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TOXICOKINETIC, TISSUE RESIDUE, AND METABOLITE STUDIES OF DEOXYNIVALENOL (VOMITOXIN) IN TURKEYS

by

Henry Charles Gauvreau

Doctor of Veterinary Medicine, University of Saskatchewan, 1980

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Toxicokinetics, Tissue Residue, and Metabolite Studies of Dexamethasone (Voltaren) in Turkey

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ABSTRACT

Deoxynivalenol (vomitoxin) a toxic secondary metabolite produced by the fungi *Fusarium graminarium*, contaminates food sources of animals and humans when infection of crops by the fungal organism occurs. The most common toxicity symptoms observed in animals that consume deoxynivalenol contaminated feedstuffs are feed refusal, reduced weight gain and vomition. At a biochemical level deoxynivalenol inhibits protein synthesis producing lesions in many systems of the body. Previous toxicological studies indicate wide species variation in the toxicity of deoxynivalenol. Poultry in general have been shown to be more tolerant of deoxynivalenol in their rations than other species and data on the toxicokinetics, metabolism and tissue accumulation of deoxynivalenol residues in chickens is available. Very little is known about the biological fate of deoxynivalenol in turkeys.

The purposes of this study were (1) to determine the toxicokinetics of deoxynivalenol in turkeys (Meleagris Gallopava) that have been dosed orally and intravenously, (2) to examine turkey tissues for the presence of deoxynivalenol or its metabolites, and (3) to determine the chemical structures of deoxynivalenol metabolites produced by turkeys.

Statistical analysis of deoxynivalenol blood concentration versus time profiles from intravenous dosing could best be described

as a three-compartment open toxicokinetic model. Deoxynivalenol blood levels declined very rapidly producing a terminal elimination half-life (t 1/2β) of approximately 44 minutes. Oral studies revealed deoxynivalenol to be poorly absorbed from the gastrointestinal tract (0.96% of the dose was absorbed). Elimination of deoxynivalenol follows first order kinetics with the majority of the toxin appearing in the excreta within three hours. In turkeys deoxynivalenol and its metabolites are rapidly excreted by the kidney into the urine. Deoxynivalenol metabolites were observed in the excreta and urine but they were not chemically identified. Tissue residues of deoxynivalenol and/or metabolites were apparent but they declined rapidly down to trace levels.

In memory of my parents

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INTRODUCTION

Fungi are a diverse group of parasitic organisms, generally saprophytes, that grow and reproduce on a wide variety of nutrient sources including many human foods and animal rations. Under specific growth environments several genera of fungi are known to produce metabolites toxic to animal and humans. These toxic metabolites originate from secondary fungal metabolic pathways: a series of biochemical reactions that result in the production of specialized biomolecules (Lehninger, 1982). The specific function of toxic metabolites is not clear although Jenzen (1977) speculates they may have evolved as the microorganism's strategy to preserve sources of nutrition, giving these organisms a method of competing for food sources.

Mycotoxicosis, the development of undesirable biological effects due to exposure to mycotoxins, may develop from direct ingestion, skin contact or inhalation of these toxins. Secondary exposure to mycotoxins may occur through the consumption of animal tissues containing residues of mycotoxins or their metabolites. The undesirable biological effects vary from symptoms associated with acute toxicity (vomiting, diarrhea, skin irritation, sudden death) to symptoms associated with more chronic toxicity (neoplasia, teratogenesis, and mutagenesis). Incidences of mycotoxicosis have been reported from many areas of the world as a result of the ability of fungi to grow and develop under a variety of environmental conditions.

In addition, mycotoxicosis has been reported throughout history. However, it has only been in recent years with the development of modern analytical chemistry and fungal culture techniques that the production and identification of specific toxins has been possible and definitive toxicological studies undertaken. Many aspects of mycotoxin toxicity need to be elaborated to establish the significance of the various toxins to both human and animal health. The information generated by research efforts in Canada and other countries has demonstrated the potential of specific mycotoxins to adversely affect the various systems of the body when exposed to small quantities of these toxins. It is the work of current researchers to pursue these initial findings and expand the toxicological information to cover the many aspects of mycotoxins that are still unknown.

In Canada, surveys of crops for mycotoxins have demonstrated the presence of deoxynivalenol (DON) contamination in cereal grains used for animal ration and human food (Scott et al., 1981; Trenholm et al., 1983). However, only a limited number of toxicology studies using deoxynivalenol have been carried out on farm animals such as sheep, dairy cattle, chickens and swine. These investigations provided preliminary information on the biological fate of DON in these species and the accumulation of DON or its metabolites in the tissues. Previous investigations have determined the accumulation of residues in eggs and meat of laying hens, the effect of DON on the health and productivity of adult laying hens and growing chicks and poults, and DON toxicokinetics in chickens.

The biological fate of DON in turkeys has not been studied. Considering poultry tend to be comparibly tolerant to the effects of DON could the consumption of DON contaminated feeds lead to the accumulation of tissue residues and what is the chemical nature of these residues? Knowing the kinetics of DON and its metabolites is important to an understanding of the risks to the health of the birds and potentially to humans who consume the meat.

Therefore, the purpose of this study is to investigate the biological fate of DON in broiler turkeys. Kinetic studies have been undertaken to determine the absorption, distribution, metabolism and excretion of DON. Tissue samples were analyzed to determine the chemical nature of the major metabolites of DON in turkeys. In addition, a residue study was carried out to determine if DON or its metabolites accumulate in the tissues of turkeys.

Mycotoxins in General

There exists a great diversity of mycotoxins, over one hundred at the present time, have been identified. In his work on mycotoxins prevalent in cereal grains, Osweiler (1990) placed the major mycotoxins into eight major groups based on similarities of chemical structures and properties and summarized several general but noteworthy characteristics of mycotoxins and mycotoxicosis: (a) several genera of fungi produce toxic metabolites, (b) these fungi can grow and develop in a wide variety of substrates that serve as foods for both animals and humans, (c) many species of animals are

susceptible to mycotoxins, and (d) mycotoxins can be toxic to many systems of the body. These mycotoxins' properties have resulted in incidences of severe mycotoxicoses occurring in humans and a variety of animal species in many locations throughout the world.

With the worldwide movement of agricultural commodities, it is possible for countries to both import and export mycotoxin problems with their trading partners. Analysis of feed samples to determine the levels of mycotoxins present in Canadian crops have been conducted periodically. Prior (1976) screened samples of food-stuffs for aflatoxins and found mostly negative results. The National Research Council (1985) reported that 1% of all peanut butter samples screened contained aflatoxin above the 15 mg/kg tolerance level and detectable quantities of aflatoxin were found in 10 of 60 imported cheese samples. In Canada, ochratoxin has been detected in mouldy grain samples at levels of up to 4000 mg/kg and is suspected as a source of toxicosis in broiler chicks, dairy cattle and laying hens (Abramson et al., 1983). Ergot alkaloids have been found at low levels in only a very few feed samples (Prior, 1976). Zearalenone may affect all farm animal species and produces a number of changes in reproductive physiology; prolonged estrus, vaginal prolapse, testicular atrophy, infertility, stillbirths, and fetal mummification. It is considered a major mycotoxicosis in Canada because it is regularly detected at potentially toxic levels in suspect feed samples. Fennell (1979) reported that, of the 2000 suspect feed samples analyzed in Ontario from 1972 to 1977, levels of zearalenone were detected between 0.07-141 mg/kg. Sutton (1980) found

up to 29% fungal contaminated corn samples contained detectable amounts of zearalenone. Citrinin, tremorgenic mycotoxins and fumonisins are not considered to be significant sources of mycotoxicosis in Canada at this time.

Trichothecene Mycotoxins

As a group, the trichothecene mycotoxins, which includes deoxynivalenol, represent a major global health problem for both humans and domestic animals. In this group of chemically related sesquiterpenoid compounds approximately eighty different isolates have been identified (Ueno, 1987). Based on their chemical properties Ueno (1987) classified the major trichothecenes into four distinct groups (A, B, C, and D). Within group A of the trichothecenes mouldy corn toxicoses and alimentary toxic aleukia, due to T-2 toxin has resulted in mortality of animals and man. Group B trichothecenes which include deoxynivalenol will be discussed in the next section. Crotocin, group C, is an antibiologic agent and is not a significant trichothecene (Ueno, 1987). Group D mycotoxins are classified as macrocyclic trichothecenes because of their unique chemical structure and in general they are the most toxic with the contaminating fungi frequently colonizing the straw bedding of the animals. *Fusarium* spp. are the major fungi producing the trichothecene mycotoxins, although *Cephalosporium, Trichothecium, Myrothecium, Stachybotrys, Cylindocarpin* and *Verticimonosporim* spp. produce the group C and D trichothecenes. Mycotoxicoses due to *Fusarium* contamination of cereal grain crops develop from the consumption of these food sources by animals and humans.

Deoxynivalenol

Sporadic deoxynivalenol toxicoses associated with *Fusarium graminarium* (Schwabe telemorph *Gibberella zeae*) contamination of animal and human food have been identified for many years. In Japan and Korea a toxicoses identified as red mould intoxication has occurred as a sporadic health problem in humans and domestic animals in several years; 1901, 1914, 1958, 1963 and 1970 (Ueno, 1980). This toxicosis was attributed to *Fusarium graminarium* contamination of the local cereal crops such as oats, corn, barley and wheat. Similarly in the United States, corn ear toxicosis due to *Fusarium graminarium* parasitized cereal corn has been reported. In 1923 in Russia, a major outbreak of Fusarioses in humans and animals associated with *Fusarium graminarium* contamination of cereal crops was reported (Doceman et al., 1930). Dickson et al. (1930) published a report of illness in swine characterized by emesis as a result of feeding barley that was contaminated by *Gibberella zeae*.

In many of the cereal growing regions of Canada, DON has been identified as a major contaminant of the cereal crops. The trichothecene found most commonly and at the highest levels in Canadian feedstuffs is DON. Extensive screening work by Scott (1981, 1984) and Trenholm in 1981 found significant levels of DON in the 1980 and 1981 wheat crops in eastern Canada. Scott et al. (1981) surveyed the 1980 wheat crop from Ontario and Quebec after noting extensive infection by *Fusarium* spp. resulting in head blight. The results of the survey of seventy-two wheat samples found DON

concentrations ranging from 0.01 - 4.3 mg/kg. Samples from western Canada were found to be free of deoxynivalenol that year. Trenholm et al. (1981) in 1980 analyzed 41 feed winter wheat samples from Ontario and reported deoxynivalenol levels of 0.38 - 0.98 mg/kg. In surveying the 1982 and 1983 wheat crops, Scott et al., (1985) produced the following data: for Ontario in 1981 the average of all samples analyzed contained 0.18 mg/kg DON and in 1982 the average was 0.70 mg/kg. In 1981 for Quebec, the average contamination of all the wheat samples analyzed for DON was 2.27 mg/kg and in 1982 the samples averaged 0.28 mg/kg. In general, the analysis of wheat samples from western Canada in 1982 detected trace levels of DON (0.1 mg/kg). Samples of white winter wheat from Alberta had levels of DON ranging from 0.02 to 0.07 mg/kg. The survey of corn samples in Ontario reported DON present at the following levels: 1981 - 0.33 mg/kg, in 1982 - 0.22 mg/kg and in 1983 the average sample contained 0.17 mg/kg. Of interest, a single feed wheat sample from Quebec contained 7.71 mg/kg deoxynivalenol (Scott et al., 1981) and Trenholm et al. (1981) reported a single feed wheat sample containing 8.5 mg/kg DON.

Production Conditions

The existence of environmental conditions that support mould growth and toxic production are critical for the development of mycotoxin problems. The sporadic nature of mycotoxicosis relates to there being suitable conditions in the field and/or on storage facilities, that favor fungal growth and mycotoxin formation. In the

field the major source of spore contamination of crops, seeds and soil is old crop debris; previously infected stalks, stubble and ears of corn (Warren et al., 1973). Crops become contaminated and infected by spores that originate from the soil through a number of mechanisms; as growing seedlings, aerosols, and spores carried on insects and birds (Attwater and Busch, 1983, Sutton et al., 1980). Hesseltine (1976) identified a number of factors that would lead to the production of mycotoxins during the harvest period - moisture, humidity, temperature and weathering. Physical damage to the plants by insects, birds, heavy rains, wind and hail which give the spores access to the deeper plant tissues will aid the parasitism of crop plants by the fungal organisms. In the field, warmer temperatures (15-35°C) and persistent wet conditions favor progressive development of the *Fusarium* life cycle stages (Sutton, 1982, Liang et al., 1981). Field crops are not readily susceptible at the flowering stage while corn ears are most susceptible when the silks have first emerged (Sutton 1982, Anderson 1948). Mechanical injury to the green kernels at harvest and spore inoculation due to soil contamination of grain helps establish fungal growth in stored grain.

In storage facilities critical factors influencing *Fusarium* growth and toxin formation are degree of spore contamination, moisture content of the grain, temperature and relative humidity. Marten and Gilman (1976) found that the moisture content of grain critical for *Fusaria* development was between 22 and 33% moisture. A temperature of 12-20°C will support mycelial growth but optimum growth occurs at

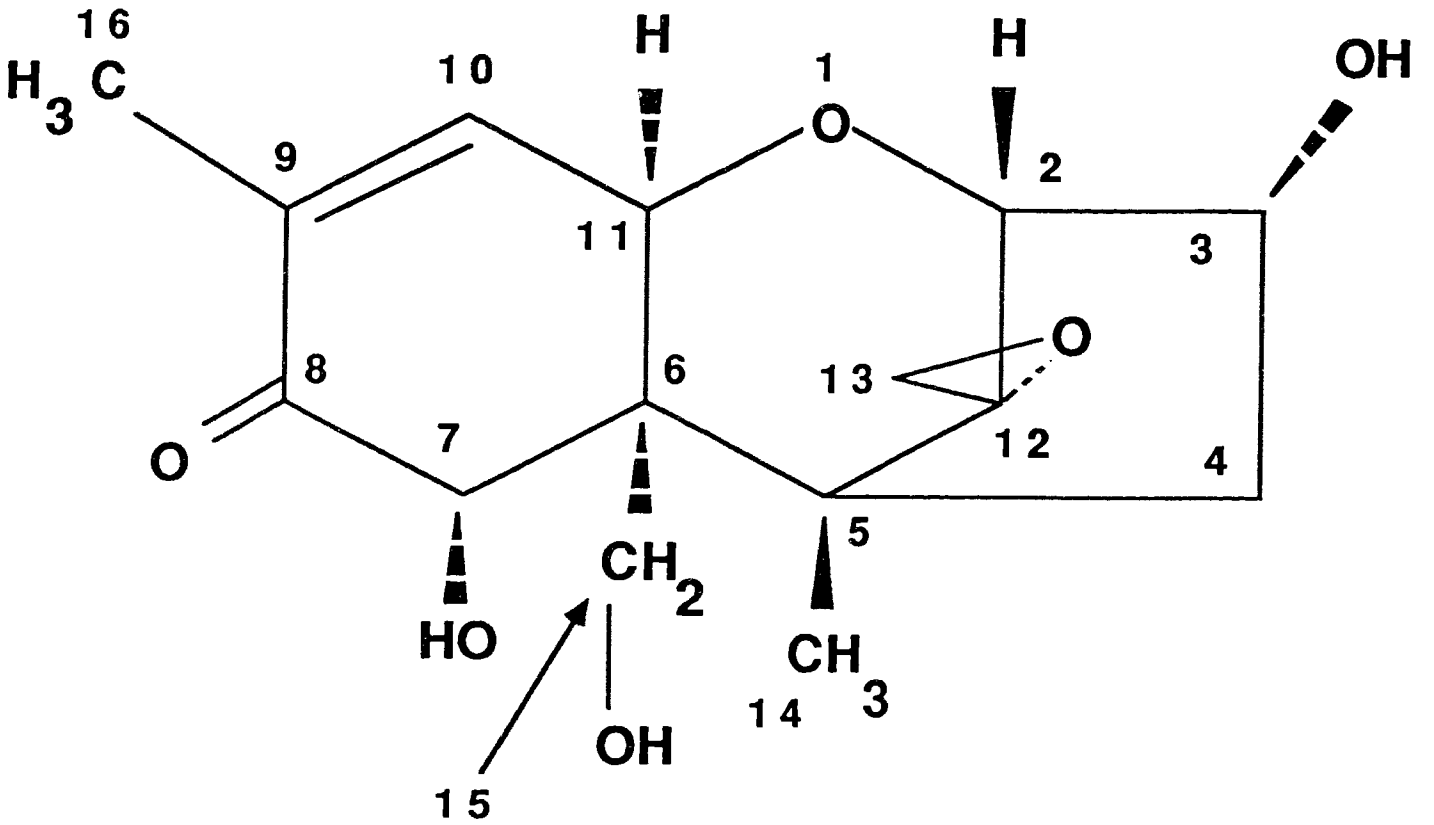
20-30°C (Joffee 1963). Under specific laboratory conditions *Fusarium* growth occurred at temperatures as low as 7°C and a moisture content of 18% (Sherwood and Peabody 1974). Vesonder et al. (1982) reported that *Fusarium roseum* developed in corn resulted in optimum levels of DON being produced at 30°C, 30% moisture content and after 41 days of incubation.

Chemical/Physical Properties

Trichothecenes are classified as sesquiterpenoids because they contain the tetracylic 12, 13 epoxytrichothecene skeleton. Chemically these mycotoxins can be separated into four distinct groups based on their functional groups and specific fungal origin (Ueno, 1977). Group A toxins (T-2 toxin, diacetoxyscripenol, etc.) are characterized by a functional group other than a ketone at C-8. Group B trichothecenes (nivalenol, deoxynivalenol, fusarenon-X) are identified by the carbonyl group at C-8. Crotocin is the major trichothecene found in Group C and is chemically identified by a second epoxide found at C-7, 8. The macrocyclic trichothecenes comprise Group D (*verrucarins, roridins, satratoxins* etc.) and contain a macrocyclic ring structure between C-4 and C-15. The structure and numbering system of deoxynivalenol, a keto-derivative trichothecene is illustrated in Figure 1.

