# Diazinon Metabolism in the Dog

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Diazinon, [0,0-diethyl 0-(2-isopropyl-4-methyl pyrimidin 6-yl)phosphorothioate] is a widely used and effective insecticide. Metabolic studies in the rat have shown that the parent compound is degraded rapidly, yielding diethyl phosphoric acid (DEP), diethyl phosphorothioic acid (DETP) and at least 3 known pyrimidinols (MUCKE et al. 1970; YANG et al. 1971; NAKATSUGAWA et al. 1969). Recently, SHISHIDO et al. (1972) have also shown, in vitro, that a glutathione (GSH) dependent aryl transferase in rat liver is responsible for cleavage of minor amounts of Diazinon, yielding a glutathione conjugate containing the pyrimidine ring. Other workers, JANES et al. (1973) and MIAZAKI et al. (1969) have reported that esterase inhibiting metabolites have been found in the urine of sheep and mice dosed with Diazinon. One of these compounds, hydroxydiazinon, was also found as a metabolite in field sprayed kale, and as a photoalteration product of Diazinon (PARDUE et al. 1970).

In view of these investigations, the present study was designed to determine the urinary metabolites of Diazinon in the dog. This species was chosen since little previous work has been done in the determination of individual metabolites, although MILLAR (1963) has determined the presence of unchanged Diazinon, DEP and DETP in several canine tissues and EARL et al. (1971) described the toxic effects observed at various levels of the organophosphate.

## Methods

Intravenous studies: Two female beagles weighing 7 and 9 kg were fitted with a jugular cannula connected to a 3-way stopcock. Diazinon ($^{14}$C ethoxy) 0.2 mg/kg in 0.7 ml ethanol, was injected into the femoral vein. Specific activity of the ethoxy Diazinon was 3.4 µc/mg. Blood



Bulletin of Environmental Contamination & Toxicology,
Vol. 13, No. 5 © 1975 by Springer-Verlag New York Inc.

samples ca. 2 ml, were drawn from the cannula at times ranging from 5 min. to 7 hr. after injection. Duplicate 0.5 ml aliquots of blood were bleached with 0.2 ml 30% hydrogen peroxide and digested in 0.25 ml 60% perchloric acid for 1 hr at 70°C. A commercial scintillator (Aquasol) was added to the digest and the samples counted in a Beckman liquid scintillation counter with external standard quench correction. The remainder of the blood sample was used to prepare serum for the spectrophotometric determination of cholinesterase using butyrylthiocholine as substrate (ELLMAN et al. 1961). Throughout the blood sampling period, the dogs were housed in metabolism cages and the urine collected for analysis at the end of 24 hours.

Oral dosing: Two female beagle dogs 6 and 7 kg, were given ring labelled Diazinon, 4.0 mg/kg, which had been dissolved in ethanol and placed in a gelatin capsule just prior to dosing. Specific activity of the ring labelled compound was 3.6 µc/mg. The dogs were held in metabolism cages for 24 hours with free access to food and water. Ring labelled Diazinon was also hydrolysed in 6N HCl and the labelled pyrimidinol extracted with chloroform. The recovered material (metabolite 1, Figure 5) was given orally to a female beagle at a dose level of 2 mg/kg.

Determination of urinary metabolites: The urine was filtered through Whatman #1 paper and an aliquot counted for total radioactivity. Another aliquot was extracted with hexane for determination of diazinon by gas chromatography (glc) on a Varian 2100 equipped with an alkali flame ionization detector and a 3'x1/8" (o.d. glass) column of 5% DEGS on Chromosorb W, HP, 80-100 mesh. The column temperature was 155°C; nitrogen flow 35 ml/min; compressed air 200 ml/min; and hydrogen 27 ml/min. The remaining urine was freeze dried and stored until use. Reconstituted, concentrated urine samples were then subjected to thin-layer chromatography (tlc) on silica gel G, 0.25 and 0.5 mm layers, with and without fluorescent indicator. The solvent systems were A; Acetonitrile:water:Ammonia, 80:18:2.B; Ethyl Acetate:Ethanol:Ammonia, 80:15:5 and C; Acetone:Hexane, 1:4.

