The Metabolism of Arylazoisoxazolones by Rats and Dogs

By J. W. DANIEL

*Imperial Chemical Industries Ltd., Industrial Hygiene Research Laboratories,
 Alderley Park, Cheshire*

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1. When rats were given a single oral dose of the lipid-soluble fungicide 4-(2-chlorophenylhydrazono)-3-methyl-4-¹⁴Cisoxazol-5-one ([¹⁴C]drazoxolon), about 75% of the label was excreted in the urine and 13% in the faeces in 96 hr. An additional 7% of the radioactivity was recovered as ¹⁴CO₂ in 48 hr. 2. About 8% of the label was excreted by rats in the bile in 0–24 hr. and an additional 6% was excreted by the same route in 24–48 hr. 3. When dogs were given a single oral dose of [¹⁴C]-drazoxolon about 35% of the label was excreted in the urine and a similar amount was excreted in the faeces in 96 hr. 4. The major metabolites in the urine of the rat and the dog were identified as 2-(2-chloro-4-hydroxyphenylhydrazono)acetoacetic acid (dog, 14%), the corresponding ether glucosiduronic acid (dog, 12%; rat, 13%) and ester sulphate (rat, 65%). 5. When rats were given a single oral dose of 3-methyl-4-([U-¹⁴C]phenylhydrazono)isoxazol-5-one about 75% of the label was excreted in the urine and 15% in the faeces in 96 hr. The major metabolite in the urine was identified as the ester sulphate conjugate of 2-(4-hydroxyphenylhydrazono)-acetoacetic acid. 6. Reduction of the azo link was of minor quantitative significance. 7. These results are discussed in their relation to species differences in the toxicity of these compounds.

4-(2-Chlorophenylhydrazono)-3-methylisoxazol-5-one (I; R = Cl; also known as drazoxolon) is one of a series of lipid-soluble arylazoisoxazolones that are valuable for the control of powdery mildew and a number of seed and soil-borne diseases (Geoghegan, Summers & Turner, 1965; Geoghegan, 1967). The acute oral toxicity of drazoxolon in rats (LD₅₀ 125 mg./kg.) is appreciably less than in dogs (LD₅₀ 17 mg./kg.) although in both species it evokes convulsant activity. In contrast, 3-methyl-4-(phenylhydrazono)isoxazol-5-one (I; R = H) is of low toxicity to rats (LD₅₀ > 2 g./kg.) and is, moreover, devoid of convulsant activity.

This paper describes the metabolism of drazoxolon by rats and dogs and that of 3-methyl-4-(phenylhydrazono)isoxazol-5-one by rats; the toxicological implications of the results are discussed.

MATERIALS

All melting points, which are uncorrected, were determined on a Koffler block.

3-Methyl-4-(phenylhydrazono)isoxazol-5-one. This compound, m.p. 190°, was obtained from Plant Protection Ltd., Bracknell, Berks.

4-(2-Chlorophenylhydrazono)-3-methylisoxazol-5-one. This compound was obtained as a technical-grade sample from Plant Protection Ltd. It was purified by repeatedly washing with methanol at 50° until the residue showed the presence of only a single coloured component when chromatographed on thin-layer plates of silica gel in benzene-ethanol (9:1, v/v). Two recrystallizations from ethanol gave drazoxolon (m.p. 166°) as pale-yellow needles.

4-(2-Chloro-4-hydroxyphenylhydrazono)-3-methylisoxazol-5-one and 4-(4-hydroxyphenylhydrazono)-3-methylisoxazol-5-one. These two compounds, m.p. 212° (from ethanol) (Found: C, 47.5; H, 3.6; N, 16.6; C₁₀H₈ClN₃O₃ requires C, 47.5; H, 3.2; N, 16.4%) and m.p. 232° (from ethanol) (Found: C, 54.8; H, 4.11; N, 19.2; C₁₀H₉N₃O₃ requires C, 54.8; H, 4.10; N, 19.2%) respectively, were prepared by the general procedure of Summers, Freeman & Shields (1965) whereby an aqueous solution of NaNO₂ was added to a cold mixture of ethyl acetoacetate, hydroxylamine and the appropriate aminophenol in HCl. After standing for 1–2 hr. the precipitate was collected by filtration and recrystallized.