Noteworthy structures within the molecule are; an olefinic bond at C-9~C-10, an epoxy ring at C-12~C-13, and the hydroxy groups at

Fig 1. Chemical Structure of Vomitoxin



DEOXYNIVALENOL
(VOMITOXIN)

(Rd Toxin)

C-3, 7 and 15. DON ($C_{15}H_{20}O_6$) has an m.p. of 153-154°C and is soluble in ethyl acetate, methanol, ethanol, chloroform, acetonitrile and is insoluble in hexane. It is only slightly soluble in water (Bamburg et al., 1971). While only marginally water soluble it has a low water-lipid partition ratio (Prelusky et al., 1991). The other trichothecenes are more soluble in chloroform, acetone and ethyl acetate. Under severe acidic conditions (pH 1) the epoxy ring is stable to nucleophilic attack but becomes unstable if heated (pH 1 for 6 hours at 90°C). The conjugated carbonyl system allows for both U-V detection (absorbance at 220 nm) and fluorescence detection. Common methods of analysis include TLC, GC, HPLC and mass spectrometry. Combination GC-MS analysis of the trimethylether of DON using a selected ion monitoring system results in diagnostic mass fragments at M/Z 422, 325, 295, 235, and 197 (M+ = 512) (Yoshizawa, 1983).

Toxicology DON

In general, similar chemical signs of toxicity have been observed for most of the trichothecenes including DON; with varying degrees of potentcy depending on the toxin. Ueno (1973) reported the major symptoms of DON toxicity as vomiting, nausea, feed refusal, diarrhea, bone marrow destruction and hemorrhage in the intestines, brain, vagina, adrenals and uterus. These symptoms were observed in cows, pigs, sheep, goats and chickens. Ogasarwa (1965) reported observing similar clinical signs in humans due to the consumption of wheat flour which was contaminated by *Fusarium graminarium* mould. Chung (1975) observed these signs in humans and animals that consumed

grain contaminated by *Gibberella zeae* in Korea. Trenholm et al. (1984) and Pollman et al. (1985) reported that DON in the diet of swine at 1-2 mg/kg produced reduced weight gain and stimulated feed refusal. Swine ingesting higher concentrations of DON (20 mg/kg) can result in emesis (Trenholm et al., 1988). In rats, a diet containing DON at 2 mg/kg stimulated feed refusal (Vesonder et al., 1979). Mice exhibited feed refusal and reduced weight gain at a dose of 1 mg/kg (Tryphoras et al., 1986). Acute toxicity studies in mice fed deoxynivalenol contaminated rations resulted in gastrointestinal necrosis and intestinal hemorrhage (Forsell et al., 1987). Coppock et al. (1985) dosed swine intravenously (0.5 mg DON/kg of body weight) and reported necrosis of pancreatic acinar and Islet cells causing hypoglycemia. Pestka et al. (1987) and Robbara-Barnet et al. (1986) reported deoxynivalenol to be immunosuppressive in mice at 5 mg/kg of the diet.

In reproductive and teratological studies in mice fed DON, Khera et al. (1984) reported an increased incidence of fetal death, malformation, and neonatal death.

Deoxynivalenol has been shown to be cytotoxic (Newberne and Rogers 1981, Ueno 1980) and at a cellular level there is inhibition of protein and deoxyribose nucleic acid synthesis through disruption of ribosomal functions. Ueno and Fukishima (1968) noted the inhibition of amino acid conversion into protein in tumor cells after treatment with nivalenol at a concentration of 10μg/ml. Tatsuma et al. (1968)

reported that protein synthesis was disrupted due to inhibition of ribosomal synthetic enzyme activity. Wei et al. (1974) reported that trichodermin interacted with the 60S subunit of the ribosome. Although two proposed modes of action of inhibition of protein synthesis have been identified, that is, initiation inhibition of protein synthesis and elongation - termination inhibition of protein synthesis (Ueno, 1987), the exact mechanism of ribosomal dysfunction has not been elaborated. DNA and RNA synthesis is inhibited by trichothecenes but this is considered to be secondary to disruption of protein synthesis since a higher level of toxins was required and the extent of inhibition was less (Iwahashi et al., 1982).

The inhibition of protein synthesis explains the radiomimetic-like injuries commonly observed in animals suffering from DON intoxication, ie., damage to the immune, haematologic, gastrointestinal and dermal tissue. All these tissues depend on rapidly dividing cells to maintain the function and with the inhibition of protein synthesis the normal division and growth of cells is impaired. Bone marrow destruction results in immunosuppression due to decreased white blood cell production, anemia due to decreased cell formation, and hemorrhage may develop from reduced platelet formation (Sato et al., 1978).

Poultry seem relatively insensitive to the toxic effects of DON. Huff et al. (1981) reported no mortality in broiler chicks dosed with DON (70 mg/kg of body weight). Moran et al. (1982) reported no decrease in feed consumption or growth in 6 - 11 day old broilers fed

DON up to 116 mg/kg of ration. Hamilton et al. (1983) fed a naturally contaminated wheat ration containing 4.9 mg/kg to Leghorn chicks for 24 weeks and reported no significant effects. Feeding a diet containing 5 mg/kg to young chicken and turkey poults was well tolerated by these birds (Hamilton et al., 1985). Trenholm et al. (1984) fed a DON contaminated ration to Leghorn chickens for 10 weeks and reported no significant changes to weight gain, feed consumption or egg production. When laying hens were fed wheat rations naturally contaminated with DON (up to 4.9 mg DON/kg of feed) Hamilton et al. (1985) reported small but significant decrease in shell weight, shell thickness, and percentage shell. Kubana et al. (1987) fed mature laying hens a naturally contaminated wheat ration containing 18 mg DON/kg for 16 weeks and reported no significant effects on body weights, efficiency of feed production, and ten-day egg production. There was a slight significant decrease in eggshell weight.

El-Banna et al. (1983) fed groups of laying hens a ration containing 4-5 mg DON/kg of ration for 28 to 190 days and could not detect DON residues in eggs or tissues of the chickens. Kubana (1985) fed two levels of DON contaminated wheat ration (9 mg and 18 mg DON/kg) for one to 35 days and found no detectable DON in the tissues in hemoglobin or hematocrit of the birds fed the high dose ration between day 18 and 35 of the experiment, at a detection limit of 10 μg/kg of tissue.

Metabolism/Kinetics/Elimination of Trichothecenes

The metabolism of trichothecenes involves four principle biochemical pathways; deacylation, hydroxylation, de-epoxidation and conjugation, with many possible metabolites resulting from the combinations of those reactions on the parent molecule. The metabolism of T-2 toxin has been intensively studied and deacylation and hydroxylation reactions are very important in its metabolism. The deacylation of T-2 toxin and its intermediate metabolites has been observed in a variety of animals; rats (Ueno, 1987), chickens (Chi etal., 1978) (Yoshizawa, 1980) and lactating cows (Yoshizawa, 1981). The biotransformation of T-2 toxin occurs in the microsomol fraction of the hepatocytes and are enzyme catalyzed reactions (Ohta et al., 1977). The T-2 metabolites produced are similar to T-2 metabolites produced by fungi (Visconti, 1985). Other trichothecenes that are subject to deacylation reactions are fusarenon-X and 3-acetyldeoxynivalenone (Matsumoto et al., 1978)

Hydroxylation reactions are another significant process in the metabolism of trichothecenes. Kobayaski (1985) concluded that the cytochrome P-450 enzyme system was an important mechanism in the hydroxylation reactions for the metabolism of T-2 in mice. The major metabolite of T-2 and HT-2 toxin that has been identified in animals (rodents, chickens, cows) are the hydroxylated moieties 3'-hydroxy T-2 toxin and 3'-hydroxy HT-2 toxin (Ueno, 1987).

In de-epoxidation reactions the 12,13 epoxide ring common to all trichothecenes is converted to a vinyl bond which is an important step in the detoxification of these compounds since the epoxy ring is responsible for much of the toxicity. Swanson et al. (1988) isolated, extracted, and analyzed for the de-epoxy metabolites of T-2 toxin, diacetoxyscripenol, monacetoxyscripenol and scripenol. The de-epoxy metabolite of DON (DOM-1) was initially identified in the urine and feces of rats which had been dosed orally with DON (Yoshizawa et al., 1983). King (1984) reported the conversion of DON to DOM-1 by rumen microorganisms. Other trichothecenes subject to de-epoxidation reactions are diacetoxyscripenol, (Sakamoto, 1986), T-2 toxin (Yoshizawa, 1985), T-2 tetraol (Chattergee 1986), and nivalenol (Onji et al., 1989).

The major metabolite of DON identified in the urine and feces of animals is the de-epoxide metabolite DOM-1. Lake et al. (1987), Worrell et al. (1989), Yoshizawa et al. (1983) found that DOM-1 was excreted in the urine and feces of rats. Prelusky et al. (1985) noted the presence of this metabolite in sheep urine and feces. *In vitro* investigations using bovine rumen fluid maintained under strict anerobic conditions reported the conversion of DON to DOM-1 (King, 1984; Swanson et al. 1987). The role of anerobic microflora of intestinal origin in the formation of the DON de-epoxide metabolite was further confirmed by Worrell et al. (1989) using both *in vitro* and *in vivo* methodology. Studies designed to show the role of the liver enzymes in the formation of DOM-1 metabolite have been inconclusive (Worrell, 1989) and this metabolite is believed to be

microbial in origin.

Conjugate metabolites have been identified for the following trichothecenes; T-2 toxin, HT-2 toxin, DON and DOM-1. Prelusky et al. (1986) and Worrell et al. (1989) identified DON glucuronide conjugates in sheep bile and rat bile respectively. The microbial conjugation of HT-2 and T-2 toxin involves 3-0-acetyl conjugation pathway and is carried out by *Fusarium, Ulocladium* and *Mucor* sp. (Yoshizawa, 1980).

The pharmacokinetics of DON has been studied in several species. Prelusky et al. (1988) administered ^{14}C-DON I.V. to swine and determined that the plasma distribution of DON corresponded to a three-compartment open model with the half-life values (t 1/2) for the α phase of 5.8 min (rapid distribution), β phase of 96.7 min (slow distribution) and γ phase of 510 min (terminal distribution). Coppock (1985) found swine plasma concentration values (I.V. dosing) gave a one-compartment model with an elimination phase half-life of 125-219 min. Other pharmacokinetic parameters reported by Prelusky et al. (1988) for swine include; volume of distribution 1.34 liter/kg, volume of the central compartment 0.166 liter/kg, and plasma clearance 1.81 ml/min/kg. From the same study data collected following intragastric dosing gave a t 1/2 for the elimination phase of 7.1 hrs; DON plasma levels reached peak concentrations with 15-30 min of administration, and DON plasma levels plateaued between 730-130 ng/ml for approximately 9 hrs, before they slowly declined. Bioavailability of DON in swine was 48-65%, and it is excreted primarily in the urine

(80-104%) unchanged (> 95%), Prelusky et al. (1988). Friend et al. (1986) found the bioavailability of DON and its metabolites in swine to be 65%. The estimated bioavailability in some other species is 4% in dairy cattle (Prelusky et al., 1984) and 6-10% in sheep (Prelusky et al., 1985).

Prelusky et al. (1985) reported a two phase exponential decrease of DON in plasma levels of sheep dosed intravenously; the rapid distribution phase (t 1/2 α 12-23 min) and a slower elimination phase (t 1/2 β 57-78 min). There was no evidence of significant tissue binding of DON. Oral dosing resulted in t 1/2 β of 100-125 min with DON completely eliminated in 20-30 hours. When dosed orally, approximately 75% of the DON was converted to the DON glucuronide metabolite which was slowly eliminated from the body (6.1-7.1 hr). Only a small amount of the de-epoxide conjugate was detected (< 2%).

In another study in which sheep that were dosed intravenously with DON (0.5 mg DON/kg), it was found to be eliminated in the urine in several forms; unchanged DON or the metabolites DON glucuronide conjugate, DOM-1 and DOM-1 glucuronide conjugate (Prelusky et al., 1986). With I.V. dosing the t 1/2 (elimination phase) for DON in the urine is 1.2 hr, while the two major metabolites were found to have t 1/2 as follows: DON glucuronide - 2.2 hr, DOM-1 glucuronide - 3.1 hr. Prelusky et al. (1986) found that 63% of the total dose was eliminated in the urine with maximum urinary excretion occurring 0.5-1.5 hr after dosing. Further, approximately 3.5% of the I.V. dose

was excreted into the bile as conjugated DOM-1, with peak biliary excretion being established within 1 hr of dosing. In the oral dosing studies, approximately 55-75% of the oral dose was recovered from the feces unchanged (Prelusky et al., 1986). Approximately 7.1% of the administered DON was recovered in the urine (7%) and bile (0.11%). Urinary excretion (t 1/2) of metabolites were as follows - DON - 3.2 hr, conjugated DOM-1 - 5.0 hr, conjugated DON - 4.0 hr, with maximum rate of excretion occurring 6-9 hr, after treatment. DON and its metabolites are rapidly excreted from the body with metabolism being a major mechanism of elimination in sheep.

Few studies have been carried out to determine if mycotoxin residues are accumulating in animal tissues. Yoshizawa (1981) reported for dairy cattle that 0.2% of the administered dose of T-2 toxin could be found in the milk. Robinson (1979) in a similar study using T-2 toxin, found a small portion of the dose accumulated in the milk. Prelusky et al. (1984) found trace amounts ($\leq$ 4 ng/ml) of DON in the milk of dairy cows administered 1.9 mg of DON per kg of body weight. El-Banna (1983) fed DON to layer hens and broiler chickens (4-5 mg/kg of body weight for 28-190 days) and was unable to detect DON in the tissues or eggs. The presence of only trace amounts of radioactivity in tissue samples of swine fed low levels of DON contaminated rates over an extended length of time suggests sequestration of DON or its metabolites in tissues may be insignificant (Prelusky, 1991).

§ MATERIALS - METHODS

Chemicals

Unlabelled deoxynivalenol (DON) and labelled (^{14}C-DON) was produced from methods described by Miller and Arnison (1986) Agriculture Canada, Ottawa, Ontario. The chemical purity of the unlabelled DON was determined by TLC and HPLC analysis (97% pure); similarly the ^{14}C-DON was found to be 96% pure with a specific radioactivity of 1.3μCi/mg. The ^{14}C-DON was produced by a biosynthetic process using ^{14}C-acetate and resulted in non-specific labelling of the DON.

All solvents were HPLC grade or better.

β-glucuronidase (Sigma Type IX from *E. coli*) was obtained from Sigma Chemical Co., St. Louis, MO. for enzymatic hydrolysis studies.

For the clean-up columns, non-activated carbon (Darco G-60, 7-E343, J.T. Baker Chemical Co., Phillipsburg, N.J.) and chromatographic grade neutral alumina (70-230 Mesh, Merck M 01077-36, BDH Chemicals, Toronto, Ontario) were used.

In the preparation of the HPLC mobile phase both solvents were filtered under vacuum. Methanol was filtered through a FH - 0.5 mM filter (Millipore Ltd., Mississauga, ON.). The water was double distilled (Sybron-Barnsted distiller) and then filtered through a HA - 0.45 mM Millipore filter.

Biofluor (New England Nuclear, Lachine, Que.) was used for liquid scintillation counting.

Apparatus

The HPLC utilized a variety of components: Spectra-Physics Model SP-8000-03 L.C. containing a temperature programmed oven, autoinjector with 100 µl sample loop, Spectro-Flow 773 UV detector (Kratos Analytical Instruments, Westwood, N.J.) and a cathode ray tube display unit. Sample separation was obtained using a stainless steel L.C. column (25 cm x 4.6 mm id) packed with reverse phase RP-18, 5µm OD-5A Sphere-5 (Brownlee Labs, Santa Clara, CA) and a guard column packed with Spherisorb S5 ODS, 5µm OD-5, (Phase Separations, Hauppage, N.Y.) inserted between the injector and the L.C. column.

Measurements of radioactivity were carried out by two methods, direct liquid scintillation counting (L.S.C.) and oxygen combustion followed by L.S.C. Beckman 8000 liquid scintillation counter (Beckman Instrument Co.) was used. Determination tissue radioactivity was accomplished using a Tri-Carb Model 306 autosampler oxidizer, (Packard, Downers Grove, Ill.).

T.L.C. plates were Merck pre-coated 20 x 20 cm silica gel 60 F254 plates (Terochem, Unit 3, 2740A Slough Street, Mississauga, ON.).

Clean-up columns were made from 10 ml (18 mm id x 30 cm) disposable glass pipettes (Fisher Scientific, Pittsburgh, PA.).

All glassware was washed in distilled water and heated in a muffle furnace (500°C for 2 hours) prior to use to remove all organic material.

Animals and Treatments

One-day old broiler poults (Meleagris Gallopavo) were obtained from a commercial turkey hatchery (Hybrid Turkey Incorporated, Dorval, Quebec) and moved to the brooder room, Agriculture Canada Research Farm, Nepean, Ontario. Groups of 24 birds were received every three weeks for the duration of the project to facilitate having birds of three to four kilograms for the various studies.

During the eight-week growing period the groups of turkeys were housed in poultry floor pens and were given feed and water *ab libetum*. For the first two weeks the poults were fed a turkey starter crumble ration and then they were switched to a turkey grower ration. All feeds were formulated by Agriculture Canada nutritionists following N.R.C. guidelines and processed on the farm. Samples of rations were analyzed to check the nutritional content of the feed and to check for the presence of the mycotoxins deoxynivalenol and zearalenone.

The following environmental conditions were maintained: relative humidity (ambient), temperature (20°C) and day length (12 light-12 dark).

Routine gross post-mortem examinations were conducted on all birds that died. All systems were systematically examined for evidence of gross pathology and tissues showing evidence of disease were submitted for further microbiological and histological examination.

In preparation for the various experiments, groups of eight turkeys were transferred to the radioactive studies wing of the pathology laboratory. At this stage, the birds weighed between three and four kilograms and were approximately eight weeks old. They were individually identified and caged and assigned to a specific treatment group.

The turkeys were given free access to feed and water and environmental conditions maintained similar to those in the growing area.

In studies where repeated blood sampling and/or intravenous dosing was necessary, the brachial veins were catheterized according to the following procedure. Turkeys were administered a general anesthetic using a non-rebreath anesthesia system. Anesthesia was induced and maintained with halothane (Ayerst 0.5%-2%) and a O_2 flow of 2 litres/minute. Once stabilized under anesthesia the brachial vein in the proximal ulnar region was plucked free of feathers and the skin swabbed with alcohol in preparation for catheterization. Intravenous teflon catheters (22 ga x 1 in - Angiocath) were inserted into the vein and anchored in position by folding cloth tape across the hub of the

catheter and suturing the tape to the skin. To maintain the patentcy of the catheters they were flushed with 0.5 ml of sodium citrate solution (3.9%). After both brachial veins had been catheterized, the turkeys were re-weighed and allowed to recover from anesthesia. The turkeys were given 24 hours to recover from anesthesia and surgery prior to dosing.

