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BIOLOGICAL INTERACTIONS OF 2,4-DICHLOROPHENOXYACETIC ACID (2.4-D) WITH 2-METHYL-4-CHLOROPHENOXYACETIC ACID (MCPA) AND DIAZEPAM IN THE DOG

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

by

Laurene M. Dickow B.S., D.V.M.

* * * * *

The Ohio State University

2000

Dissertation Committee:

Dr. Paul Stromberg
Dr. Diane Gerken
Dr. Richard Sams
Dr. M. Judith Radin

Approved by

Paul Stromberg
Department of Veterinary Biosciences
ABSTRACT

The phenoxy herbicide 2.4-dichlorophenoxyacetic acid (2.4-D) is often used in combination with other structurally similar phenoxy and benzoic acid compounds in a single product. Limited studies have been reported indicating toxicokinetic and toxicodynamic interactions of 2.4-D with other structurally similar compounds. Knowledge about the interactions of mixtures is important in the assessment of risk from occupational and environmental exposure to combination products. It was the goal of this research to investigate the potential biological interaction of two structurally similar compounds, 2.4-D and 2-methyl-4-chlorophenoxyacetic acid (MCPA) by evaluating toxicodynamic and toxicokinetic responses and to investigate the potential for 2.4-D to alter liver metabolism by evaluating the pharmacokinetics of a highly metabolized therapeutic agent, diazepam, in the canine model.

Subclinical myotonia, determined electromyographically, was used as a sensitive indicator of phenoxy toxicity. Beagle dogs were evaluated for signs of toxicity after 2.4-D administration. No obvious clinical signs of myotonia were evident, but slight decreases in serum calcium and potassium, vomiting, diarrhea, percussion dimpling of the
tongues of anesthetized dogs, and insertional myotonia on electromyography were observed. The gastrointestinal effects and possibility of decreased electrolyte concentrations contributing to muscle membrane instability prevented further study of the toxicodynamic interaction of 2.4-D and MCPA.

As individual compounds, 2.4-D and MCPA bound to canine albumin and plasma in a concentration dependent manner at two classes of binding sites. Each compound was capable of displacing the other from plasma proteins in a concentration dependant manner. 2.4-D was capable of inhibiting the renal elimination of MCPA when administered in a 10 fold higher dose. No differences were observed in plasma AUCs, despite the observed increase in free fraction and decreased renal elimination.

A statistically significant increase in the in vivo clearance of diazepam was seen when pretreatment with 2.4-D occurred. This appeared to be due to an increase in metabolism of diazepam to temazepam and subsequently oxazepam. Furthermore, 2.4-D was capable of increasing, in vitro, the free fraction of diazepam in canine albumin solution.
To my husband Ron. for his patience and support
ACKNOWLEDGEMENTS

I would like to thank Diane Gerken for her guidance of all aspects of my PhD program including the development of my research and presentation skills. I would also like to thank Diane for "looking out for my best interests". I appreciate both the personal and professional relationship we have developed.

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I thank the support staff in Veterinary Biosciences including Lisa Wilcoz, Elaine Bletz, Georgia Porcelli, Sherry Frisch. They have helped with a variety of tasks from ordering supplies, making travel arrangements, and reserving rooms. I also thank Margie
Maxwell for sorting through the never ending financial aid saga that complicates returning to school. I appreciate the efforts of the biomedical media staff especially Tim Vojt. I thank the Analytical Toxicology Laboratory personnel, especially Sue Ashcraft, for both their friendship and analytical and computer help. Lastly I would like to thank my colleagues and friends who have supported me through this endeavor, after all much of this experience is personal.
VITA

1993-1996 Associate Veterinarian. Wright Veterinary Medical Center. Bethlehem. Pennsylvania
1996-present Graduate Research Associate. Ohio State University. Columbus. Ohio
1996-present Veterinary Toxicology Resident. Ohio State University. Columbus. Ohio

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<td>9-anthryldiazomethane</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AST</td>
<td>aspartate aminotransferase</td>
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<tr>
<td>AUC</td>
<td>area under the curve</td>
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<tr>
<td>BUN</td>
<td>blood urea nitrogen</td>
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<tr>
<td>b.w.</td>
<td>body weight</td>
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<td>C_F</td>
<td>free drug concentration</td>
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<td>CK</td>
<td>creatine kinase</td>
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<td>CI</td>
<td>clearance</td>
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<td>C_P</td>
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<td>4-CPA</td>
<td>4-chlorophenoxyacetic acid</td>
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<td>EPN</td>
<td>o-ethyl-p-nitrophenylphosphorothionate</td>
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<td>g</td>
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<td>GC</td>
<td>gas chromatograph</td>
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<tr>
<td>GFR</td>
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<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>ILACUC</td>
<td>Institutional Laboratory Animal Care and Use Committee</td>
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<td>ILLOD</td>
<td>instrument lower limit of detection</td>
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<td>intraperitoneal</td>
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<tr>
<td>i.v.</td>
<td>intravenous</td>
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<td>dissociation constant for the i^{th} class of binding sites</td>
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<tr>
<td>K_{dinh}</td>
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kg .............................. kilogram
L ................................ liter
LD_{50} ................................ lethal dose to 50 % of test animals
LDH ................................ lactate dehydrogenase
LLOQ ............................. lower limit of quantitation
MCPA ............................ 2-methyl-4-chlorophenoxyacetic acid
MCPP ............................. 2-methyl-4-chlorophenoxypropionic acid
mg ............................... milligram
µg .............................. microgram
mL ............................... milliliter
µL ............................... microliter
MSB .............................. mean square between residual
MSR ............................. mean square residual
n.a. .............................. not applicable
n_i ................................. capacity constant for the i^th class of binding sites
NIEHS ........................ National Institute of Environmental Health and Safety
n.d. .............................. none detected
ND ................................ nordiazepam
NOEL ........................... No Observed Effect Level
n.s. .............................. no sample
OX .............................. oxazepam
[P] ............................... total protein concentration
PAH ............................ para aminohippuric acid
PBS ................................ phosphate buffered saline
Q ................................ organ blood flow
QC ............................... quality control
s.d. ............................. standard deviation
2.4.5-T .......................... 2.4.5-trichlorophenoxyacetic acid
T_{max} ........................... time at maximal plasma concentration
V_{dss} ........................... volume of distribution at steady state
Why study interactions?

In reality, humans are not exposed to individual agents, but to a variety of compounds at any given time. Scientists and government have been aware and interested in the study of interactions as early as the beginning of this century, however it was not until the 1980's that substantial advancements were made (Calabrese, 1991). The advances of the 1980's often revolved around the concept of risk assessment (Calabrese, 1991). Risk assessment can be defined as the “process whereby relevant biological, dose-response, and exposure data are combined to produce a qualitative or quantitative estimate of adverse outcome from a defined activity or chemical agent” (Scala, 1991). The study of mixtures is critical to achieve a realistic assessment of environmental pollutants and occupational hazards (El-Masri et al. 1997). Traditional models for the systematic study of interactions have been based on statistical or mathematical determinations of these interactions with a variety of terms used to describe the interactions: additivity (1+1 = 2), co-synergy (1+1 > 2), potentiation (0+1 > 1) and antagonism (1+1 < 2) (Calabrese, 1991). These terms however do not describe the
mechanism or degree of response (Calabrese. 1991). Furthermore, systematic testing is impractical due to the high number of compounds involved and the large number of animals required (El-Masri et al. 1997). Recent advances in science have allowed for the study and emphasis of mechanisms and biological processes and for the development of models used in the analysis and risk assessment of chemical interactions (i.e. physiologically based pharmacokinetic models) (Calabrese. 1991).

Chemicals can biologically interact through a variety of mechanisms including effect (dynamic) and pharmacokinetic interactions. Examples of such interactions are as follows (Calabrese. 1991):

Effect (dynamic): Blockage of acetylcholine receptors by atropine in organophosphate toxicity results in decreased effect of organophosphates.

Pharmacokinetic:
Absorption: Calcium binding to tetracylines in the intestine forming nonabsorbable complexes leads to decreased absorption and efficacy of the tetracycline.

Plasma protein binding: Phenylbutazone displacing warfarin from albumin which leads to life threatening bleeding due to higher free warfarin concentrations.

Excretion: Probenecid competitively inhibiting the renal excretion of penicillin which results in decreased elimination of penicillin and longer therapeutic effect.

Metabolism: EPN (o-ethyl p-nitrophenylphosphorothioate) inhibiting carboxyesterase enzymes responsible for the detoxification of malathion which results in increase toxicity of malathion.
It is apparent that altering the pharmacodynamics and/or pharmacokinetics by chemical interactions can lead to increased or decreased effect of a given compound. In some cases, calcium and tetracyclines, these compounds are not structurally similar, while in other cases, probenecid and penicillin, there are at least partial structural similarities. Such similarities often result in interactions at physiologic processes that require certain chemical structures or characteristics, (i.e. renal organic anion transport).

The time, effort, and cost of performing an interaction study can be substantial. The optimal study design for the investigation of an interaction of toxicity requires that 4 different treatment groups be evaluated: 1) an untreated control group, 2) a combination of the two compounds (A and B) treated group, 3) a compound A treated group, and 4) a compound B treated group (Calabrese, 1991). It is further believed that this investigation will not always determine the synergy of the interaction if performed at only one dose level, and therefore multiple dose levels are needed to fully evaluate the interaction (Calabrese, 1991). The previous discussion assumes that an appropriate biological quantitative variable can be used for the study of the interaction of compounds A and B. Without this endpoint, the studies cannot be performed. The use of pilot studies to investigate the potential for interaction to occur would be prudent and necessary based on the extensive requirements of interaction studies. These pilot studies can determine the feasibility of the use of specific endpoints as well as evaluate the potential for the interaction. The structurally similar phenoxy herbicides have the potential for a biological interaction and studies should be performed to assess the interaction and to determine a proper biological variable.
2.4-Dichlorophenoxyacetic acid (2.4-D), a member of the phenoxy class of herbicides, was first introduced in the 1940's. Since then it has become the third most widely used herbicide in the United States. It and other structurally similar phenoxy herbicides are used on crops, lawns, pastures, and right of ways to control broadleaf weeds. In order to increase the spectrum of broadleaf weed control, 2.4-D is often combined with other structurally similar phenoxy (MCPA, MCPP) or benzoic acid compounds (dicamba, trichlopyr) in the same product. The particular use and residues of lawn care and agricultural products containing multiple phenoxy compounds exposes animals and people to potential interactions on a regular basis. Many cases of intentional or accidental exposure to the products containing the phenoxy or phenoxy/benzoic acid compounds in both man and dog have been reported in the literature (Harrington et al. 1996, Jorens et al. 1995, Keller et al. 1994, Prescott et al. 1979, Schmoldt et al. 1997), however limited information exists pertaining to potential interactive effects. Recently, the National Institute of Environmental Health Sciences (NIEHS) has been interested in evaluating mixtures encountered in environmental and occupational settings and that share a common mechanism of action (Bucher et al. 1998).

The limited studies reported suggest a biological interaction between the phenoxy herbicides and other compounds. In the rat, co-administration of probenecid, an organic anion transport inhibitor, has been shown to lower the LD\textsubscript{50} of the phenoxy herbicides, 2.4-D, 2-methyl-4-chlorophenoxyacetic acid (MCPA), and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) to 71, 30, and 80 %, of respective individual compound treated controls.
This finding demonstrates an altered dynamic or toxic effect from combined exposure of phenoxy herbicides with another compound of similar structure. The authors further found that the addition of probenecid increased the *in vitro* plasma free fraction and *in vivo* brain concentrations of all three compounds, but did not alter the total plasma concentrations (Ylitalo *et al.* 1990). Ylitalo *et al.* (1990) concluded that the increased toxicity was the result of both an increased plasma free fraction of the phenoxy compound and probenecid inhibition of the active transport of the individual phenoxy from the brain.

Additional interaction studies with phenoxy compounds include the percent reduced uptake of 2,4,5-T into renal cortical slices from dogs and rats by para-aminohippuric acid (PAH) and probenecid (Hook *et al.* 1974). The consequences of this inhibition is to delay elimination of the phenoxy herbicide resulting in increased plasma concentration over a longer time and in a biological effect. *In vivo.* PAH and probenecid administration to rats inhibited the renal elimination of another phenoxy herbicide, 2-methyl-4-chlorophenoxyacetic acid (MCPA), by approximately 75% compared to MCPA only treated controls (Bräunlich *et al.* 1989). The dose of MCPA administered (5 mg/kg) in the Bräunlich *et al.* (1989) study was non-toxic, hence dynamic effects could not be evaluated. Similar results could be expected with phenoxy-phenoxy interactions, however there are no investigations of these interactions in the literature.
Toxicity of 2,4-D (Pharmacodynamics)

In most mammals the oral dose lethal to 50% of the animals (LD$_{50}$) ranges from 300 to over 1000 mg 2,4-D/kg body weight (mg/kg) (Stevens et al. 1991). In contrast, the dog was reported to be the most sensitive with a reported oral LD$_{50}$ of 100 mg/kg (Drill et al. 1953). More recently, findings conflicting with Drill’s conclusions suggest that the LD$_{50}$ in the dog is greater (> 2 fold) than 100 mg/kg (Arnold et al. 1991b, Beasley et al. 1991, Steiss et al. 1987) and closer to the other species. Clinical signs of 2,4-D toxicity observed in all species, including man, are similar.

Initial clinical signs observed in acute 2,4-D toxicity in the dog are often described as lethargy, myotonia, and gastrointestinal irritation. Myotonia is characterized as an inability to rise, muscle stiffness resulting in abnormal gait, and percussion dimpling of musculature. In the study by Drill et al. (1953), mongrel dogs orally administered 2,4-D showed signs of toxicity at dosages $\geq$ 100 mg/kg but not at 25 mg/kg ($n \leq 4$ dogs per dose). Clinical signs in that study were anorexia with weight loss, ataxia, stiffness and myotonia with severity increasing with increased dosage from 100 - 400 mg/kg (Drill et al. 1953). There were also occasional signs of ocular irritation (rubbing of eyes), sneezing, and diarrhea at dosages $\geq$ 100 mg/kg (Drill et al. 1953). At necropsy most dogs that died ($\geq$ 100 mg 2,4-D/kg) had hepatic congestion, while some had redness of the intestinal mucosa and pneumonia (Drill et al. 1953). In more recent studies, awake English pointer dogs given 2,4-D dimethylamine dosages between 8.8 and 86.7 mg/kg orally had no overt clinical signs, but had signs of myotonia on electromyographic (EMG)
evaluation (n = 1 dog per dose) which included prolonged insertional activity and bizarre, high frequency, harmonic (myotonic or pseudomyotonic) activity (Beasley et al. 1991). When the same author administered 175 and 220 mg 2,4-D dimethylamine/kg orally, clinical signs of vomiting, lethargy, knuckling of feet, hypermetria, and incoordination (myotonia) were evident as early as 45 minutes post administration (n = 1 dog per dose) (Beasley et al. 1991). EMG observations also included bizarre harmonic activity but of a more intense and frequent occurrence than at dosages that did not result in observable clinical signs (Beasley et al. 1991). No alterations in clinical chemistry parameters were associated with observable 2,4-D toxicity after administration of 175 and 220 mg 2,4-D dimethylamine/kg (n = 1 dog per dose) (Arnold et al. 1991). Steiss et al. (1987) evaluated mongrel dogs (n = 4 per dose) and found EMG evidence of myotonic discharges at dosages ≥ 50 mg 2,4-D/kg in anesthetized dogs and muscle dimpling in 25% of dogs at each dosage between 50 - 125 mg 2,4-D/kg and clinical stiffness in 50% of dogs at 100 and 125 mg 2,4-D/kg. In the above mentioned studies, observable clinical signs were not evident until doses were equal to or greater than 50 mg 2,4-D/kg. however deaths did not occur at the highest dosages of 220 mg/kg. 220 mg/kg or 125 mg/kg in the Beasley et al. (1991), Arnold et al. (1991). or Steiss et al. (1987) studies, respectively, while they did occur in the Drill et al. (1953) study at 100 mg/kg.

Chronic administration of 2,4-D to the dog produced changes including weight loss, renal and liver changes. Chronic 2 year feeding of 2,4-D acid to dogs at doses ranging from 10 to 500 ppm/day failed to shown any clinical or histopathological effects.
with the exception of one dog being emaciated (Hansen et al. 1971). In a later study dogs administered ≤7.5 mg 2.4-D/kg/day for one year showed slight decreases in body weight gain at the highest dose with intermittent decrease in feed consumption (Charles et al. 1996a). Treatment related increases in BUN, creatinine, cholesterol, and ALT and decreases in glucose were observed in dogs fed 5 and 7.5 mg 2.4-D acid/kg/day, but not 1 mg 2.4-D acid/kg/day (Charles et al. 1996a). Perivascular chronic active liver inflammation and increase pigment in the tubular epithelium were the only histopathologic changes found at the conclusion of the study (Charles et al. 1996a). The No Observable Effect Dose (NOEL) for the was determined to be 1 mg 2.4-D/kg/day (Charles et al. 1996a).

The acute toxicity found in the dog is similar in signs and dosages to what is observed in other species. Rats given 100 or 200 mg 2.4-D/kg intraperitoneally had decreased locomotion by 4 hours with abnormal gait, posture and muscle stiffness maximal at 3 to 6 hours lasting up to 24 hours (n = unknown) (Bernard et al. 1985). EMG evaluations of the rats receiving 200 mg 2.4-D/kg also revealed signs of myotonia (Bernard et al. 1985). Calves (n = 2 animals per dose) orally administered 50 mg 2.4-D amine/kg had slight dysphagia while 100 mg/kg caused some anorexia, and 200 mg/kg administration resulted in anorexia, thirst and hindquarter weakness lasting 3-4 days (Björklund et al. 1966). When Björklund et al. (1966) administered 100 mg 2.4-D/kg as the ester and Na-K salt, no effects and dysphagia, respectively, were seen. Adult cattle given 2.4-D dimethylamine had weakness, lethargy, and increased lactate dehydrogenase
(LHD). 2 - 24 hours and creatine kinase (CK) 4 - 24 hours after dosing when administered 600 mg/kg, but not at doses ≤300 mg/kg (n = 4 animals per dose) (Paulino et al. 1995). Increases in liver and kidney enzymes have been observed in rats (Paulino et al. 1996), while these changes have not occurred in dogs (Arnold et al. 1991). The increases in biochemistry parameters observed in rats were not dose dependent and decreased to normal within 4 days after oral exposure to 2,4-D dimethylamine (Paulino et al. 1996). It is unknown if any histopathological changes were associated with these biochemical changes. Both a decrease (600 mg 2.4-D dimethylamine/kg) in rats at 5, 8, and 24 hours post administration (Paulino et al. 1996) and a dose dependent (300 and 600 mg 2.4-D dimethylamine/kg) increase in cattle (Paulino et al. 1995) of serum glucose at 24 hours post administration have been reported after acute 2,4-D toxicity. Pigs administered 2.4-D amine had anorexia at 50 mg/kg, diarrhea, stilted gait, and transitory depression at 100 mg/kg, and severe muscular weakness, vomiting, and depression at doses above 500 mg/kg (n = 2 animals per dose) (Björklund et al. 1966). When these animals were necropsied, gastrointestinal irritation was evident at all dosages, and pneumonia and renal and hepatic congestion were seen at dosages above 500 mg/kg (Björklund et al. 1966). In conclusion the clinical and histopathological signs seen in the dog and other species after acute 2,4-D exposure are similar and are produced at similar doses. Signs were clinically observed at ≥50 mg 2.4-D/kg in dogs while clinically observable signs in other species occurred at doses between 50 and 600 mg/kg.
Chronic administration of 2.4-D to rodent species resulted in findings similar to those observed after chronic administration to the dog. Decreased weight gain was observed in rats fed \( \geq 45 \) mg 2.4-D acid/kg/day for 2 years (Charles et al. 1996b). Retinal degeneration and cataracts were observed in rats fed 150 mg 2.4-D acid/kg/day for 2 years, but not at doses less than 150 mg 2.4-D acid/kg/day (Charles et al. 1996b). This finding was not observed in dogs (Charles et al. 1996a). Statistically significant decreases in liver weights (5\%) were observed in rats fed 75 and 150 mg 2.4-D acid/kg/day (Charles et al. 1996b). A statistically significant increase in alanine aminotransferase (ALT) and alkaline phosphatase (ALP) was observed in male rats and an increase in ALP was observed in female rats at 2 years after 75 and 150 mg 2.4-D/kg/day administration, but not 5 mg 2.4-D/kg/day (Charles et al. 1996b). The NOEL determined for the rat in the Charles et al. (1996b) study was 5 mg/kg/day based on lack of renal changes observed in an earlier study at 15 mg/kg, which is slightly higher than that determined for the dog (1 mg/kg/day) by Charles et al. (1996a) based on mild histopathologic and serum chemistry changes. Rats exposed to 200 ppm 2.4-D dimethylamine in drinking water for 180 days had no observable, macroscopic, or histologic signs of toxicity, but clinical chemistry parameters were 122\% (ALT), 130\% (ALP), 163\% (LDH), 94\% (amylase), and 89\% (glucose) of control values (Paulino et. al. 1996).