Urine from the ethoxy-labelled Diazinon was also applied to a 2x31 cm column of Dowex 1-X8 and eluted with the gradient systems given by YANG et al. (1971). DEP and DETP were quantitated by extracting the individually pooled radioactive peaks with derivatization according to SHAFIK et al. (1973) prior to glc on the Varian 2100 equipped with a dual flame photometric detector operating in the phosphorus and sulfur modes. Other conditions were essentially as described by SHAFIK et al. (1973).

Additional confirmation was obtained by running urine samples on tlc in solvent A, scanning the plates with a Panax thin-layer scanner and scraping off the radioactive zones into Aquasol for scintillation counting. Urine from the ring labelled diazinon was run on tlc solvent B, and on a silica gel column 2.5x30 cm. prepared in solvent A and eluted with solvent A. Aliquots of 0.1 ml from each fraction were counted for total radioactivity. The peaks were pooled and concentrated on a rotary evaporator. Identification of the metabolites was made by co-chromatography with known metabolite standards and by comparison of the mass spectra of the tetramethyl silyl derivatives of the standards and isolated urinary metabolites.

Hydroxy diazinon was isolated after U.V. irradiation of Diazinon following the procedure of PARDUE et al. (1970). Using the 5% DEGS column described above, the relative retention times for diazinon, diazoxon and hydroxydiazinon were 1, 1.83 and 3.05 respectively.

## Results

Figure 1 shows the decay of radioactivity in the blood of a dog receiving 0.2 mg/kg ($^{14}C$ ethoxy) Diazinon, I.V. The curve was biphasic with the second slower phase yielding a half life of 363 min (mean of 2 experiments).

The effect of 0.2 mg/kg Diazinon on serum cholinesterase is shown in Figure 2. Maximal depression, to 50% of control values in one case, is reached after 150 min. but there is a very rapid loss to 61% of control levels within 10 min. of administration.

Figure 3 shows a typical elution pattern obtained from a Dowex 1-X8 column loaded with urine collected from the dogs receiving the I.V. $^{14}C$ ethoxy Diazinon. Two well defined peaks were present and were identified as DEP and DETP. Recovery of radioactivity in urine was 58% of the administered dose, after 24 hours, with DEP representing 16% and DETP 42% of the total dose. Column recovery was 96% of the applied radioactivity. These values represent the mean of 2 experiments.

The tlc separation of ring labelled Diazinon metabolites from a dog receiving 4 mg/kg, orally, is shown in Figure 4. A large poorly defined peak at the origin contained the "water soluble fraction" as defined by MUCKE et al. (1970). The two large peaks were identified

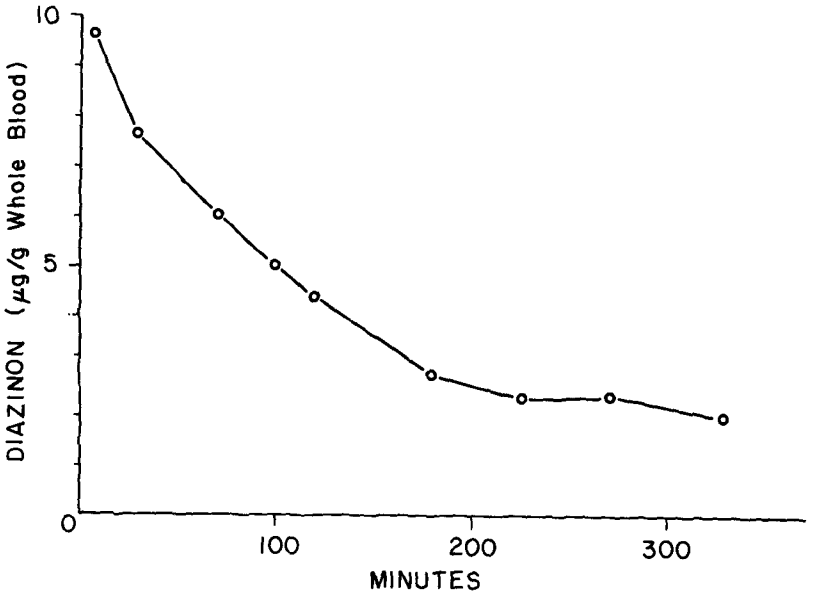


Figure 1. Decline of radioactivity in blood of a female beagle receiving intravenous ethoxy labelled Diazinon, 0.2 mg/kg