Urine was collected by cannulating the ureters. The birds were anesthetized as previously described, the openings of the ureters in the cloaca identified and plastic cannula (50 cm) were inserted into the ureters approximately 2.0 cm from the orifice. The cannula were secured in place using a purse string suture (3-0 Dexon). After recovery from anesthesia, birds were placed in narrow cages to restrict their movement. The cannula for collecting urine extended from the cloaca to 15 ml screw-top vials in test tube racks securely attached to the outside of the cages. The urine samples were identified and frozen (-20°C) immediately after collection.

For the tissue residue study, tissue samples (breast muscle, thigh muscle, spleen, brain, proventricules, gizzard, kidney, liver, adrenal, heart, abdominal fat, subcutaneous fat, bile, small intestine) were collected immediately after euthanasia. The birds were euthanized by intravenous injection of T-61 (Hoechst - 1 ml) for necropsy. Each tissue sample was collected and stored in an individual plastic bag. All the individual samples from a single bird were kept together in a large container and frozen (-20°C) until analyzed.

Analytical Techniques

1. DON Administration

For the kinetic studies, DON was administered orally and intravenously at two doses - 5 mg/kg and 1 mg/kg body weight. To facilitate dosing DON was dissolved in a 3% ethanol:sterile water solution at a concentration of 10 mg/ml for the high dose studies (5 mg/kg) and at a concentration of 2 mg/ml for the low dose studies (1 mg/kg). For those birds dosed with ^{14}C-DON in addition to cold DON, the quantity of DON contained in the ^{14}C-DON material was calculated to maintain the total dose administered at 5 mg DON/kg or 1 mg DON/kg.

For I.V. dosing, the dissolved DON was drawn up into a 3 ml syringe (Monoject) injected into the right brachial vein using the implanted catheter which was then flushed with 0.5 ml of saline. For oral dosing, the dose of DON was placed directly into the crop by tubing the birds (*per os*) using a neonatal stomach feeding tube (size 8 Fr. x 10" long). After administering the DON solution with a syringe, the tube was flushed with 1 cc of sterile water.

In those experiments used to determine the urinary excretion of DON and/or its metabolites, the birds were given a total dose of 1 μCi ^{14}C-DON intravenously or orally.

The birds used for the tissue residue studies were dosed

orally at two levels; 5 mg DON/kg body weight (0.5 μCi - ^{14}C-DON/kg and 1 mg DON/kg of body weight 0.5 μCi - ^{14}C-DON/kg). These birds were then sacrificed at the following intervals: 0.75, 1.5, 3, 16, 12, 24 and 72 hours.

2. Sample Collection

Blood samples (1 ml) were collected via the left brachial vein catheter into 1 ml syringes. The samples were transferred to 1.5 ml disposable polypropylene centrifuge vials (Eppendorf) containing 100 μL of 3.9% sodium citrate as anticoagulant. After adding the blood to the vials, they were capped, shaken to mix the anticoagulant and blood, and then refrigerated (3°C) until they were prepared for analysis. For the intravenous dosing studies, blood was collected at the following intervals: 0, 2.5, 5, 10, 15, 20, 30, 40, 60, 80, 100, 120, 150, 180, 210, 240, 300, 360 minutes. For the oral dosing studies, blood was collected at 0, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 260, 280, 300, 320, 340 and 360 minutes.

Excreta samples (containing both urine and feces) were collected at 3, 6, 24, 36, 48 and 72 hour intervals from birds dosed with ^{14}C-DON. The excreta was collected on plastic wrapped fiberglass trays placed under the wire cages. At the proper time interval the excreta was removed, placed in plastic containers and frozen (-20°C) until analyzed.

3. Extraction, Clean-up and HPLC Analysis of Blood Samples

The HPLC technique used for the analysis for DON in blood was a modification of methods developed by Trenholm et al. (1985) for the extraction, clean-up and analysis of cereal grain samples.

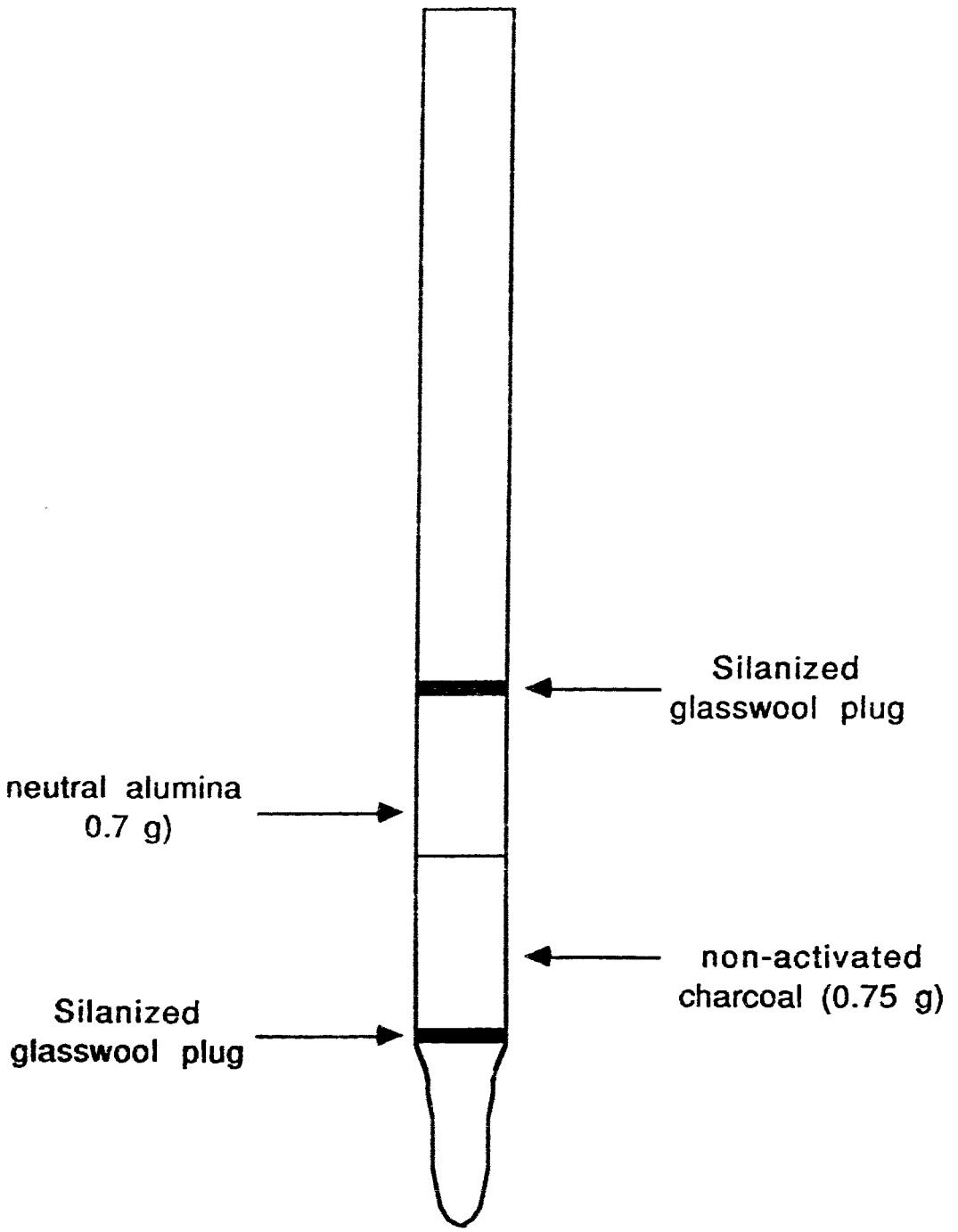
In brief, the extraction, clean-up and analytical procedure is outlined below. Clean-up columns (Fig 2) were packed in the lab according to the following procedure.

The top portion of the pipette was removed and the distal tip was plugged with a small amount of silanized glass wool. Added into the pipette was 0.75 g non-activated charcoal which was packed under vacuum to approximately 2/3 of its original volume. Added on top of this layer was 0.7 g of neutral alumina which was packed under vacuum and then a glass wool plug was lightly tamped into place to retain contents.

Prior to the extraction of blood samples, clean-up columns were attached to a multiple sample vacuum system (Trenholm et al., 1985) and pre-washed with 15 ml of an acetonitrile water solution (84:16). Eluant flow rate was adjusted by vacuum to approximately 1 drop every 10 seconds. Vacuum was removed before the pre-washing solution reached the level of pipette contents to prevent the contents from drying.

Figure 2

Clean-Up Column



For extraction of DON from the stored blood samples the samples were mixed (30 sec) and 1 cc of sample was transferred to a 15 ml screw-top vial. Acetonitrile (5 ml) was added and the mixture was mixed for 1 hour at 1000 rpm (IKA-Bibrax-VXR). This mixture was centrifuged (5 min at 1500 rpm) and the supernatant was pipetted into the pre-washed clean-up columns, and vacuum started to facilitate filtration of sample. The remaining blood sample was re-extracted with acetonitrile (1 ml), mixed for 30 min, centrifuged (5 min), and the supernatant was added to the clean-up column. An additional wash of 8 ml of acetonitrile:H_2O (84:16) was added to the column, and when the clean-up column was dry, vacuum was removed and the eluant that had been collected was removed from drying. Eluant samples were dried using a gentle flow of N_2 and a heating block (50°C). The residues remaining were dissolved in 1 ml of HPLC mobile phase solvent (methanol:water - 91:9) transferred to HPLC vials, capped and refrigerated until analyzed.

For samples obtained from oral dosing studies the residue that remained after drying with N_2 was dissolved in 300μL of HPLC solvent, transferred to 300μL HPLC vials, capped and refrigerated.

The concentration of DON in the eluant was determined by HPLC analysis using the instrumentation described previously. Analysis was performed under the following conditions: mobile phase - methanol:water (91:9); detector wave-length - 220 nm; solvent flow rate - 1.2 ml/min, column temperature -30°C and injection volume - 100μL.

Figure 3 shows a typical HPLC chromatogram of an extracted blood sample.

DON standard solutions were prepared and standard curves derived using peak heights. An external standard method of sample analysis was used. Peak height was linearly related to DON concentrations between 5 ng/100μl to 100 ng/100μl (Fig 4).

4. Liquid Scintillation Counting (L.S.C.)

Liquid scintillation counting was used to determine the total radioactivity in blood, urine and T.L.C. extracts. The radioactivity in the sample was determined by liquid scintillation counter (Beckman 8000 L.S. counter, Beckman Instrument Co., Irvine, CA). Measurements made by L.S.C. were automatically corrected for dilution, quenching, background, and counting efficiency.

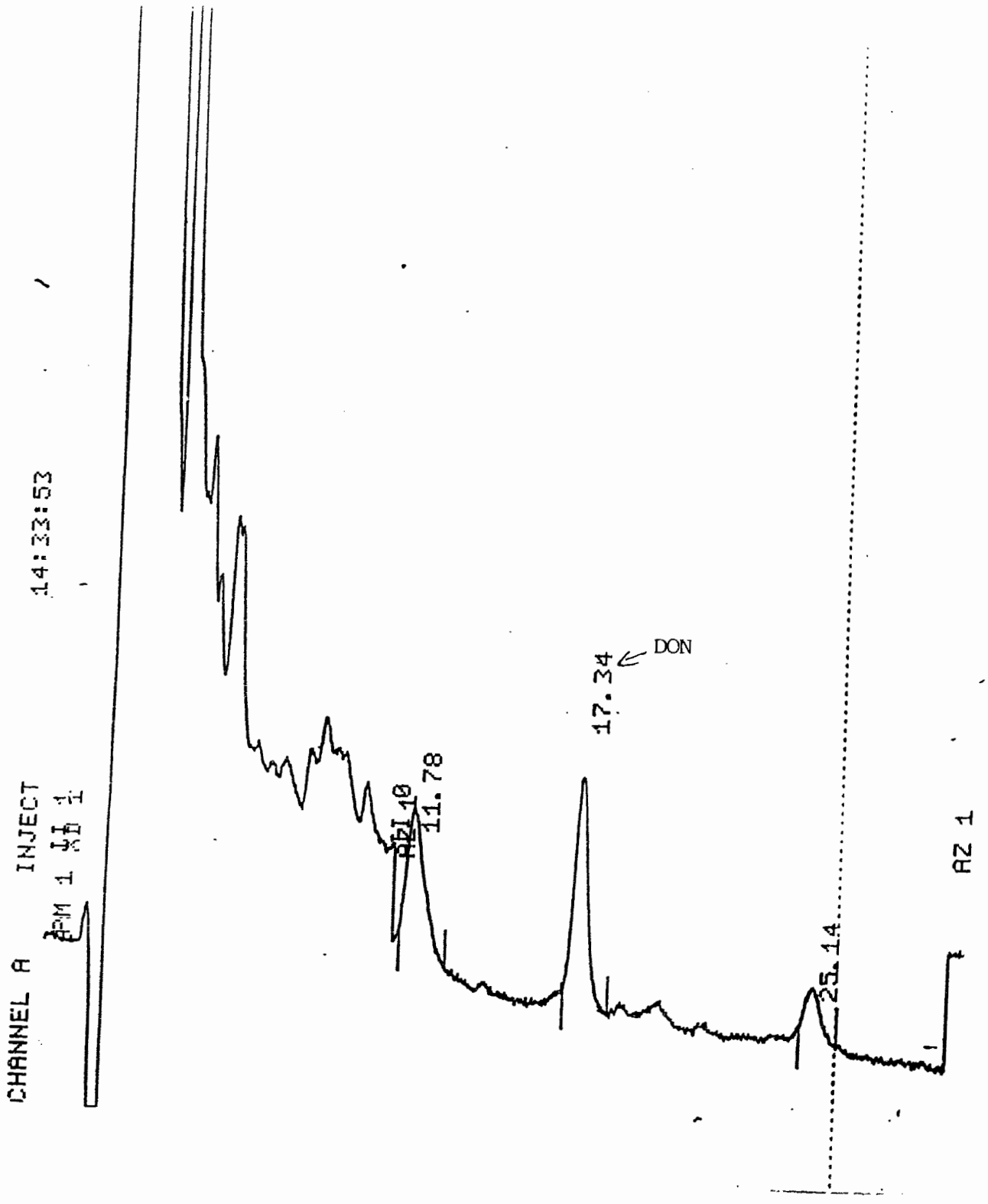
Whole blood samples (100μL) were counted in triplicate. The blood sample was pipetted into plastic scintillation vials and 15 ml of Biofluor was then added into the vial, mixed and counted.

Urine samples (200μL) were also counted in triplicate. These samples were air-dried in the scintillation vials before 15 ml of Biofluor was added. The vials were then mixed, allowed to stabilize for 12 hours and counted.

The T.L.C. extracts of the excreta studies were counted in duplicate (2 ml) by L.S.C. after the extract was air-dried in scintillation vials. Biofluor (15 ml) was added before L.S.C. For

Figure 3

Typical HPLC Chromatogram of Extracted Blood Sample



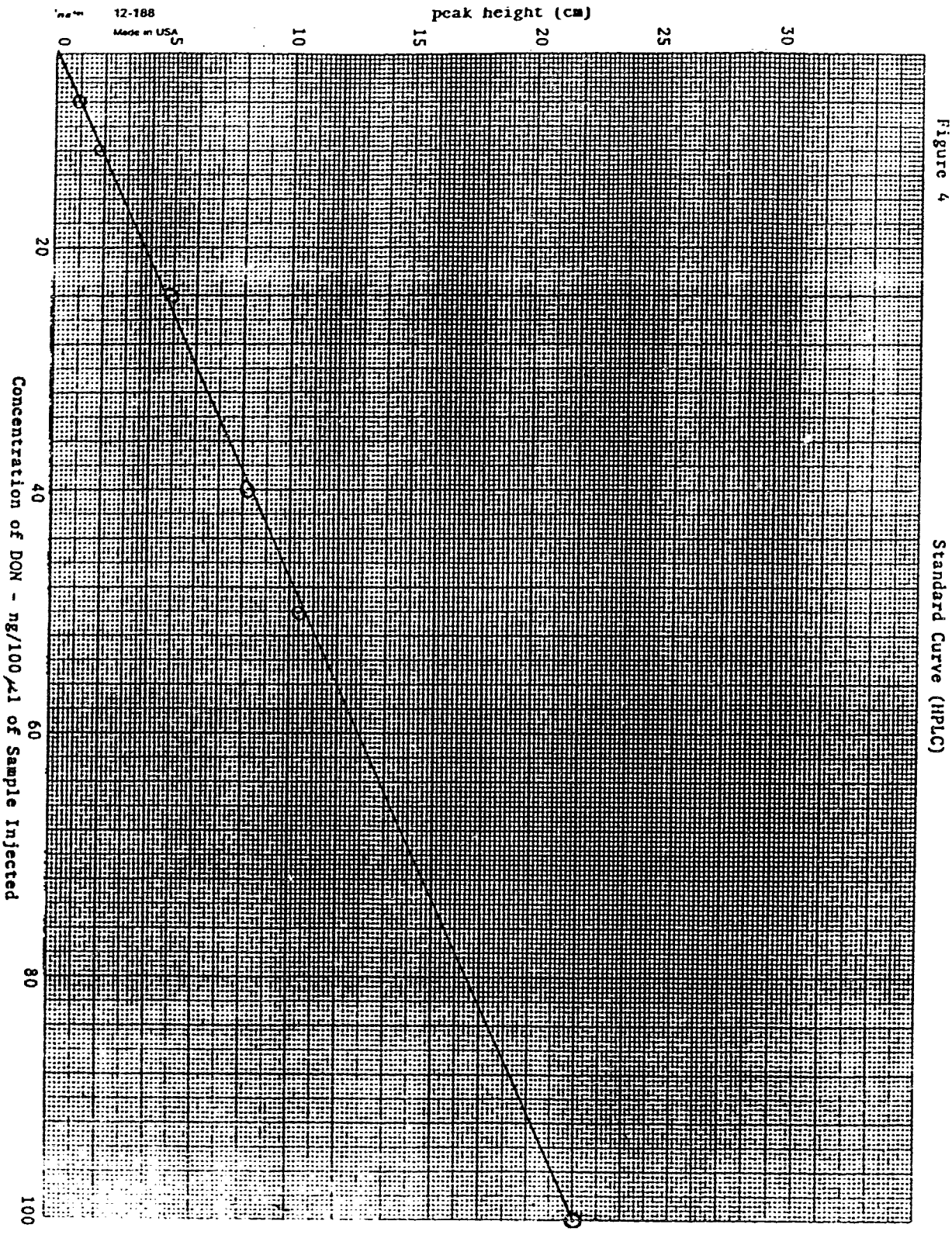


Figure 4 Standard Curve (HPLC)

the T.L.C. extracts of the urine studies, triplicate 50 µL samples of extract were air-dried in the vials, 15 ml of Biofluor added and the mixture was counted in L.S.C.