Similar signs of 2.4-D toxicity observed in laboratory animals have been reported in humans. Exposure is often oral and to products containing multiple compounds (both
phenoxy and non-phenoxy) and at excessive dosages. However, there are a few reported cases of 2.4-D only toxicity. After ingestion of approximately 240 g 2.4-D, a 60 year old man presented to a hospital agitated, tachycardic, and hypothermic (Jorens et al. 1995). Clinical signs progressed to metabolic acidosis, cardiogenic shock, respiratory failure and death (Jorens et al. 1995). Prior to death, the patient developed hematemesis from hemorrhagic esophagitis and gastritis with necrotic lesions in the esophagus (Jorens et al. 1995). Similar signs were observed after a 49 year old man ingested an unknown amount of 2.4-D. Upon admission the man vomited, had bloody diarrhea, and was disoriented (Keller et al. 1994). Endoscopy revealed hemorrhage and necrosis of the stomach (Keller et al. 1994). This patient progressively worsened and developed acute renal failure by the second day and subsequently died from multiple organ failure (Keller et al. 1994). The large amounts of acid ingested most likely were the cause of the clinical signs observed. In a different case in which the patient survived, a 39 year old man ingested a combination of 2.4-D and mecoprop (a phenoxy herbicide) with observed signs similar to the previous cases (Prescott et al. 1979). However, when the patient was stabilized, a marked myotonia was present (Prescott et al. 1979). It is possible that myotonia may have been present in the first two cases, however the severity of the other signs obscured these observations. There is considerable concern about the neuromuscular effects of 2.4-D and other phenoxy herbicides in humans including polyneuropathy, demyelination, decreased nerve conduction velocity, and behavioral disorders. These findings are based on case reports and epidemiological studies including studies of exposure to multiple phenoxy herbicides and have not been repeatedly confirmed in laboratory animals (Munro et al. 1992).
Effects of 2,4-D on hepatic enzymes

It has been shown that 2,4-D induces cytochrome P450s of the CYP4A family in the human and rat (Bacher et al. 1988, Hietanen et al. 1985, Pineau et al. 1996). This family of enzymes plays a role in metabolizing endogenous fatty acids. Limited in vitro information exists pertaining to the other P450 families, which are important to the metabolism of xenobiotic compounds including therapeutic drugs. It has been shown, in rat livers, that 2,4-D increased the CYP 1A1 activity, by increased ethoxyresorufin O-deethylation (~2 fold), but not CYP 2B1 activity with no changes in benzphetamine N-demethylation (Bacher et al. 1988). Increases in ethoxyresorufin O-deethylase (135 - 185%), as well as, ethylmorphine N-demethylase (125%) activity in rat liver has been observed by Knopp et al. (1992). Aniline hydroxylase activity has also been seen to increase in a dose dependent manner after 2,4-D administration to rats (Cristae et al. 1981). After 100 and 1000 ppm 2,4-D fed for 90 days aniline hydroxylase activity increased 139 and 152 %, respectively (Cristae et al. 1981). It appears that 2,4-D is capable on inducing, in vitro, drug metabolizing enzymes of the P450 family, however the existing literature is limited and more studies, both in vitro and in vivo are needed to further assess the overall potential of 2,4-D to affect the metabolism of drugs by the induction of specific P450 enzymes.

Pharmacokinetics of 2,4-D

Absorption. The oral absorption of 2,4-D has been shown to be rapid. In pigs peak plasma concentrations were observed at 5 hours after administration of the amine form
(Björklund et al. 1966) and between 4 and 7 hours in pigs, rats, and calves after administration of the amine and ester forms (Erne 1966). In man the time to peak plasma concentration ($T_{\text{max}}$) was found to be slightly longer and ranged between 7 and 24 hours (Kohli et al. 1974). Administration of 2,4-D dimethylamine to Fisher rats resulted in very rapid ($T_{\text{max}} = 20$ minutes) and almost complete ($\geq 90\%$) absorption (Pelleiter et al. 1989). Plasma concentration-time curves for the dog have not been published in the literature.

Distribution. The apparent volume of distribution (Vd) has been shown to be 0.10 L/kg in man (Kohli et al. 1974). Erne (1966) reported the Vd in pigs and calves to be between 25 and 50% of body weight, which is slightly larger than extracellular fluid volume. The herbicide does distribute into tissue with the highest concentrations being found in the kidney and liver (Elo et al. 1979, Erne 1966, Pelletier et al. 1989, Sandberg et al. 1996). The percent of the Area Under the Curve (AUC) in the kidney from 0 to 2 hours of 2,4-D to plasma AUC was found to be approximately 100, 130, and 67% after 1, 10, and 40 mg 2,4-D/kg intravenous administration to rabbits (Sandberg et al. 1996). The percentage of tissue to plasma ratios of $^{14}$C activity was 75.3, 68.2, and 15.0 for kidney, liver, and brain, respectively, after 3.5 to 4 hours post administration of 250 mg 2,4-D sodium salt/kg intravenously to rats (Elo et al. 1979). Erne (1966) found that 2,4-D levels in the liver and kidney approached plasma levels at 6 hours, but by 24 hours had often exceeded plasma levels after 100 mg 2,4-D amine/kg administration in rats and pigs. Brain concentrations in pigs were approximately 5% of plasma concentrations.
after 100 mg 2,4-D amine/kg (Erne, 1966). The amount of 2,4-D in skeletal muscle is ≤20% of that found in plasma (Elo et al. 1979, Erne 1966, Pelletier et al. 1989). The specific mechanism of 2,4-D uptake in skeletal muscle has not been described.

The protein binding of any compound plays a role in its distribution in the body since only unbound chemical can distribute to the tissues. 2,4-D has been shown to be highly plasma protein bound in a concentration dependent manner. Binding in goat plasma ranged from 97 to 65% for plasma 2.4-D concentrations between 20 and >1000 µg/mL (Örberg, 1980). 2.4-D is reported to bind to albumin in a variety of species including human, rat, bovine, guinea pig, sheep, horse, and rabbit (Fang et al. 1980, Kolberg et al. 1973, Mason, 1975, Rosso et al. 1998). The binding of 2,4-D to dog plasma or albumin has not been described. The potential protein binding interaction of 2,4-D with other structurally similar compounds has been limited to studies involving probenecid. (Ylitalo et al. 1990). The Ylitalo et al. (1990) study did show, in vitro, a 41% increase in the unbound portion of 2,4-D by probenecid which partially contributed to higher brain concentrations of 2,4-D and a decrease in the LD₅₀ observed.

Metabolism and Elimination. The phenoxy herbicides undergo very little metabolism with the majority of compound being eliminated primarily unchanged in the urine. Over 90% of a 1.0 mg 2,4-D/kg dose was found to be excreted unchanged in the urine of rats within 6 hours after oral administration (Pelletier et al. 1989). In humans, 76.5% of a
5.0 mg 2.4-D/kg oral dose was eliminated unchanged in the urine by 96 hours post-administration (Kohli et al. 1974). In goats 2.4-D was shown to be excreted essentially unaltered in the urine (Örberg, 1980).

Renal clearance of 2.4-D in the goat was shown to be greater than glomerular filtration rate (GFR) at plasma 2.4-D concentration < 31 μg/mL and less than GFR at concentrations above 40 μg/mL (Örberg, 1980). The author suggests that this was due to altered renal blood flow, however the resorption of 2,4-D by the kidney was not investigated as a possible cause of the lower than GFR clearance. Alkaline diureses increased the renal clearance of 2.4-D from 0.14 mL/min to 63 mL/min and subsequently decreased the plasma t½, in a case of self poisoning (Prescott et al. 1979).

Kim et al. (1994) described the elimination of 2.4-D from the rat as saturable after oral and intravenous administration of doses ranging from 5 - 150 mg/kg. The active transport of 2,4-D has been demonstrated in vitro using rat renal cortical slices, and has been shown to be saturable and subject to competitive inhibition by probenecid and PAH (Koschier et al. 1978. Villalobos et al. 1996). The transport of 2.4-D has been determined to be mediated by an α-ketogluterate exchange, which functions to transport organic anions (Villalobos et al. 1996). A similar transport mechanism works to remove organic acids from the brain. The transport of 2.4-D from the kidney has not been evaluated in the dog, however the dog has been shown to accumulate 60% of the amount of 2,4,5-T in renal slices compared to the rat, suggesting the transport of 2,4,5-T in to the
kidney cell, and subsequently out of the body, is less efficient in the dog (Hook et al. 1974). This finding would suggest the dog would be a sensitive indicator of the renal elimination interactions of 2.4-D with other phenoxy compounds.

**MCPA**

The phenoxy herbicide 2-methyl-4-chlorophenoxyacetic acid (MCPA) is structurally similar to 2.4-D (Figure 1.1). There is a paucity of information in the literature about MCPA compared to 2.4-D. however the pharmacodynamic and pharmacokinetic effects of MCPA and 2.4-D appear to be similar.

![Chemical structure of 2.4-D and MCPA](image)

*Figure 1.1. Chemical structure of 2.4-D and MCPA*

Clinical signs associated with MCPA toxicity are similar to those observed with 2.4-D and are similar across species. The acute oral LD$_{50}$ in Wistar rats has been determined to be 2250 mg/kg (2.4-D LD$_{50}$ = 980 mg/kg) with signs of myotonia, ataxia, inactivity.
and muscular weakness (Ylitalo, 1990). The herbicide causes myotonia and lethargy in rats at doses above 250 mg/kg, ataxia at 100 mg/kg and no observable signs at 25 mg/kg (Elo et al. 1979). A young man presented with vomiting, weakness, and burning of the mouth in a case of intentional human MCPA ingestion (Schmoldt et al. 1997). Clinical signs progressed to hypotension, painful spasms of the extremities and slurred speech (Schmoldt et al. 1997). An accidental case of ingestion of MCPA and bromoxynil in the dog was reported with clinical signs evident as depression, lethargy, ptyalism, panting, conjunctival congestion, and myotonia (Harrington et al. 1996). The common clinical signs of myotonia and structural similarity of 2,4-D and MCPA suggests a similar mechanism of action and a potential pharmacodynamic (effect) interaction.

The pharmacokinetic characteristics of 2,4-D and MCPA are also similar. Peak plasma concentrations occurred 1 hour after administration when 15 µg MCPA/kg was orally administered to human volunteers (Kolmodin-Hedman et al. 1983). Blood and tissue MCPA concentrations reached a maximum 2 hours after gastric administration of 11.5 mg/kg to rats (Elo. 1976). The rapid absorption of MCPA is similar to what is observed with 2,4-D exposure, which in laboratory species, ranged from 20 minutes (Pelleiter et al. 1989) to 7 hours (Björklund et al. 1966. Erne. 1966).

Similarities are also observed with distribution and elimination of MCPA and 2,4-D. The volume of distribution of MCPA was determined to be 17 -18 % of body weight in rats after 5 and 10 mg/kg i.p. administration (Bräunlich et al. 1989). As with 2,4-D, this
approximates extracellular fluid volume. Tissue distribution of MCPA is similar to 2.4-D with the liver and kidney having high concentrations and skeletal muscle relatively low concentrations. MCPA could be detected in a variety of tissues after gastric administration of 11.5 mg/kg with the tissue/blood ratio percentages highest in the kidney, approximately 60 and 75 % at 8 and 4 hours post administration (Elo. 1976). Kidney MCPA concentrations approximated blood concentrations by 24 hours post administration (Elo, 1976). In that same study, muscle MCPA concentrations were ≤ 16 % of blood concentrations from 4 to 24 hours post MCPA administration (Elo. 1976). There was a dose dependent increase in the tissue/plasma ratio when MCPA was subcutaneous administered to rats (Elo et al. 1979). At 25 mg MCPA/kg, kidney, liver, and muscle to plasma ratios were 0.62, 0.25, and 0.08, respectively (Elo et al. 1979). These values increased to 0.70, 0.95, and 0.35 after 500 mg MCPA/kg administration (Elo et al. 1979). The apparent differences in tissue uptake as a result of increased dose were not explained.

Like 2.4-D, MCPA has been shown to be highly plasma protein bound. When rat plasma MCPA concentrations were ≤80 μg/mL, plasma protein binding was 85% (Bräunlich et al. 1989). Rat plasma incubated with 200 and 2200 μg MCPA/mL had plasma protein binding of 77 and 36 %, respectively (Elo et al. 1979). This concentration dependent binding approximated that found with 2.4-D (97-65 % at 20 - 1000 μg/mL)(Örberg, 1980). Furthermore, co-incubation of 0.05 mg MCPA/mL with 1 mg/mL probenecid, an organic acid transport inhibitor, decreased the binding of MCPA
by 73% (Ylitalo et al. 1990), demonstrating the potential for a protein binding interaction. As with 2,4-D, MCPA has been shown to bind to bovine albumin *in vitro* (Mason 1975). There is a potential for a pharmacokinetic protein binding interaction since 2,4-D and MCPA have similar plasma protein and albumin binding characteristics and are both displaced by probenecid. There are no binding studies in which combinations of 2,4-D and MCPA have been studied.

The excretion of 2,4-D and MCPA are also similar with both compounds shown to be primarily eliminated unchanged in the urine. Approximately 92% was excreted in the urine of rats within the first 24 hours after administration of 11.5 mg MCPA/kg gastrically (Elo. 1976). This is similar to the > 90% elimination of 2,4-D in the rat (Pelletier *et al.* 1989). In man, on average, 40% of a 15 μg MCPA/kg oral dose was eliminated in the urine within 24 hours post administration (Kolmodin-Hedman *et al.* 1983). Renal excretion of MCPA could be increased by alkalinization of the urine (Bräunlich *et al.* 1989. Schmoldt *et al.* 1997).

Bräunlich *et al.* (1989) found no dose dependent renal elimination of MCPA in rats at dosages below 10 mg/kg and dose dependence at 20 mg/kg. Furthermore probenecid and α-aminohippurate (PAH) inhibited renal excretion of MCPA to approximately 25% of controls *in vivo* (Bräunlich *et al.* 1989). The similar and saturable elimination of MCPA and 2,4-D suggest another potential site of pharmacokinetic interaction.
In summary the similarities in the pharmacodynamic (myotonia) and pharmacokinetic (renal elimination and protein binding) of 2.4-D and MCPA suggest multiple potential biological interactions, but no studies exist investigating these interactions. The relationships between protein binding, elimination, and biological effect are complex, making the development of a model in which to study these interactions difficult but desirable.

In addition to direct interactions, there is potential for indirect effects such as drug metabolizing induction effects.

**Diazepam**

Diazepam, a benzodiazepine, is a commonly used anxiolytic/hypnotic agent in both veterinary and human medicine. It is extensively metabolized by N-demethylation and hydroxylation to desmethyldiazepam (nordiazepam), oxydiazepam (temazepam), and oxazepam (Figure 1.2). *In vivo,* diazepam is rapidly converted to nordiazepam in the dog (Klotz *et al.* 1976. Löscher *et al.* 1981. Vree *et al.* 1979). man, and rat (Klotz *et al.* 1976). In the dog, 51-83% of the total benzodiazepines in the blood was nordiazepam, with oxazepam accounting for 7-28% (Löscher *et al.* 1981). Only trace amounts of temazepam were found in that same study (Löscher *et al.* 1981). Similar metabolic profiles for the dog were observed by Vree *et al.* (1979). In man approximately 50% of diazepam is metabolized to nordiazepam and oxazepam and temazepam are the other (minor) metabolites (Greenblatt *et al.* 1988).
Figure 1.2. Chemical structure of diazepam and its metabolites

*In vitro* hepatic metabolism of diazepam is similar to *in vivo* metabolism. The main metabolite found in isolated canine, human, and monkey cultured hepatocytes incubated with diazepam was nordiazepam (Seddon *et al.* 1988). Furthermore oxazepam was a significant metabolite in man and dog with temazepam being a minor metabolite in the dog (Seddon *et al.* 1988). The rat appeared to have a different metabolic profile with a 4'-hydroxy diazepam produced as the predominant metabolite (Seddon *et al.* 1988). Similar findings with cultured rat, human, and canine hepatocytes were found by Chenery *et al.* (1986). Chenery *et al.* (1986) further found in the dog that temazepam was quickly converted to oxazepam and that both temazepam and oxazepam were glucuronidated. This is consistent with the *in vivo* findings by Vree *et al.* (1979). Chenery *et al.* (1986)
identified nordiazepam as the principle metabolite and temazepam as a lesser metabolite in cell cultures from a single human subject. When specific cytochrome P450's (CYP P450) were evaluated for their contribution to diazepam metabolism in the human, predominate enzymes were CYP 3A4/5, 2B6, and 2C sub-family (Yang et al. 1998, Yang et al. 1999). In summary, the metabolic profile of diazepam is similar between the human and the dog. The functions of N-demethylation and hydroxylation are the same for both species, suggesting information from the effects of 2,4-D on diazepam metabolism in the dog can be applicable to humans.

Diazepam has been shown to be bound mainly to human albumin in a concentration independent manner within the therapeutic concentration range (Divoll et al. 1981). The binding is extensive with < 2% existing as free diazepam (Allen et al. 1981, Gardner et al. 1997). The free fractions of diazepam were not statistically different demonstrating concentration independence in dogs with plasma diazepam concentrations ranging from 0.5 - 20.0 µg/mL (Wala et al. 1995). Like most therapeutic drugs, concentration independent binding of diazepam is observed at therapeutic ranges. Diazepam and 2,4-D have been shown to bind to the same site on human albumin (Rosso et al. 1998), thus a potential exists for a protein binding interaction on human albumin. The albumin binding site for diazepam in the human is similar to the binding site for diazepam in the dog, but not the bovine, rat, or rabbit (Kosa et al. 1997). It would be expected that 2,4-D also binds to this same site in the dog. Since both diazepam and 2,4-D are highly protein bound to albumin, the plasma protein binding of diazepam needs to be investigated as a potential site of interaction.