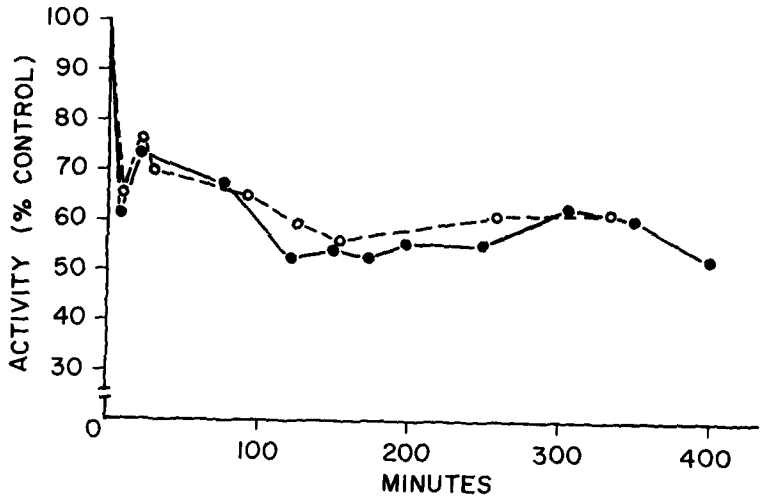


Figure 2. Effect of 0.2 mg/kg Diazinon on serum cholinesterase after intravenous administration.

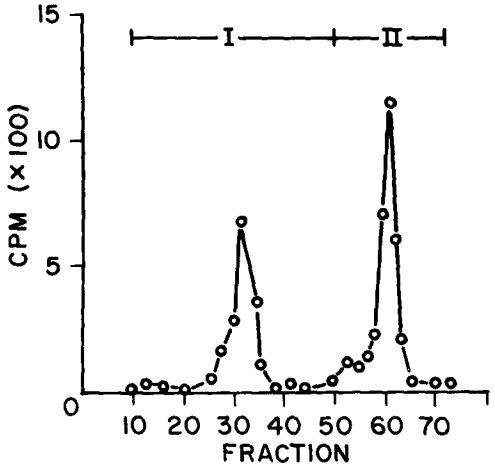


Figure 3. Separation of ethoxy labelled Diazinon metabolites from dog urine by ion-exchange chromatography: I and II refer to the gradient elution systems.

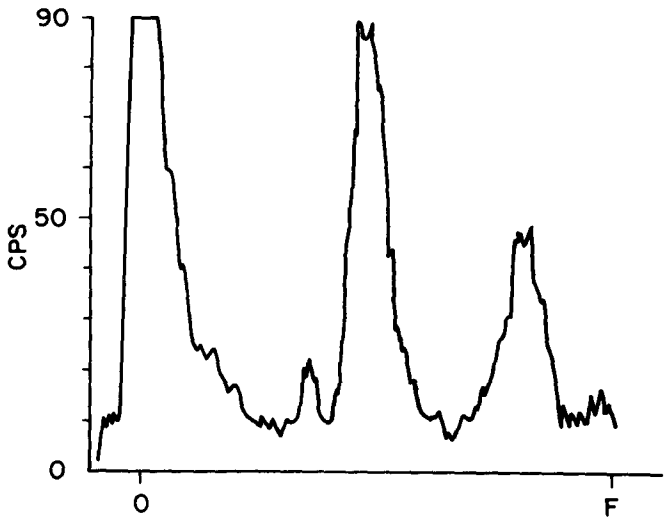


Figure 4. TLC separation of ring labelled Diazinon metabolites on slice gel using solvent system. B. O, origin: F, solvent front.

as metabolites 1 and 2 (see figure 5). Recovery of radioactivity was 85% of the administered dose. The water soluble fraction contained 53% of the total radioactivity given while metabolites 1 and 2 accounted for 10% and 23% of the total, respectively. Unchanged Diazinon was not detected by glc analysis of the hexane extracts of urine. Scintillation counting of these extracts yielded only background values, confirming the absence of radiolabelled hexane extractable compounds. The pattern of metabolites found in urine after administration of the Diazinon hydrolysis product was essentially similar to that of the parent compound. Recovery

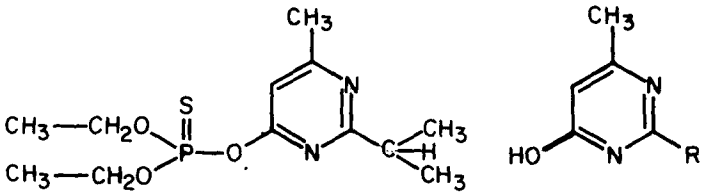


Figure 5. Structure of metabolites discussed in text.