5. Oxidizing Techniques

Triplicate tissue samples containing ^{14}C-DON or its metabolites were weighed (100 - 200 mg) and then burned in an autosampler oxidizer (Tri-Carbo Model 306 autosampler oxidizer). The $^{14}C-CO_2$ released from the tissues was captured in Carbo-Sorb, mixed with Permafluor (scintillation cocktail) and counted on the L.S.C. Tissues from control birds that had been administered ^{14}C-DON were oxidized as a source of background samples. Excreta samples were oven-dried, weighed and pulverized with mortar and pestle prior to being oxidized. Quenching was determined by external standard method.

6. Thin Layer Chromatography Studies

(a) General Procedures

Precoated silica gel plates (20 x 20 cm) were etched vertically into several channels for a variety of widths according to the number of samples being analyzed (Fig 5). Plates were developed in a solution of $CHCL_3$:acetone:isopropanol (40:30:10). The origin was located 2 cm from the edge of the plate and the total distance from the origin to the top of the sample channel was 14 cm. The plates were developed in an equilibriated T.L.C. tank and took approximately 1 hour to develop. At the completion of development, the T.L.C. plates

A Typical TLC Plate

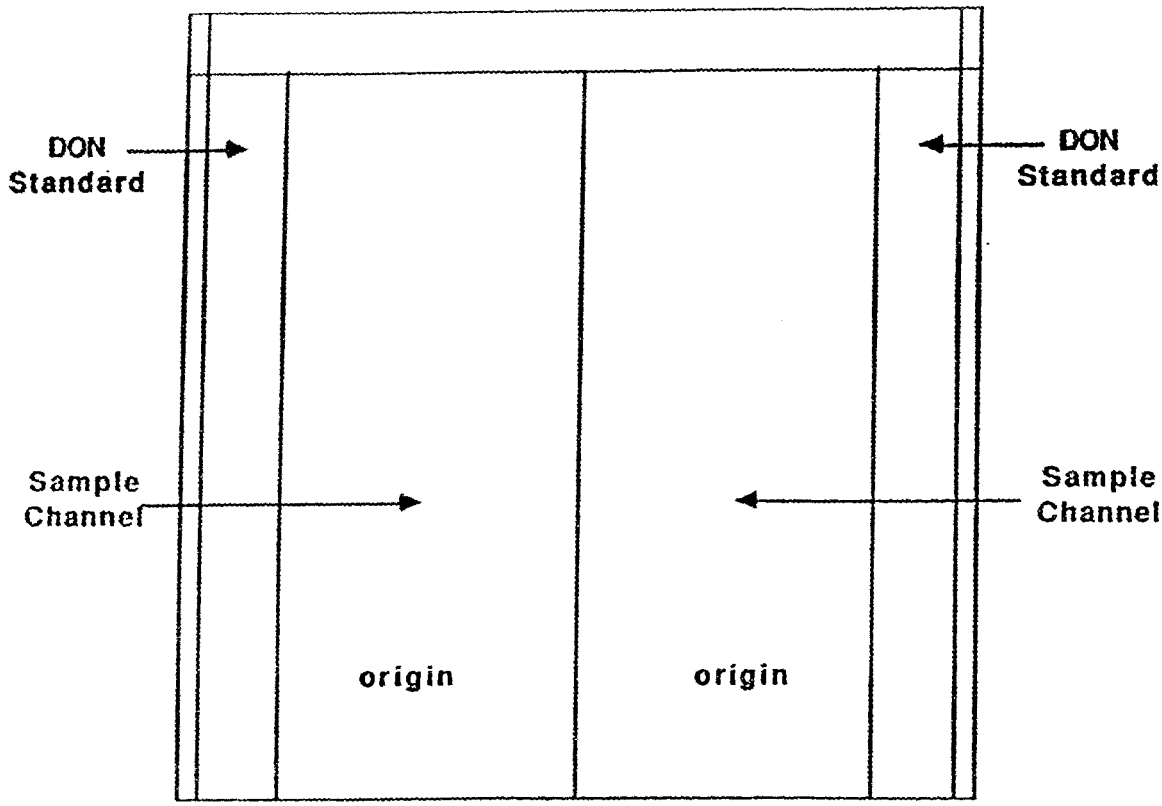


Figure 5

were air-dried in a fume hood and then examined in a UV viewing cabinet using UV long wave radiation (365 nm). DON was observed as a spot of blue fluorescence approximately 10 cm from the origin. All plates included two reference DON channels that were run concurrently.

(b) T.L.C. of Excreta Samples

These studies were carried out with excreta obtained from birds dosed orally with 5 mg DON/kg body weight. Air-dried excreta samples (50 mg) were rehydrated for 24 hours in 2 ml of sodium phosphate monobasic buffer (pH 5) prior to extraction in 15 ml screw-top glass vials. Blank excreta samples and samples from birds dosed with DON were extracted. The hydrated feces were extracted twice; initially with 5 ml of ethyl acetate followed by 5 ml of methylene chloride. After the addition of extraction solvent, the tubes were vortexed for 30 min, centrifuged (10 min at 1500 rpm) and the supernatant removed. The supernatant from both extractions was combined, dried slowly using a gentle flow of N_2 and a heating block (30°C). The residues were redissolved with 200μL of ethyl acetate:methanol (50:50) and spotted (100μL) onto the plates. Each TLC plate contained two DON standards. After the spotted areas on the plates were dried and the plates were developed in the T.L.C. solvent system described previously, the plates were examined under UV radiation (365 nm). These studies revealed two major zones of fluorescence on the T.L.C. plate, one approximately 5 cm from the origin, the other at approximately 10 cm.

These studies were performed with the following extraction solvents: (a) ethyl acetate and methylene chloride

(b) acetone

(c) acetonitrile, and

(d) methanol and ethyl acetate

Ethyl acetate and methylene chloride were found most efficient in extracting the radioactivity from the excreta.

(c) T.L.C. (Excreta Samples Containing ^{14}C-DON)

These studies were carried out with excreta samples (50 mg) obtained from turkey dosed with 5 mg/kg which included 0.5μCi ^{14}C-DON/kg. The T.L.C. plates were developed as outlined in section (b) and were then scanned to observe areas of radioactivity. The T.L.C. plate showed two areas of radioactivity - one corresponding to DON, the other an area containing an unidentified metabolite(s). Autoradiography of these same plates revealed three bands of radioactivity; the origin, a zone 5 cm from origin, and a zone 10 cm from origin. UV scanning of the plate established DON at 10 cm from the origin.

(d) T.L.C. - Analysis of Enzyme-Treated Excreta

In preparation for the enzymatic studies, air-dried excreta samples (200 mg) were rehydrated in 2 ml of sodium phosphate monobasic buffer (pH 5) in 15 ml screw-top vials for 24 hours.

β-glucuronidase (2000 units) was added to the rehydrated excreta sample and incubated at 37°C for 6 hours. A second rehydrated excreta sample which did not contain the enzyme was subjected to the

same incubation conditions. At the end of the incubation period, samples were extracted and analyzed by the T.L.C. methods outlined in section (b). After development, the T.L.C. plates were scanned for radioactivity and autoradiographed. Each vertical channel was sequentially divided into 1 cm wide horizontal strips, starting at the origin. The silica scraped from the 1 cm strips was added into a pre-washed scintered glass filter sitting on top of a 15 ml screw-top vial and washed with 13 ml of methanol. Aliquots of the methanol were used for HPLC analysis and L.S.C. For analysis of DON, 0.5 ml of the methanol eluant was dried down in HPLC vials using N_2 gas and then HPLC solvent (1 ml) was added to the vials. Standard procedures for HPLC analysis of DON were then followed.

For L.S.C. analysis of aliquots (2 ml) of the methanol eluant from each strip were pipetted into scintillation vials, air-dried, and Biofluor (15 ml) added. The prepared samples were then counted to determine the quantity of radioactivity using an automatic L.S. counter, as outlined previously.

Mass spectral analysis of eluant containing metabolite(s) from strips 4 to 7 was performed (no record of conditions utilized).

§ RESULTS

Recovery Study of DON from Whole Blood - HPLC Analysis

A recovery study was carried out with pure crystalline DON dissolved in a solution of methanol:water (9:91). Various concentrations of DON (Table 1) were prepared to facilitate the addition of 25μL of DON to the blood samples (975μL). The spiked blood samples were then mixed, extracted and analyzed by HPLC according to the methods outlined earlier. The results indicated that recovery of DON from whole blood samples was greater than 87%.

A repeatability study was initiated to determine the accuracy of the HPLC technique. The blood samples (1 ml) from two birds were split into two equal proportions (0.5 ml), extracted and analyzed according to the procedures outlined previously. The analysis of the samples containing trace concentrations of DON/ml of blood show an acceptable level of variation since the analysis of the paired samples was similar (Table 2).

Table 1

Recovery of DON from Spiked Blood Samples Prepared for HPLC Analysis

Concentration of Spiked Blood (ng DON/ml of Whole Blood	Final Volume (cc)	Injection Volume (μL)	Amount of DON Injected (ng)	Peak Height of (cm)	Peak Height of DON Standard (cm)	% Recovery
1000	4	100	25	5.50	5.6	98
500	2	100	25	5.10	5.6	91
1000	1	100	100	19.4	22.0	88
500	1	100	50	9.1	10.5	87
250	1	100	20	4.05	4.20	96
125	1	100	12.5	2.16	2.25	95
50	1	100	5	1.0	1.1	90

Table 2

Repeatability Study of HPLC Analysis Using Split Blood Samples
(dose - 5 mg DON/kg - orally)

Time (min)	Study 1 (ng DON/ml of Blood)		Study 2 (ng DON/ml of Blood)	
	Analysis 1(ng)	Analysis 2(ng)	Analysis 1(ng)	Analysis 1(ng)
0	0	0	0	0
30	15	20	9	6
60	36	40	18	20
90	29	28	41	43
120	17	22	27	25
150	13	18	19	17
180	12	17	18	19
210	7	11	20	17
240	4	8	12	14
270	2	7	9	9
300	0	5	9	8
330	0	5	9	4
360	4	9	7	4
420	2	7	0	1

To determine the accuracy of L.S.C. a study was undertaken comparing the results of L.S.C. and HPLC analysis of split blood samples containing DON. The HPLC analysis is specific for DON only, whereas L.S.C. counting measures total radioactivity which can originate from unchanged DON and/or DON metabolites. Therefore, it is necessary to investigate the accuracy of the DON blood concentration determined by L.S.C. The accuracy of analysis is determined by comparing the results obtained by the two methods of analysis (Table 3).

The data in Table 3 is derived from two separate studies. The radioactivity measurements of blood DON (L.S.C.) are similar to the results determined by HPLC analysis indicating that L.S.C. of blood samples for DON concentrations under these circumstances, is an appropriate technique. This study indicates that little or no metabolites are present in the blood in the initial 60 minutes. Therefore, L.S.C. data for the time period studied, provides a good estimation for the DON profile in blood.

Pharmacokinetic Data

The blood DON concentrations versus time (C-T) profile for the turkeys dosed intravenously are shown in Figure 6, 7 and 8. The semilogarithmic plots of the declining DON concentrations follow a

Table 3

Comparison of Blood DON Values Utilizing Two Methods of Analysis - HPLC and L.S.C.

dose - (0.5 μCi/kg - ^{14}C-DON, 5 mg DON/kg)

Sample Time (min)	HPLC Analysis (ng DON/ml of Blood)	L.S.C. Analysis (ng DON/ml of Blood)	% Change of Averages
5	5557 $\pm$ 123.5	5571 $\pm$ 352	0.25
15	2068 $\pm$ 103	1897 $\pm$ 366	8.26
60	636 $\pm$ 61	655 $\pm$ 109	2.90

*HPLC - $\pm$ S.D. four samples analyzed/unit time

*L.S.C. - $\pm$ S.D. for 10 samples analyzed/unit time

Figure 6

Blood concentration of DON in Turkeys
Following single intravenous administration of 1 mg of DON/kg

(HPLC analysis)

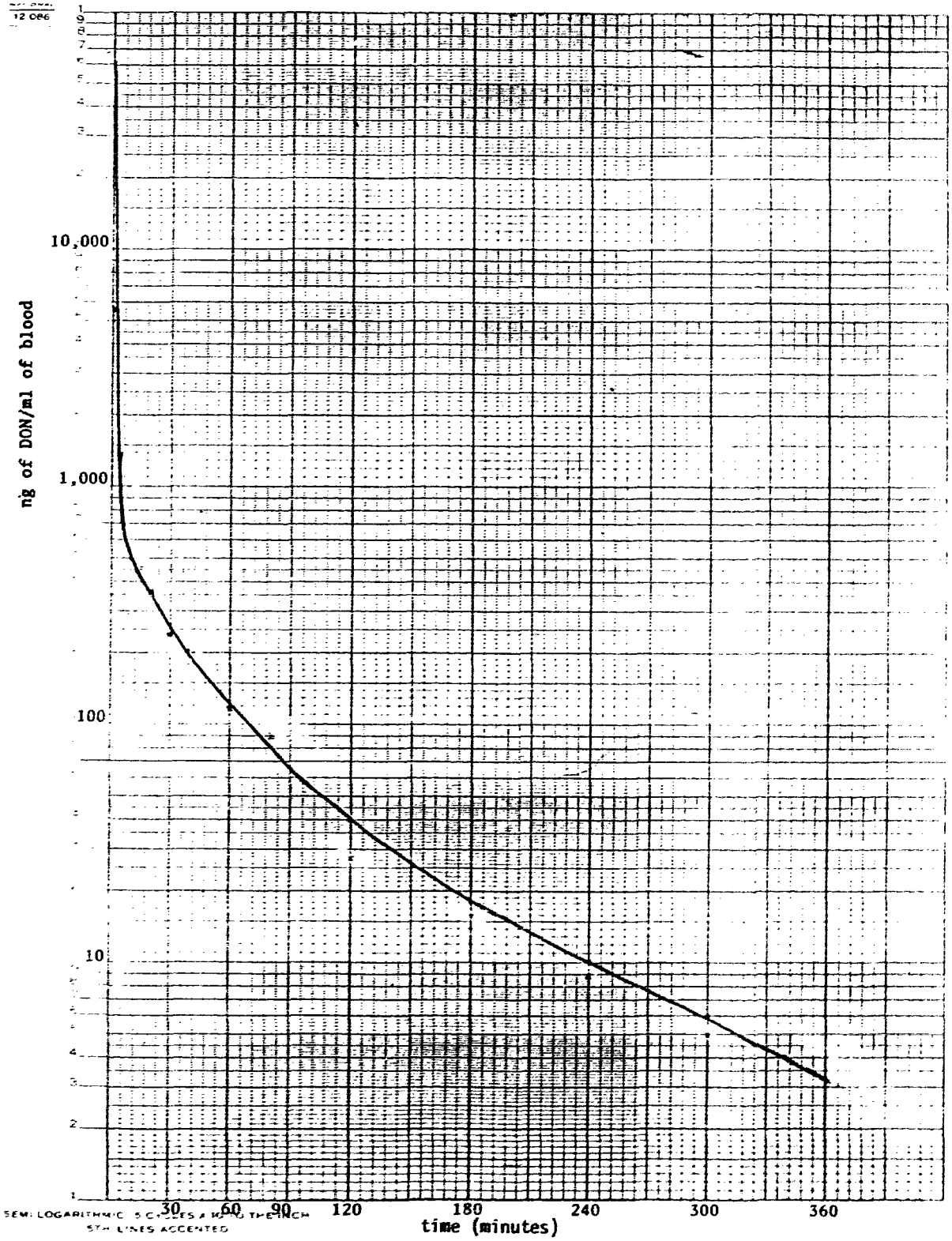


Figure 7

Blood concentration of DON in Turkeys Following single intravenous administration of $0.5\mu Ci/kg$ ^{14}C-DON, total dose DON - 1 mg/kg