In summary, in order to study the effect that 2,4-D has upon in vivo hepatic metabolism (via induction) of other xenobiotic compounds, a model compound was
chosen. Diazepam is an extensively metabolized compound with a similar metabolic profile and plasma protein binding in man and dog. Furthermore it has been suggested that the albumin binding site of diazepam in man is most similar to the dog. The pharmacokinetics of diazepam can be studied in the dog as a model of man for the potential effects of altered hepatic metabolism by 2,4-D.

**Proposed Investigation**

The phenoxy herbicide 2,4-D is often found in combination with other structurally similar phenoxy and benzoic acid compounds in a single product. There have been only limited studies performed to investigate potential interactions of 2,4-D with other compounds, despite the potential for exposure to combination products. With the current interest in the study of mixtures and the paucity of information about 2,4-D interactions, the following is a brief outline of the proposed investigation of the interaction of 2,4-D with MCPA and 2,4-D with diazepam.

1) Development of a HPLC method to simultaneously determine 2,4-D and MCPA in biological matrices. Currently, there are no published methods for the simultaneous determination of these two compounds in biological matrices. Simultaneous determination will be critical in analyzing concentration-effect relationships and pharmacokinetic parameters after combined administration of 2,4-D and MCPA to the dog.

2) Investigation of the pharmacodynamic effect of 2,4-D and MCPA in the dog. Myotonia was selected as the indicator of phenoxy toxicity. Determinations will be made using EMG and visual observation of individual compound and combined exposure.
3) Investigation of the pharmacokinetic effect of 2.4-D and MCPA in the dog. These studies will focus on the plasma concentration-time profiles, renal elimination, and protein binding of 2.4-D and MCPA after individual compound and combined exposure.

4) Investigation of the effect of 2.4-D on diazepam metabolism. Plasma concentration-time profiles and plasma protein binding of diazepam before and after 2.4-D administration will be evaluated.
References


Cristea E., Dinu V., Dinu I., Mihăilescu I., Boghainu L. (1981) 2,4-Dichloro-phenoxyacetic acid interactions with the detoxification hepatic systems. *Physiologie.* 18, 199-203.


Kim C.S., Gargas M.L., Andersen M.E. (1994) Pharmacokinetic modeling of 2,4-dichlorophenoxyacetic acid (2,4-D) in rat and in rabbit brain following single dose administration, *Toxicol. Lett.* 74, 189-201.


CHAPTER 2

Currently, there are no published methods for the simultaneous determination of 2,4-D and MCPA in biological matrices, although there can be simultaneous exposure to these compounds. Simultaneous determination, which will allow for accurate identification of plasma and urine 2,4-D and MCPA concentrations, will be critical in analyzing concentration-effect relationships and pharmacokinetic parameters after combined administration of 2,4-D and MCPA to the dog.
SIMULTANEOUS DETERMINATION OF 2,4-D AND MCPA IN CANINE PLASMA AND URINE BY HPLC WITH FLUORESCENCE DETECTION UTILIZING 9-ANTHRYLDIAZOMETHANE (ADAM)

Introduction

2,4-Dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) (Figure 2.1) are classified as phenoxy acid herbicides and are widely used for the control of broad-leaf weeds. Often they are used in combination with other phenoxy or benzoic compounds such as mecoprop and dicamba, respectively. There have been numerous accounts of intentional or accidental poisoning with these products of both humans and dogs (Flanagan et al. 1990, Fraser et al. 1984, Harrington et al. 1996, Jorens et al. 1994, Keller et al. 1994, Osterloh et al. 1983, Prescott et al. 1979, Schmoldt et al. 1997.). Recently, a dog was reported to have ingested MCPA in sufficient quantities to produce clinical signs of myotonia (Harrington et al. 1996).

Figure 2.1. Chemical Structure of 2,4-D and MCPA

The phenoxy herbicides undergo limited biotransformation and are eliminated primarily unchanged in the urine. Approximately 75% of administered 2,4-D is eliminated unchanged in the urine of man after a 5 mg/kg oral dose (Kohli et al. 1974).
Further, approximately 90% of a 1.0 mg 2,4-D/kg oral dose has been shown to be present in the urine of rats (Pelletier et al. 1989). When 2,4-D was orally administered to dogs at varying dosages, it was detected in urine within 1 hour of administration (Arnold et al. 1991). There are, however, no reports of the percentage of 2,4-D excreted in the urine of the dog over a given time. Since the phenoxy compounds are eliminated primarily unchanged in the urine, analytical determination of these compounds in both plasma and urine could be performed. Few validated methods have been reported for the determination of phenoxy acid herbicides in blood (Flanagan et al. 1989, Miki et al. 1998, Olivera et al. 1995) and urine (Aprea et al. 1997, De Felip et al. 1988, Flanagan et al. 1989, Grover et al. 1985, Miki et al. 1998, Smith et al. 1979, Thompson et al. 1996, Vural et al. 1984). However, with the exception of one method (Flanagan et al. 1989), the reported methods were used for the analysis of single phenoxy herbicides. Flanagan et al. (1989) described a method to quantitate multiple compounds utilizing HPLC with ultraviolet detection, but were unable to separate 2,4-D from MCPA chromatographically.

A method described by Suzuki and Watanabe for analyzing phenoxy compounds in ground water samples utilized 9-anthryldiazomethane (ADAM) for derivatization to fluorescence label the carboxyl portion of the phenoxy compounds prior to HPLC analysis (Suzuki et al. 1991). This allowed for detection of concentrations of 0.5 µg/L. Our objective was to develop a validated HPLC method for the simultaneous determination of 2,4-D and MCPA in canine plasma and urine using derivatization with ADAM.
Materials and Methods

The concentrations of 2.4-D and MCPA in canine plasma samples were determined by high-performance liquid chromatographic (HPLC) analysis of derivatized extracts of acidified samples. 4-Chlorophenoxyacetic acid (4-CPA) was added to the samples prior to acidification as an internal standard.

Chemicals and reagents

The following reagents were used: HPLC grade acetonitrile, ethyl acetate, hexane, and water (Burdick & Jackson Laboratories, Muskegon, MI, USA), analytical grade hydrochloric acid (Mallinckrodt, Paris, KE, USA), 0.9% sodium chloride solution, USP (Baxter, Deerfield, IL, USA), 9-Anthryldiazomethane (ADAM) (Research Organics, Cleveland, OH, USA), analytical standards of 2.4-D, MCPA, and 4-CPA (Aldrich Chemical Co., Milwaukee, WI, USA), drug-free canine plasma (Harlan Bioproducts for Science, Indianapolis, IN, USA), and drug-free canine urine collected from laboratory beagles.

Individual standard solutions of 2.4-D and MCPA at a concentration of 10.0 g/L were prepared in acetonitrile. The initial standard solutions were serially diluted with acetonitrile to produce additional standard solutions of 1.00 g/L and 0.100 g/L. The standard solution of 4-CPA was prepared at a concentration of 1.00 g/L in acetonitrile. Standard solutions were stored in the dark at approximately -17°C. A working solution of 4-CPA was prepared by diluting the standard solution with acetonitrile to a final concentration of 0.100 g/L. Concentrations of standards were verified spectrophotometrically.
**Procedure**

*Preparation of calibrators.* Two sets of plasma calibrators were prepared by adding 2,4-D and MCPA standard solutions to drug-free canine plasma. One set was prepared by adding appropriate volumes of 1.00 g/L stock solution of 2,4-D and MCPA to produce concentrations of 0.50, 1.00, 2.00, 3.00, 4.00, and 5.00 mg/L. Another set was prepared by adding appropriate volumes of the 10.0 g/L stock solution of 2,4-D and MCPA to produce concentrations of 5.0, 10.0, 30.0, 50.0, and 100 mg/L. A set of urine calibrators was prepared by adding the appropriate volumes of 1.00 g/L or 10.0 g/L 2,4-D and MCPA stock standard solutions to drug-free canine urine to produce concentrations of 5.00, 10.0, 30.0, 50.0 and 70.0 mg/L. Calibrators were stored at approximately -17°C.

*Quality Control Samples.* One set of quality control samples was prepared for each set of calibration standards. A set of samples was prepared by adding the appropriate volumes of the 1.00 g/L stock solutions of 2,4-D and MCPA to drug-free canine plasma to produce concentrations of 1.00, 3.00 and 5.00 mg/L. A second set of samples was prepared by adding the appropriate volumes of the 10.0 g/L stock solutions of 2,4-D and MCPA to drug-free canine plasma to produce concentrations of 10.0, 50.0, and 100 mg/L. A third set of samples was prepared by adding the appropriate volume of the 1.00 and 10.0 g/L 2,4-D and MCPA stock solutions to drug-free canine urine to produce concentrations of 7.00, 10.0, and 50.0 mg/L. Quality control samples were stored at approximately -17°C.

*Plasma extraction procedure.* For calibrators, quality control (QC), or test samples expected to be 5.00 mg/L or greater, 100 µL was diluted with 900 µL of 0.9% sodium chloride to produce a final volume of 1.00 mL. Plasma calibrators, quality control, and test samples less than 5.00 mg/L were extracted undiluted. An 8.0 µL aliquot of the 4-CPA working solution was added to 13 × 100 mm screw-cap tubes followed by an 100
μL aliquot of calibrator, QC, or test sample and vortexed. The mixture was acidified with 0.5 mL of 0.1 N hydrochloric acid and briefly vortexed. The sample was then extracted with 4.0 mL of ethyl acetate-hexane (70/30, v/v), mixed by end-over-end inversion for 5 minutes, and centrifuged at approximately 1000 g for 5 minutes. The organic phase was transferred to a conical tube and evaporated under nitrogen at 40 - 45°C.

**Derivatization of plasma samples.** The dry extracted residue was dissolved in 70 μL of 0.025% ADAM in acetonitrile and an additional 30 μL of acetonitrile was added. This mixture was incubated at room temperature for 10 minutes. After the addition of 700 μL mobile phase (acetonitrile-water, 75/25, v/v) and brief mixing, a 25 μL aliquot of each extracted derivatized sample was injected onto the high performance liquid chromatographic column.

**Urine extraction procedure.** 100 μL of the calibrator, quality control, or test sample was diluted with 900 μL of 0.9% sodium chloride to produce a final volume of 1.00 mL. A 7.0 μL aliquot of the 4-CPA working solution was added to 13 x 100 mm screw-cap tubes followed by an 100 μL aliquot of calibrator, QC, or test sample and vortexed. The mixture was acidified with 0.5 mL of 0.1 N hydrochloric acid and briefly vortexed. The sample was then extracted with 4.0 mL of ethyl acetate-hexane (70/30, v/v), mixed by end-over-end inversion for 5 minutes, and centrifuged at approximately 1000 g for 5 minutes. The organic phase was transferred to a conical tube and evaporated under nitrogen at 40 - 45°C.

**Derivatization of urine samples.** The dry extracted residue was dissolved in 200 μL of 0.025% ADAM and incubated at room temperature for 10 minutes.
addition of 600 μL of mobile phase (acetonitrile-water, 70/30, v/v) and brief mixing, a 25 μL aliquot of each extracted derivatized sample was injected onto the high performance liquid chromatographic column.

*High-performance liquid chromatography.* The HPLC system consisted of a dual-piston reciprocating pump (ConstaMetric IIIG pump, LDC/Milton Roy, Riviera Beach, Fl. USA), an automatic sample injector (WISP 712 autosampler, Waters, Milford, MA. USA), and a fluorescence detector (Kratos FS-970, Applied Biosystems, Ramsey, NJ. USA). Chromatographic separations were performed using a 4.6 mm × 150 mm reverse phase chromatographic column (Zorbax SB-C18, 5 μm particle size, Mac-Mod Analytical, Inc., Chadds Ford, PA. USA). A guard column (Perisorb RP-18, 30-40 μm particle size, Upchurch Scientific, Inc., Oak Harbor, WA. USA) was placed between the injector and the chromatographic column. The mobile phase for plasma sample analysis was acetonitrile-water (75/25, v/v) at a flow rate of 1.0 mL/min. The mobile phase for urine sample analysis was acetonitrile-water (70/30, v/v) at a flow rate of 1.0 mL/min. Fluorescence was measured at an excitation wavelength of 365 nm and an emission wavelength of 389 nm (Kratos FS-970, Applied Biosystems, Ramsey, NJ. USA). A reporting integrator (Spectra Physics 4270, Spectra Physics, San Jose, CA. USA) detected peak heights at a chart speed of 0.25 cm/min.

If the plasma concentration of either 2,4-D or MCPA was determined to be greater than 100 mg/L plasma, an aliquot of plasma sample was diluted with drug-free plasma such that the expected value was within the calibration range, and the sample reanalyzed. If the urine concentration of either 2,4-D or MCPA was determined to be greater than 70.0 mg/L urine, an aliquot of urine sample was diluted with saline such that the expected value was within the calibration range, and the sample reanalyzed.
**Calibration Curves.** Calibration curves were obtained by calculating the ratios of the peak heights (height of the 2.4-D peak divided by height of the 4-CPA peak, or height of the MCPA peak divided by height of the 4-CPA peak) of calibration standards and plotting them against the known analyte concentration of 2.4-D and MCPA standards respectively. The slope, intercept, and coefficient of determination of each curve was determined by linear regression analysis. The concentrations of test samples were calculated from the peak height ratio and the slope and intercept of the appropriate calibration curve.

**Validation**

The HPLC method was evaluated for linearity, specificity, stability, lower limit of quantification, instrument lower limit of detection, precision, and accuracy. Linearity was demonstrated by evaluating the relationship between peak height ratios and the fortified analyte concentrations of prepared standards using non-weighted linear regression, and is defined as having a coefficient of determination, $r^2$, greater than 0.990. Specificity was defined as the ability of the assay to distinguish 2.4-D, MCPA, and 4-CPA from each other, as well as other interfering substances found in drug-free canine plasma or urine. Stability was defined as the ability to reanalyze derivatized samples after 3 days with acceptable accuracy. The lower limit of quantification (LLOQ) was the concentration of analyte found to have precision $\leq 20\%$ and accuracy between 80 - 120\%, while the instrument lower limit of detection (ILLOD) was the lowest amount found at a signal to noise ratio of 3. Inter- and intra-day precision was defined as the distribution of individual measurements around the mean value between and within days, respectively, and was calculated as:
Eq. 1

\[
\text{percent interday precision} = \frac{\sqrt{\text{MSB}}}{\text{mean}} \times 100
\]

and

Eq. 2

\[
\text{percent intraday precision} = \frac{\sqrt{\text{MSR}}}{\text{mean}} \times 100
\]

with MSB and MSR being the mean squared between residual and mean squared residual respectively, obtained from one way ANOVA (Sigma Stat 2.0, Jandel Scientific, San Rafael, CA, USA). Accuracy was defined as the closeness of a measured value to the real value and is calculated as recovery:

Eq. 3

\[
\text{percent recovery} = \frac{\text{mean observed}}{\text{expected}} \times 100
\]

Accuracy was considered acceptable if percent recovery was \(\geq 85\%\) and \(\leq 115\%\) except at the LLOQ in which accuracy between 80 - 120\% was acceptable. Precision was acceptable at \(\leq 15\%\) except at the LLOQ when \(\leq 20\%\) was acceptable.

**Application**

Healthy beagle dogs were administered either 100 mg 2.4-D/kg body weight (bw) \((n=1)\), 100 mg MCPA/kg bw \((n=1)\), or 100 mg 2.4-D plus 100 mg MCPA/kg bw \((n=1)\)
orally as a single dose. Blood was collected into EDTA tubes at 0, 1, 2, 4, 6, 8, 24, 36, 48, 72, 96, 120, 144, 168, 360, and 528 hours. After centrifugation for 20 minutes, plasma was harvested and frozen at -17°C until analysis. Free catch urine was collected at 0, 6, 8, 24, 48, 168, 360, and 528 hours. Urine samples were frozen at -17°C until analysis. The dogs were observed for clinical signs of phenoxy acid toxicity: ataxia, muscular tremors, weakness, inability or difficulty in rising, and stiffness at a walk.

Results and Discussion

Validation

Chromatographs from plasma and urine sample analyses are shown in figure 2.2 and 2.3, respectively. Retention times of 4-CPA, 2.4-D, and MCPA extracted from plasma samples were approximately 7.8, 10.2 and 11.4 minutes, respectively. The retention times of 4-CPA, 2.4-D, and MCPA extracted from urine samples were approximately 13.2, 17.7 and 20.0 minutes, respectively. The mean calibration curves for plasma and urine calibrators are shown in Tables 2.1 and 2.2, respectively. Mean coefficients of determination for all equations were greater than 0.992. The instrument’s lower limit of detection (LLOD), defined as a signal to noise ratio of 3 was 1.56 ng for both 2.4-D and MCPA. For each analyte in plasma, a lower limit of quantification (LLOQ) of 5.00 mg/L was determined for the standards between 5.00 mg/L to 70.0 mg/L, and 0.50 mg/L for standards between 0.50 mg/L to 5.00 mg/L. The LLOQ for 2.4-D in urine was 5.00 mg/L, and the LLOQ for MCPA in urine was 10.0 mg/L. There were no significant interfering peaks detected from blank dog plasma or urine.
Figure 2.2. Chromatograms from plasma analysis of (A) blank plasma, (B) a supplemented sample containing 30.0 mg 2,4-D/L and 30.0 mg MCPA/L, and (C) a test sample obtained 6 hours after a single oral dose administration of 100 mg 2,4-D/kg plus 100 mg MCPA/kg. The measured 2,4-D and MCPA concentrations in the test sample were 57.1 and 63.0 mg/L, respectively. Peak identification: (1) 4-CPA, (2) 2,4-D, and (3) MCPA.
Figure 2.3. Chromatograms from urine analysis of (A) blank urine, (B) a supplemented sample containing 10.0 mg 2.4-D/L and 10.0 mg MCPA/L, and (C) a test sample obtained 6 hours after a single oral dose administration of 100 mg 2.4-D/kg plus 100 mg MCPA/kg. The measured 2,4-D and MCPA concentrations in the test sample were 24.0 and < 10.0 mg/L, respectively. Peak identification: (1) 4-CPA, (2) 2.4-D, and (3) MCPA.
<table>
<thead>
<tr>
<th>compound</th>
<th>concentration range (mg/L)</th>
<th>$y = mx + b$</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4-D</td>
<td>0.50 - 5.00</td>
<td>$y = 0.0728x - 0.0039$</td>
<td>0.995</td>
</tr>
<tr>
<td>2.4-D</td>
<td>5.00 - 100</td>
<td>$y = 0.0067x - 0.0031$</td>
<td>0.998</td>
</tr>
<tr>
<td>MCPA</td>
<td>0.500 - 5.00</td>
<td>$y = 0.0925x - 0.0028$</td>
<td>0.994</td>
</tr>
<tr>
<td>MCPA</td>
<td>5.00 - 100</td>
<td>$y = 0.0092x - 0.0021$</td>
<td>0.998</td>
</tr>
</tbody>
</table>

* data shown is average of single curves from 3 different days
† x, concentration added; y, peak height ratio of analyte to internal standard; b, intercept; m, slope.
‡ $r^2$, coefficient of determination

Table 2.1. Linear regression analysis data of 2.4-D and MCPA in canine plasma*

<table>
<thead>
<tr>
<th>compound</th>
<th>$y = mx + b$ †</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4-D</td>
<td>$y = 0.0062x + 0.0152$</td>
<td>0.995</td>
</tr>
<tr>
<td>MCPA</td>
<td>$y = 0.0092x + 0.0093$</td>
<td>0.997</td>
</tr>
</tbody>
</table>

* data shown is average of single curves on 3 different days
† x, concentration added; y, peak height ratio of analyte to internal standard; b, intercept; m, slope.
‡ $r^2$, coefficient of determination

Table 2.2. Linear regression analysis data of 2.4-D and MCPA in canine urine*

Two sets of 3 plasma quality control samples (1.00, 3.00, and 5.00 mg/L and 10.0, 50.0 and 100 mg/L) were analyzed in duplicate on five different days. One set of 3 urine quality control samples (7.00, 10.0 and 50.0 mg/L) was analyzed in duplicate on five different days. In urine, MCPA was found to have unacceptable accuracy (< 80 or >120%) below 10 mg/L. Accuracy of the remaining quality control samples in urine and plasma was within the acceptable limits of 85 - 115%. Precision values calculated for the analytes in plasma and urine are reported in tables 2.3 and 2.4, respectively. Standard and quality control samples re-analyzed three days after initial concentration determinations were on average within 90% of initially determined values.
### Table 2.3. Precision of 2,4-D and MCPA in plasma*

<table>
<thead>
<tr>
<th>compound</th>
<th>calibration range (mg/L)</th>
<th>concentration (mg/L)</th>
<th>percent intraday precision</th>
<th>percent interday precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D</td>
<td>0.50 - 5.00</td>
<td>1.00</td>
<td>10.5</td>
<td>11.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.00</td>
<td>6.66</td>
<td>11.8</td>
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<tr>
<td></td>
<td></td>
<td>5.00</td>
<td>5.64</td>
<td>5.05</td>
</tr>
<tr>
<td>MCPA</td>
<td>0.50 - 5.00</td>
<td>1.00</td>
<td>8.80</td>
<td>7.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.00</td>
<td>3.93</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.00</td>
<td>4.11</td>
<td>7.32</td>
</tr>
<tr>
<td>2,4-D</td>
<td>5.00 - 100</td>
<td>10.0</td>
<td>3.76</td>
<td>8.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50.0</td>
<td>1.46</td>
<td>4.76</td>
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<tr>
<td></td>
<td></td>
<td>100</td>
<td>2.37</td>
<td>3.19</td>
</tr>
<tr>
<td>MCPA</td>
<td>5.00 - 100</td>
<td>10.0</td>
<td>1.84</td>
<td>7.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50.0</td>
<td>1.62</td>
<td>2.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>2.12</td>
<td>3.74</td>
</tr>
</tbody>
</table>

*n=2 samples per day, analyzed on 5 different days.