2-(2-Chloro-4-hydroxyphenylhydrazono)acetoacetic acid. 4-(2-Chloro-4-hydroxyphenylhydrazono)-3-methylisoxazol-5-one (1 g.) was added to water (150 ml.) and the suspension

boiled vigorously for 18 hr. The solution was then concentrated under reduced pressure at 40° and the residue recrystallized from aq. methanol to give yellow needles, m.p. 221° (Found: C, 46.7; H, 3.6; N, 10.6; $C_{10}H_9ClN_2O_4$ requires C, 46.7; H, 3.5; N, 10.9%). Mass spectrometry showed the parent ion to exist at m/e 256, and by accurate measurement it was shown to have the composition $C_{10}H_9ClN_2O_4$. The ion at m/e 212 is derived from the parent ion as a result of decarboxylation, and the base peak at m/e 141.5 is derived from that at m/e 212 by fission of the azo link. The nuclear-magnetic-resonance (n.m.r.) spectrum in $[D_6]$ dimethyl sulphoxide obtained at 100 Mc/sec. was consistent with the structure proposed for this material. Hydrolysis of drazoxolon gave 2-(2-chlorophenylhydrazono)-acetoacetic acid, m.p. 132° (from aq. methanol) (Found: C, 49.9; H, 3.8; N, 11.6; $C_{10}H_9ClN_2O_3$ requires C, 49.8; H, 3.7; N, 11.7%). 4-Amino-3-chlorophenol, m.p. 160°, was prepared by the catalytic reduction of the corresponding nitro compound obtained as described by Hodgson & Moore (1925).

4-(2-Chlorophenylhydrazono)-3-methyl[4- ^{14}C]isoxazol-5-one ([^{14}C]drazoxolon). Ethyl [3- ^{14}C]acetoacetate (390 mg.; 1.8 mc) and ethanol (0.5 ml.) were added to Na_2CO_3 (175 mg.) in water (2.5 ml.) and the mixture was stirred at 0–5° while a solution of hydroxylamine hydrochloride (230 mg.) in water (0.5 ml.) was added over a period of 5 min. Stirring was continued for 10 min. and for an additional 20 min. after the temperature of the reaction mixture had been raised to 20°. The mixture was extracted with ether (4 × 2 ml.) and a solution of morpholine (265 mg.) in ethanol (0.5 ml.) added to the combined extracts. The ether was removed by evaporation, the residue heated at 65° for 10 min. and the morpholine-isoxazolone complex precipitated with ether. The complex was dissolved in ethanol (1.5 ml.), chilled and added to a freshly prepared solution of diazotized *o*-chloroaniline (282 mg.). The yellow precipitate that formed was recovered by filtration and purified on thin-layer plates of silica gel developed with methylene chloride. The product, m.p. 167°, was obtained in 70% yield and had a specific radioactivity of 0.7 mc/m-mole.

3-Methyl-4-([U- ^{14}C]phenylhydrazono)isoxazol-5-one. This compound was similarly prepared from [U- ^{14}C]aniline hydrogen sulphate (0.1 mc). The product was obtained in 84% yield.

R_F values and spectral characteristics of these compounds are given in Table 1.

Radiochemicals. Ethyl [3- ^{14}C]acetoacetate (10 mc/m-mole) and [U- ^{14}C]aniline hydrogen sulphate (40 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks.

Enzymes. β -Glucuronidase (3000 units/mg.) and arylsulphatase (10000–15000 units/mg.) were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks.

METHODS

Animals and dosing. A solution of either [^{14}C]drazoxolon or 3-methyl-4-([U- ^{14}C]phenylhydrazono)isoxazol-5-one in polyethylene glycol 300 (1 mg./ml.) was administered by stomach tube to adult male albino Wistar rats of the Alderley Park strain (specific-pathogen-free). During the experiments the animals were kept in individual glass metabolism cages permitting the separate collection of

urine and faeces. Unrestricted food and water were supplied, and the excreta were collected daily and stored at –20°. When the expired air was analysed for $^{14}CO_2$, air was drawn through the cages at 1 l./min. and passed through two traps in series containing 2 M-NaOH.

The bile duct was cannulated in rats that had been previously given a solution of [^{14}C]drazoxolon by stomach tube. After recovery from the anaesthetic they were placed in individual restraining cages and provided with water containing glucose (1%) and NaCl (0.9%). Bile was collected for either 24 or 48 hr.

In experiments involving the isolation and characterization of metabolites in the urine, rats were given by stomach tube an aqueous suspension (5%, w/v) of unlabelled drazoxolon or 3-methyl-4-(phenylhydrazono)isoxazol-5-one. Groups of five animals were housed in metabolism cages and urine was collected for 24 hr.

In experiments with dogs, adult beagle hounds (13–15 kg. body wt.) were given a gelatin capsule containing a solution of [^{14}C]drazoxolon in polyethylene glycol 300. They were then transferred to individual metabolism cages and unrestricted food and water were allowed. Urine and faeces were collected daily and stored at –20°. The experiments were terminated 96 hr. after dosage.

Details of the amount and radioactivity of the doses are given in the appropriate Tables.

Measurement of radioactivity. Liquid-scintillation counting of the biological samples and of subsequent fractions was made according to the methods described by Daniel & Gage (1965), with a Packard Tri-Carb model 3002 liquid scintillation spectrometer (Packard Instrument Co. Ltd. Wembley, Middx.).