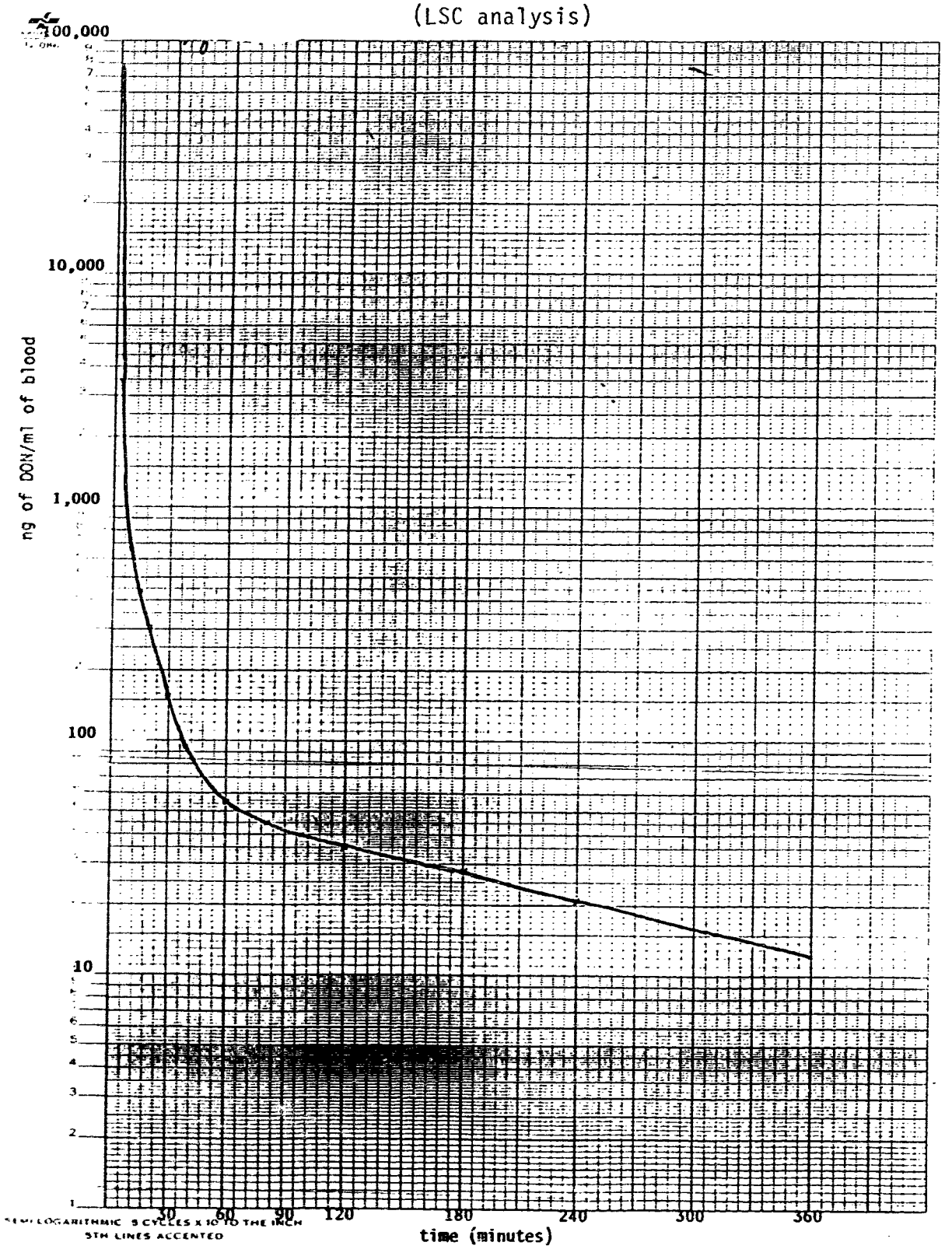
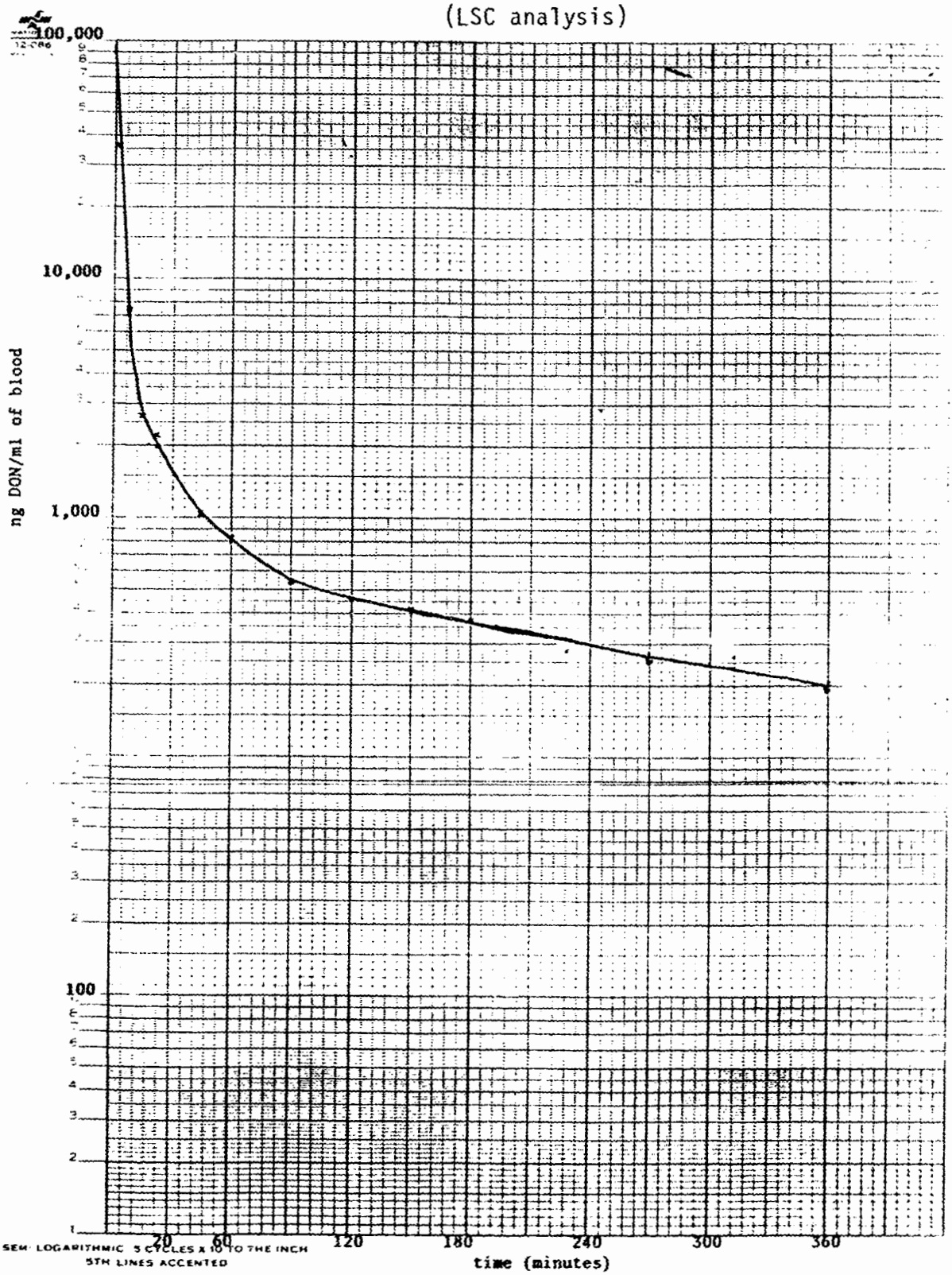


Figure 8

Blood concentration of DON in Turkeys Following single intravenous administration of 0.5 μCi ^{14}C-DON. total dose of DON - 5 mg/kg

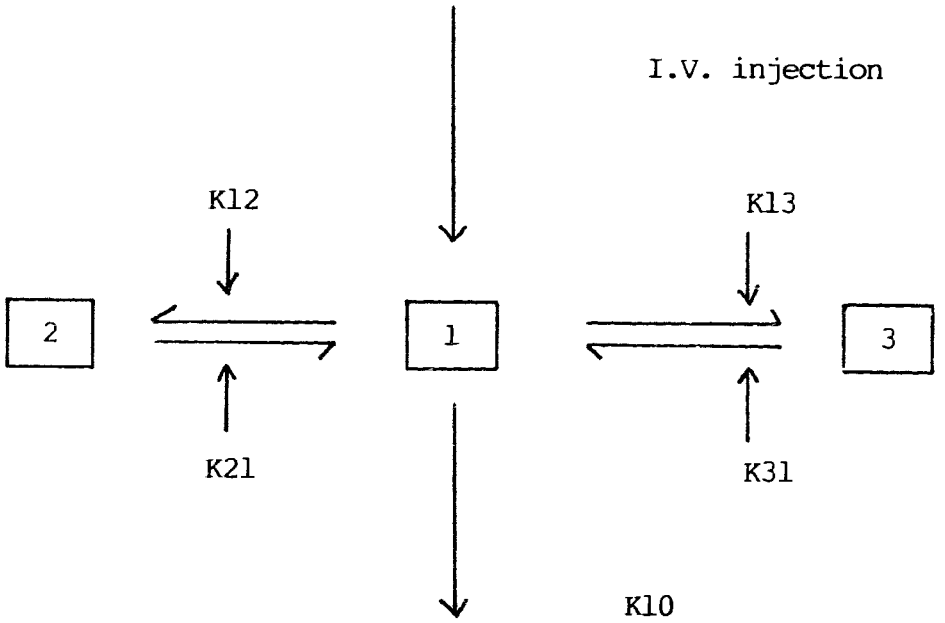


triexponential profile best described by a three-compartment open toxicokinetic model. The C-T profiles for I.V. and oral studies represent the best fitted computer model of the data. The empirical data was calculated using the means of each group of birds, either three or six (see appendix 1).

Figure 9 illustrates an open three-compartment toxicokinetic model described by the I.V. disposition of DON in turkeys.

Figure 9

Three-Compartment Toxicokinetic Model



In utilizing the model it should be noted that the intravenous injection results in DON entering the central compartment (1) and a number of rate constants (K) describe the distribution, redistribution, and elimination of DON into and out of the other compartments (2 and 3) of the model.

Analysis of the blood C-T curves indicates there is a rapid two phase distribution phase (α,β) followed by a slower prolonged elimination phase (γ).

For the high dose I.V. study (5 mg DON/kg) (Fig 8), the following kinetic values were obtained (Table 6) t 1/2α - 0.84 min t 1/2β - 9.31 min and t 1/2γ - 141.63 min respectively. For the low dose I.V. study (Table 4), based on HPLC analysis, the distribution figures are t 1/2α - 0.44 min, t 1/2β - 5.27 min and elimination half-life (t 1/2γ) is 44.35 minutes. Low plasma levels of DON were detectable at 6 hours post-dosing, the time period for which sequential blood samples were taken. Blood samples collected at 12 hours post-dosing did not show any measurable amounts of DON. The high dose I.V. data was determined entirely by L.S.C. counting of samples (6 birds) and the low dose data was derived by a combination of techniques, L.S.C. for 3 birds and HPLC analysis of blood for 3 birds. Previous studies indicated that the blood radioactivity counted essentially is measuring unchanged DON for the initial 60 minutes of the experiment.

The toxicokinetic parameters for both I.V. dosages are given in Tables 4 (1 mg/kg), 5 (1 mg/kg) and 6 (5 mg/kg).

Table 4

Pharmacokinetic Parameters of DON in Turkeys

1.0 mg/kg I.V. dose of DON - average values for 3 turkeys - HPLC

DON

A (ng/ml)	167,999.0
B (ng/ml)	2,079.9
C (ng/ml)	299.0
α ($min.^{-1}$)	1.55
β ($min.^{-1}$)	0.13
γ ($min.^{-1}$)	0.01
t 1/2α (min.)	0.44
t 1/2 β (min.)	5.27
t 1/2 γ (min.)	44.35
AUC ($ng/min.\ ml^{-1}$)	143,045.0
V_{dss}	0.34 L/kg
$Cl_{(p)}$	6.8 ml $min.^{-1}kg^{-1}$

Table 5

Pharmacokinetic Parameters of DON in Turkeys

1.0 mg/kg I.V. dose of DON - average values for 3 turkeys - LSC

DON

A ($ng\ ml^{-1}$)	603,544.54
B ($ng\ ml^{-1}$)	1,459.66
C ($ng\ ml^{-1}$)	63.54
α ($min.^{-1}$)	1.49
β ($min.^{-1}$)	0.08
γ ($min.^{-1}$)	0.00
t 1/2α (min.)	0.46
t 1/2 β	7.81
t 1/2 γ	152.44
AUC	434,296.4
$V_{d(ss)}$	0.192 l/kg
Cl(p)	2.3 ml $min^{-1}kg^{-1}$

Table 6

Pharmacokinetic Parameters of DON in Turkeys

5 mg/kg I.V. dose of DON - average values for 6 birds - LSC

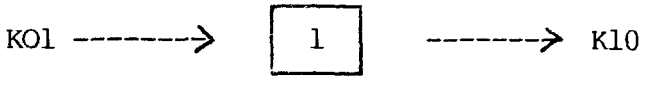
DON Parameter	Value
A (ng ml^{-1})	241,338.53
B (ng ml^{-1})	4,169.50
C (ng ml^{-1})	666.7
α $min.^{-1}$	0.81
β $min.^{-1}$	0.07
γ $min.^{-1}$	0.07
t 1/2α (min.)	0.84
t 1/2β (min.)	9.31
t 1/2γ (min.)	141.63
AUC (ng min ml^{-1})	487,020.32
$V_{d(ss)}$	0.66 l/kg
Cl	10.41 ml $min^{-1}kg^{-1}$

The low dose I.V. study (1 mg/kg) data is presented as two separate tables reflecting two methods of analysis of the samples HPLC, (3 birds) and LSC (3 birds). Although both analytical methods resulted in a three-compartment model, the rate constants of the terminal phase of the blood C-T curve are very different; the C-T curve decline is faster in HPLC. The HPLC measures unchanged DON and LSC measures unchanged DON + metabolite.

The DON blood concentration versus time profiles for oral studies are shown by Figures 11 (1 mg/kg) low dose study and 12 (5 mg/kg) high dose study. This data best fits a one-compartment model as illustrated below (Figure 10).

Figure 10

One-Compartment Toxicokinetic Model



The DON plasma profile observed has two components, an absorptive phase followed by a monoexponential phase of declining plasma DON levels. Table 7 and 8 give the toxicokinetic parameters calculated from the profile data collected.

Figure 11

Blood concentration of DON in Turkeys
Following single oral administration of 1 mg DON/kg

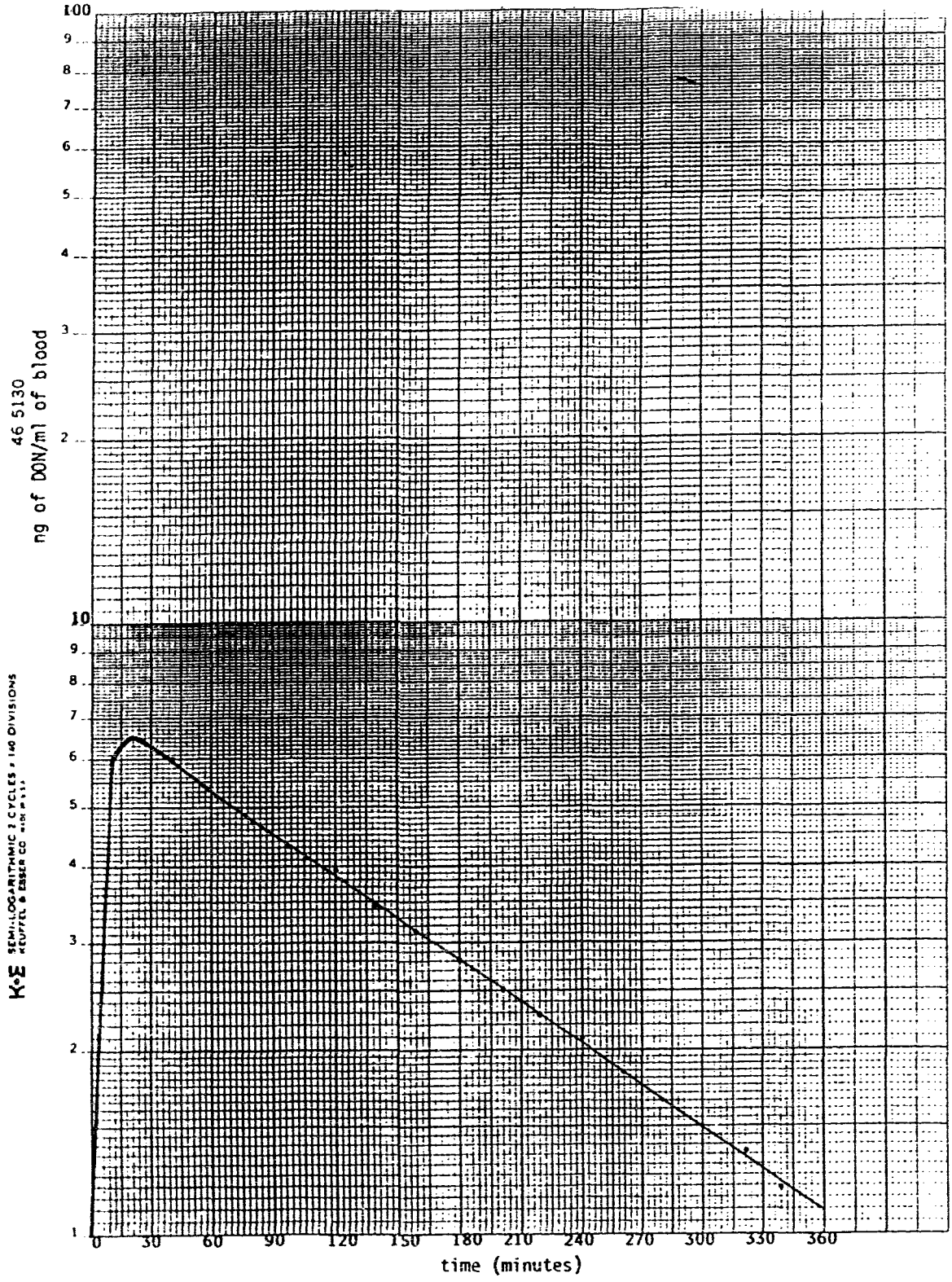


Figure 12

Blood concentration of DON in turkeys
Following single oral administration of 5 mg DON/kg

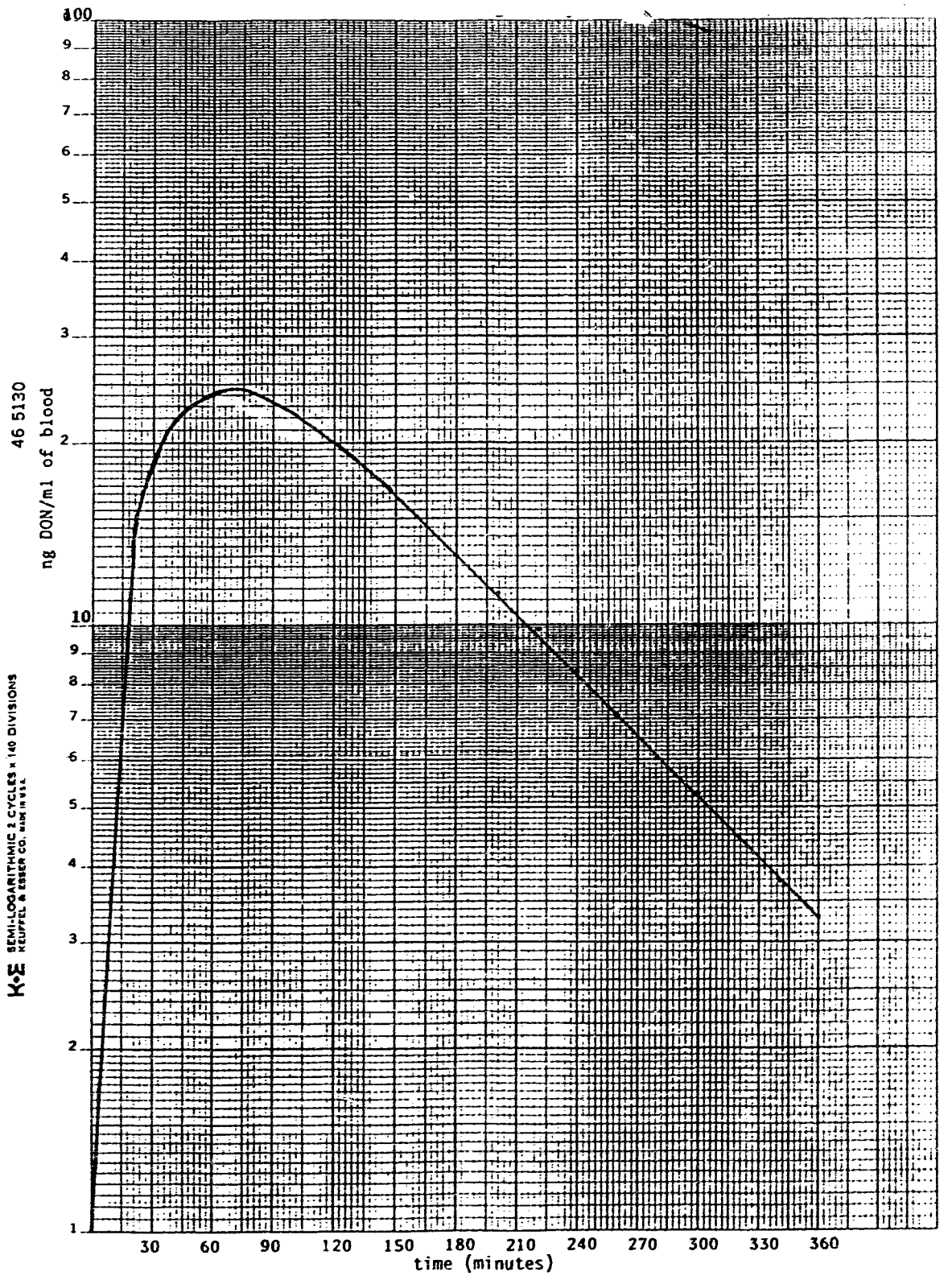


Table 7

Pharmacokinetic Parameters of DON in Turkeys (HPLC)