### Table 2.4. Precision of 2,4-D and MCPA in urine*

<table>
<thead>
<tr>
<th>compound</th>
<th>concentration (mg/L)</th>
<th>percent intra-day precision</th>
<th>percent inter-day precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D</td>
<td>7.00</td>
<td>3.32</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>9.93</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>3.87</td>
<td>9.97</td>
</tr>
<tr>
<td>MCPA</td>
<td>10.0</td>
<td>8.35</td>
<td>8.52</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>2.39</td>
<td>4.71</td>
</tr>
</tbody>
</table>

*n=2 samples per day, analyzed on 5 different days

Table 2.4. Precision of 2,4-D and MCPA in urine*
Application

The method was both sensitive and specific for the analysis of samples obtained from beagle dogs. Figures 2.4-2.6 shows plasma concentration of 2.4-D and MCPA plotted against time after a single oral dose of 100 mg 2.4-D/kg bw. 100 mg MCPA/kg bw. and 100 mg 2.4-D/kg plus 100 mg MCPA/kg bw. The peak plasma 2.4-D concentrations alone or in the presence of MCPA were 390 and 300 mg/L at 6 and 8 hours, respectively. Plasma 2.4-D concentration-time curves were similar following single compound and combination exposure, and concentrations were quantifiable above 5.0 mg/L until 360 hours post administration. Peak plasma MCPA concentrations were 266 and 315 mg/L at 8 and 6 hours after administration of MCPA alone and with 2.4-D, respectively. MCPA was quantifiable in plasma above 5.0 mg/L until 360 hours after administration.
Figure 2.4. Plasma 2.4-D concentrations in one dog after oral administration of 100 mg 2.4-D/kg bw.
Figure 2.5. Plasma MCPA concentrations in one dog after oral administration of 100 mg MCPA/kg bw.
Figure 2.6. Plasma 2,4-D and MCPA concentrations in one dog after a single oral administration of 100 mg 2,4-D/kg bw plus 100 mg MCPA/kg bw.
The time period of detectable 2,4-D and MCPA in plasma and urine samples was similar. Urinary 2,4-D and MCPA are detectable until 48 hours in 4 of 4 samples and 168 hours post administration in 3 of 3 samples (table 2.5). There were no clinical signs of phenoxy herbicide toxicity in any of the dogs in this study. These dogs were given 100 mg 2,4-D/kg, the cited LD₅₀ for 2,4-D in the dog (Drill et al. 1953). 100 mg MCPA/kg, or 100 mg 2,4-D/kg plus 100 mg MCPA/kg with both 2,4-D and MCPA quantitated until at least 48 hours in both plasma and urine samples. This method should be adequate to determine 2,4-D or MCPA exposure.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>2,4-D (mg/L)</th>
<th>MCPA (mg/L)</th>
<th>2,4-D (mg/L)</th>
<th>MCPA (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>6</td>
<td>n.s.</td>
<td>n.s.</td>
<td>24.0</td>
<td>&lt;10.0</td>
</tr>
<tr>
<td>8</td>
<td>93.5</td>
<td>511</td>
<td>n.s.</td>
<td>n.s.</td>
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<tr>
<td>24</td>
<td>49.5</td>
<td>152</td>
<td>541</td>
<td>258</td>
</tr>
<tr>
<td>48</td>
<td>127</td>
<td>33.6</td>
<td>250</td>
<td>88.6</td>
</tr>
<tr>
<td>168</td>
<td>72.9</td>
<td>n.s.</td>
<td>34.1</td>
<td>&lt;10.0</td>
</tr>
<tr>
<td>360</td>
<td>&lt;5.00</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n = 1 dog per administration; * n.d., none detected (the limit of detection for a 100 µL urine sample is 3 mg 2,4-D/L and 3 mg MCPA/L at a signal to noise ratio of 3); † n.s., no sample

Table 2.5. Urine concentrations in dogs after oral administration of 100 mg 2,4-D/kg, 100 mg MCPA/kg or 100 mg 2,4-D plus 100 mg MCPA/kg.

Conclusion

HPLC methods (Aprea et al. 1997, Flanagan et al. 1990) have been developed for analysis of single phenoxy herbicides in plasma or urine. One method simultaneously detected multiple phenoxy compounds in plasma and urine samples utilizing HPLC with UV detection, but separation of 2,4-D and MCPA was not possible. We have developed an HPLC method to simultaneously measure the concentrations of 2,4-D and MCPA in canine plasma or urine using fluorescence detection. The method uses a sample volume of 100 μL of plasma or urine, while maintaining an acceptable level of quantification of 0.50 mg/L plasma and 5.00 mg/L urine for 2,4-D, and 0.50 mg/L plasma and 10.0 mg/L urine for MCPA. Our method allows for rapid and reliable analysis of 2,4-D and MCPA in canine plasma and urine applicable to pharmacokinetic studies or toxicology conformation. This method has potential use in analyzing human plasma and urine samples with the same considerations.
References


CHAPTER 3

To evaluate the toxicodynamic interaction of 2.4-D and MCPA, a sensitive and measurable indicator or toxicity was necessary. As stated in Chapter 1, electromyography (EMG) has been used to identify and grade subclinical myotonic changes. Based on the cost and extensiveness of such a study, it was prudent to do pilot studies to determine the appropriate dosage in which myotonic discharges, both insertional and spontaneous, would be observed in 2.4-D and MCPA treated dogs. An initial dosage of 25 mg 2,4-D/kg b.w. was chosen, based on the reported information by Steiss et al. (1987) and Beasely et al. (1991). There was no data in the literature concerning the EMG changes after MCPA exposure, or 2.4-D and MCPA co-exposure.

These pilot studies were performed and a brief description of the study designs and results follows. Dogs were administered 25 mg 2,4-D/kg b.w. (n = 2), 25 mg MCPA/kg b.w. (n = 2), with no myotonic changes found. Subsequently dogs were administered 50 mg 2,4-D/kg b.w. (n = 1), 50 mg MCPA/kg b.w. (n = 1), again with no myotonic changes observed. Due to the lack of clinical or EMG signs of myotonia, the dosages were increased to 75 mg 2,4-D/kg b.w. (n = 1), 75 mg MCPA/kg b.w. (n = 1). 75 mg 2,4-D/kg b.w. plus 75 mg MCPA/kg b.w. (n = 1). At this dosage administration, EMG evaluations revealed prolonged insertional myotonic activity only in the combined administration, but no observable clinical signs. Based on the lack of myotonic activity in the 2,4-D or MCPA only treated animals, the dosage was increased to 100 mg 2,4-D/kg b.w. (n = 1), 100 mg MCPA/kg b.w. (n = 1), and 100 mg 2,4-D/kg b.w. plus 100 mg MCPA/kg b.w. (n = 1), with insertional myotonia, but not clinical myotonia, observed in all three dosage
groups (200 - 500 ms for 2,4-D, 100 - 200 ms for MCPA), with the combined being most severe (500 - 1000 ms). In summary, the following conclusions were reached based on these EMG pilot studies:

1) No myotonic changes, clinical or electromyographic, were found in any dogs at dosages below 50 mg/kg of either compound.

2) Prolonged insertional myotonic activity was seen at the combined dosage of 75 mg 2,4-D/kg b.w. plus 75 mg MCPA/kg b.w.

3) Prolonged insertional myotonic activity was observed in all dose groups at 100 mg/kg b.w. administration with somewhat longer activity recorded in the animal administered 2,4-D and MCPA.

4) No spontaneous myotonia (defined as myotonic discharges occurring after cessation of needle movement) were observed in any of the dogs at any of the dosages administered.

5) Clinical (observable) myotonia was not evident at any dose administration.

The insertional myotonic activity was indicative of muscle membrane instability and changes of function of the muscle membrane. It appeared that this finding was 2,4-D dose dependant and co-administration of MCPA enhanced this effect. However at 100 mg/kg b.w. dose administration, statistical comparisons of 2,4-D alone and 2,4-D with MCPA would not produce significance without spontaneous activity occurring. Based on this information, it was decided to increase the dosage to 200 mg 2,4-D/kg b.w. (n = 1). 200 mg MCPA/kg b.w. (n = 1) and 200 mg 2,4-D/kg b.w. plus 200 mg MCPA/kg b.w. (n = 1). The dog receiving 2,4-D had visible signs of myotonia with mild ataxia, muscle dimpling, and slight stiffness on initial rising and slight decrease in appetite. The dog receiving MCPA had no observable signs of myotonia but loose stool. Based on these observations, a combined administration of 200 mg 2,4-D/kg plus 200 mg MCPA/kg was
given to one dog. Observable myotonia was not found in the combined administration
dog, however the dog vomited twice in 96 hours. Because of the apparent muscular
response in the 200 mg 2.4-D/kg treated dog, this dose of 2.4-D was selected for the
study. The following presentation of results summarize the response in six dogs
administered 200 mg 2.4-D/kg b.w. orally. Unfortunately the concurrent confounding
gastrointestinal effects of 2.4-D as this dose precluded the completion of the interaction
study.
CLINICAL EFFECTS AND PLASMA CONCENTRATION DETERMINATION AFTER 2.4-DICHLOROPHENOXYACETIC ACID/200MG/KG ADMINISTRATION IN THE DOG

Introduction

2.4-Dichlorophenoxyacetic acid (2.4-D) is classified as a phenoxy herbicide and is extensively used for post emergent control of broadleaf weeds. It is often found in commercial and at home lawn care products applied during the summer season in North America. Because of its widespread use, toxicity to both humans and dogs is a concern. Oral toxicity to most species ranges from 300 - 1000 mg/kg (Stevens et al. 1991) however the dog was reported to be the most sensitive with an LD$_{50}$ of 100 mg/kg (Drill et al. 1953). A few researchers have administered 2,4-D orally to dogs at doses approximately 1 - 2 fold greater than the reported LD$_{50}$ without deaths occurring (Steiss et al. 1987. Beasley et al. 1991. Arnold et al. 1991). Thus controversy exists regarding the lethal dose in the dog.

Initial signs of acute toxicity in all animal species are vomiting, diarrhea, and myotonia. Beasley et al. (1991) dosed English pointer dogs with 1.3 to 225 mg/kg of the dimethylamine formulation of 2.4-D (n = 1 dog/dose). Clinical signs of vomiting, hypermetria, incoordination, and knuckling occurred as early as 45 minutes post administration of 175 and 225 mg/kg and resolved by 24 hours. Steiss et al. (1987) evaluated mongrel dogs (n=4 dogs/dose) after oral administration of 2.4-D dosages ranging from 25 - 125 mg/kg and observed muscle dimpling in one dog at each dosage between 50 - 125 mg/kg and muscle stiffness in 2 of 4 dogs in each of the 100 and 125 mg/kg dose groups. Similar findings of gait abnormalities and gastrointestinal disturbance have been observed in cattle and pigs (Bjorklund et al. 1966. Paulino et al. 1995).
Myotonia has a characteristic electromyographic (EMG) appearance of high frequency waxing and waning bursts of electrical activity (Duncan, 1989). Beasley et al. (1991) found EMG abnormalities in dogs approximately 24 hours after administration of doses as low as 8.8 mg/kg. These changes consisted of prolonged insertional and increased bizarre harmonic (myotonic) activity. Steiss et al. (1987) reported myotonic discharges of <200 ms to >1000 ms in dogs 24 hours post administration of 50 to 125 mg/kg. Myotonic discharges, with an average maximal grading of 500 - 1000 ms in duration, were also seen in rats after intraperitoneal administration of 2.4-D 200 mg/kg (Bernard et al. 1985).

Few studies have evaluated clinical chemistry parameters in any species after acute 2.4-D exposure. Arnold et al. (1991) found no changes in clinical chemistry parameters in dogs at doses that clinically produced myotonia. Cattle dosed with 100 to 600 mg/kg dimethylamine were evaluated for changes in clinical chemistry parameters, with only changes in glucose and glucose and BUN at 300 and 600 mg/kg, respectively (Paulino et al. 1995).

Limited information is available about the plasma 2.4-D concentrations in acute cases of toxicity. Average total plasma 2.4-D concentrations in pigs 24 hours after a single oral administration of 2,4-D amine 50 and 1000 mg/kg were reported to be 35 and 580 mg/mL (mg/L), respectively (Bjorklund et al. 1966). At approximately 24 hours, single dogs dosed with 175 or 220 mg/kg of 2.4-D dimethylamine were reported to have 2.4-D concentrations of 718 and 934 ppm (mg/L), respectively (Arnold et al. 1991). In reported hospitalized attempted suicide cases of 2.4-D toxicity after ingestion of unknown amounts, blood or plasma concentrations have been measured at 520 mg/L (Fraser et al. 1984), and 369.8 mg/g (mg/L) (Osterloh et al. 1983). In none of the animal or human
studies has the unbound 2,4-D concentration been measured. Since only the unbound fraction of a drug is considered to be biologically active, it may be a better measure of toxicity than total plasma 2.4-D concentration.

The objective of the present investigation was to study the clinical effects, and to determine the 2.4-D plasma concentration at the time of the EMG (24 hours) following a dose, twice the reported LD$_{50}$, orally administered to six beagle dogs. Investigation of clinical effects were focused on the electromyographic evaluations and biochemical parameter analyses.

**Materials and Methods**

**Animals**

The experimental protocol was approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee. Six female beagle dogs weighing between 9.6 and 12.7 kg were individually housed in climate controlled runs and had free access to food and water. Food was withheld 12 hours prior to 2.4-D as acid (98% purity, Aldrich, Milwaukee, WI, USA) administration. The dogs were orally administered 2.4-D 200 mg/kg body weight (b.w.) in gelatin capsules and monitored hourly for 6 hours for the development of clinical signs of toxicity. Dogs were walked for 2-5 minutes to evaluate the presence of gait abnormalities and muscle dimpling. The number of bouts and time of any emesis or defecations were recorded. Blood was collected via the jugular vein one day prior to 2.4-D administration and at the time of the EMG, 24 hours post administration. Plasma total and unbound 2.4-D concentration determinations and clinical chemistry parameter analyses were performed using the collected blood.
**2,4-D analysis**

Blood was collected in EDTA anticoagulant and centrifuged. The plasma was harvested and divided into two portions. The first portion was analyzed for total 2,4-D. The unbound 2,4-D was separated from the second plasma portion using the Centrifree micropartition centrifugation system (Millipore, Danvers, MA, USA). Approximately 1.0 mL of plasma was placed in the top chamber of the device. The device was centrifuged at 37°C for 7 minutes at 1000 g resulting in 100 - 150 μL of ultrafiltrate. The harvested plasma and ultrafiltrate were immediately frozen at -17°C until chemical analysis. The total and unbound 2,4-D concentrations were determined by HPLC with fluorescence detection.

**HPLC**

A 8.0 μL aliquot of the internal standard, 4-chlorophenoxyacetic acid (4-CPA), working solution (0.1 g/L) was added to screw-cap tubes followed by the addition of an 100 μL sample or calibrator. The mixture was briefly vortexed and acidified with 0.5 mL of 0.1 N hydrochloric acid. The sample was then extracted with 4.0 mL of ethyl acetate-hexane (70/30, v/v), mixed by end-over-end inversion for 5 minutes, and centrifuged at approximately 1000 g for 5 minutes. The organic phase was transferred to a conical tube and evaporated under nitrogen at 40 - 45°C. The dry residue was dissolved in 70 μL of 0.025% 9-anthryldiazomethane (ADAM) in acetonitrile and an additional 30 μL of acetonitrile and incubated at room temperature for 10 minutes. After the addition of 700 μL mobile phase and brief mixing, a 25 μL aliquot of each extracted derivatized sample was injected onto the high performance liquid chromatographic column.

The HPLC system consisted of a dual-piston reciprocating pump (ConstraMetric IIIG pump. KDC/Milton Roy. Riviera Beach, FL, USA), an automatic sample injector (WISP
712 autosampler, Waters, Milford, MA, USA), and a fluorescence detector (Kratos FS-970, Applied Biosystems, Ramsey, NJ, USA). Chromatographic separations were performed using a 4.6 x 150 mm column (Zorbax SB-C18, 5 μm particle size, Mac-Mod Analytical, Inc., Chadds Ford, PA, USA). A guard column (Perisorb RP-18, 30 - 40 μm particle size, Upchurch Scientific, Inc., Oak Harbor, WA, USA) was used to protect the chromatographic column. The mobile phase was acetonitrile-water (75/25, v/v) at a flow rate of 1.0 mL/min. Fluorescence was measured at an excitation wavelength of 365 nm and an emission wavelength of 389 nm (Kratos FS-970, Applied Biosystems, Ramsey, NJ, USA). A reporting integrator (Spectra Physics 4270, Spectra Physics, San Jose, CA, USA) detected peak heights at a chart speed of 0.25 cm/min. Calibration curves were obtained by calculating the ratios of the peak heights of the calibrated standards to the internal standard and plotting them against the known analyte concentration of 2.4-D standard.