Measurement of spectra. Ultraviolet spectra of solutions of purified metabolites and of reference compounds in Spectrosil-grade methanol were measured with a Unicam SP.800 spectrophotometer. The i.r. spectra of materials dispersed in liquid paraffin were determined with a Perkin-Elmer 21 spectrometer. Mass spectra were obtained with an MS9 double-focussing spectrometer (Associated Electrical Industries Ltd., Manchester) and the n.m.r. spectra were determined with a Varian HA-100 spectrometer.

Chromatography. Fractions were chromatographed on thin-layer plates of silica gel G and developed with the solvent systems shown in Table 1. The plates were then examined by radioautography with Ilford Industrial G X-ray film. Non-radioactive reference compounds and metabolites were either detected visually or by spraying successively with 2 M-HCl, 1% (w/v) $NaNO_2$ and aq. 1% (w/v) *N*-1-naphthylethylenediamine. Glucuronic acid conjugates were detected by spraying with a solution of 2% (w/v) naphtharesorcinol in 33% (w/v) trichloroacetic acid and heating the plate at 135° for 5 min. Areas of the support corresponding with radioactivity were scraped off the plate and transferred to a vial containing liquid scintillator. For the purification of metabolites before identification, a solution was applied as a band to a thin-layer plate and, after development, the radioactive area was located and removed. The radioactivity was extracted from the support with methanol and the chromatography repeated on a second plate, with a different solvent system if necessary.

Estimation of 4-amino-3-chlorophenol in urine. Sufficient conc. HCl was added to urine to give a solution molar with respect to the acid. The solution was heated at 100° for 30 min., cooled, adjusted to pH 7 with M-NaOH and

Table 1. *R_F* values and absorption spectra of reference compounds

Solvent mixtures (by vol.; upper phases): *A*, benzene-ethanol (9:1); *B*, di-isopropyl ether saturated with water; *C*, butan-1-ol saturated with water; *D*, butan-1-ol-acetic acid-water (4:1:5); *E*, butan-2-ol saturated with water.

Solvent system ...	<i>R_F</i>					Light-absorption in ethanol	
	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>E</i>	$\lambda_{\text{max.}}$ (m μ)	$10^{-3} \epsilon_{\text{max.}}$
Drazoxolon	0.83	0.83	0.89	0.83	0.72	254, 260, 392	8.9, 6.8, 20.6*
4-(2-Chloro-4-hydroxyphenyl-hydrazono)-3-methylisoxazol-5-one	0.49	0.50	0.90	0.83	0.75	258, 425	7.6, 19.8
2-(2-Chloro-4-hydroxyphenyl-hydrazono)acetoacetic acid	0.05	0.0	0.64	0.78	0.56	253, 305, † 317, 390	9.1, 3.5, 3.8, 21.9
2-(2-Chlorophenylhydrazono)-acetoacetic acid	—	0.0	0.61	0.71	0.46	244, 361	10.5, 22.6
4-Amino-3-chlorophenol	0.54	0.51	0.80	0.83	0.75	235, 308	7.5, 3.0
3-Methyl-4-(phenylhydrazono)-isoxazol-5-one	0.79	0.63	0.86	0.83	0.76	248, 255, 394	9.9, 8.4, 23.8
2-(4-Hydroxyphenylhydrazono)acetoacetic acid	0.06	—	0.65	0.80	0.35	257, 302, 312, 396	7.5, 0.4, 0.4, 17.8
4-(4-Hydroxyphenylhydrazono)-3-methylisoxazol-5-one	0.48	0.32	0.83	0.84	0.78	252, 422	10.1, 27.4

* See also Summers *et al.* (1965).

† Inflexion.

extracted twice with ethyl acetate (10–15ml.). Amino compounds in the combined extracts were extracted into *m*-HCl and 4-amino-3-chlorophenol was estimated colorimetrically after diazotization and coupling with *N*-1-naphthylpropylenediamine as described by Daniel (1961). When the amine (100 μ g.) was added to normal urine (50ml.) the recovery was 98% (range 95–101%).

Enzymic hydrolysis of ¹⁴C-labelled metabolites. (a) With β -glucuronidase. Solutions of plate-purified ¹⁴C-labelled metabolites were incubated in 0.1M-sodium acetate buffer, pH 4.5 (5ml.), with β -glucuronidase (200 units/ml.) at 37° for 18hr. Neutral and weakly acidic metabolites were extracted with ethyl acetate (10ml.); unhydrolysed starting materials were extracted with ethyl acetate after acidification of the reaction mixture to pH 3.