1 mg/kg oral dose of DON - average values for 6 birds

*Volume (l/kg)	.20
*K O → 1 (α)	0.42
K 1 → O (β)	0.005
T(lag) min	1.61
*AUC (ng min ml^{-1})	1379.18
*t 1/2 - K O → 1 (min.)	1.62
t 1/2 - K 1 → O (min.)	131.85
*t max. min.	10.43
*C max. (ng ml^{-1})	6.86

*Large Standard Error

Table 8

Pharmacokinetic Parameters of DON in Turkeys (HPLC)

5 mg/kg oral dose of DON - average values for 6 birds

Volume (L/kg)	.19
K O → 1 (α)	0.029
K 1 → O (β)	0.0076
T(lag) min	3.59
AUC (ng min ml^{-1})	5025.92
t 1/2 K O → 1 (min.)	23.46
t 1/2 K I → O (min.)	90.65
t max. min.	65.36
C max. (ng ml^{-1})	23.96

Absorption of the orally administered dose was rapid with maximum blood levels occurring within 65 minutes of dosing. A monoexponential profile characterized the elimination process with (t 1/2 for 5 mg DON/kg being 90.65 minutes and t 1/2 for 1 mg DON/kg being 131.8 minutes). Only low levels of blood DON were detectable after oral administration C (max.) - 23.9 ng for the high dose study and 6.8 ng for the low dose study. Bioavailability of DON to turkeys based on the comparison of the low dose intravenous and oral AUC calculations was 0.96%. Similarly, bioavailability calculated by comparison of the high dose intravenous and oral AUC figures is 1.04%.

From the oral studies, the lag time absorption observed was 1.6 minutes in the low dose study to 3.6 minutes in the high dose study. This short period of lag time between dosing and when blood concentration of DON began to increase to measurable levels and occurs as a result of the birds being dosed into the crop. The crop is a storage compartment of the digestive system and does not have an absorption function. The lag time of absorption reflects the transit time it takes the DON to pass from the crop, through the gizzard and into the proventriculus where absorption begins.

The calculated volumes of distribution Vd_{β} from the low and high dose oral studies were quite similar (0.195 - 0.206 L/kg). The results from the low dose oral study were very near the limits of detection of the HPLC analytical technique but the similarities of the volume of distribution helps confirm the accuracy of the low dose

data. Although representative of no specific physiological compartment in the body, it does indicate the DON was confined to cardiovascular and extracellular water fluid compartment.

Excretion Data

The elimination of DON from turkeys was studied using ^{14}C-DON. The amount of ^{14}C-DON in the excreta and urine was measured over time. Three separate studies were utilized to determine the elimination profile. In the first study (Table 9) the quantity of radioactivity present in the excreta, when dosed intravenously was determined. These values include radioactivity eliminated in both the urine and feces. In the second study (Table 10), the quantity of radioactivity eliminated through the kidneys and excreted in the urine was determined. The birds were dosed intravenously and had both ureters catheterized which provided a means of physically separating the urine and feces. In the third study (Table 11), the birds had been catheterized to facilitate urine collection but were dosed orally. The radioactivity present in the urine was determined.

Table 9

Elimination of Radioactivity in Excreta

dose - (5 mg DON/kg - I.V.)

($0.5\,\mu$Ci - ^{14}C-DON/kg)

Time Period (hours)	% Dose Excreted	Cumulative % Excreted
0 - 3	75.2	75.2
3 - 6	6.2	81.4
6 - 12	9.2	90.6
22 - 24	5.2	95.8
24 - 36	1.0	96.8
36 - 48	.2	97.0
48 - 72	.3	97.3

*4-5 birds/unit time

*excreta samples counted in triplicate

Table 10

Elimination of Radioactivity in Urine

dose - 1μCi - ^{14}C-DON I.V. (0.77 mg)

Time Period (hours)	% Dose Excreted	Cumulative % Excreted
0 - .33	19.5	19.5
.33 - 1.0	39.3	58.8
1.0 - 2.0	7.0	65.8
2.0 - 3.0	2.5	68.3
3.0 - 4.0	3.3	71.6
4.0 - 5.0	1.4	73.0
5.0 - 6.0	1.4	74.4
6.0 - 18.0	.2	74.6

- data collected from two birds

- each sample counted in triplicate

Table 11

Elimination of Radioactivity in Urine

dose - 1 μCi - ^{14}C-DON Orally (0.77 mg)

Time Period (hours)	% Dose Excreted	Cumulative % Excreted
0 - .33	0	0
.33 - 1.0	.16	.16
1.0 - 2.0	.67	.83
2.0 - 3.0	.44	1.27
3.0 - 4.0	.20	1.47
4.0 - 5.0	.15	1.62
5.0 - 6.0	.14	1.76
6.0 - 18.0	.06	1.82

- data collected from two birds

- each sample counted in triplicate

A large proportion (75%) of radioactivity is found in the excreta within 3 hours and approximately 90% of the dose was eliminated by 12 hours. At the end of the study period (72 hours) 97.3% of the radioactivity had been accounted for in the excreta.

Excreta in birds is a mixture of wastes eliminated by the urinary system and the digestive system. To more accurately determine the quantity of radioactivity eliminated from each system it was necessary to collect the urine separate from the feces. Birds that had the ureters catheterized, allowing for the collection of urine separately from the feces, eliminated a large proportion of the radioactivity into the urine when dosed intravenously (Table 10). Within 0.33 hours of injection 19.5% of the dose could be accounted for in the urine and this value rapidly increased 58.8% of radioactivity in 1 hour and 68.3% in 3 hours. In total approximately 74.6% of the total dose administered was accounted for in the urine. When the catheterized birds were dosed orally (1 μCi of DON) only a small portion of the radioactivity could be accounted for in the urine (Table 11). At the end of 0.33 hours, no radioactivity was measured; at 1 hour, 1.6% of the total dose was measured in the urine; at the end of 18.0 hours only 1.82% of the total radioactivity administered was accounted for in the urine.

Metabolism Data

Preliminary T.L.C. work established a T.L.C. technique which

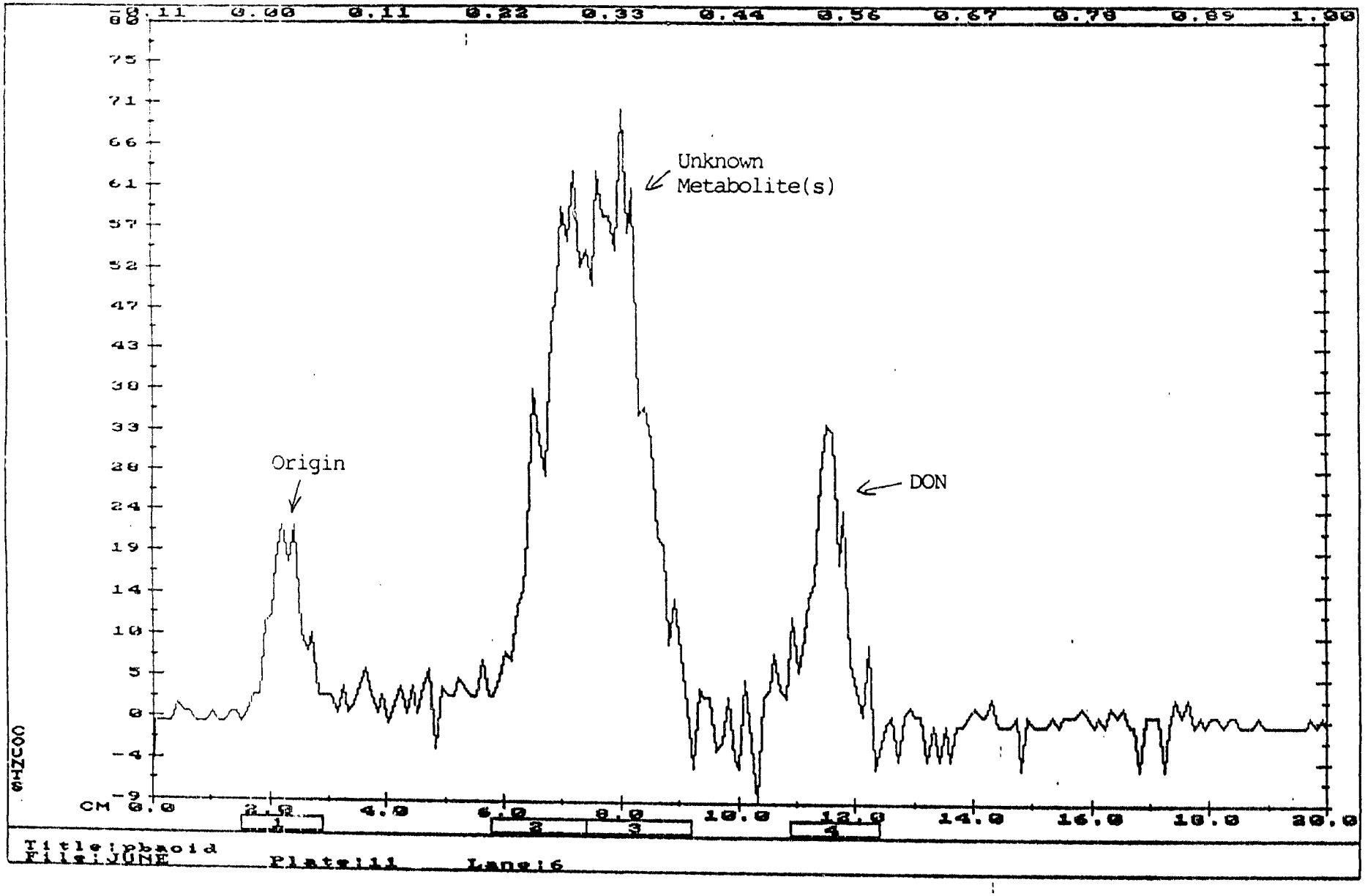
eluted DON a distance of approximately 10 cm from the origin (R_f - 0.67) in the developed plate. Further T.L.C. studies using extracts from excreta of birds dosed with ^{14}C-DON produced a zone of fluorescence and radioactivity beginning 5 cm from the origin (R_f - 0.33). This zone was fairly broad and could cover several centimeters (Fig 13). The zone with an R_f value of 0.33 was interpreted as a region of unknown metabolite(s) of DON since the only source of radioactivity available to turkeys was the ^{14}C-DON administered to them. Autoradiography of urine samples subjected to T.L.C. revealed three zones of radioactivity - zone 1 at the origin, zone 2 at approximately 5 cm from the origin corresponding to unknown metabolites, and zone 3 at 10 cm from the origin which corresponds to ^{14}C-DON (autoradiograph 1). L.S.C. of eluant from 1 cm horizontal strips revealed levels of radioactivity which corresponded to the visual observation seen in the autoradiography results, and HPLC analysis of these eluants established that only the radioactivity at 10 cm on the T.L.C. plate is associated with the parent toxin, DON (Table 12).

Studies were undertaken to chemically characterize the unknown metabolite(s). Samples of excreta extract subjected to enzymatic hydrolysis (6 hours) by β-glucuronidase and sulphatase did not show substantial observable differences from non-hydrolyzed sample. (Autoradiograph 2).

In another study acid treatment of excreta extract using a variety of conditions (Table 13) to attempt to hydrolyze possible

Figure 13

Typical TLC Radioactivity Scan - Urine TLC



Autoradiograph 1

TLC of Urine Sample

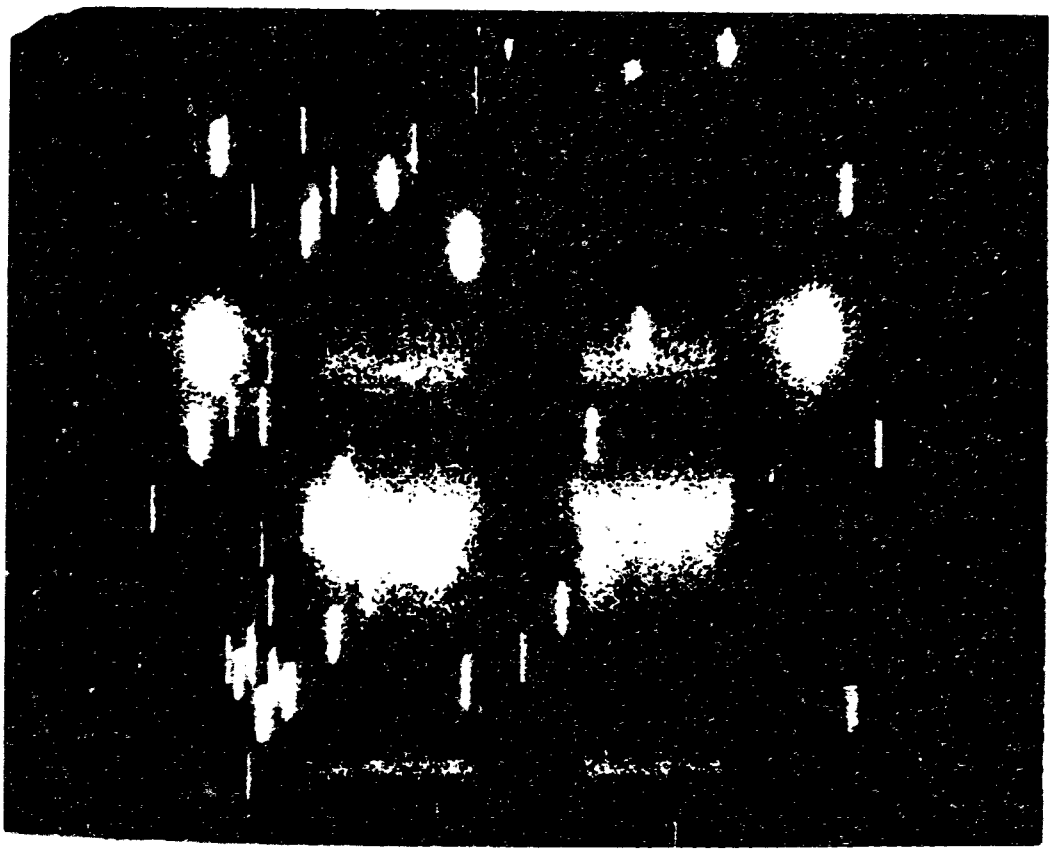


Table 12

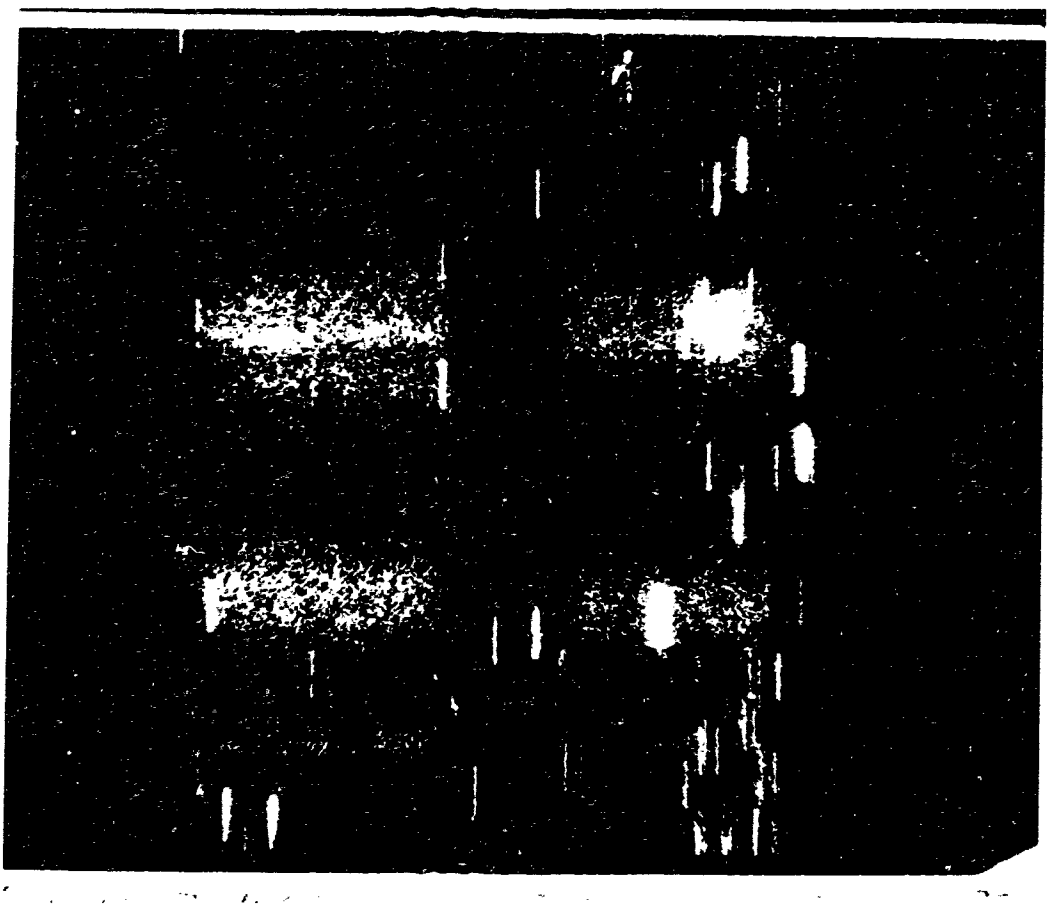
Analysis of T.L.C. Plate Extracts

T.L.C. Strip	L.S.C. DPM/μl of Extract	HPLC (ng/100 μl of Extract)
1	67	0
2	23	0
3	55	0
4	212	0
5	277	0
6	315	0
7	300	0
8	42	0
9	19	0
10	22	0
11	168	5.2 ng
12	96	0
13	14	0
14	12	0
15	13	0
16	7	0

- analysis of strips begins at the origin

Autoradiograph 2

Enzymatic Hydrolysis of Metabolite(s)



Column 1
Non-hydrolysed extract

Column 2
Hydrolysed extract

conjugated metabolites resulted in partial hydrolysis in the region of unknown metabolite(s) (autoradiograph 3) but also decomposed the free DON.