**Clinical chemistry analysis**

Blood was collected without anticoagulant, centrifuged, and the harvested serum was submitted to the Clinical Pathology Laboratory, Veterinary Hospital, The Ohio State University (OSU) College of Veterinary Medicine. The following clinical chemistry parameters were determined using the Hitachi 911 (Boehringer Mannheim, Indianapolis, IN, USA): CO₂, calcium, phosphorus, glucose, creatinine, total bilirubin, sodium, potassium, chloride, total protein, BUN, albumin, cholesterol, alkaline phosphatase (ALP), creatine kinase (CK), alanine aminotransferase (ALT), and aspartate aminotransferase (AST).
**Electromyographic studies**

Needle EMG was performed under general anesthesia for all dogs to permit complete evaluation of insertional and spontaneous activity (Duncan, 1989). At 24 hours after 2.4-D administration, dogs were anesthetized with 0.1 mg/kg diazepam (Valium. Roche Laboratories Inc., Nutley, NJ, USA) followed by 10 - 15 mg/kg pentothal (Abbott Laboratories, North Chicago. IL, USA) to effect and endotracheally intubated. Animals were maintained on 1.5 - 2.0% isoflurane (Isoflo. Solvay Animal Health. Mendota Heights. MN, USA). Monopolar needle (26 gauge. Neurosupplies. Watertown. CT. USA) EMG testing was performed on the dogs in lateral recumbency throughout the procedure by one of the authors (MP) with intramuscular potentials recorded and displayed on an electromyograph instrument (Nihon-Kodon. 4-channel Minipack. Irvine. CA, USA). Muscles for EMG testing were grouped into the following sites: proximal thoracic limb muscles (supraspinatus, infraspinatus, deltoideus, and long and lateral heads of the triceps), proximal pelvic limb muscles (superficial and middle gluteals. biceps femoris, semitendinosus and the vastus lateralis), distal thoracic limb muscles (extensor carpi radialis. flexor carpi radialis. and flexor carpi ulnaris). distal pelvic limb muscles (extensor digitorum longus. cranial tibial and gastrocnemius). cervical epaxial muscles, and remaining expaxial muscles. Prolonged insertional activity was defined as any activity present that outlasted the cessation of needle movement (Kimura. 1989). Insertional myotonia was graded according to the duration of discharge as: 100-200 ms (+1). 200 - 500 ms (+2). 500 - 1000 ms (+3). and >1000 ms (+4).

Spontaneous activity was defined as post-insertional electrical activity recorded from muscle without any voluntary contraction (Kimura. 1989). The types of spontaneous activity reported in this paper were fibrillation potentials, positive sharp waves (PSWs). and myotonic discharges. Each of these waveform types was identified according to the
parameters described by Kimura (1989). Fibrillation potentials were identified as biphasic spikes of usually short duration (< 5 ms) with an initial positive phase associated with a high-pitched regular sound ("rain on a tin roof"). Positive sharp waves were identified as biphasic potentials with a rapid initial positive deflection (< 1 ms) followed by a low amplitude, longer duration negative phase (10 - 100 ms) associated with a variety of sounds often resembling engine noises. Myotonic discharges were identified as waxing-waning biphasic or positive waves with repetitive discharges at rates between 20 to 80 Hz upon needle insertion that were associated with "dive bomber" sound. The minimum criteria used to define the presence of spontaneous activity was persistence of an abnormal waveform greater than 3 seconds after complete cessation of needle movement that was reproducible with redirection of the EMG needle. Activity was graded from absent (0), to mild (+1) to severe (+4). The muscular electrical activity was evaluated based on the duration of insertional activity, myotonic discharges, and the presence and frequency of fibrillations and positive sharp waves.

Statistical analysis

Statistical analyses of the clinical chemistry parameters were performed using a paired t-test with an overall α of 0.05 utilizing Sigma Stat 2.0 statistical software package (Jandel Scientific, San Rafeal, CA, USA).

Results

Clinical Effects

All dogs developed semi-liquid feces by 24 hours after administration of 2,4-D. Between 2 and 3 hours after administration one dog vomited once, and between 6 and 24 hours a second dog had vomited once. At no time did any of the dogs exhibit gait abnormalities or difficulty in rising. Mean (± s.d.) values of the clinical chemistry
parameters obtained pre- and 24 hours post- administration are presented in table 3.1. Statistically significant decreases are seen in serum calcium and potassium after 2.4-D, but were not severe enough to have required medical treatment. Statistically significant decreases in total bilirubin were seen after administration of 2.4-D however, the mean value was within the OSU Veterinary Teaching Hospital’s normal range for dogs.

Mild percussion dimpling of the tongue was observed in 5 out of 6 anesthetized dogs at the time of EMG testing. Results of the electromyographic studies are shown in table 3.2 with an example of an EMG in figure 3.1. Insertional myotonic activity was recorded in all muscle groups in 5 of the 6 dogs. In those five dogs, two dogs were graded as (+2), one dog as (+3), and two dogs as (+4). The remaining dog showed (+1) activity only in the proximal pelvic musculature. Spontaneous activity in the form of fibrillation potentials and complex repetitive discharges was evident in multiple muscles of only one dog.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>metric SI</th>
<th>Pre mean (+s.d.)</th>
<th>24 hrs post administration mean (+s.d.)</th>
<th>OSU normal canine range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO₂</td>
<td>mEq/L mmol/L</td>
<td>22.8 (1.7)</td>
<td>25.3 (2.6)</td>
<td>18 - 30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22.8 (1.7)</td>
<td>25.3 (2.6)</td>
<td>18 - 30</td>
</tr>
<tr>
<td>calcium</td>
<td>mg/dL mmol/L</td>
<td>9.78 (0.51)</td>
<td>8.58 (0.21)*</td>
<td>9.4 - 12.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.44 (0.13)</td>
<td>2.14 (0.05)*</td>
<td>2.35 - 2.99</td>
</tr>
<tr>
<td>phosphorus</td>
<td>mg/dL mmol/L</td>
<td>3.82 (0.63)</td>
<td>4.32 (1.22)</td>
<td>2.7 - 7.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.23 (0.20)</td>
<td>1.39 (0.39)</td>
<td>0.87 - 2.45</td>
</tr>
<tr>
<td>Glucose</td>
<td>mg/dL mmol/L</td>
<td>90.0 (7.24)</td>
<td>86.3 (13.46)</td>
<td>63 -120</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.00 (0.40)</td>
<td>4.79 (0.75)</td>
<td>3.50 - 6.66</td>
</tr>
<tr>
<td>creatinine</td>
<td>mg/dL μmol/L</td>
<td>1.17 (0.16)</td>
<td>0.90 (0.11)</td>
<td>0.7 - 1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>103 (14.1)</td>
<td>79.6 (9.72)</td>
<td>61.9 - 124</td>
</tr>
<tr>
<td>Total bilirubin</td>
<td>mg/dL μmol/L</td>
<td>0.22 (0.04)</td>
<td>0.10 (0)*</td>
<td>0.1 - 0.5</td>
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<tr>
<td></td>
<td></td>
<td>3.76 (0.68)</td>
<td>1.71 (0)*</td>
<td>1.71 - 8.55</td>
</tr>
<tr>
<td>sodium</td>
<td>mEq/L mmol/L</td>
<td>144 (1.10)</td>
<td>138 (2.93)</td>
<td>144 - 160</td>
</tr>
<tr>
<td></td>
<td></td>
<td>144 (1.10)</td>
<td>138 (2.93)</td>
<td>144 - 160</td>
</tr>
<tr>
<td>potassium</td>
<td>mEq/L mmol/L</td>
<td>4.2 (0.37)</td>
<td>3.27 (0.25)*</td>
<td>4.0 - 5.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.2 (0.37)</td>
<td>3.27 (0.25)*</td>
<td>4.0 - 5.5</td>
</tr>
<tr>
<td>chloride</td>
<td>mEq/L mmol/L</td>
<td>111 (1.72)</td>
<td>105 (3.39)</td>
<td>105 - 119</td>
</tr>
<tr>
<td></td>
<td></td>
<td>111 (1.72)</td>
<td>105 (3.39)</td>
<td>105 - 119</td>
</tr>
<tr>
<td>total protein</td>
<td>gm/dL g/L</td>
<td>5.87 (0.25)</td>
<td>5.33 (0.32)</td>
<td>5.6 - 8.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>58.7 (2.5)</td>
<td>53.3 (3.2)</td>
<td>56 - 80</td>
</tr>
<tr>
<td>B.U.N.</td>
<td>mg/dL mmol/L urea</td>
<td>19.2 (4.54)</td>
<td>22.0 (2.83)</td>
<td>7 - 31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.85 (1.62)</td>
<td>7.85 (1.01)</td>
<td>2.50 - 11.1</td>
</tr>
<tr>
<td>albumin</td>
<td>gm/dL g/L</td>
<td>3.37 (0.23)</td>
<td>3.23 (0.25)</td>
<td>3.1 - 4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33.7 (2.30)</td>
<td>32.3 (2.50)</td>
<td>31 - 44</td>
</tr>
<tr>
<td>cholesterol</td>
<td>mg/dL mmol/L</td>
<td>261 (89.2)</td>
<td>188 (32.7)</td>
<td>106 - 330</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.75 (2.31)</td>
<td>4.86 (0.85)</td>
<td>2.74 - 8.53</td>
</tr>
<tr>
<td>ALP</td>
<td>IU/L</td>
<td>33.0 (16.0)</td>
<td>45.0 (16.6)</td>
<td>20 - 130</td>
</tr>
<tr>
<td>CK</td>
<td>IU/L</td>
<td>180 (107)</td>
<td>274 (246)</td>
<td>14 - 350</td>
</tr>
<tr>
<td>ALT</td>
<td>IU/L</td>
<td>57.8 (20.5)</td>
<td>24.5 (5.09)</td>
<td>15 - 110</td>
</tr>
<tr>
<td>AST</td>
<td>IU/L</td>
<td>32.8 (9.66)</td>
<td>31.8 (11.7)</td>
<td>7 - 44</td>
</tr>
</tbody>
</table>

* p < 0.05 overall; post values compared with pre values

Table 3.1. Mean (±s.d.) values of clinical chemistry parameters obtained 24 hours after oral administration of 200 mg 2,4-D/kg. (n = 6) Data was analyzed using paired-t tests.
<table>
<thead>
<tr>
<th>Dog</th>
<th>Insertional EMG activity*</th>
<th>Spontaneous EMG activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(+2) insertional myotonia in all muscles tested</td>
<td>none</td>
</tr>
<tr>
<td>2</td>
<td>(+2) insertional myotonia in all muscles tested</td>
<td>none</td>
</tr>
<tr>
<td>3</td>
<td>(+4) insertional myotonia in all muscles tested.</td>
<td>Fibrillation potentials (+1) and complex repetitive discharges (+1)</td>
</tr>
<tr>
<td>4</td>
<td>(+1) insertional myotonia in proximal pelvic muscles only</td>
<td>none</td>
</tr>
<tr>
<td>5</td>
<td>(+3) insertional myotonia in all muscles tested</td>
<td>none</td>
</tr>
<tr>
<td>6</td>
<td>(+4) insertional myotonia in all muscles tested</td>
<td>none</td>
</tr>
</tbody>
</table>

*Insertional myotonia gradations are: 100 - 200 ms (+1), 200 - 500 ms (+2), 500 - 1000 ms (+3), >1000 ms (+4). Spontaneous activities were graded from mild (+1) to severe (+4).

Table 3.2. Electromyographic evaluation of six dogs 24 hours after oral administration of 200 mg 2,4-D/kg.
Figure 3.1. Electromyographic recording from the thoracic epaxial muscle of dog 2 demonstrating typical insertional waxing-waning myotonic discharges.
2,4-D Plasma Concentrations

Total and unbound 2,4-D concentrations in individual dogs are shown in table 3.3. Mean total and unbound plasma 2,4-D concentrations at the time of electromyographic evaluation were 511± 72.6 mg/L and 129 ± 24.7 mg/L, respectively. The percent of unbound 2,4-D in the plasma ranged from 23.1 to 28.1 percent.

<table>
<thead>
<tr>
<th>Dog</th>
<th>Total 2.4-D (mg/L)</th>
<th>Unbound 2.4-D (mg/L)</th>
<th>percent unbound 2.4-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>526</td>
<td>148</td>
<td>28.1</td>
</tr>
<tr>
<td>2</td>
<td>516</td>
<td>114</td>
<td>22.9</td>
</tr>
<tr>
<td>3</td>
<td>633</td>
<td>166</td>
<td>26.2</td>
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<td>4</td>
<td>455</td>
<td>119</td>
<td>26.2</td>
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<tr>
<td>5</td>
<td>514</td>
<td>127</td>
<td>24.7</td>
</tr>
<tr>
<td>6</td>
<td>421</td>
<td>97.4</td>
<td>23.1</td>
</tr>
</tbody>
</table>

Table 3.3. Total and unbound plasma 2.4-D concentration in dogs 24 hours after oral administration of 200 mg 2.4-D/kg.

Discussion

This study demonstrated that the LD$_{50}$ of 2,4-D is above 200 mg/kg in beagles dogs. No deaths occurred and limited clinical signs of toxicity were observed, mainly vomiting and diarrhea. Myotonia was only visibly evident as percussion dimpling of the tongue while the dogs were under anesthesia. Electromyographic studies revealed the presence of myotonic discharges in all dogs. Thus, the beagle dog is not as sensitive to the acute effects of 2,4-D toxicity as previously reported. The total or unbound plasma 2,4-D concentrations were consistent between dogs and may be useful in predicting acute toxicity in the future, but additional correlation studies are necessary.
In our study, dogs had been dosed with 2.4-D/200 mg/kg with no subsequent deaths. Our finding is consistent with other researchers who have orally dosed dogs 1 - 2 fold greater than the reported LD$_{50}$ of 100 mg 2.4-D/kg (Drill et al. 1953, Steiss et al. 1987, Beasley et al. 1991). Furthermore, in the original report by Drill and Hiratzka (1953), 3 of 3 dogs dosed with 250 mg 2.4-D/kg died. Although the unspecified form of 2.4-D used in the original study was stated as 98.5 % pure (Drill et al. 1953), contamination with other products may have played a role in deaths observed. More recent findings by us and other researchers suggest the true oral LD$_{50}$ of 2.4-D in the dog is unknown but much greater than 200 mg 2.4-D/kg (Beasley et al. 1991, Arnold et al. 1991). This would indicate that the dog is not the most sensitive species and the canine dose-response is not appreciably different from other species.

Clinical signs observed in this study were diarrhea and vomiting, consistent with findings by other researchers (Drill et al. 1953, Beasley et al. 1991, Arnold et al. 1991, Bjorklund et al. 1966). Statistically significant alterations in the biochemical parameters calcium, bilirubin, and potassium were found in our study, however they were small and probably not of clinical significance. and were considered secondary to the gastrointestinal effects of 2,4-D. However, the physiological effects of the observed ion disturbances on the muscle membrane are unknown. The insertional myotonia seen in our study is similar to findings by others in both the dog (Steiss et al. 1987, Beasley et al. 1991) and rat (Bernard et al. 1985). The findings of spontaneous activity (fibrillation potentials and complex repetitive discharges) indicate muscle membrane instability, the significance of which is unknown. Both of these potential types can be seen with primary disturbances of muscle electrical activity or denervating processes. While EMG findings were similar, the lack of observable gait changes seen by us is in conflict with findings by other authors. When Arnold et al. (1991) administered 175 and 225 mg/kg 2.4-D
dimethylamine to dogs, clinical signs of toxicity, vomiting, ataxia and incoordination, were seen within one hour after administration. In the same study total plasma 2.4-D concentrations at approximately 24 hours were reported as 718 and 934 ppm (mg/L), respectively (Arnold et al. 1991). This is higher than our reported total 2.4-D concentrations, which may explain why the decreased severity of clinical signs was observed in our study. The reason for the difference in plasma concentrations is unknown, but may be a result of lower bioavailability to our dogs.

The determination of 2.4-D plasma concentrations may aid in comparison of toxicity across species, but further work is needed. In pigs average 2.4-D plasma concentrations at 24 hours post administration of 500 mg/kg were lower (400 mg/L) (Bjorklund et al. 1966) than those determined from our dogs after 200 mg/kg. This may be due to a slower elimination of 2.4-D in the dog resulting in higher plasma concentrations at later time points. Plasma 2,4-D values at the time of observed clinical signs are reported in cases of human toxicity. These values (389.6 and 520 mg/L) (Fraser et al. 1984. Osterloh et al. 1983) are less than or similar to those seen by us suggesting the dog is not more sensitive than the human. However multiple compound exposures occurred in these cases and the effects of combination exposures are unknown.

Only the unbound portion of a drug or chemical is available to elicit clinical effects (Svensson et al. 1986), thus knowledge of the unbound concentration is likely to be a better determinant of dose response relationships. In our study, the average unbound plasma 2.4-D concentration was 129 mg/L. Unfortunately, the unbound concentration of 2.4-D was not determined in any of the reported dog, pig, or human exposure situations.
In conclusion, our findings support the hypothesis that the oral LD$_{50}$ for 2.4-D in the dog is greater than the previously reported value of 100 mg/kg, and likely to be similar to that reported in other species. The only clinically significant signs detected after a single oral administration of 200 mg/kg were vomiting and diarrhea. Furthermore our EMG findings were similar to those seen by other authors in other canine breeds and the rat, suggesting the beagle is not unique. Plasma total or unbound 2.4-D may be a useful indicator in comparing toxicity of 2.4-D across species.
References


CHAPTER 4

The purpose of the 2.4-D interaction studies was to investigate both the toxicodynamic and toxicokinetic effects of combined phenoxy compound administrations to the beagle dog. The compounds 2.4-D and MCPA are structurally very similar, however no increase in toxicity was observed during pilot toxicodynamic studies. Investigations of the toxicokinetic interaction of 2.4-D and MCPA is important to understanding the lack of biological interactions of these compounds. These studies were performed in conjunction with the toxicodynamic interaction portion of this research.
Introduction

Phenoxy herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) (Figure 4.1) are widely used for post-emergent broadleaf weed control. Often the phenoxy herbicides are used in combination with other structurally similar phenoxy and benzoic acid compounds to increase the spectrum of herbicide activity. Currently there is an extensive public and government interest and concern about the potential biological affects of combination exposures to structurally similar compounds (Bucher and Lucier, 1998). In addition, many cases of intentional or accidental exposure to 2,4-D and MCPA in both man and dog have been reported in the literature (Fraser et al. 1984, Harrington et al. 1996, Jorens et al. 1995, Keller et al. 1994, Osterloh et al. 1983, Prescott et al. 1979, Schmoldt et al. 1997). These exposures often involve products containing multiple compounds (Fraser et al. 1984, Harrington et al. 1996, Osterloh et al. 1983, Prescott et al. 1979). There are no data describing the biological interactions of combined exposure to phenoxy compounds, but there are multiple areas for potential toxicokinetic interactions of phenoxy herbicides such as protein binding and saturation of active transport processes in the kidney and brain.
Plasma protein binding of the individual compounds, 2.4-D and MCPA, has been shown to be extensive and concentration dependent in a variety of species including rat (Elo and Ylitalo, 1979), bovine (Kohlberg et al., 1973, Fang and Lindstrom, 1980), horse, human, guinea pig (Fang and Lindstrom, 1980), and goat (Örberg, 1980). The free fractions of 2.4-D and MCPA in the rat have been shown to increase 41% and 73%, respectively, by co-incubation with probenecid, a common organic anion transport inhibitor (Ylitalo et al., 1990). This displacement provides evidence that a protein binding interaction can occur, however phenoxy-phenoxy protein binding interactions studies have not been performed. It would be expected that 2.4-D and MCPA would bind to the same binding sites, due to the structural similarity of these compounds, resulting in increased free fractions of both compounds.