(b) With arylsulphatase. A portion of the methanol extract (2ml.) of freeze-dried urine (see below) was evaporated and the residue redissolved in 0.2M-sodium acetate buffer, pH 5.0 (5ml.). Incubation was for 18hr. at 37° with arylsulphatase (4000 units/ml.). Solutions were then extracted with ethyl acetate (10ml.).

With both enzymes, control digests were prepared in the same way but were heated at 100° for 5min. before addition of the metabolite solution.

Fractionation and separation of metabolites from the urine and bile of rats and the urine of dogs dosed with [¹⁴C]drazoxolon.

(a) Rat urine. A portion (one-fifth) of the combined urines (48hr.) of five rats dosed with [¹⁴C]drazoxolon was freeze-dried and the residue triturated with methanol (10ml.). Solids were removed by filtration and the filtrate was stored at –20° overnight. The solution was again filtered and the methanolic extract was concentrated under reduced pressure.

The rest of the urine was acidified (pH 2) and extracted continuously with ether for 24hr. The ether was separated and extracted with 2M-NaHCO₃ (25ml.). The aqueous phase was collected and extracted successively at pH 4 and

pH 2 with ethyl acetate. The extracts were separately concentrated under reduced pressure at 40° and the residues dissolved in a small volume of methanol. The urine remaining after extraction of all ether-soluble acidic metabolites was mixed with an equal volume of conc. HCl and the solution boiled vigorously under N₂ for 2hr. The solution was then neutralized with Na₂CO₃ and extracted continuously with ether for 18hr. The extract was separated, washed once with an equal volume of water and then extracted with 2M-HCl (20ml.). The aqueous phase was separated, neutralized with Na₂CO₃ and re-extracted by shaking with ether (50ml.). The ethereal solution was concentrated to low volume by blowing N₂ over the surface of the solution. It was then applied as a band to a plate (20cm. x 20cm.) of silica gel and the chromatogram developed with solvent system *B*. The plate was partially covered, leaving a strip along one edge, which was sprayed to reveal amino compounds. The area of gel corresponding to the major component was removed and extracted with methanol.

(b) Rat bile. The 24hr. bile of two rats dosed with [¹⁴C]drazoxolon were combined and added to a column (2cm. x 20cm.) of DEAE-cellulose (Cl[–] form) (Whatman DE-11 grade). The column was eluted with a gradient of 0.05M-HCl into water (250ml.), 35 fractions (each 10ml.) being collected. The column was then eluted with 0.1M-HCl, a further 15 fractions being collected. Fractions 21–27, containing the bulk of the radioactivity, were combined and extracted with butan-1-ol (50ml.). The extract was then concentrated and the residue redissolved in methanol (1ml.).

(c) Dog urine. The urine samples (48hr.) from two dogs dosed with a solution of [¹⁴C]drazoxolon in polyethylene glycol were combined and centrifuged, and (NH₄)₂SO₄ was added (50g./100ml.). The urine was then extracted three times with an equal volume of ether-ethanol (3:1, v/v). The combined extracts were dried over anhydrous Na₂SO₄

and concentrated under reduced pressure, and the residue was dissolved in methanol (10ml.).

The residual urine was acidified (pH2) and extracted three times with ether-ethanol (3:1, v/v). A neutral aqueous solution of this extract was applied to a column (1.5cm. $\times$ 25cm.) of DEAE-cellulose (Cl⁻ form), which was eluted with a gradient of 0.05M-HCl into water (250ml.). Fractions (5ml.) were collected on an automatic fraction collector and those containing the major proportion of the radioactivity (fractions 11-19) were combined and extracted with ethyl acetate (50ml.). The extract was concentrated and the residue redissolved in methanol (10ml.).

Fractionation and separation of metabolites from the urine of rats dosed with 3-methyl-4-([U-¹⁴C]phenylhydrazono)-isoxazol-5-one. The combined 24hr. urine samples of four rats dosed with 3-methyl-4-([U-¹⁴C]phenylhydrazono)-isoxazol-5-one were freeze-dried and the residue was extracted with methanol (15ml.). The extract was stored at -20° overnight, filtered and concentrated under reduced pressure.

Isolation of metabolites from urine of rats dosed with 3-methyl-4-(phenylhydrazono)isoxazol-5-one. The urine samples (24hr.) of five rats dosed with 3-methyl-4-(phenylhydrazono)isoxazol-5-one (500mg./kg.) were combined and centrifuged. One portion was brought to pH5 by the addition of acetic acid, and a solution of saturated lead acetate (0.5 vol.) was added. The precipitate was collected, washed twice with water and gassed with H₂S. The PbS was removed by filtration and the filtrate concentrated under reduced pressure. A little methanol was added and the precipitate that formed was collected, washed with ether and air-dried.

The remaining urine was hydrolysed for 1 hr. with 2M-HCl, cooled and extracted continuously with ether. The ether was evaporated under reduced pressure and the residue recrystallized from aq. methanol.