Table 13

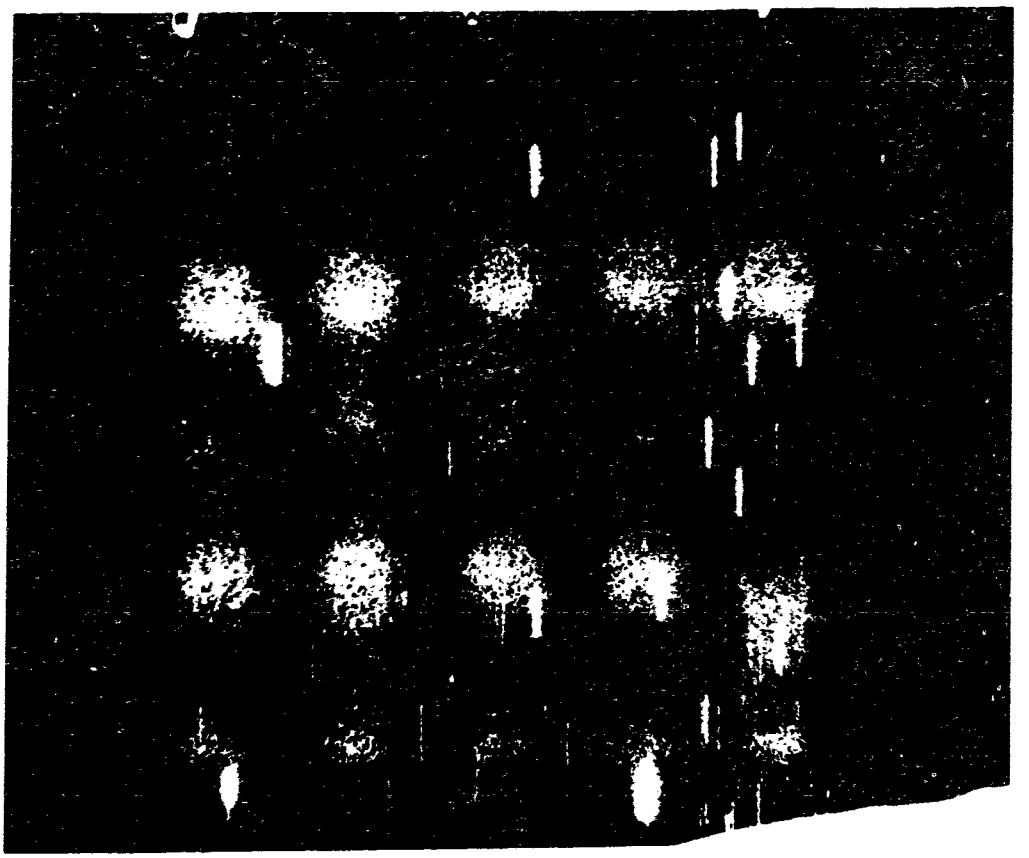
Conditions Used for Acid Hydrolysis of Metabolite

Sample 1	- control	- only extracted
2	- control	pH 8 - no heat - extracted
3		pH 1 - heated 30 min. at 60°C
4		pH 1 - heated 120 min. at 60°C
5		pH 1 - heated 120 min. at 90°C

Radioactivity scanning of excreta samples subject to β-glucuronidase hydrolysis for varying lengths of time (2, 3, 4, 6, 18 hours) and varying quantities of enzyme (2000, 4000, 6000 units) did not appear to change the amount of radioactivity found in the region of unknown metabolite(s) when compared to non-hydrolyzed samples. Furthermore, enzymatic hydrolysis studies of putative conjugate metabolites using blood samples drawn from turkeys at 5, 15 and 60 minutes (Table 14) revealed slightly but not significantly higher levels of DON in the hydrolyzed samples. Mass spectrometry analysis of samples of unknown metabolite(s) obtained from the scrapings from the T.L.C. plates was unsuccessful due to the presence of interferring ocmpounds. Attempts to utilize the HPLC column as a means of separating and purifying the unknown metabolite(s) demonstrated that the metabolite(s) were very polar compounds. No separation or purification of compounds could be achieved and mass spectral analysis of unknown metabolite(s) samples obtained after passing through the HPLC column were unrewarding.

Autoradiograph 3

Acid Hydrolysis of Metabolites



Column 1 2 3 4 5

(see table 13)

Table 14

Lack of Effect on β-glucuronidase on DON levels in Blood

Time (min.)	Non-Hydrolyzed (ng DON/ml)	Hydrolyzed (ng DON/ml)
5	4355 ± 137.9	4435 ± 70
15	1467 ± 22.1	1527 ± 52.7
60	489.8 ± 22.8	556.5 ± 13.9

* ± S.D. four samples analyzed (HPLC) per unit time

Tissue Residue Study

To determine if there are significant tissue residues (DON) or its metabolites accumulating in the various tissues of the turkeys, two residue studies were carried out. In the high dose study the total dose of DON administered was 5 mg/kg including 0.5μCi ^{14}C-DON/kg; and in the low dose study 1 mg/kg including 0.5μCi ^{14}C-DON/kg. The various tissues were analyzed for total radioactivity. The distribution and elimination of DON or its metabolites is shown in Table 15 (1 mg DON/kg) and Table 16 (5 mg DON/kg). By the first sampling time (0.75 hours) radioactivity was found in all the tissues analyzed and had reached its maximum level except for the brain, kidney, liver, abdominal fat, subcutaneous fat, and bile. These tissues all showed peak levels of radioactivity by 1.5 hours post dosing. Once maximum radioactivity levels had been reached in the tissues, there was a rapid, steady decline of tissue radioactivity with the exception being the bile levels following the high dose which fluctuated over time. The tissues with the highest levels of radioactivity were the kidney, liver, spleen, and adrenal with the exception of the gastrointestinal tract which reflected the effects of oral dosing. The elimination of radioactivity from the tissues was rapid with most tissues being essentially cleared by 6 hours (1 mg/kg) or 24 hours for the 5 mg DON/kg birds. Those tissues still containing significant levels of radioactivity at this time period were kidney, liver, spleen, adrenal and small intestine.

TABLE 15

Elimination Rates of Radiocarbon in Turkey Poults Following a single dose of ^{14}C-DON

LOW DOSE STUDY -1 MG/KG TOTAL DON
.5 µCI - ^{14}C-DON/KG

	TIME (MIN.)							ELIMINATION KINETICS	
	.75 HRS.	1.5 HRS.	3 HRS.	6 HRS.	12 HRS.	24 HRS.	72 HRS.	K_{el} (Hour -1)	$t_{1/2}$ (hr.)
	Specific Radioactivity (ng Equivalents DON/gr. Wet Tissue)								
Breast Muscle	145± 318	122± 41	94± 20	38± 32	33± 11	18± 10	4± 6	0.0326	21.251
Thigh Muscle	173± 835	125± 336	99± 19	34± 18	25± 22	9± 3	2± 5	0.03485	19.881
Spleen	253± 114	147± 22	124± 28	94± 33	47± 10	24± 12	12± 3	0.01264	54.835
Brain	55± 22	85± 89	88± 23	34± 28	49± 9	20± 7	5± 9	0.026029	19.568
Proventriculus	198± 358	159± 285	99± 15	52± 25	38± 16	61± 41	10± 9	0.023371	29.658
Gizzard	221± 173	127± 352	80± 10	53± 16	37± 17	19± 9	15± 7	0.0091196	76.108
Kidney	1556± 584	9352± 2734	525± 104	207± 111	158± 68	66± 42	16± 10	0.033859	20.472
Liver	508± 1848	292± 944	212± 59	91± 31	65± 23	34± 14	23± 11	0.012235	56.855
Adrenal	296± 1405	189± 621	144± 48	71± 50	98± 32	43± 21	12± 10	0.02864	24.205
Heart	167± 16	94± 102	80± 16	45± 31	53± 14	24± 9		0.041338	16.763
Abdominal Fat	172± 305	219± 907	170± 34	101± 37	118± 20	64± 31	3± 5	0.056912	12.179
Subcutaneous Fat	167± 532	205± 75	164± 42	80± 44	65± 17	29± 14	1± 2	0.067451	10.276
Bile	24,416± 10,441	57,983± 6759	62,968± 16,020	33,639± 8934	8071± 7,870	2385± 1415	292± 158	0.10446	6.636
Small Intestine	501± 107	546± 142	391± 139	163± 64	66± 20	136± 115	14± 12	0.026887	25.893

* 4-5 birds/unit time

* mean ± S.D.

TABLE 16

Elimination Rates of Radiocarbon in Turkey Poults Following a single intravenous dose of ^{14}C-DON
HIGH DOSE STUDY - 5.4MG/KG TOTAL DON
0.5 µCi - ^{14}C-DON/KG

	TIME (MIN.)							ELIMINATION KINETICS	
	.75 HRS.	1.5 HRS.	3 HRS.	6 HRS.	12 HRS.	24 HRS.	72 HRS.	K_{el} (Hour $^{-1}$)	$t_{1/2}$ (hr.)
	Specific Radioactivity (ng Equivalents DON/gr. Wet Tissue)								
Breast Muscle	1567± 515	2004± 331	589± 249	103± 57	87± 14	41± 14	14± 9	0.0252	27.511
Thigh Muscle	2113± 482	1587± 347	764± 173	127± 78	71± 15	34± 4	14± 17	0.02206	31.422
Spleen	2279± 447	1683± 512	786± 32	398± 175	168± 26	48± 17	32± 27	0.00881	78.716
Brain	488± 123	699± 49	309± 49	119± 51	79± 55	33± 11	32± 5	0.00157	43.768
Proventriculus	1616± 344	2566± 629	1081± 269	154± 60	117± 38	67± 27	49± 22	0.00916	75.65
Gizzard	2275± 245	1877± 318	1220± 189	301± 85	283± 79	73± 13	20± 9	0.031687	21.96
Kidney	6744± 904	13008± 1861	3254± 452	912± 334	516± 198	278± 64	115± 24	0.02078	33.357
Liver	3362± 1020	3806± 144	1537± 273	459± 75	341± 65	135± 38	87± 21	0.01342	51.641
Adrenal	1344± 326	3742± 1509	2356± 658	156± 60	249± 90	46± 26	77± 33	0.085476	8.109
Heart	1988± 539	1332± 253	429± 60	124± 52	106± 35	44± 17	37± 6	0.010531	65.823
Abdominal Fat	850± 319	1451± 137	400± 125	178± 88	130± 54	101± 48	71± 46	0.008288	83.633
Subcutaneous Fat	1036± 177	1132± 350	407± 68	226± 61	230± 75	154± 43	50± 10	0.022957	30.194
Bile	275,621± 89,985	94,080± 24,576	91,802± 72,889	33,988± 12,114	118,150± 17,475	109,345± 68,553	1481± 1077		
Small Intestine	4389± 943	5184± 1008	2355± 513	366± 127	164± 63	123± 56	59± 23	0.015088	45.942

* 4-5 birds/unit time
* Mean ± S.D.

DISCUSSION

For accurate blood DON concentration-time profiles and subsequent toxicokinetic analysis it is necessary to ensure that the blood DON can be accurately quantitated. In the initial stages of the project the use of purified DON (>97% pure) to establish HPLC analytical techniques was undertaken to ensure accuracy. In the recovery study pure DON solutions of known concentrations were added to blood samples. These samples were then extracted and analyzed according to established protocol to ensure accuracy of DON concentrations in the blood. The other method used for analysis of DON in the blood was LSC. LSC while simple, rapid and relatively accurate has the major disadvantage of not being able to distinguish between radiolabelled parent compounds and possible metabolites that may retain the radiolabel.

The low dose intravenous study analyzed by LSC illustrates the limitations of a technique which measures total radioactivity as a method of analysis for kinetic studies. Comparison of the C-T profile derived from HPLC analysis versus LSC analysis shows some differences concerning the prolonged elimination phase determined by LSC analysis. (Fig 6 and 7). In the C-T profile generated by HPLC analysis there is a fairly rapid decline of blood DON levels; with no DON detectable at approximately 6 hours. The C-T profile generated by LSC shows a prolonged elimination phase with trace quantities of DON (21 ng at 240 min., 16 ng at 300 min., and 12 ng at 360 min.) present towards the end of the sample collection period. One possible explanation for

this could be that the LSC technique is measuring both DON and DON metabolites together and therefore an elevated and kinetically inaccurate profile of DON would be generated. A second factor involved could be that basic insensitivity of the LSC technique in accurately assessing DON values at extremely low levels, levels very close to background levels (< 30 ng DON/ml).

In studying the raw data (Table 17) the blood DON concentration values derived from LSC in two of the three birds showed quite erratic blood concentrations at later sampling times, dropping to zero at 100-120 minutes before "rebounding" to 30-60 ng DON/ml of blood, 30 minutes later. While not discounting the possibility of DON metabolites resulting in the prolonged C-T value derived by LSC counting since the analysis of excreta do indicate the presence of metabolites, the ability of LSC to accurately measure the lower concentrations of DON should be considered as well. In the study undertaken to compare blood DON concentrations using HPLC analysis versus LSC, the results indicate the two methods of analysis were comparable in accuracy during the first hour of the study, and if there were ^{14}C-DON metabolites present up to that time, they were found in such small quantities as to not significantly affect the results. In addition when using ^{14}C-DON as a marker for the low dose I.V. study (1 mg DON/kg-0.5μCi ^{14}C-DON/kg) each dpm is equivalent to 1.6 ng of DON and variability in counts and therefore blood DON values will be magnified by this factor. Also, some of the increased variability in LSC arose from the procedure of counting whole blood instead of serum or plasma.

Table 17

Summary of Blood DON Concentration Data From Low Dose I.V. Study

(ng DON/ml of Blood)

	BIRDS I.D.					
Time (min)	**1***	**2***	**3***	**4#**	**5#**	**6#**
2.5	3,163	4,214	8,471	30,159	6,704	10,018
5	1,220	1,418	1,401	807	1,510	1,708
10	970	725	1,375	452	527	1,117
15	321	518	428	366	250	709
20	268	491	312	380	210	220
30	193	264	267	170	146	180
40	137	243	164	113	127	106
60	75	164	119	60	61	91
80	57	135	80	47	11	26
100	34	105	99	50	60	0
120	21	N.S.	34	46	0	32
150	13	37	37	N.S.	66	0
180	9	26	14	56	13	0
210	9	32	12	25	28	21
240	3	14	10	20	0	0
300	4	9	2	0	0	0
360	60	4	0	10	0	0

*Determined by HPLC single sample analysis
#Determined by LSC triplicate 100μL samples

N.S. - no sample

The intravenous blood concentration versus time (C-T) profile provides information about DON distribution and elimination. Using the three-compartment model the DON is injected into the central compartment which normally consists of the cardiovascular system and extracellular water component of the body. The distribution of DON through this compartment is an extremely rapid event, ($t_{1/2\alpha}$ 0.8 - 0.4 minutes), the decrease in blood concentration is very rapid, resulting in a C-T profile that has a very steep slope. In turkeys, there is a second distribution phase ($t_{1/2\beta}$ 5.27 - 9.3 minutes) which corresponds to the movement of DON into some tissues of the body at a slightly slower rate. This phase lasts approximately 40-60 minutes. The distribution of DON to the tissues is a more rapid process than elimination of DON and therefore, is the major factor in determining the shape of C-T profile during the early stages. As DON reaches equilibrium between the serum and the various tissues of the body, then it is the elimination processes that will determine the rate of decline. For DON in turkeys, half-life of the elimination phase is 44 min. DON appears to be eliminated from the blood of the turkeys very rapidly.

The C-T profile observed with oral dosing are comparatively simpler than the I.V. dosing profiles. From the data gathered it appears to best fit a one-compartment model, with only an absorption and elimination process - no observable distribution. This apparent inconsistency between the two models could reflect the dynamics of the distribution process in that distribution to the tissues is so rapid that it was not being detected by the sampling times used. A possible

explanation for this based on AUC calculations is that absorption of DON from the digestive tract is quite limited, approaching the limits of detection and therefore analytical variability may be hindering observing distribution phases in the blood profile. The elimination processes in the oral studies have elimination half-lives (90-151 min) in comparison to the I.V. study elimination half-lives.

Although limited absorption explain the features of the oral C-T profile and kinetics, it may also be possible that DON is being extensively eliminated from the body before reaching systemic circulation resulting in low blood concentrations. This could occur if the DON that is absorbed by the small intestine is carried directly to the liver via the portal vein where it would be eliminated from the body. If this was the case it would account for the apparent low absorption from the gut, low blood levels following oral dosing, and low levels eliminated in the urine following oral dosing. However, this doesn't correspond with the findings that with I.V. dosing urinary excretion appears to be the major route of elimination, not through the liver. Therefore, it would appear that there is in fact very limited absorption of DON from the gut.

The blood samples collected from the oral studies were analyzed by HPLC only. Oral administration of the DON resulted in very low blood levels which were close to accurate detection limits (1 ng/ml of blood). However, in comparing the data between the high dose oral and low dose oral studies, the volumes of distribution obtained are

in agreement (0.195-0.206 L/kg). Also, the AUC for the two studies (1,379:5,025) gave approximately a 1:4 ratio which is close to what would be expected for the two doses of 1 mg/kg:5 mg/kg. In considering these factors, even though the low dose studies values are very close to the detection limit and many of the parameters did display individual variation, the data generated appears to be consistent.

