Aliquots from fractions in the effluent from one chromatographic run were assayed by the phenylhydrazone and nitromethane procedures (Table IV). Three components, 4, 6, and 8, gave a positive response, indicating they contained the nitrofurfural moiety. Component 4 responded to both assay methods and was the only component responding to the nitromethane assay. Thus the nitromethane assay of the original urine gave a direct measure of component 4.

With some columns the three components were pooled and assayed by the phenylhydrazone method. Recoveries of material in the effluent measured by the phenylhydrazone assay were 76, 75, and 96% for three individual chromatographic runs.

Table IV. Comparison of the Assay of Individual Chromatographic Components by the Phenylhydrazone Procedure and the Nitromethane Procedure

	Equivalents of nifuratrone	
Component	Phenylhydrazone procedure, μmol	Nitromethane procedure, μmol
4	0.33	0.32
6	0.53	0.00
8	0.65	0.05

The absorption spectra of fractions of column effluent were determined. The maxima in the spectra of the individual components are summed in Table III. Five of the seven metabolites had an absorption band with a maximum at 365 nm, which was a characteristic of the parent compound. The remaining chromatographic components had spectra with maxima at lower wavelengths. The chromatographic procedure separated the metabolites so they can be measured. The monitor of column effluent by measure of absorbance at 320 nm or 375 nm did not show resolution of the components 1, 2, and 3. The absorption spectra of individual fractions across the broad peak were needed to show that three individuals were definitely present.

The evidence was firm that component 4 was nifuratrone. When nifuratrone was chromatographed in water or was added to control urine, it appeared in the same elution position as that of component 4. No other components were detected when nifuratrone was added, except those normally found in control urine. In the chromatography of nifuratrone, recoveries of 86 to 95% were obtained.

The metabolites which were measured by the phenylhydrazone assay of urine were eliminated rapidly. The ion exchange chromatography detected the same rapid elimination of all metabolites. Successive urine samples from pig 3 from Experiment 3 were all chromatographed. Period D had only traces of a few of the components due to medication.

Chemical Stability of Metabolites. Nifuratrone and the metabolites of nifuratrone detected by the phenylhydrazone procedure were stable in solution in urine. Analyses of urine held 12 hr at either 5 or 25°C were the same as analyses of freshly collected urine. However, the same metabolites were unstable in procedures involving freezing of the urine. Metabolite 3 was degraded by a simple freezing–thawing. When a portion of urine was frozen, stored for a week at −25°C, thawed, and chromatographed in the same manner, one major change occurred in the chromatographic pattern: there was almost complete disappearance of component 3.

The routine procedure of freezing–drying of urine induced degradation of metabolites and nifuratrone. The changes observed occurred while the dried urine was held at room temperature exposed to air. For example, a portion of urine (20 ml) containing 0.22 μmol equivalents of nifuratrone per ml by the phenylhydrazone assay was freeze-dried. The flask was removed from the freezer-drier and after 5 hr at room temperature the dry material was redissolved in 20 ml of water. The reconstituted urine then contained only 0.15 μmol equivalents/ml. The solution again was freeze-dried, this time in the dark. The dry material was held at room temperature in the dark for 5 days, and then was redissolved in water and assayed. Less than 0.01 μmol equivalents/ml remained, showing extensive degradation of the dry material in the dark. Degradation of the freeze-dried material was reduced by immediately redissolving the preparation or by storing the material under nitrogen.

Table V. The Amounts of Metabolites[a] Remaining in the Body Related to Time after Dosing

Time, hr	Experiment 2		Experiment 3	
	Pig 2, μmol	Pig 3, μmol	Pig 2, μmol	Pig 3, μmol
Zero	18.0	31.3	24.0	44.5
2	18.0	31.3	16.0	20.5
4	6.3	15.2	16.0	20.5
6	0.8	0.8	0.5	0.5
8	0.2	0.2	0.0	0.0

[a] Metabolites were measured by the phenylhydrazone method using nifuratrone as a standard.

Urine containing metabolites was freeze-dried and stored at room temperature in the dark for 12 days. When the dry material was redissolved in water and chromatographed, the pattern of the chromatogram showed almost complete loss of components detected at 375 nm.

Urinary Excretory Half-Life. The amounts of metabolites measured by the phenylhydrazone procedure remaining in the pig body at specific times were calculated for Experiments 2 and 3 (Table I) and summed in Table V. To make the calculations it was assumed that the total amount of metabolites excreted was equal to the amount of metabolites present in the body at zero time or the time of dosing. The values for each pig were plotted on semilog paper. The number of values for each pig allow only an estimate of excretory half-life for the metabolite group. In each case it was less than an hour. The chromatography showed that when the components measured by phenylhydrazone assay were not detected in the urine, the other metabolites with an intact furan ring were absent.

DISCUSSION

Nifuratrone excreted in the urine would be measured by the phenylhydrazone and nitromethane assays and would be detected in the chromatography. The evidence is strong then that component 4 was nifuratrone: it was measured by the two assays; its elution position was proper; and it had the same absorption band, with a maximum at 365 nm. Among the chromatographic components, only component 4 responded to the nitromethane assay and it was concluded that the method measures only the parent compound and none of the metabolites. Thus, the nitromethane method is a specific assay of nifuratrone in urine.

The nitromethane assays show that less than 2% of the nifuratrone of the dose was eliminated intact in the urine. It is evident that unless the compound was trapped by body tissues in some manner it was extensively metabolized. In addition the present work shows that the amounts of drug-related metabolites eliminated in urine are a minor portion of the dosage. In the case of those metabolites measured by the phenylhydrazone procedure, we have an average of 5.0% of the dose. The metabolites detected by chromatography but not measured by the phenylhydrazone assay were not present in greater concentration unless there was some drastic change in absorptivity of which we are unaware.

In general, nitrofurans given orally are metabolized very rapidly by animals and birds (Paul and Paul, 1964). The metabolism takes two general pathways: (a) rupture of the furan ring leading to simple compounds and eventually to normal body components; and (b) conversion to metabolites which retain the furan ring structure. The metabolites with a furan ring are eliminated rapidly in the urine and have not been detected in tissues with other nitrofurans. It is apparent from the data presented here that nifuratrone is absorbed and metabolized by pigs in the same patterns as nitrofurans in general. The compound and metabolites with a furan ring appeared in the urine shortly after administration of the compound to pigs. The elimination of the metabolites was rapid and they were not detected after 8 hr. Although the excretory half-life data were not precise, they suggested that the half-life of all metabolites was less than 1 hour. The total amounts of components with a furan ring did not account for major portions of the dose, so it is probably that conversion to normal body components was extensive. In the chromatography used here, lutidine was present in all effluent. Since lutidine absorbs strongly at wavelengths below 300 nm, it is difficult to detect chromatographic components which do not have absorption bands in the region above 300 nm. To our knowledge compounds with the nitrofuran ring and compounds with an amino group in the place of the nitro group do have significant absorbance bands above 300 nm. It has thus been assumed that metabolites of nifuratrone where the furan ring has opened would not be detected by this chromatography.

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