RESULTS

Elimination of radioactivity by rats dosed with [¹⁴C]drazoxolon (Table 2). After a single oral dose of [¹⁴C]drazoxolon rats excreted 73.6% (68.8-81.0%) of the label in the urine and 12.2% (9.2-15.4%) in the faeces. In three experiments 3.7% (2.7-5.3%) was excreted as ¹⁴CO₂ in the expired gases in 24hr., and in two of these experiments an additional 1.6% and 1.8% was similarly excreted

in 24-48hr. The total recovery of radioactivity in 96hr. was about 90% (81-102%).

The rate of elimination of radioactivity in the urine was somewhat variable: 41.0-74.2% of the ¹⁴C was excreted during 0-24hr. and 6.4-28.5% in 24-48hr. The low excretion of ¹⁴C in the faeces during the first 24hr. after dosage indicates almost complete alimentary absorption. The excretion of ¹⁴CO₂ from [¹⁴C]drazoxolon during 24-48hr. suggests that a proportion of [¹⁴C]drazoxolon is only slowly metabolized. Only a small amount of radioactivity was excreted in bile: 8.3% (7.6-9.4%) in 0-24hr. and 6.3% (4.6-9.2%) during 24-48hr. (Table 3). These values correlate well with the ¹⁴C excreted in the faeces in 24-48hr. and 48-72hr. (Table 2) respectively and suggest the absence of any significant reabsorption of metabolites excreted in the bile.

Elimination of radioactivity by dogs dosed with [¹⁴C]drazoxolon (Table 4). When [¹⁴C]drazoxolon was administered as a solution in polyethylene glycol 300 about 70% of the ¹⁴C was excreted in 96hr. The amount of ¹⁴C excreted in the urine was about equal to that in the faeces. The low faecal excretion of label during 0-24hr. is presumptive evidence of almost quantitative alimentary absorption. The ¹⁴C that subsequently appeared in the faeces probably represented material excreted into the intestine, presumably in bile. In both experiments a similar amount of ¹⁴C was excreted in the urine on the first and second days. This could be due to intestinal reabsorption. If it is assumed that all ¹⁴C in the urine on the second day was so derived then it can be calculated that about 30% of the material excreted into the intestine was reabsorbed.

Elimination of radioactivity by rats dosed with 3-methyl-4-([U-¹⁴C]phenylhydrazono)isoxazol-5-one (Table 5). A single dose of 3-methyl-4-([U-¹⁴C]phenylhydrazono)isoxazol-5-one was excreted by rats largely (73.4-84.8%) in the urine, the remainder (13.9-15.8%) being excreted in the faeces. Urinary excretion was rapid, 75% (66.6-77.9%) of the label being excreted during 0-24hr. and 5.1% (4.6-5.5%)

Table 2. *Elimination of ¹⁴C in the urine and faeces of rats dosed orally with 4-(2-chlorophenylhydrazono)-3-methyl[4-¹⁴C]-isoxazol-5-one*

Dose		Percentage of dose excreted									
		Urine					Faeces				
		0-24hr.	24-48hr.	48-72hr.	72-96hr.	Total	0-24hr.	24-48hr.	48-72hr.	72-96hr.	Total
(mg./kg.)	(disintegrations/sec.)										
4.3	81400	74.2	6.4	0.3	0.1	81.0	0.9	8.3	1.8	0.7	11.7
13.0	29500	53.6	13.6	1.2	0.4	68.8	1.4	4.4	4.4	1.7	11.9
13.0	29500	41.0	28.5	3.7	0.4	73.6	0.2	5.0	6.3	1.4	12.9
15.8	33800	48.5	21.0	2.0	0.6	72.1	0.2	0.8	5.5	2.7	9.2
15.8	33800	43.2	24.6	4.1	0.6	72.5	0.7	4.4	8.7	1.6	15.4

during 24–48 hr. There was no evidence of any $^{14}\text{CO}_2$ being produced.

Identification of metabolites of drazoxolon in rat urine and bile, and in dog urine. (a) Rat urine. Most (85–90%) of the radioactivity in the freeze-dried urine was recovered in the methanol extract. Chromatography of this solution in solvent systems *C* and *D* gave poor resolution, only one radioactive component being detected, with R_F 0.3–0.35 in both solvents. Chromatography in solvent system *A* gave no evidence for the presence of drazoxolon or for that of the hypothetical intermediate, 4-(2-chloro-4-hydroxyphenylhydrazono)-3-methylisoxazol-5-one. Hydrolysis of a portion of the methanol extract, either with arylsulphatase or with 0.2 M-hydrochloric acid for 30 min. at 100°, gave a

material that was readily extracted into ethyl acetate at pH 5. Chromatography in solvent systems *C*, *D* and *E* showed this material to be identical with authentic 2-(2-chloro-4-hydroxyphenylhydrazono)acetoacetic acid. There was no evidence for the formation of this material from the control enzyme digests. The metabolite was also isolated from the acid-hydrolysed urine of five rats dosed with unlabelled drazoxolon. The identity of the compound was established by n.m.r. spectroscopy and by comparing the i.r.- and u.v.-absorption spectra with that of the authentic material.