The residue study traced the specific radioactivity of the major tissues of the body over a 72 hour time period (Table 15, 16). The highest levels of radioactivity were found in the kidney, liver and adrenal glands with the kidney containing 9300 dpm in the low dose study and 13,000 dpm in the high dose study. The clearance of the radioactivity from the tissue in the low dose study resulted in a wide range of elimination half-lives - 10-12 hr for the fat to 76.1 hr for the gizzard. Further, the major tissues of the body had the following elimination half-lives; muscle (20.5 hr), kidney (20.5 hr), liver (56.8 hr) and fat (11.2 hr). In the high dose study the range of elimination half-life was 8.1 hr for the heart to 83.6 hr for abdominal fat. In comparing the major tissues, the following results were observed; muscle (29.5 hr), kidney (33.3 hr), liver (51.6 hr), fat (56.8 hr). Although the higher dose did take marginally longer to be cleared from the tissues, it would appear that the clearance processes are not dose dependent. The clearance processes appear to be relatively efficient in removing the radiocarbon as the levels of radioactivity in the organs dropped to very low levels within 24 hours of dosing.

The distribution of DON to tissues will depend on several factors, the physio-chemical properties of DON, concentrations gradients, ratio of blood flow to the tissue mass and the affinity of the DON for tissues components. The tissue distribution of DON or its metabolites was very high to the liver and kidney in part because these tissues receive a large portion of the cardiac output and also in part because they are involved in the elimination process. Other tissues with high blood flow to mass ratio; brain, heart, and endocrine glands (adrenal) were the other tissues to show high levels of radioactivity, DON, or its metabolites. The adipose tissues of the body showed persistent levels of radioactivity which, although quite low, were slow to be eliminated from the body. Adipose tissue has a low systematic perfusion so lipid soluble compounds, in general, are slowly released back into circulation.

In a similar study using ^{14}C-DON Prelusky et al. (1986) found that laying hens dosed orally retained residues the longest in the kidney, liver and fat. These results are similar to the results obtained in this study when dosing at 5 mg/kg. Further in a study using swine Prelusky et al. (1991) similarly found that residues persisted the longest in these three particular tissues; liver, kidney and fat. The elimination half-lives of DON in swine (dose 1 mg/kg I.V.) are considerably shorter (range from 1.16 hrs - 5.5 hrs) than in turkeys (10.3 hr - 76.1 hr).

After DON distribution has reached an equilibrium, then elimination from the body is the primary process that results in the decline of blood concentration. Elimination of DON from the turkeys involved both the liver and the kidney. Using ^{14}C-DON, it was determined that a large proportion of the dose (81%) could be found in the excreta within 6 hours and that most of this radioactivity was being eliminated through the kidneys (74%). Unchanged DON and a large amount of unknown metabolite(s) were present in the urine. A smaller portion of DON or its metabolite(s) were excreted into the bile.

In summary, the calculated kinetic parameters support the conclusion that DON and/or its metabolites are rapidly eliminated from turkeys following I.V. administration. Analysis of the excreta revealed 75% of the dose was eliminated within 3 hours and 95.8% within 24 hours. On intravenous dosing (1 mg/kg), the blood C-T time curve showed a two-stage distribution process followed by the rapid elimination of DON to trace levels within 6 hours of administering the DON. Similarly, the disappearance of tissue radioactivity to trace levels within 12 hours of dosing after peaking at 1.5 hours, indicates that the radioactivity was rapidly removed to trace levels. However, both tissue residue studies have detectable levels of radioactivity through to 72 hours, indicating there is a slower elimination process with temporary retention of trace amounts of radioactivity in the tissues. On oral administration, the characteristic blood DON C-T profile is prolonged by on-going absorption compared to the I.V. data. Also, the distribution phase of DON on oral administration does not correspond as closely to the intravenous kinetic data as absorption

from the gut may be too slow to allow observation of the various stages. The elimination kinetics are important in producing relatively low peak blood levels that occurred shortly after administration (t max - 65 min).

Physiological based pharmacokinetics modelling may be used as an alternative to compartment modelling. This approach attempts to define the physiological processes that determine drug disposition in the body and develop from that information a model that explains the physiological structure of the body. For the development of the physiological-based pharmacokinetic model, the body is depicted as a member of organs interconnected by the circulatory system. The tissues important in the disposition of the drug are identified separately and the remaining tissues are pooled in general tissue compartments. In utilizing the principles of physiological modelling for this study, the following data are needed; cardiac output, organ weights, partitioning coefficient and blood distribution to the various organs (Tables 18, 19, 20 and 21). Data for both chickens and turkeys has been included to illustrate the similarities between the two species. The partitioning coefficients of DON for the various organs in turkeys and chickens is unknown.

If all the necessary data was available then the distribution of DON to the various organs of the body and the organism as a whole could be calculated. Elimination of DON from the liver and kidney would be calculated and it would be possible to establish the kinetics

Table 18

Cardiac Output

Chickens (1)	172 ± ml/min/kg
Turkeys (2)	164 ± ml/min/kg

(1) from Sturkie, P.D.
(2) from Martine Boulianne, PhD thesis, University of Guelph, 1991

Table 19

Organ Weights
(mean % of live weight)

Organ	Turkeys (1) (3.674 kg)	Chicken (2) (1.850 kg)
Muscle	57.0	61.0
Proventriculus	0.16	0.18
Brain	0.15	-
Liver	1.12	2.50
Gizzard	1.95	2.05
Heart	0.33	0.45
Kidneys	0.29	0.80
Intestines	2.62	3.0

(1) from Marsden, S.J.
(2) from Sturkie, P.D.
- no data available

Table 20

Distribution of Blood Flow in Chickens (average weight 1.850 kg)

Organ	% Cardiac Output (1)	Per Organ (ml/min)
Kidney	9.96	38.1
Heart	5.78	18.2
Liver	7.05	22.7
Duodenum	4.81	15.9
Colon	0.40	1.6
Pancreas	0.70	3.0
Spleen	3.37	10.3

(1) from Sturkie, P.D.

Table 21

Distribution of Blood Flow in Turkeys (average weight 3.674 kg)

Organ	% Cardiac Output (1)	Per Organ (ml/min)
Kidney	15.8	90.3
Heart	5.3	31.9
Liver	8.8	68.7
Duodenum	-	-
Colon	-	-
Pancreas	-	-
Spleen	-	-

- no data available
(1) from Bell, D.J. and Freeman, B.M.

of DON in these species based on the physiological and anatomical information available. Of interest the tissue residue studies have shown the highest levels of radioactivity in the heart, liver and kidney, which also are the organs which are receiving the highest percentage of blood flow.

The comparison of DON kinetics between species is one of the advantages of physiologic modelling over the traditional compartment modelling. From the data found in the tables, it appears that chickens and turkeys are very similar physiologically and anatomically. Using physiological modelling techniques, it may be possible to determine DON kinetics in turkeys from the quantity of data already available on chickens.

Studies undertaken to chemically identify the unknown metabolites were unsuccessful. The unknown metabolite(s) are more polar molecules than DON as evidenced by the distance removed on TLC and how rapidly they moved through the HPLC column. Treatment of the metabolite(s) with β-glucuronidase, sulfatase and acid hydrolysis were unrewarding. Similarly the enzymatic hydrolysis of blood with β-glucuronidase did not reveal the presence of glucuronide metabolites. From these studies it was ascertained that the metabolite(s) were not sulfate or glucuronic acid metabolites. The acid hydrolysis study did decrease the concentration and therefore it was impossible to observe the expected increase in DON after acid hydrolysis. These enzymatic hydrolysis results indicated that DON metabolism in turkeys does not

produce the common glucuronide or sulfatase conjugates found in other species. The acid hydrolysis studies showed that hydrolysis of a conjugate metabolite(s) was possibly occurring but since the DON concentration also decreased any definite conclusion regarding the chemistry of the metabolite cannot be made. The metabolites of DON produced in the turkeys may be chemically unique of fowl, perhaps ornithinine or taurine amino acid conjugates that have been reported to occur in birds (Casarett et al., 1986).

Visconti (1985) attempted to identify the metabolites of T-2 toxin, a closely related trichothecene, in chicken excreta. In total ten different metabolites were identified with 3'-hydroxy HT-2 toxin being the major metabolite found in excreta and organs. These metabolites are produced by deacetylation and hydroxylation of the parent compound. For DON, however, only a limited number of metabolites have been observed in the various species: DON glucuronide conjugate, the de-epoxide DOM, DOM glucuronide conjugate. These are products of conjugation or de-epoxidation reactions. The chemical nature of the possible metabolites that are produced in turkeys remain unknown.

Turkeys can metabolize DON extremely rapidly and excrete it into the urine very efficiently, as significant quantities of DON metabolites were not isolated from the blood. The presence of the unknown metabolite(s) does make it more difficult to definitely interpret the tissue residue study where total radioactivity is measured. The ratio of radioactivity due to DON and to the metabolite(s) is unknown,

however the elimination pattern of tissue radioactivity is very similar to the elimination profile for the intravenous DON C-T profile.

The absorption of DON given orally results in an increasing C-T profile, but the nature of the absorption profile will depend on these simultaneously occurring processes - absorption, distribution, and elimination. The C-T profile for the orally administered DON was best represented as a one-compartment model with two rate constants; absorption and elimination. The observed difference between the one-compartment oral model and the three-compartment I.V. model could be a function of the rapid sequence of distribution processes with the distribution phase of DON being lost in the absorption processes. Further, these rapidly occurring processes may have been missed in the time spacing between samples. The elimination phase for the oral studies (t 1/2 elimination 90-130 minutes) is very similar to the results obtained for the intravenous studies. Only very low blood levels of DON were observed in the oral studies, with maximum concentrations occurring within 65 minutes of dosing. Bioavailability on a measure of the total quantity of drug absorbed on oral administration of DON in turkeys is 0.96%. The low bioavailability of DON is consistent with laying hens where the bioavailability was shown to be less than 1% (Prelusky et al., 1986).

The C-T profile is commonly used to calculate several other variables; volume of distribution and clearance. Volume of distribution is an estimate of toxin distribution expressed commonly as L/kg. Although the V_d does not refer to any particular physiological

space in the body it can offer useful information on distribution. For the C-T profile determined in these studies, the dynamic processes led to a large amount of variation between birds and therefore the pharmacokinetic parameters calculations based on the C-T profiles. The V_d based on the 5 mg/kg I.V. dose C-T profile was 0.66 L/kg and Cl was 10.4 ml/min $^{-1}$kg $^{-1}$, an extremely high value in comparison to other species.

In this study the volume of distribution and clearance values were quite variable. The volume of distribution figures indicate that DON is not being extensively bound to plasma protein or tissues of the body. If binding to tissues were occurring then excretion and elimination processes would be slower since less of the DON would be available in the plasma. However, the calculated clearance value of DON from the turkeys were quite high values indicating the DON is being removed from the tissues and eliminated from the body rapidly.

Lun et al. (1989) orally dosed chickens with ^{3}H-DON and determined that the majority of the radioactivity was found in the urine within 3 hours of dosing. In their study, 60% of the dose appeared in the urine in 6 hours, while 2% of the radioactivity appeared in the feces. The comparable data for this study are 74% and 1.76%, respectively. In the study by Lun et al., 82% of the total radioactivity was accounted for in the excreta of chickens after 24 hours, the comparable data for turkeys was 95.8%. Similarly, both

studies found low blood levels of radioactivity after oral dosing with radiolabelled DON, with blood levels peaking before maximum urinary excretion occurred after which the rate of renal excretion was greater than the rate of absorption, leading to declining blood concentrations of DON.

Urinary excretion is the major route of elimination (>90% of the administered dose) of DON or its metabolites from sheep (Prelusky, 1986) and swine (Prelusky, 1988) with bile being a minor route of elimination.

Prelusky et al. (1986), in a tissue distribution and excretion study of ^{14}C-DON in chickens, found that an oral dosing DON was poorly absorbed and reached plasma levels in 2-2.5 hours, slightly slower than the 0.2-1.0 hours found in turkeys. In chickens, maximum tissues levels of radioactivity occurred in 3 hours (except for 6 hours in fat, muscle, oviduct) which in turkeys most of the levels of radioactivity occurred within 45 minutes of dosing with kidney, liver, fat, brain tissues reaching maximum in 1.5 hours. Similarly, the kidney, liver, and spleen had the highest levels of activity. Clearance of radioactivity from tissues occurred rapidly with only trace levels being found within 24 hours, as would be expected from data from C-T profiles and excretions. El-Banna et al. (1983) and Kubena et al. (1985) who conducted tissue residue studies in chickens using cold DON were unable to detect measurable amounts of DON residues. This study using ^{14}C-DON would account for both DON and

its metabolite(s) in tissues and found only small quantities of DON or its metabolite(s) present for short periods of time, similar to results obtained by Prelusky (1986) in study with chickens.

In turkeys it appears that DON is metabolized by the liver shortly after absorption to a polar metabolite(s) that is then rapidly and very efficiently excreted into the urine and eliminated from the body. The low levels of tissue residues that occur are removed, with fat having the slowest rate of elimination.

APPENDIX I

Table 22

Data Summary of Blood Concentration in Turkeys
Following Single Intravenous Administration of 1 mg of DON/kg

Time (min.)	Bird 1	Bird 2	Bird 3	Mean ± S.D.
0	0	0	0	0
2.5	3,163	4,214	8,471	5283 ± 2810
5	1,220	1,418	1,401	1346 ± 109
10	970	725	1,375	1023 ± 328
15	321	518	428	422 ± 98
20	268	491	312	357 ± 118
30	193	164	267	241 ± 41
40	137	243	164	181 ± 55
60	75	114	119	102 ± 24
80	57	135	80	90 ± 40
100	34	105	99	79 ± 39
120	21	N.S.	34	27
150	13	37	37	29 ± 13
180	9	26	14	16 ± 9
210	9	32	12	17 ± 12
240	3	14	10	7 ± 3
300	4	9	2	5 ± 3
360	N.S.	4	0	2

* N.S. - No sample recorded.

Table 23

Data Summary of Blood Concentration of DON in Turkeys Following Single Intravenous Administration of .5μ Ci/kg ^{14}C-DON

Total Dose DON - 1 mg/kg

Time (min.)	Bird 1	Bird 2	Bird 3	Mean ± S.D.
0	0	0	0	0
2.5	30,159	6,704	10,018	15627 ± 12693
5	807	1,510	1,708	1341 ± 473
10	452	527	1,117	698 ± 364
15	366	250	709	441 ± 238
20	380	210	220	270 ± 95
30	170	146	180	165 ± 17
40	113	127	106	115 ± 10
60	60	61	91	70 ± 17
80	47	11	26	28 ± 18
100	50	60	0	36 ± 32
120	46	0	32	26 ± 23
150	N.S.	66	0	33
180	56	13	0	23 ± 29
210	25	28	21	24 ± 3
240	20	0	0	6 ± 11
300	0	0	0	3 ± 5
360	10	0	0	3 ± 5

* N.S. - No sample recorded.

Table 24

Data Summary of Blood Concentration of DON in Turkeys Following Single Intravenous Administration of .5μ Ci ^{14}C-DON

Total Dose of DON - 5 mg/kg

Time (min.)	Bird 1	2	3	4	5	6	Mean ± S.D.
0	0	0	0	0	0	0	
2.5	50,124	44,018	35,143	25,679	21,384	32,619	34827 ± 10842
5	7,240	8,459	10,086	5,516	6,517	9,324	7857 ± 1740
10	2,380	3,970	1,517	2,329	3,050	3,121	2727 ± 842
15	2,158	3,494	848	1,816	2,761	2,448	2254 ± 895
20	1,029	2,056	838	1,222	1,545	1,525	1269 ± 435
30	890	1,775	444	1,143	931	1,974	1192 ± 578
40	790	566	447	1,300	1,080	760	825 ± 318
60	650	380	560	697	531	598	569 ± 110
80	505	330	309	590	580	430	457 ± 121
100	445	500	228	411	514	325	403 ± 109
120	494	309	213	380	456	354	367 ± 101
150	253	184	N.S.	350	338	293	283 ± 67
180	192	185	235	358	283	238	256 ± 65
210	550	36	135	312	297	165	249 ± 180
240	146	0	121	192	315	102	146 ± 104
300	182	0	0	0	0	86	44 ± 75
360	140	0	30	142	145	52	84 ± 65

Table 25

Data Summary of Blood Concentation of DON in Turkeys Following Single Oral Administration of 1 mg DON/kg

Time (min.)	Bird 1	2	3	4	5	6	Mean ± S.D.
0	0	0	0	0	0	0	0
20	2	7	10	9	7	7	7.0 ± 2.7
40	3	8	9	4	4	10	6.3 ± 3.0
60	6	15	7	4	3	6	6.8 ± 4.2
80	2	6	4	3	4	N.S.	3.8 ± 1.5
100	4	7	3	5	4	4	4.5 ± 1.3
120	3	4	3	3	3	4	3.3 ± 0.5
140	3	2	4	2	2	2	2.5 ± 1.0
160	4	5	3	3	3	4	3.6 ± .8
180	3	4	4	3	1	4	3.1 ± 1.2
200	0	7	3	1	2	2	2.5 ± 2.4
220	4	5	1	0	3	2	2.5 ± 1.8
240	3	2	2	0	0	0	1.2 ± 1.3
260	0	0	2	0	0	0	.3 ± .8
280	2	3	0	2	0	0	.8 ± 1.3
300	1	1	0	0	0	1	.5 ± .5
320	3	0	0	0	1	0	.7 ± 1.2
340	3	0	0	0	0	0	.5 ± 1.2
360	0	0	1	0	0	1	.3 ± .5

* N.S. - No sample recorded.

Table 26

Data Summary of Blood Concentration of DON in Turkeys Following Single Oral Administration of 5 mg DON/kg

Time (min.)	Bird 1	2	3	4	5	6	Mean ± S.D.
0	0	0	0	0	0	0	0
20	6	4	6	11	18	15	10.0 ± 5.6
40	20	18	21	14	39	31	23.8 ± 9.3
60	44	44	36	20	48	32	37.3 ± 10.3
80	19	18	48	57	37	43	37.0 ± 15.7
100	22	25	30	19	16	28	23.3 ± 5.3
120	15	17	19	18	11	14	15.6 ± 2.9
140	15	14	19	6	11	13	13.0 ± 4.3
160	14	14	22	9	13	12	14.0 ± 4.5
180	12	11	17	8	11	10	11.5 ± 3.0
200	N.S.	5	16	5	4	10	8.0 ± 5.0
220	11	10	18	8	6	8	10.1 ± 4.2
240	10	13	12	N.S.	6	8	9.0 ± 3.2
260	16	14	7	3	7	7	9.0 ± 4.9
280	8	9	13	4	3	2	6.5 ± 4.2
300	5	5	5	3	3	4	4.1 ± .9
320	5	4	7	0	4	1	3.5 ± 2.6
340	4	3	6	0	0	0	2.1 ± 2.5
360	N.S.	0	6	4	0	0	2.0 ± 2.8

* N.S. - No sample recorded.

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