2.4-D and MCPA, as individual compounds, undergo limited metabolism and are excreted primarily unchanged in the urine (Elo, 1976. Kohli et al. 1974, Kolmodin-Hedman, 1983, Pelletier et al. 1989). Gorzinski et al. (1987) found, in rats, that as the dose of 2.4-D increased, the amount of 2.4-D excreted in the urine was less than expected, suggesting saturation of transport. The excretion of 2.4-D has also been shown
to be mediated by active transport in rat renal slices and was inhibited by probenecid and para-aminohippuric acid (PAH) (Koschier et al., 1978. Villalobos et al., 1996). Furthermore 2,4-D inhibited the transport of PAH in rat renal slices (Koschier et al., 1978. Villalobos et al., 1996). The renal excretion of MCPA was found to be dose dependant in the rat and inhibited by co-administration of PAH or probenecid (Bräunlich et al., 1989), again suggesting saturation of transport. The effects of 2,4-D on MCPA transport and MCPA on 2,4-D transport have not been evaluated. It would be expected however, that inhibition of renal elimination would result in prolonged plasma concentrations with the potential for increased toxicity.

The ultimate interest in the toxicokinetic interactions is the resulting potential for an increase in toxicity. When Ylitalo et al. (1990) co-administered probenecid with 2,4-D or MCPA to rats, a 29 and 70 %, respectively, decrease in the LD$_{50}$ compared to phenoxy only administration was observed. This increase in toxicity was associated with increased brain concentrations of 2,4-D and MCPA (Ylitalo et al. 1990). Ylitalo et al. (1990) attributed the increased brain concentrations to displacement of 2,4-D and MCPA from plasma proteins and inhibition of transport out of the brain by probenecid.

In summary, the concentration dependent plasma protein binding, saturable renal elimination of the phenoxy compounds, and studies with probenecid suggest a pharmacokinetic interaction could occur. This interaction has the potential to alter the biological effect and increase toxicity of the individual phenoxy compounds. Using 2,4-D and MCPA as representative phenoxy compounds, the purpose of this study was to evaluate the toxicokinetic interaction of phenoxy herbicides in the dog by comparing the plasma concentration-time curves, urinary excretion, and plasma protein binding of 2,4-D alone, MCPA alone, and 2,4-D plus MCPA.
Materials and Methods

**Compounds.** 2,4-Dichlorophenoxyacetic acid (2,4-D, 98%). 2-methyl-4-chlorophenoxyacetic acid (MCPA, 95%). 4-chlorophenoxyacetic acid (4-CPA, 98%) were purchased from Aldrich Chemical Co. (Milwaukee, WI) and canine albumin (fatty acid free, Fraction V) was purchased from Sigma Chemical Co. (St. Louis, MO). 9-Anthryldiazomethane (ADAM) was obtained from Research Organics (Cleveland, OH). All other chemicals were HPLC grade and purchased from Burdick & Jackson Laboratories (Muskegon, MI).

**Animals.** Six female Beagle dogs (10-14 kg) (Harlan, Indianapolis, IN) were acquired under an Ohio State University ILACUC approved protocol and maintained in accordance with ILAR guidelines for the care and use of laboratory animals. Dogs were individually housed in runs with free choice food and water, except during kinetic studies when metabolism cages were used. During kinetic studies, food was withheld 12 hours before dose administration and returned 8-24 hours later.

**In vivo study design.**

**Experiment I.** A triple cross over design study was used with the following three treatment groups of 2 dogs per group: 1) single dose of 25 mg MCPA/kg b.w. orally in gelatin capsules, 2) single dose of 25 mg 2,4-D/kg b.w. orally in gelatin capsules, and 3) a single dose of 25 mg MCPA/kg b.w. co-administered with 25 mg 2,4-D/kg b.w. orally in gelatin capsules. There was a 3 week washout period between dose administrations. Blood samples were collected into EDTA anticoagulant immediately before and at 1, 2, 4, 6, 8, 12, 24, 36, 48, 72, 96, 120, and 144 hours and at 7, 8, 9, and 12 days after dose administration. The samples were centrifuged and the harvested plasma was frozen at -17°C until 2,4-D and MCPA analysis.
Experiment 2. A cross over design study was used with the following two treatment groups of 3 dogs per group: 1) a single intravenous dose of 0.5 mg 2.4-D/kg b.w. and 2) a single oral dose of 5.0 mg MCPA/kg b.w. administered in gelatin capsules followed 12 hours later by 0.5 mg 2.4-D/kg b.w intravenously. The 2.4-D solution was prepared in water and buffered with NaOH until in solution with a final concentration ranging from 0.9 to 1.0 mg 2.4-D/mL. There was a 3 week washout period between dose administrations. Intravenous 2.4-D was administered through an 18 gauge catheter placed in the cephalic vein. Blood samples from the jugular vein were collected into EDTA anticoagulant immediately before and at 2, 4, 12, 16, 20, 24, 36, 48, 60, 72, 84, and 96 hours post administration. Plasma samples from this and subsequent experiments were processed using the same procedures as described in experiment 1. Free catch urine was collected before and at 12, 20, 36, 48, 60, 72, 84, and 96 hours after administration. Urine pH and volume were measured and samples were stored at -17°C until analysis.

Experiment 3. Six dogs were intravenously administered 2.0 mg MCPA/kg b.w. (5 mg MCPA/mL in NaOH buffered water) through an 18 gauge catheter placed in the cephalic vein. Blood samples from the jugular vein were collected in EDTA anticoagulant immediately before and at 1, 2, 4, 6, 8, 12, 20, and 24 hours after administration. Free catch urine was collected at the time of blood sample collection. At 24 hours post MCPA administration, all dogs were orally administered 200 mg 2.4-D/kg in gelatin capsules. Two weeks after 2,4-D administration, dogs were again intravenously administered 2.0 mg MCPA/kg b.w with blood and urine samples collected. Blood and urine samples were processed using the same procedures as previously mentioned.

Plasma Protein Binding. Solutions were prepared using freshly obtained, pooled heparinized beagle plasma or commercially obtained fatty acid free canine albumin in
phosphate buffered saline (PBS, 0.067 M, pH 7.4) supplemented with 2.4-D or MCPA at concentrations between 40 and 600 μg/mL. Solutions of 2.4-D plus MCPA were similarly prepared with 2.4-D concentrations between 50 and 600 μg/mL and MCPA concentrations between 0 and 200 μg/mL. Separation of bound and unbound analyte was performed using a micropartition centrifugation system (Centrifree Centrifugal Filtration Devices, Millipore, Danvers, MA). Approximately 1.0 mL of plasma (n = 3 at each concentration) was placed in the top chamber of the device. The devices were centrifuged at 1000 g at 37°C so that approximately 100 - 150 μL of ultrafiltrate was collected. Experiments indicated no detectable binding of either chemical to the devices at the limit of analytical detection, 5.0 μg/mL. Plasma and PBS albumin concentrations were determined using the Hitachi 911 Automatic Analyzer (Boehringer Mannheim, Indianapolis, IN) by The Ohio State University Veterinary Teaching Hospital’s Clinical Pathology Laboratory.

**Chemical Analysis of plasma and ultrafiltrate and urine samples.** Samples were analyzed using the methods described in Chapter 2. Urine samples from Experiments 2 and 3 were analyzed as hydrolyzed and non-hydrolyzed samples. Hydrolysis was performed by incubating the urine samples (100 μL) with 2.0 mL of patella vulgaris (Sigma Chemical Co., St. Louis MO) for 3 hours at 65°C. The samples were extracted with 6.0 mL of ethyl acetate/hexane (70/30, v/v) and remainder of the analyses were performed according to the method described in Chapter 2.

**Pharmacokinetic calculations.** Area under the plasma concentration-time curves to the last quantifiable concentration (AUC<sub>0-τ</sub>) and to infinity (AUC<sub>τ-∞</sub>) were determined by the trapezoidal method. Time to maximum plasma concentration (T<sub>max</sub>) and maximum plasma concentration (C<sub>max</sub>) were determined from actual data. Clearance (Cl) to time
and Volume of distribution at steady state (Vdss) were determined non-compartmentally. Urine samples from individual time points were pooled for each dog and the total amount of 2,4-D or MCPA excreted in the urine of that dog was calculated as the total volume of urine multiplied by the concentration in that pooled volume. Renal clearance was calculated as the amount excreted in urine (Ae) / plasma AUC.

The concentration of bound analyte was calculated by subtracting the unbound concentration in the ultrafiltrate from the total analyte concentration in the original plasma or canine albumin solution sample. The percentage of protein binding of 2,4-D and MCPA was calculated as follows:

\[
\text{Eq. 1} \quad \% \text{ unbound} = \frac{C_f}{C_p} \times 100
\]

where \( C_p \) and \( C_f \) are the total and free analyte concentrations (\( \mu g/mL \)), respectively. The molar concentrations (M) of the analyte was calculated by dividing \( C_p \) or \( C_f \) by the molecular weight of the analyte. The molar bound concentration was obtained by subtracting the molar free concentration from the molar total concentration.

Rosenthal plots (Rosenthal, 1967) were used to determine if one (linear plot) or multiple (hyperbolic plot) binding sites existed after supplementation of either 2,4-D or MCPA to 4 % albumin solution or pooled plasma. The data were then analyzed using the following equation:

\[
\text{Eq. 2} \quad \frac{[DP]}{[P]} = \sum \frac{n_i \cdot [D]}{K_{d_i} - [P]}
\]
where $[DP]$ is the bound molar analyte concentration, $[P]$ is the total molar protein concentration, $n_i$ is the number of binding sites per molecule of protein for the $i$th class of sites (capacity constant), $K_{d,i}$ is the equilibrium dissociation constant (Molar) for the $i$th class of sites (affinity constant), and $[D]$ is the unbound molar concentration of the analyte.

The data from the interaction between 2.4-D and MCPA were analyzed using a competitive interaction model with the following equation:

$$\frac{[DP]}{[P]} = \sum \frac{n_i \cdot \langle n \rangle}{K_{d,i} \cdot \langle n \rangle}$$

Eq. 3

where $[I]$ and $K_{d,i}$ are the free concentration and dissociation constant of the inhibitor, respectively.

**Statistical analysis.** Statistical differences in pharmacokinetic parameters of the *in vivo* studies were assessed using a paired t-test. Concentration dependent protein binding of 2,4-D and MCPA was determined by One Way ANOVA. The interactions between 2,4-D and MCPA were analyzed by One Way ANOVA at each total 2,4-D or MCPA concentration with Tukey’s post-test used to evaluate specific differences. Analyses were performed using Sigma Stat 2.03 (Jandel Corporation, San Rafael, CA) with p values < 0.05 reported.

**Results**

**Experiment 1.**

When 2,4-D was orally administered alone, plasma 2,4-D concentrations initially increased, peaked between 4 and 8 hours, then declined to concentrations less than the
limit of quantitation (5.0 µg 2,4-D/mL) by 288 hours (Table 4.1. Figure 4.2A). After co-
administration with MCPA, plasma 2,4-D concentrations peaked between 4 and 12 hours
and, with the exception of a single dog, were below the limit of quantitation by 288 hours
(Figure 4.2A. Table 4.1). An 131 % increase (p<0.05) in T_{max}, was seen after co-
administration of 2,4-D with MCPA compared to 2,4-D alone (Table 4.1). No
differences in the mean AUC\textsubscript{0-\textinfty} were observed when 2,4-D was administered alone or
with MCPA (Table 4.1).

When MCPA was administered alone, plasma MCPA concentrations rose initially.
with a peak plasma concentration occurring between 2 and 8 hours and declined to below
the limit of quantitation (5.0 µg MCPA/mL ) by 288 hours (Figure 4.2B. Table 4.1).
When MCPA was co-administered with 2,4-D, the peak plasma MCPA concentrations
ranged between 4 and 8 hours (Figure 4.2B, Table 4.1). At 288 hours MCPA was still
detectable in two of the six dogs (Figure 4.2B). A 19 % decrease (p<0.05) in the mean
C_{max} and a non-significant 28% increase in the mean AUC\textsubscript{0-\textinfty} was observed when MCPA
was administered with 2,4-D compared to MCPA administered alone (Table 4.1).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>2.4-D alone (25 mg/kg)</th>
<th>2,4-D (25 mg/kg) with MCPA (25 mg/kg)</th>
<th>MCPA alone (25 mg/kg)</th>
<th>MCPA (25 mg/kg) with 2.4-D (25 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC\textsubscript{0-\textinfty} (µg*hr/mL)</td>
<td>10716 ± 5723</td>
<td>13102 ± 6115</td>
<td>6307 ± 2956</td>
<td>8014 ± 3259</td>
</tr>
<tr>
<td>T_{max} (hours)</td>
<td>5.7 ± 1.5</td>
<td>7.5 ± 2.7*</td>
<td>4.7 ± 2.1</td>
<td>5.7 ± 1.5</td>
</tr>
<tr>
<td>C_{max} (µg/mL)</td>
<td>137 ± 21.3</td>
<td>118 ± 17.0</td>
<td>129 ± 21.8</td>
<td>105 ± 22.6*</td>
</tr>
</tbody>
</table>

* p < 0.05; combined compared to individual compound administration

Table 4.1. Results of Experiment 1. Mean (± s.d.) Toxicokinetic parameters of 2,4-D
and MCPA after 25 mg 2.4-D/kg, 25 mg MCPA/kg or 25 mg 2.4-D/kg co-administered
with 25 mg MCPA/kg orally. (n = 6)
Figure 4.2. Mean (± s.d.) plasma 2,4-D concentrations in six dogs after A) 25 mg 2,4-D/kg b.w. oral administration with (●) and (○) without 25 mg MCPA/kg b.w. co-administration and B) 25 mg MCPA/kg b.w. oral administration with (●) and (○) without 25 mg 2,4-D/kg b.w. co-administration.
**Experiment 2.**

The mean plasma concentration-time data for six dogs intravenously administered 0.5 mg 2.4-D/kg with and without 5.0 mg MCPA/kg pretreatment are shown in Figure 4.3. When 2.4-D was administered alone, plasma 2.4-D concentrations were below the limit of quantitation in all dogs by 60 hours. In contrast, when 2.4-D was administered with MCPA, 2.4-D plasma concentrations were below the limit of quantitation in all dogs by 72 hours (Figure 4.3). The AUC for 2.4-D increased, on average, 27% (not significant) when 5.0 mg MCPA/kg b.w. was orally administered 12 hours before 2.4-D administration compared to 2.4-D only administration (Table 4.2A). Clearance and \( V_d \) were not statistically different when 2.4-D was administered alone and in the presence of MCPA (Table 4.2A). The mean total amount of 2.4-D excreted, renal clearance, and \% of dose excreted were not different between 2.4-D only and 2.4-D plus MCPA administration (Table 4.2A). Hydrolysis of urine samples did not increase the amount excreted in the urine. Plasma MCPA was detected until 96 hours in 4 dogs, 84 hours in one dog, and 72 hours in one dog (Figure 4.3). The concentration of MCPA in the pooled urine samples ranged from 8.7 \( \mu g/mL \) to 15.7 \( \mu g/mL \). Urine pH ranged from 5.47 to 9.01. Mean total urine volumes collected by 96 hours were not significantly different between 2.4-D only and 2.4-D plus MCPA treatments (Table 4.2A).
Figure 4.3. Mean (± s.d.) plasma 2.4-D concentrations in six dogs after 0.5 mg 2.4-D/kg b.w. i.v. administration with (●) and (○) without 5 mg MCPA/kg b.w. pretreatment. Mean plasma MCPA concentrations (▼) are also shown.
**A) Experiment 2**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>2.4-D (0.5 mg/kg) alone</th>
<th>2.4-D (0.5 mg/kg) with MCPA (5 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{AUC}_{0-\infty}$ (µg*hr/mL)</td>
<td>78.1 ± 39.2</td>
<td>99.1 ± 37.9</td>
</tr>
<tr>
<td>Clearance $\text{Cl}_{\text{ur}}$ (mL/hr/kg)</td>
<td>8.67 ± 5.89</td>
<td>5.75 ± 2.34</td>
</tr>
<tr>
<td>Renal clearance $\text{Cl}_{\text{ur}}$ (mL/hr)</td>
<td>37.1 ± 25.1</td>
<td>27.3 ± 12.8</td>
</tr>
<tr>
<td>$V_d$ (mL/kg)</td>
<td>143 ± 37.2</td>
<td>122 ± 58.4</td>
</tr>
<tr>
<td>Urine volume (mL)</td>
<td>550 ± 132</td>
<td>451 ± 143</td>
</tr>
<tr>
<td>Total amount excreted in urine (mg) in 96 hours</td>
<td>2.28 ± 0.84</td>
<td>2.38 ± 0.58</td>
</tr>
<tr>
<td>% dose excreted in urine in 96 hours</td>
<td>36.5 ± 13.0</td>
<td>37.7 ± 7.74</td>
</tr>
</tbody>
</table>

*no significance*

**B) Experiment 3**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MCPA (2.0 mg/kg) alone</th>
<th>MCPA (2.0 mg/kg) with 2.4-D (200 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{AUC}_{0-\infty}$ (µg*hr/mL)</td>
<td>243 ± 88.2</td>
<td>239 ± 26.0</td>
</tr>
<tr>
<td>Clearance $\text{Cl}_{\text{ur}}$ (mL/hr/kg)</td>
<td>8.92 ± 2.40</td>
<td>8.48 ± 0.96</td>
</tr>
<tr>
<td>Renal clearance $\text{Cl}_{\text{ur}}$ (mL/hr)</td>
<td>12.8 ± 9.01</td>
<td>0.16 ± 0.38</td>
</tr>
<tr>
<td>$V_d$ (mL/kg)</td>
<td>166 ± 37.1</td>
<td>176 ± 23.4</td>
</tr>
<tr>
<td>Urine volume (mL)</td>
<td>204 ± 114</td>
<td>162 ± 60.5</td>
</tr>
<tr>
<td>Total amount excreted in urine (mg) in 24 hours</td>
<td>2.71 ± 1.70</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>% dose excreted in urine in 24 hours</td>
<td>11.3 ± 6.67</td>
<td>0.17 ± 0.41</td>
</tr>
</tbody>
</table>

† p < 0.05; combined compared to individual compound administration

Table 4.2. Plasma area under the curve (AUC) and urinary parameters of 2.4-D and MCPA after A) Experiment 2: 0.5 mg 2.4-D/kg i.v. administration alone and 12 hours after oral administration of 5.0 mg MCPA/kg (n = 6) and B) Experiment 3: 2.0 mg MCPA/kg i.v. administration alone and 14 days after oral administration of 200 mg 2.4-D/kg. (n = 6).
*Experiment 3.*