Continuous extraction of acidified urine with ether resulted in the recovery of 80–85% of the radioactivity as ether-soluble metabolites. Fractionation showed that most of the ether-soluble radioactivity was extractable into ethyl acetate at pH 4, and the remainder was extracted at pH 2. The pH 4 extract contained a single metabolite, which was chromatographically identical with 2-(2-chloro-4-hydroxyphenylhydrazono)acetoacetic acid. The pH 2 extract contained two metabolites, R_F 0.31 and 0.25 (solvent system *C*), corresponding to 4% and 16% of the radioactivity present in the urine. The component with R_F 0.25 gave a positive reaction for glucuronide and was chromatographically identical with one of the major metabolites in dog urine, the nature of which is discussed below. The metabolite with R_F 0.31 has not been identified.

4-Amino-3-chlorophenol was detected on chromatograms, developed in solvent system *B*, of the ether

Table 3. *Excretion of radioactivity in the bile of rats after the oral administration of 4-(2-chlorophenylhydrazono)-3-methyl[4- ^{14}C]isoxazol-5-one*

Dose		Percentage of dose excreted		
(mg./kg.)	(disintegrations/sec.)	0–24 hr.	24–48 hr.	Total
10.0	21100	8.1	—	8.1
7.9	16800	7.6	—	7.6
11.0	15600	8.3	9.2	17.5
11.1	15600	9.4	4.6	14.0
11.1	15600	8.1	5.1	13.2

Table 4. *Elimination of ^{14}C in the urine and faeces of dogs dosed orally with 4-(2-chlorophenylhydrazono)-3-methyl[4- ^{14}C]isoxazol-5-one*

Dose		Percentage of dose excreted									
(mg./kg.)	(disintegrations/sec.)	Urine					Faeces				
		0–24 hr.	24–48 hr.	48–72 hr.	72–96 hr.	Total	0–24 hr.	24–48 hr.	48–72 hr.	72–96 hr.	Total
1.6	21300	14.0	12.6	4.5	2.1	33.2	—	33.9	2.9	0.7	37.5
1.5	21300	14.5	14.5	8.8	1.5	39.3	2.6	26.9	1.8	0.4	31.7

Table 5. *Elimination of ^{14}C in the urine and faeces of rats dosed orally with 3-methyl-4-([U- ^{14}C]phenylhydrazono)isoxazol-5-one*

Dose		Percentage of dose excreted									
(mg./kg.)	(disintegrations/sec.)	Urine					Faeces				
		0–24 hr.	24–48 hr.	48–72 hr.	72–96 hr.	Total	0–24 hr.	24–48 hr.	48–72 hr.	72–96 hr.	Total
3.7	89700	66.6	5.1	0.9	0.8	73.4	5.0	8.7	1.1	0.7	15.5
4.3	103500	77.9	5.1	0.6	0.4	84.0	4.5*	7.5*	1.3*	0.6*	13.9*
4.3	103500	78.5	5.5	0.5	0.3	84.8					
4.3	103500	77.6	4.6	0.5	0.5	83.2					

* Average values based on the analysis of faeces that were collected daily and pooled.

extract of acid-hydrolysed urine and its identity confirmed by a comparison of the u.v. spectrum with that of the authentic material. Quantitative analysis showed that about 7% of the drazoxolon administered was excreted as derivatives of 4-amino-3-chlorophenol.

(b) Rat bile. Fractions 21–27 contained radioactivity equivalent to 5% of the dose. Chromatography in solvent system *D* indicated the presence of two labelled compounds. The slower-moving component (R_F 0.23) gave a positive reaction for a glucuronide (naphtharesorcinol) and was chromatographically indistinguishable from the glucuronide isolated from dog urine. About 20% of the activity in bile was due to this component. The second, faster-moving, component (R_F 0.44) was readily hydrolysed on warming with dilute mineral acid to give an ether-soluble derivative identified chromatographically as 2-(2-chloro-4-hydroxyphenylhydrazono)acetoacetic acid. Presumably this material was present in bile as a labile (e.g. sulphate) ester. The ratio of glucuronide to sulphate in bile was 4:1, corresponding to a 5:1 ratio for the urine.