metabolite 1, R = $-C(CH_3)(H)(CH_3)$ metabolite 2, R = $-C(CH_3)(OH)(CH_3)$

metabolite 3, R = $-C(CH_3)(H)(CH_2OH)$

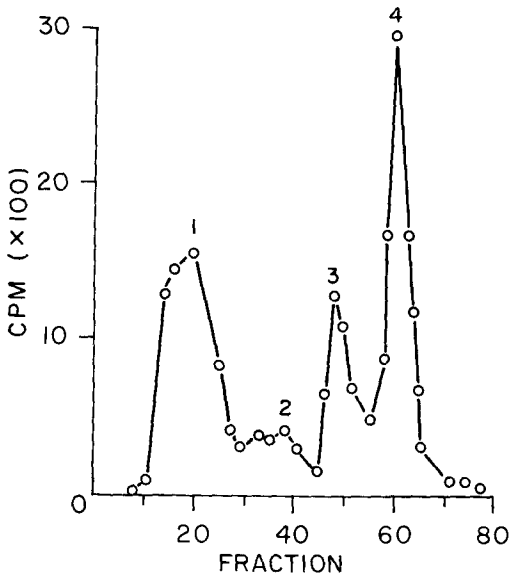


Figure 6. Column chromatography of dog urine containing ring labelled Diazinon metabolites.

was 82% of the dose administered with the water soluble fraction accounting for 59% while metabolites 1 and 2 represented 6% and 17%.

In an attempt to characterize the water soluble fraction, a partial separation was obtained by column chromatography on silica gel. Figure 6 shows that 4 peaks were obtained. Peak 1 contained metabolites 1 and 2. Peak 2 contained little radioactivity and since it was heavily contaminated with urine components, further analysis was not attempted. Peaks 3 and 4 gave a positive reaction with ninhydrin, however treatment with 6N HCl at 105° for 18 hours did not result in the appearance of any radioactive peaks with an altered rf when developed with solvents A or B.

The organic phase of a urine aliquot which had been subjected to repeated chloroform extraction was run on tlc with solvent D according to JANES et al. (1973). Only one radioactive spot was detected and it was slightly ahead of the application line. Hydroxydiazinon, prepared by U.V. irradiation, migrated with an rf of 0.27, well ahead of the peak found. Analysis of the chloroform extract by tlc with solvent B showed that only metabolites 1 and 2 were present.

## Discussion

The results of the present study suggest that Diazinon metabolism in the dog proceeds rapidly and is relatively complete. The two pyrimidinol metabolites, 1 and 2, that were found in rat urine were also present in the dog urine. A metabolite found in rat urine (metabolite 3, Figure 5) was not found in the present study. However a small peak, estimated at <1% of the applied radioactivity was noted at or near the rf approximated for the metabolite from the chromatogram shown in the report by MUCKE et al. (1970).

The relative proportions of metabolites excreted by the rat and dog are different. Metabolite 1 accounted for 22% of the total dose in the female rat but only 10% in the dog. While metabolite 2 represented about 23% of the dose in both species, the "water soluble" metabolite fraction was 53% for the dog and only 15% for the rat.

If a GSH dependent aryl transferase was responsible for a significant portion of Diazinon metabolism, it would be expected that a portion of the polar fraction would contain mercapturic acids. The lack of effect of HCl treatment and the fact that a large water soluble component occurs after dosing with metabolite 1 suggests that the water soluble fraction is probably comprised mainly of pyrimidinols more polar than metabolites 1 and 2.

The failure to detect intact organophosphate metabolites in dog urine in the present study does not imply that they are not produced. In vitro experiments with a liver microsomal enzyme system or a re-examination of the urinary metabolites after a greatly increased dose of Diazinon may yield more information on these toxic metabolites. Even at the relatively high doses (1 g/kg) given to sheep (JANES et al. 1973), less than 1% of the total dose was recovered as intact organophosphate metabolites. It is also possible that these compounds may have been produced, or have accumulated, as a result of the saturation of major enzymatic pathways of degradation.

## Acknowledgements

The authors wish to acknowledge the assistance of W. Miles, for mass spectral analysis, Ciba-Geigy for their generous gift of labelled Diazinon and the Diazinon metabolites and Dr. R.F. Willes for animal surgery.

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