Plasma MCPA concentration-time data resulting from a 2.0 mg MCPA/kg b.w. intravenous administration alone and in the presence of 2.4-D are shown in Figure 4.4. When a low dose of MCPA (2.0 mg/kg) was administered following a high dose of 2.4-D (200 mg/kg) the AUC, Cl, and Vd were not statistically different from MCPA administration alone (Table 4.2B). In contrast, the total amount of MCPA excreted in the urine and renal clearance was greater in all of the dogs when MCPA was administered alone compared to in combination with 2.4-D (Table 4.2B). In fact, MCPA was detected in the urine of only one dog when MCPA was administered after pretreatment with 2.4-D. Within the first 24 hours after MCPA administration, on the average, 11.3% of the dose was excreted in the urine when MCPA was administered alone, while less than 1% of the dose was excreted after 2.4-D pretreatment (Table 4.2B). Hydrolysis of urine samples did not increase the amount excreted in the urine. Values of urine pH ranged from 5.2 to 8.3. Mean total urine volumes, collected by 24 hours, were not significantly different between MCPA only and MCPA with 2.4-D treatments (Table 4.2B). The concentration of 2.4-D in the pooled urine after MCPA treatment ranged from 4.57 to > 70.0 μg/mL. Plasma 2.4-D concentrations 24 hours after 2.4-D administration were between 421 and 633 μg/mL. Fourteen days later, at the time of MCPA administration, 2.4-D plasma concentrations were between 121 and 333 μg/mL.
Figure 4.4. Mean (± s.d.) plasma MCPA concentrations in six dogs after 2.0 mg 2.4-D/kg b.w. i.v. administration with (●) and (○) without 200 mg 2.4-D/kg b.w. pretreatment.
**Protein binding.** Concentration dependent binding of 2.4-D and MCPA was observed in 4% albumin solution and pooled beagle plasma. The mean percent unbound of 2.4-D or MCPA in 4% albumin solution increased from 1 to 17 percent, when total concentrations ranged from 40 to 600 μg/mL (Figure 4.5). The mean percent 2.4-D unbound in plasma increased from 4 to 26 percent and the mean percent MCPA unbound in plasma increased similarly from 3 to 29 percent, when total 2.4-D or MCPA concentrations ranged from 50 to 500 μg/mL (Figure 4.5). There was an approximately 2 fold higher binding of 2.4-D and MCPA in albumin compared to plasma at equal concentrations.
Figure 4.5. Mean (± s.d.) percent unbound of 2,4-D and MCPA in 4% canine albumin solution and pooled beagle plasma. (n = 3 samples per concentration except n = 2 at 117 and 127 µg MCPA/mL plasma)
Analyses of the number of binding sites for each compound were predicted using Rosenthal plots. The hyperbolic curves of the Rosenthal plots indicated more than one binding site for both 2,4-D and MCPA in both albumin solution and plasma. In fact, the 2,4-D data were superimposable with the MCPA data when analyzed in albumin solution or plasma. These curves are indicative, based on their shape, of 2 classes of sites, a high affinity, low capacity class of site and a low affinity, high capacity class of site (Matthews, 1993). As a result, data were then fitted to a model for two classes of binding sites, with the parameter estimates and 95% planar confidence intervals for 2,4-D and MCPA displayed in Table 4.3. Capacity estimates for 2,4-D and MCPA are very similar, while there was a slightly lower dissociation (higher affinity) estimate for MCPA compared to 2,4-D in both plasma and albumin solution. In general a greater affinity (2-5 fold) for the binding of each compound to canine albumin compared to plasma was observed.
Table 4.3. Parameter estimates (and 95% planar confidence intervals) of protein binding capacity and affinity constants for 2,4-D and MCPA bound to beagle plasma and 4% fatty acid free canine albumin solution. The parameters \( n_1 \) and \( n_2 \) are the number of binding sites per molecule of protein for the 1st and 2nd class of sites, respectively. \( K_{d1} \) and \( K_{d2} \) are the dissociation constants (Molar) for the 1st and 2nd class of sites, respectively. Plasma was analyzed with albumin (3.8%) representing the major binding protein. A) Data was individually modeled using a weighting of \( 1/y \). B) Data was combined and simultaneously analyzed using a competitive interaction model using a weighting of \( 1/y \).
There was an interaction of co-supplemented MCPA and 2,4-D in both plasma and 4% albumin solution. As the MCPA concentration increased, the percent of 2,4-D unbound increased at all 2,4-D concentrations evaluated (Table 4.4). The increase in the percent of 2,4-D unbound was greatest at the lowest total 2,4-D concentration and ranged from 7 to 1.5 fold at the lowest and highest total 2,4-D concentrations, respectively. Conversely, as the 2,4-D concentration increased, the percent of MCPA unbound increased at all MCPA concentrations evaluated (Table 4.5). The degree of increased free fraction of MCPA was greatest at the lowest total MCPA concentration and ranged from about 5 fold to 1.5 fold at the lowest and highest total MCPA concentrations evaluated. It appeared that 2,4-D and MCPA were equally effective at displacing each other. Data from both 2,4-D and MCPA protein binding in plasma and albumin were simultaneously fitted to the competitive interaction model again using two classes of binding sites. Parameter estimates for capacity and affinity of sites one and two were determined (Table 4.3B).
<table>
<thead>
<tr>
<th>Matrix</th>
<th>2,4-D (μg/mL)</th>
<th>MCPA 0 μg/mL</th>
<th>MCPA 50 μg/mL</th>
<th>MCPA 100 μg/mL</th>
<th>MCPA 200 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>albumin</td>
<td>100</td>
<td>1.23 ± 0.10</td>
<td>3.35 ± 0.07*</td>
<td>4.70 ± 0.80*</td>
<td>8.79 ± 0.56^b,c</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>4.99 ± 0.17</td>
<td>6.75 ± 0.32*</td>
<td>9.56 ± 0.55^b</td>
<td>10.1 ± 0.33^b,c</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>10.8 ± 0.38</td>
<td>11.1 ± 0.52*</td>
<td>13.8 ± 0.59^b</td>
<td>18.6 ± 0.44^b,c</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>15.0 ± 0.45</td>
<td>-</td>
<td>17.5 ± 0.99</td>
<td>22.2 ± 1.49^a</td>
</tr>
<tr>
<td>plasma</td>
<td>50</td>
<td>3.50 ± 0.16</td>
<td>3.46 ± 0.35</td>
<td>7.07 ± 0.29^b</td>
<td>16.2 ± 1.09^b,c</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>6.07 ± 0.14</td>
<td>7.25 ± 1.00</td>
<td>9.33 ± 0.30^b</td>
<td>19.7 ± 0.28^b,c</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>5.57 ± 0.31</td>
<td>11.1 ± 0.95*</td>
<td>12.9 ± 0.50^b</td>
<td>23.6 ± 1.41^b,c</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>14.0 ± 0.52</td>
<td>12.0 ± 1.13</td>
<td>15.2 ± 1.12^b</td>
<td>25.6 ± 0.23^b,c</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>21.5 ± 0.90</td>
<td>28.3 ± 0.98</td>
<td>27.2 ± 3.10</td>
<td>30.7 ± 1.50^a</td>
</tr>
</tbody>
</table>

Table 4.4. Mean (± s.d.) percent unbound of 2,4-D in 4% canine albumin solution and plasma after co-supplementation (n = 3 samples per 2,4-D/MCPA combination). Differences (p< 0.05) in the percent of 2,4-D unbound were noted for the MCPA co-supplemented albumin versus albumin containing a) 0 μg MCPA/mL, b) 50 μg MCPA/mL, or c) 100 μg MCPA/mL for each total 2,4-D concentration evaluated. Differences (p< 0.05) in the percent of 2,4-D unbound were noted for MCPA co-supplemented plasma versus plasma containing a) 0 μg MCPA/mL, b) 10 μg MCPA/mL, or c) 50 μg MCPA/mL for each total 2,4-D concentration evaluated.
<table>
<thead>
<tr>
<th>Matrix</th>
<th>MCPA (µg/mL)</th>
<th>2,4-D 100 µg/mL</th>
<th>2,4-D 200 µg/mL</th>
<th>2,4-D 400 µg/mL</th>
<th>2,4-D 600 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>albumin</td>
<td>100</td>
<td>2.39 ± 0.42</td>
<td>5.96 ± 0.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.7 ± 0.4&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>13.5 ± 0.8&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>10.6 ± 0.46</td>
<td>9.71 ± 0.42</td>
<td>16.1 ± 0.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.1 ± 0.99&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>plasma</td>
<td>50</td>
<td>4.70 ± 0.24</td>
<td>6.96 ± 0.13</td>
<td>9.09 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.5 ± 0.99&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>15.3 ± 0.39</td>
<td>17.3 ± 0.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.4 ± 0.32&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>21.7 ± 0.24&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 4.5. Mean (± s.d.) percent unbound of MCPA in 4% canine albumin solution and pooled beagle plasma after co-supplementation with 2,4-D (n = 3 samples per 2,4-D/MCPA combination). Differences (p< 0.05) in the percent of MCPA unbound were noted for the 2,4-D co-supplemented albumin versus albumin containing a) 100 µg 2,4-D/mL, b) 200 µg 2,4-D/mL, or c) 400 µg 2,4-D/mL for each total MCPA concentration evaluated. Differences (p< 0.05) in the percent of MCPA unbound were noted for 2,4-D co-supplemented plasma versus plasma containing a) 50 µg 2,4-D/mL, b) 100 µg 2,4-D/mL, c) 150 µg 2,4-D/mL, or d) 200 µg 2,4-D/mL for each total MCPA concentration evaluated.
Discussion
In Experiments 1, 2, and 3, no significant differences in the AUCs were seen compared to either compound administered alone. The \( T_{\text{max}} \) values for 2.4-D had been reported to occur between 4 and 10 hours in rats (Erne, 1966), which was similar to findings in this study after alone or combined oral administration of 25 mg 2.4-D/kg. The MCPA \( T_{\text{max}} \) values when 25 mg MCPA/kg was administered either alone or with 2.4-D, were similar to a MCPA \( T_{\text{max}} \) of 2 hours reported in the rat (Elo, 1976). The maximal concentration achieved after oral administration of 2.4-D and MCPA was similar between compounds, although a slight decrease in \( C_{\text{max}} \) was observed when MCPA was co-administered with 2.4-D compared to alone. The physiological parameter \( V_d \) (~12 - 18 % body weight) calculated in the present study for both 2.4-D and MCPA were similar, with MCPA having a 136 % higher volume of distribution. For both compounds, the \( V_d \) was less than or equal to extracellular fluid volume (17 -27 % of body weight) regardless of individual or combined compound administration. The \( V_d \) of MCPA in the present study was similar to the \( V_d \) in the rat, 17 - 18 % of body weight, by Bräunlich et al. (1989). The majority of 2.4-D and MCPA is ionized at physiologic pH, which is likely a major determinant in the low volume of distribution.

It was hypothesized that the lack of difference in AUCs in Experiment 1 was due to saturation of renal transport, even in the single compound administrations. Based on this hypothesis, Experiments 2 and 3 were performed in attempt to lower the dose below saturation threshold. Plasma clearance did not change in Experiments 2 or 3 when two phenoxy compounds were administered compared to single agent administration. In contrast renal clearance of MCPA was substantially decreased after 2.4-D co-administration compared to MCPA only administration (Experiment 3). Furthermore in the MCPA only administration, renal clearance at 24 hours accounted for only ~11 % of...
the total plasma clearance. In the present study, approximately 11% of the dose was excreted by the kidney in 96 hours. In contrast, Elo (1976) found in the rat that after oral administration of approximately 11.5 mg MCPA/kg (approximately 6 fold higher dose), 92% was eliminated within 24 hours in the urine. In the present study, 2,4-D renal clearance in 96 hours accounted for <40% of the total plasma clearance for 96 hours (or <40% of the dose was excreted by the kidney), whereas at approximately 90% of a 1 mg 2,4-D/kg oral dose was excreted in 6 hours in the rat (Pelletier et al. 1989). These findings indicated the phenoxy herbicides are significantly eliminated by non-renal routes in the dog in comparison to primary renal elimination observed in the rat.

The decrease in renal elimination of MCPA in experiment 3 after co-administration with 2,4-D was likely due to saturation of active transport in the renal proximal tubules by 2,4-D, resulting in inhibition of MCPA transport. The proposed method of transport of 2,4-D is by the para-aminohippuric acid (PAH)/α-ketogluterate organic anion transport system (Villabos, 1996). This is probably the same system of transport for MCPA since PAH and probenecid have been shown to inhibit MCPA renal elimination in vivo (Bräunlich et al., 1989). In contrast, there was no change in the urinary excretion of 2,4-D when it was administered with 5.0 mg MCPA/kg. Two possible explanations exist regarding this outcome. First 2,4-D could have a greater affinity than MCPA for the transporter protein (Dawson and Renfro 1993). Secondly the plasma concentrations of MCPA may not have been high enough to saturate the transport mechanism. Saturation of transport of MCPA was not observed in the rat at a dose of 10 mg/kg, but was observed at 20 mg/kg (Bräunlich et al. 1989). The pKa for both compounds is ~ 3, therefore it is unlikely that the urine pH (>5.5) had an affect on the elimination by promoting resorption. Furthermore, volume diuresis was not apparent and subsequently
did not affect elimination. In summary it appears that the dose of the inhibitor was sufficient in experiment 3 but not experiment 2 to saturate the renal transport mechanism.

2.4-D and MCPA bind individually to canine albumin and plasma in a concentration dependent manner, although there was an approximately 1.75 fold greater binding to purified albumin than pooled plasma. The concentration dependence observed was similar to that found in plasma (Örberg, 1980. Elo and Ylitalo. 1979. Ylitalo et al. 1990) and albumin (Kolberg et al. 1973. Mason. 1975) in other species. The difference in albumin and plasma binding was likely due to competition from medium chain fatty acids in the plasma which have been shown to displace site II markers in a variety of species including dog (Panjehshahin et al. 1992) and man (Wanwimolruk et al. 1983). This was the same site shown to bind 2.4-D on human albumin (Rosso et al. 1998). Binding site II in man has been shown to be similar to binding site II in the dog (Kosa et al. 1997).

When the individual compounds 2.4-D and MCPA were co-supplemented in canine plasma and albumin solution, a concentration dependent displacement of each compound is observed, resulting in an increase fraction unbound and a potential for increased biological effect. The binding displacement along with the similarities in binding characteristics of the two compounds support MCPA and 2.4-D binding to the same site on albumin.

Modeling of the protein binding data resulted in parameter estimates consistent with Rosenthal plot analyses and postulated to be of a low affinity high capacity class and a high affinity low capacity class of binding sites. Similar binding of 2.4-D or MCPA to albumin was observed in horse, human, guinea pig, bovine and rat (Fang and Lindstrom 1980. Kolberg et al. 1973. Mason. 1975). The higher fraction unbound of 2.4-D or MCPA found with plasma proteins correlated with the observed higher dissociation
(lower affinity) parameters calculated for plasma proteins. Similar findings were observed by Lima et al. (1981) when studying the plasma protein binding of disopyramide in normal patient, ill patient, and blood bank donor plasma. Based on the information gained in the present study, it would be acceptable to use albumin as a representative in binding studies of the phenoxy compounds in plasma of the dog. The use of albumin eliminates the necessity for obtaining fresh plasma and avoids storage, which can alter drug binding (Erkmen et al. 1995, Morgan and Badaway, 1994).

In conclusion, 2,4-D and MCPA were capable of interacting at the level of renal elimination and protein binding. However the alterations in plasma concentrations resulting from such interactions do not result in statistically significant differences in the AUCs of either compound. The interaction at the renal transport site, however suggested an interaction could occur at other similar transport sites such as the organic acid transport protein in the brain, potentially increasing the toxicity of these compounds, supported by an increase in toxicity observed by Ylitalo et al. (1990) when phenoxy compounds were co-administered with the organic anion transport inhibitor, probenecid.
References


CHAPTER 5

Data present in the literature suggest an inductive effect of 2.4-D on hepatic metabolism of xenobiotic compounds. Since 2.4-D and MCPA are eliminated primarily unchanged in the urine, it was quantitatively difficult to evaluate whether or not hepatic induction was possible. In order to investigate the effect of 2.4-D on induction of hepatic enzymes, a highly metabolized model compound, diazepam was used.
EFFECT OF 2.4-D ON THE PHARMACOKINETICS AND PROTEIN BINDING OF DIAZEPAM IN THE BEAGLE DOG

The phenoxy herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), is the third most widely used herbicide in the United States and is used for post-emergent broad leaf weed control. 2,4-D has been shown to induce P450s of the 4A family (Baucher et al. 1988, Hietanen et al. 1985, Pineau et al. 1996). Limited information exists regarding 2,4-D effects on the other P450 families, which are important in metabolism of therapeutic drugs. Induction of CYP 1A1 has been demonstrated in the rat through increased ethoxyresorufin O-deethylase activity (Baucher et al. 1988, Knopp et al. 1992). In addition, increases in ethylmorphine N-demethylase (125 %)(Knopp et al. 1992), and aniline hydroxylase (139-152 %) activity (Cristae et al. 1982) have also been observed after 2,4-D administration to rats. Studies have not been performed to evaluate the interactive effects of 2,4-D on the in vivo pharmacokinetics of highly P450 metabolized therapeutic drugs.

Diazepam, a commonly used anxiolytic/hypnotic agent in both veterinary and human medicine, is extensively metabolized by N-demethylation and hydroxylation (figure 5.1). The metabolic profile of diazepam is similar between man and the dog both in vivo and in vitro, although the specific metabolic cytochromes have not been identified in the dog. Nordiazepam is the predominant metabolite in both the human and the dog, while oxazepam and temazepam are minor metabolites (Klotz et al. 1976, Löscher et al. 1981, Vree et al. 1979). Diazepam can be used as a model substrate to investigate the effect of 2,4-D on the P450's responsible for xenobiotic metabolism.
Diazepam can also be used to investigate the effects of 2,4-D on the plasma protein binding of certain therapeutic compounds. Diazepam is highly bound to albumin in both the dog and man (Allen et al. 1981, Wala et al. 1995). Research has shown that the binding site (site II) for diazepam is similar between man and dog, with the suggestion that the dog would make a good model for study of this site (Kosa et al. 1997). The phenoxy herbicide 2,4-D was found to have a greater affinity for this site on human albumin than many therapeutic compounds including diazepam (Rosso et al. 1998), although competitive binding studies were not performed.
The purpose of this study was to investigate if 2,4-D had an affect on the pharmacokinetics of diazepam in the dog as a model for metabolic induction and to investigate if 2,4-D changes the plasma protein binding of diazepam.

**Materials and Methods**

*Animals.* The experiment was performed in accordance with the Institutional Laboratory Animal Care and Use Committee (ILACUC) guidelines. Six adult female beagle dogs (Harlan, Indianapolis, IN) weighing between 9.6 and 12.7 kg were used in this study. The dogs were individually maintained in climate controlled runs and had free access to food and water, until 12 hours prior to diazepam administration, when food was withheld.

*Administered Compounds.* Diazepam (Valium, 5 mg/mL) was purchased from Roche Laboratories Inc. (Nutley, NJ, USA), and 2,4-dichlorophenoxyacetic acid (2,4-D, 98% purity) was purchased from Aldrich (Milwaukee, WI, USA).

*Compound administration and sample collection.* All dogs were administered 1 mg diazepam/kg body weight (bw) through the cephalic vein. Blood was collected pre- and 5, 10, 15, 30, 45, 60, 90, 120, 240, and 360 minutes post administration. After 6 days, all dogs were administered 200 mg 2,4-D/kg bw in gelatin capsules orally. Fifteen days after 2,4-D administration, all dogs were again given 1 mg diazepam/kg bw intravenously and samples collected. All blood samples were collected into EDTA anticoagulant and centrifuged. Plasma was harvested and stored at -17°C until analyses.

*Albumin protein binding.* Solutions of 4% fatty acid free albumin (Sigma Chemical, St. Louis, MO) in 0.067 M phosphate buffered solution (PBS)(pH 7.4) were supplemented with diazepam at 500 and 1000 ng/mL. 2,4-D was co-supplemented at 0, 50, 100, 200...
μg/mL. These concentrations represented the concentration range of 2.4-D and diazepam determined during the in vivo experiment. Separation of unbound diazepam was performed using the Centrifree micropartition system (Millipore, Danvers, MA). Approximately 1.0 mL of solution (n = 2) was placed in the top chamber of the device. The devices were centrifuged at 1000 x g at 37°C for approximately 4 minutes. Albumin solution and ultrafiltrate samples were stored at -17°C until analysis.