(c) Dog urine. The extraction of dog urine at pH 6.5 with ether-ethanol removed 55% of the radioactivity, and the remainder was extracted at pH 2. Chromatography of the material extracted at pH 6.5 showed the presence of only one compound, which was shown to be identical with 2-(2-chloro-4-hydroxyphenylhydrazono)acetoacetic acid by co-chromatography with the authentic material in solvent systems *C*, *D* and *E*.

The major radioactive fraction separated from the pH 2 extract by column chromatography also showed the presence of a single component, R_F 0.21 in solvent system *C*. The material, which gave a positive reaction for a glucuronide, was quantitatively converted into 2-(2-chloro-4-hydroxyphenylhydrazono)acetoacetic acid after incubation with β -glucuronidase, whereas in control digests only the unhydrolysed starting material was recovered by extraction of the incubation medium at pH 2. The aglycone and the conjugate were both treated with 1% sodium dithionite (1 ml.) and the solutions after adjustment to pH 7 were extracted with ethyl

acetate. Chromatography of the extracts in solvent system *C* showed the presence of 4-amino-3-chlorophenol only in the extract obtained by reduction of the aglycone. This established the metabolite to be the ether glucuronide of 2-(2-chloro-4-hydroxyphenylhydrazono)acetoacetic acid. Had it been an ester glucuronide, reduction of both the aglycone and the conjugate would have given the free aminophenol. The minor metabolite in extracts of rat urine (R_F 0.25 in solvent *C*) has been tentatively assigned the same configuration because of similar chromatographic behaviour.

The quantitative excretion of metabolites of drazoxolon in rats and dogs is summarized in Table 6.

Identification of metabolites of 3-methyl-4-(phenylhydrazono)isoxazol-5-one in rat urine. Most (89%) of the radioactivity in the freeze-dried urine was recovered in the methanol extract. Chromatography of this solution in solvent system *D* indicated the presence of four radioactive metabolites with R_F values 0.10 (6%), 0.38 (63%), 0.51 (7%) and 0.81 (8%); the values in parentheses represent the proportions of each metabolite. The material with R_F 0.38 was isolated by preparative thin-layer chromatography and a portion subjected to hydrolysis with arylsulphatase. Although less than 30% of the radioactivity could then be extracted into ether at pH 5 from the control digest, about 83% was extracted from the test digest. Chromatography of this latter extract in solvent system *D* showed the presence of only a single radioactive compound, which appeared to be identical with the material with R_F 0.81 referred to above; co-chromatography with authentic 2-(4-hydroxyphenylhydrazono)acetoacetic acid in solvent systems *C* and *D* showed the two to be identical. Moreover, the material was identical with that isolated from acid-hydrolysed rat urine. The evidence suggested that the material with R_F 0.38 was a labile sulphate conjugate of 2-(4-hydroxyphenylhydrazono)acetoacetic acid. The compound isolated by lead salt precipitation (see the Methods section) gave a positive test for sulphate (turbidimetric) after acid hydrolysis and a positive reaction

Table 6. Quantitative excretion of metabolites of [^{14}C]drazoxolon in the urine by the rat and dog

Metabolite	Percentage of dose administered	
	Rat	Dog
Drazoxolon	0	0
4-(2-Chloro-4-hydroxyphenylhydrazono)-3-methylisoxazol-5-one	0	0
2-(2-Chloro-4-hydroxyphenylhydrazono) acetoacetic acid	0	14
2-(2-Chloro-4-hydroxyphenylhydrazono)acetoacetic acid (as sulphate ester)	56	14
2-(2-Chloro-4-hydroxyphenylhydrazono)acetoacetic acid (as glucuronide)	13	12
Unidentified (R_F 0.31 in solvent system <i>C</i>)	3	0
Conjugate of 4-amino-3-chlorophenol	7	Trace
$^{14}\text{CO}_2$ in expired air	3–7	?

for sulphur (Lassaigne's test). Incubation with arylsulphatase gave the free acid (R_F 0.81), the identity of which has been described. Gel-plate purification of the metabolite with R_F 0.10 gave material that gave a positive reaction for glucuronic acid. Although not positively identified it is thought to be the ether glucuronide of 2-(4-hydroxyphenylhydrazono)acetoacetic acid. No attempt has been made to identify the component with R_F 0.51, but it seems possible that it is a derivative of *p*-aminophenol, since the original urine, after hydrolysis with dilute mineral acid, gave a positive indophenol reaction.

DISCUSSION

Drazoxolon is rapidly and virtually completely absorbed from the gastrointestinal tract of both rats and dogs. The major proportion of the absorbed ^{14}C is excreted by rats in 48 hr., mainly through the kidneys (70–80%), with smaller amounts in faeces (5–10%) and in the expired air (5–7%). In contrast, the excretion of radioactivity by the dog appears to be somewhat slower, although it was not possible to estimate the amount of ^{14}C excreted in the expired air. Moreover, in the dog the excreted label is partitioned equally between urine and faeces. The rate of excretion of ^{14}C in the urine in the periods 0–24 hr. and 24–48 hr. in both rats and dogs suggests the presence either of an enterohepatic circulation of metabolites or, alternatively, of the slow release of drazoxolon from tissues. The analysis of the bile of rats dosed with drazoxolon (Table 3) indicates that the second alternative is probably correct. Moreover, the faecal excretion of radioactivity by rats approximates to the biliary excretion, an observation that suggests that any enterohepatic circulation that may occur is small. The greater faecal excretion of ^{14}C by dogs suggests that the biliary excretion in this species is appreciably greater than that observed in the rat.

As far as the author is aware the fate of the isoxazolone moiety has not previously been described. Formation of the keto acid presumably involves hydrolysis via the intermediate ketoxime or amino derivative. Hydroxylation may precede hydrolysis, as no evidence was obtained for the presence of 2-(2-chlorophenylhydrazono)acetoacetic acid.

The major primary metabolite of drazoxolon in both species is 2-(2-chloro-4-hydroxyphenylhydrazono)acetoacetic acid. Whereas in the rat it is excreted in the urine as the corresponding sulphate ester (65%) and as the ether glucuronide (13%), in the dog there was evidence for the presence of only the glucuronide (12%) and of the free acid. The conditions used for fractionation of the dog urine appear to exclude the possibility that the free acid

was an artifact resulting from the hydrolysis of a labile conjugate.

The species differences in the excretion of radioactivity are probably to be attributed to differences in metabolism. Although little is known of the various factors that determine the extent to which a foreign compound is excreted in the bile, Millburn, Smith & Williams (1967) have postulated that when a compound is excreted by this route in appreciable amounts (5–10%) it must possess a strong polar anionic group and a molecular weight of about 300. Conjugation appears to play an important role in this respect, for bile acids (Haslewood, 1955), bilirubin (Klatskin, 1961) and thyroxine (Flock & Bollman, 1962) are excreted in the bile as conjugates. The greater faecal excretion of drazoxolon metabolites by the dog may be due to the preferential excretion in the bile of the *O*-glucuronide (mol.wt. 432.5) of the corresponding arylazoacetoacetic acid. Only a small proportion of the radioactivity in rat bile was due to this particular metabolite, the major metabolite being a labile conjugate, probably the corresponding sulphate ester (mol.wt. 335.5). However, few studies have been reported on species differences in biliary excretion, and factors additional to that of molecular weight may be involved.

The presence of 4-amino-3-chlorophenol in the urine of both rats and dogs dosed with drazoxolon and of *p*-aminophenol in the urine of rats dosed with 3-methyl-4-(phenylhydrazono)isoxazol-5-one is evidence of reductive fission of the azo link. In contrast with the behaviour of 1-phenylazo-2-naphthol (Daniel, 1962) and 4-dimethylaminoazobenzene (Stevenson, Dobriner & Rhoads, 1942), reduction of the arylazoisoxazolones is, however, only of minor importance. In this respect they resemble the lipid-soluble phenylazopyrazolones (Jones, Ryan & Wright, 1966). Daniel (1969) has shown that drazoxolon is not reduced when incubated with rat liver microsomes under conditions that effect the reduction of many other azo compounds (Fouts, Kamm & Brodie, 1957; Manchon, Gradnauer & Lowy, 1964; Daniel, 1967). There is, as yet, no explanation for the observed stability of drazoxolon. The amino compounds formed *in vivo* may be derived from those water-soluble metabolites excreted in the bile that are reduced by intestinal micro-organisms (Radomski & Mellinger, 1962).

The metabolism of 3-methyl-4-(phenylhydrazono)isoxazol-5-one by the rat is qualitatively and quantitatively similar to that of drazoxolon, the major metabolite in the urine being the sulphate ester of 2-(4-hydroxyphenylhydrazono)acetoacetic acid. The substitution of a chlorine atom into 3-methyl-4-(phenylhydrazono)isoxazol-5-one does not seem to influence the types of metabolic change

the compound undergoes, although it profoundly affects toxicity.

In the rat drazoxolon does not inhibit the activity of brain L-glutamate 1-carboxy-lyase (EC 4.1.1.15), nor does it affect the concentration of γ -aminobutyric acid, thought to act as an inhibitor of neurotransmission (J. W. Daniel, unpublished work). The mode of action of drazoxolon therefore differs from that of arylhydrazides (Davison, 1956). 2 - (2 - Chloro - 4 - hydroxyphenylhydrazono)acetic acid produces convulsions when injected into rats. The enhanced toxicity of drazoxolon to dogs may be associated with the presence in the urine of the free acid. Conjugation may therefore be considered to be a true detoxication reaction.

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