Benzodiazepine analysis. Blood was analyzed for diazepam and its metabolites nordiazepam, temazepam, and oxazepam by HPLC using the method of Vree et al. (1979). The internal standard flunitrazepam (100 ng) was added to a screw-cap tube followed by 250 μL plasma sample. After a brief mixing, the sample was extracted with 2.0 mL of toluene (Burdick and Jackson Laboratories, Muskegon, MI, USA), mixed by end-over-end inversion for 5 minutes and centrifuged at approximately 1000 g for 5 minutes. The organic phase was transferred to a conical tube and evaporated under a stream of nitrogen at 40 - 45°C. The dry residue was dissolved in 250 μL mobile phase and 80 μL was injected onto the chromatographic column.

Diazepam and its metabolites were chromatographically separated with a 4.6 x 75 mm column (Zorbax SB-C18, 3.5 μm medium, MacMod Analytical, Chadds Ford, PA, USA). A guard column (Perisorb RP-18, 30 - 40 μm medium, Upchurch Scientific, Oak Harbor, WA, USA) was placed between the autosampler and the column. Ultraviolet absorption was measured with a Spectroflow 773 detector (Applied Biosystems, Ramsey, NJ, USA) at a wavelength of 230 nm. The mobile phase consisted of water-methanol-acetonitrile (52/43/5, v/v/v) at a flow rate of 1.5 mL/min. Diazepam and its metabolites
were quantified based on the ratio of the peak heights of the analyte compared to the internal standard and plotted against the known analyte concentrations of diazepam and its metabolites.

Pharmacokinetic analysis. The area under the blood concentration time curve (AUC) for each analyte was calculated according to the trapezoidal rule to the last quantitatable plasma concentration time. The AUC was extrapolated to infinity if the AUC to the last measured time point was at least 90% of the extrapolated value. Clearance (Cl) was calculated as Dose/ AUC_{0-\infty}. The volume of distribution at steady state, Vd_{ss}, was calculated as Dose * AUMC/AUC^2, with AUMC representing the area under the moment curve. Half life was calculated from the terminal elimination phase of the plasma concentration time curve. Time to peak plasma metabolite concentration (T_{max}) and peak metabolite concentration (C_{max}) were determined from actual data. The percent unbound of diazepam was determined as

\[ \%\ free = \frac{C_f}{C_p} * 100 \]

where \( C_f \) and \( C_p \) are the unbound and total diazepam concentration, respectively.

Statistical analysis. Pharmacokinetic parameters of diazepam and its metabolites before and after 2.4-D administration were compared using paired t tests with the exception of \( T_{max} \) and half life. \( T_{max} \) was compared using the signed rank test because data were not normally distributed. Harmonic means and pseudostandard deviations of half lives were determined and compared using a t-test. Differences were determined to be significant at a p level of < 0.05. All comparisons were made using Sigma Stat 2.03 (Jandel Corporation, San Rafael, CA).
Results

Pharmacokinetics of diazepam. After intravenous administration of 1.0 mg diazepam/kg, the only active metabolites present in plasma were nordiazepam and oxazepam. The mean plasma concentration time profiles for diazepam (DZ), nordiazepam (ND), and oxazepam (OX) before and after 2.4-D administration are displayed in Figures 5.2-5.4. Mean (± s.d.) calculated pharmacokinetic parameters for diazepam and its active metabolites are displayed in Table 5.1. The AUC from 0 to 120 values for diazepam, nordiazepam, and oxazepam after 2.4-D administration were 54.3, 60.8, and 77.4 percent of the pre-administration values (Table 5.1). Diazepam clearance and volume of distribution were 1.8 and 2.5 fold greater, respectively, after 2.4-D administration compared to before (Table 5.1). There was no change in diazepam half-life (Table 5.1). The nordiazepam Cmax was 31 % lower after 2.4-D administration compared to the Cmax without 2.4-D pre-treatment (Table 5.1). When the ratios of the AUCs were determined, DZ/OX and ND/OX were 30 and 24 % lower after 2.4-D administration compared to before (Table 5.2).
Figure 5.2. Mean (± s.d.) plasma diazepam concentration time curves in six dogs after 1.0 mg diazepam/kg b.w. i.v. administration (●) before and (○) 15 days after 200 mg 2.4-D/kg oral administration.
Figure 5.3. Mean (± s.d.) plasma nordiazepam concentration time curves in six dogs after 1.0 mg diazepam/kg b.w. i.v. administration (●) before and (○) 15 days after 200 mg 2.4-D/kg oral administration.
mean oxazepam

Figure 5.4. Mean (± s.d.) plasma oxazepam concentration time curves in six dogs after 1.0 mg diazepam/kg b.w. i.v. administration (●) before and (○) 15 days after 200 mg 2.4-D/kg oral administration.
Table 5.1. Mean (± s.d.) Pharmacokinetic parameters of diazepam (DZ), nordiazepam (ND) and oxazepam (OX) after 1.0 mg diazepam/kg intravenous administration in six dogs before (pre) and after (post) 200 mg 2.4-D/kg administration.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DZ pre</th>
<th>DZ post</th>
<th>ND pre</th>
<th>ND post</th>
<th>OX pre</th>
<th>OX post</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{\text{max}}$ (min)</td>
<td>n.a.*</td>
<td>n.a.</td>
<td>7.5 (4.18)</td>
<td>6.67 (4.08)</td>
<td>37.5 (12.6)</td>
<td>15 (15.2)</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng/mL)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>616 (108)</td>
<td>426* (95.9)</td>
<td>114 (20.7)</td>
<td>98.6 (15.8)</td>
</tr>
<tr>
<td>AUC$_{0-120}$ (ng*min/mL)</td>
<td>23530 (4484)</td>
<td>12783* (2144)</td>
<td>41577 (7866)</td>
<td>25295* (4793)</td>
<td>11317 (2000)</td>
<td>8756* (777)</td>
</tr>
<tr>
<td>AUC$_{0-\infty}$ (ng<em>mL/min)</em></td>
<td>24460 (4466)</td>
<td>13500* (2014)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>$t\frac{1}{2}$ (min)</td>
<td>29.0 (3.7)</td>
<td>30.8 (4.2)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Vd ss (L/kg)</td>
<td>1.08 (0.37)</td>
<td>2.65* (0.89)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Cl (mL/min/kg)</td>
<td>42.1 (7.80)</td>
<td>75.6* (12.1)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

† n.a. not applicable; ‡ p < 0.05 post 2.4-D administration compared to pre 2.4-D administration; # AUC 0 to last measured concentration - time was ≥ 90 % of calculated AUC$_{0-\infty}$.

Table 5.2. Mean (± s.d.) AUC$_{0-120}$ ratios of diazepam and its active metabolites from six dogs.

<table>
<thead>
<tr>
<th>Ratio</th>
<th>pre</th>
<th>post</th>
</tr>
</thead>
<tbody>
<tr>
<td>DZ/OX</td>
<td>2.12 (0.50)</td>
<td>1.48* (0.33)</td>
</tr>
<tr>
<td>DZ/ND</td>
<td>0.51 (0.10)</td>
<td>0.58 (0.13)</td>
</tr>
<tr>
<td>ND/OX</td>
<td>3.82 (1.23)</td>
<td>2.92* (0.74)</td>
</tr>
</tbody>
</table>

‡ p < 0.05 post 2.4-D administration compared to pre 2.4-D administration
**Protein binding.** Less than 1 percent of the diazepam was unbound at both 500 and 1000 ng diazepam/mL (Table 5.3). An increase in the unbound diazepam, ranging from <2 fold to >7 fold, was observed when 1000 ng diazepam/mL was co-supplemented with 50 or 200 μg 2.4-D/mL, respectively, compared to diazepam only (Table 5.3).

<table>
<thead>
<tr>
<th>Diazepam (ng/mL)</th>
<th>2,4-D (μg/mL)</th>
<th>% diazepam unbound</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>500</td>
<td>200</td>
<td>3.6</td>
</tr>
<tr>
<td>1000</td>
<td>0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>1000</td>
<td>50</td>
<td>&lt;1.4</td>
</tr>
<tr>
<td>1000</td>
<td>100</td>
<td>2.8</td>
</tr>
<tr>
<td>1000</td>
<td>200</td>
<td>7.0</td>
</tr>
</tbody>
</table>

Table 5.3. Albumin binding of Diazepam (n = 2 samples per diazepam/2.4-D combination)

**Discussion**

The percent of unbound diazepam in 4% albumin solution was <1.0 % at both 500 and 1000 ng diazepam/mL, consistent with concentration independent binding at therapeutic concentrations. The low percentage of free diazepam is similar to that observed (4 - 5 %) in canine plasma or serum by other authors (Klotz et al. 1976. Löscher et al. 1981). At least a 2.8 fold increase when 100 μg 2.4-D/mL was co-supplemented compared to no 2.4-D supplementation. The approximate 2.4-D concentration was 100 μg/mL at the time of diazepam administration in the present study, resulting in a predicted approximate 2.8 % increase in the fraction of unbound plasma diazepam compared to diazepam only administration. An increase of the fraction unbound would then increase the \( V_d \) of diazepam according to the equation

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\[ V_{ds} = V_P + V_t \left( \frac{f_{up}}{f_{ut}} \right) \]

where \( V_P \) and \( V_t \) are the plasma and tissue volumes, respectively, and \( f_{ut} \) is the fraction of drug unbound in the tissue. When the \( V_{ds} \) of diazepam was calculated, a 2.5 fold increase was found after 2,4-D administration compared to before. The increase in volume resulted in an overall decrease in the plasma concentrations, which was most evident in the initial plasma concentration-times, when distribution is a dominating factor in the shape of the curve. Similar changes in plasma concentration were observed by Bjornsson et al. (1979) when clofibrate was shown to displace warfarin from its plasma proteins resulting in an increased free fraction and \( V_{ds} \) of warfarin of similar magnitude.

The protein binding of nordiazepam or oxazepam was not determined in the present study, however both compounds have been shown to be highly protein bound in dogs (Wala and Sloan, 1995). Nordiazepam, above therapeutic concentrations, has been shown to increase the free fraction of diazepam in human plasma, suggesting they are binding to the same site on human albumin (Divol and Greenblatt, 1981). Koso et al. (1997) found that the diazepam binding site in man is similar to the diazepam binding site in the dog, therefore, nordiazepam should be displaced by 2,4-D resulting in an increased free fraction of nordiazepam in the dog. This would result in an increase in the \( V_{ds} \), similar to that observed with diazepam, and a subsequent decrease in plasma concentrations, consistent with the decrease in ND \( C_{max} \) and overall plasma ND concentration-time curve observed in the present study.

The dog has been shown to have diazepam clearance values between 20 - 60 mL/min/kg (Boxenbaum 1982, Klotz et al. 1976, Papich and Alcorn, 1995), consistent with the diazepam clearance values observed in the present study before 2,4-D.
administration. After 2.4-D administration, diazepam clearance increased in the present study. Clearance after 2.4-D administration was approximately 180% of the pre-administration value, which is consistent with the 55% observed decrease in AUC_{0-\infty} of diazepam. Clearance is the only physiologic determinant of AUC, thus alterations in AUC are a result of changes in clearance, since the dose of diazepam administered did not change. Changes in clearance may be due to changes in protein binding or changes in intrinsic clearance (Cl_{int}). The concurrent increases in clearance and volume observed in the present study after 2.4-D administration resulted in no change in the plasma half-life of diazepam.

In the present study, oxazepam (OX) and nordiazepam (ND) were the only active metabolites found both before and after 2.4-D administration, while the metabolite temazepam was not found. The metabolic profile in the present study is consistent with that observed by Vree et al. (1979) and Löscher et al. (1981) in the dog. The predominant metabolite found in both studies was nordiazepam with oxazepam found at much lower concentrations (Lösch et al. 1981, Vree et al. 1979). Although the metabolic profile did not change with 2.4-D administration, the AUCs of DZ, ND, and OX were decreased after 2.4-D pretreatment compared to before. The percent of decrease for the compounds DZ, ND, and OX were not equal. This is evident as a decrease in the DZ/OX and ND/OX ratios after 2.4-D administration compared to before. Diazepam can undergo two different routes of metabolism (DZ -> ND -> OX or DZ -> Temazepam -> OX). Preferential increase in the metabolism rate of one of the pathways (DZ -> temazepam -> OX) over the other can explain the differences in AUC ratios observed.

The intrinsic clearance (Cl_{int}) of diazepam in the dog, using cultured hepatocytes, was determined to be high (Chenery et al. 1987). Seddon et al. (1989) also reported the
elimination of diazepam in cultured hepatocytes to be high. Diazepam is preferentially metabolized in vitro to nordiazepam and to a lesser amount to temazepam in the dog (Chenery et al. 1987, Seddon et al. 1989). Since nordiazepam is the predominate product, it is unlikely that increases in the metabolism of diazepam to nordiazepam would significantly influence diazepam clearance or AUC. Furthermore, nordiazepam has been shown to undergo little to no metabolism to oxazepam in cultured canine hepatocytes (Chenery et al. 1987, Seddon et al. 1989). Therefore, it would be expected, based on the above information, that the ratio of DZ/ND would change minimally. This expectation is consistent with the findings observed in the present study.

In contrast to the metabolism of diazepam to nordiazepam, diazepam is slowly converted to temazepam in cultured canine hepatocytes (Chenery et al. 1987, Seddon et al. 1989) and then temazepam is quickly converted to oxazepam or glucuronidated (Chenery et al. 1987). The conversion to oxazepam is so rapid in vivo that temazepam is not detected in plasma (Klotz et al. 1976, Löscher et al. 1981, Vree et al. 1979). Increases in drug clearance can be due to an increase in the drug’s Clint (Rowland and Tozer, 1995). If the metabolism of diazepam to temazepam is increased, a subsequent increase in oxazepam would be observed, since diazepam to temazepam is the rate limiting step. This increase could be observed with or without increased metabolism of temazepam to oxazepam. As a result, a decrease in the DZ/OX ratio would be predicted and did occur in the present study after 2.4-D administration. The lack of change in the DZ/ND ratio with the decrease in the ND/OX ratio also support the comparative increase in oxazepam formation as a result of the increase temazepam metabolism to oxazepam.

In conclusion, 2.4-D increases the clearance and volume of distribution of diazepam in the dog. The increased volume of distribution of diazepam is likely a result
of a displacement of diazepam from albumin by 2.4-D. The change in diazepam clearance is likely due to an increased metabolism of diazepam to temazepam. It cannot be determined, however, if the increase in clearance is due to an increase in the free fraction of diazepam or the induction of metabolic enzymes, since $C_{l_{\text{int}}}$ is a function of both the unbound intrinsic clearance ($C_{l_{\text{int}}}$) and the fup. Further studies need to be performed using *in vitro* systems to evaluate the $C_{l_{\text{int}}}$ and $C_{l_{\text{int}}}$ of diazepam and each of its metabolites, nordiazepam, temazepam, and oxazepam after 2.4-D pretreatment to confirm this effect.
References


Cristea E., Dinu V., Dinu I., Mihăilescu I., Boghainu L. (1981) 2.4-Dichloro-phenoxyacetic acid interactions with the detoxification hepatic systems. Physiologie., 18, 199-203.


FINAL CONCLUSIONS

The phenoxy herbicide 2.4-D is the third most widely used herbicide in the US. It is often used in combination with other structurally similar compounds in a single product, although limited studies have been performed evaluating the potential interactions of 2.4-D with other structurally similar phenoxy and non-phenoxy compounds. The limited studies performed suggest a toxicokinetic and dynamic interaction occurs. It was the goal to investigate the potential for interactions of two structurally similar phenoxy herbicides, 2,4-D and MCPA, by evaluating toxicodynamic and toxicokinetic responses and to investigate the potential for 2,4-D to alter metabolism by evaluating the toxicokinetics of a highly metabolized therapeutic agent, diazepam, in a canine model. The conclusions from our studies are following:

1) An analytical method was successfully developed which allowed for simultaneous determination by HPLC of 2.4-D and MCPA in canine plasma and urine and was utilized for the toxicodynamic and toxicokinetic studies.

2) The dog is not as sensitive to the effects of 2.4-D as previously reported and the reported LD$_{50}$, 100 mg/kg, is incorrect.
Oral administration of a toxic low dose of 200 mg 2,4-D/kg resulted in insertional myotonia ranging from +1 to +4, percussion dimpling of the tongue, decreased serum calcium and potassium, and vomiting and diarrhea, but NOT obvious clinical myotonia and NO deaths.

3) Electromyographic evaluations did not provide enough sensitivity to determine an interaction between 2,4-D and MCPA. There are no other appropriate toxicodynamic parameters available to study the interaction.

- Plasma total and unbound concentrations at the time of EMG ranged from 421 - 633, and 97.4 - 166 µg/mL, respectively, but did not directly correlate with the severity of insertional myotonia observed in each dog.
- Gastrointestinal effects and the possibility of decreased electrolyte concentrations contributing to muscle membrane instability prevented the further study of the toxicodynamic interaction of 2,4-D and MCPA.

4) Both 2,4-D and MCPA individually bind to canine plasma proteins in a concentration dependant manner at two classes of binding sites. Albumin represented the primary binding protein.

5) A statistically significant increase in the free fraction of both 2,4-D and MCPA and decrease in renal elimination of MCPA occurred, however they were not significant enough to affect the AUCs of 2,4-D and MCPA.

- 2,4-D and MCPA were capable of displacing each other from plasma protein in a concentration dependant manner resulting in increased free concentrations and the potential for altered therapeutic effect.
• A decrease in the amount and % of a 2 mg/kg dose of MCPA excreted in the urine was observed after a 200 mg 2,4-D/kg co-administration suggesting saturation of transport processes, while a decrease in the amount or % of a 0.5 mg/kg dose of 2,4-D excreted in the urine was NOT observed after a 5 mg MCPA/kg co-administration suggesting either a higher affinity of 2,4-D and/or a lack of saturation at the transport process.

• A statistically significant difference in the plasma AUCs of single (2,4-D or MCPA) or combined (2,4-D plus MCPA) was NOT observed when 2,4-D and MCPA were administered in either equal (25 mg 2,4-D/kg with 25 mg MCPA/kg) or unequal (0.5 mg 2,4-D/kg with 5 mg MCPA/kg; 200 mg 2,4-D/kg with 2 mg MCPA/kg) doses.

6) 2,4-D increases the clearance of diazepam from plasma and displaces diazepam from albumin, although determination of the mechanism was not possible.

• The AUCs of diazepam and its metabolites, nordiazepam and oxazepam, were lower after 2,4-D administration compared to before administration.

• *In vitro.* 2,4-D, at 100 μg/mL (which is representative of plasma concentrations 15 days after administration of 200 mg 2,4-D/kg) increased the free fraction of diazepam.

• Because of the multiple pathways involved and the extrahepatic metabolism of diazepam, it cannot be concluded that the increased clearance was from hepatic induction.

A toxicokinetic interaction of 2,4-D and MCPA was observed with plasma protein binding and renal elimination. These interactions were not enough to produce a significant interaction, thus rejecting the hypothesis that an interaction would occur.
These studies have provided valuable information about the use of products containing multiple phenoxy compounds. It was not possible to determine if 2,4-D induced hepatic enzymes responsible for diazepam metabolism, however 2,4-D did increase the clearance and decrease the protein binding of diazepam. Further studies are warranted to determine the mechanisms and extent of effect 2,4-D has on diazepam.
BIBLIOGRAPHY


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