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Pharmacokinetic Study of Diazepam Administered in IV bolus and IV Infusion to Dogs and Cats

by

Ileana Antoaneta Ionita

Faculté de Pharmacie

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This thesis entitled:

Pharmacokinetic Study of Diazepam Administered

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written by Ileana Antoaneta Ionita

has been approved for the Faculté de Pharmacie

Dr. Jean-Guy Besner (director)

Dr. Anthony Chilton (co-director)

Dr. France Varin

Dr. Louis Cartilier

The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.

Sommaire

L'objectif de cette étude était de démontrer qu'une perfusion intraveineuse continue de diazépam est plus appropriée que l'administration de bolus I.V répétés pour le traitement des status épilepticus et des convulsions groupées chez le chat et le chien,

Le diazépam est le médicament de premier choix pour le traitement de ces deux urgences neurologiques parce qu'il posséde d'excellentes propriétés anticonvulsivantes et surtout parce qu'il agit très rapidement. Comme le but du traitement est d'atteindre des concentrations sériques thérapeutiques de benzodiazépines dans les plus brefs délais et de les maintenir constantes pour plusieurs heures, le deuxième objectif de cette étude était de déterminer la pharmacocinétique du diazépam et de ses métabolites actifs chez le chien et le chat après des bolus I.V. répétés et après un bolus I.V. suivi d'une perfusion I.V. Ceci pourra permettre d'améliorer les protocoles de traitement quant à la dose, la durée et le mode d'administration, utilisés actuellement à l'Hôpital d'enseignement de la Faculté de Médecine Vétérinaire.

Une méthode analytique robuste et spécifique a été mise au point par chromatrographie liquide de haute pression pour déterminer le diazépam et ses métabolites, desméthyldiazépam, oxazépam et témazépam dans le sérum de chien et de chat. Cette methode a été employée pour analyser les échantillons de sérum de huit chiens et sept chats qui ont reçu le diazépam par chaqun des deux types d'administration. Les résultats ont suggéré qu'une partie du médicament n'atteint pas la circulation sanguine et des tests pour déceler le niveau d'absorbtion du diazépam par le matériel utilisé pour la perfusion ont été effectués. On a trouvé que le nécessaire utilisé pour la perfusion pendant cette étude absorbe beaucoup le diazépam et est la cause de l'importante différence entre les deux modes d'administration, étant entendu que la perfusion est la moins efficace.

On peut conclure que pour utiliser la perfusion I.V. constante comme mode d'administration pour le diazépam pour le traitement des status épilepticus et des convulsions groupées, un sac en polyoléfine et des tubulures à faible absorption devraient être utilisées pour garder les concentrations sériques thérapeutiques de benzodiazépines. De plus, la concentration de diazépam ne devrait pas depasser la limite de solubilité de 80 mg/L dans la solution de Ringer.

Summary

The first objective of this study was to demonstrate that the administration of an IV bolus followed by a constant IV infusion of diazepam is more appropriate than repeated IV bolus for the treatment of status epilepticus and clustered seizures in dogs and cats.

The treatment of choice for these two serious emergency situations in small animals is the administration of anticonvulsant drugs, and diazepam is preferred by most of the veterinarians. Because successful anticonvulsant therapy is dependent on the maintenance of plasma drug concentrations within the therapeutic range, understanding the pharmacokinetics of the drug is paramount to therapeutic success. Therefore the second objective of this study was to determine the pharmacokinetics of diazepam and its metabolites in dog and cat, that will allow to adapt the present treatment protocols used at l'Hôpital d'Enseignement de la Faculté de Médecine Vétérinaire. (the Hospital for Small Animals of the Faculty of Veterinary Medicine of University of Montreal).

To reach these objectives, a sensitive high performance liquid chromatography method for the quantification of diazepam and its three metabolites, desmethyldiazepam, temazepam and oxazepam in dog and cat serum was developed and validated. This rugged method was successfully used for the analysis of the clinical samples from eight dogs and seven cats that underwent a two-way crossover study. The results as well as the pharmacokinetic profiles indicated that after the administration of the diazepam as a constant infusion, the plasma concentrations are lower than expected, this suggesting that only a small portion of the drug was actually administrated to the subjects. Therefore a test of absorption of the diazepam solution on different types of plastic tubing and burettes was performed, and it was found that the kit used for the constant IV infusion in the present study showed a very important absorption effect. Due to these facts, a significant difference between the two treatments was found.

It is concluded that administration of diazepam in a constant IV perfusion, for the treatment of status epilepticus and cluster seizures in dogs and cats, requires the use of a different administration set, in order to keep the diazepam concentration within the therapeutic range. Therefore a solution of diazepam not exceeding 80 mg/L should be administered using a polyolefine mixing bag and low sorbing tubing.

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LIST OF ABBREVIATIONS

AUC_{0-t}:	Area under the curve between zero to t hours.
$AUC_{0-INF} = AUC_{0-\infty}$:	Area under the curve from zero to infinity.
BZ:	Benzodiazepine.
CFS:	Cerebrospinal fluid.
CL:	Total body clearance.
CNS:	Central nervous system.
CV:	Coefficient of variation.
DMD:	Desmethyldiazepam.
DZ:	Diazepam.
GABA:	Gamma amino butyric acid.
GC:	Gas chromatography.
HPLC:	High pressure (performance) liquid chromatography.
IV:	Intravenous.
IM:	Intramuscular.
FDA:	Food and Drug Administration.
FPIA:	Fluorescence polarization immunoassay.
kg:	kilogram.
Lambda_z:	Rate constant.
LLOQ:	Lower limit of quantitation.
mcg:	microgram.
min:	minute.
mL:	milliliter.
mg:	milligram.
ng:	nanogram.

LIST OF ABBREVIATIONS (CONT'D)

Nr.:	number.
Ox:	Oxazepam.
PK:	Pharmacokinetic.
PO:	Polyolefine.
PVC:	Polyvinyl chloride.
QC:	Quality control.
RE:	Relative error.
rpm:	rotations per minute.
SE:	Status epilepticus.
STD:	Calibration standard.
Std Dev:	Standard deviation.
Subj.	Subject.
V:	Volume.
Vd:	Volume of distribution.

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CHAPTER 1: LITERATURE REVIEW AND STUDY OBJECTIVES

1.1. INTRODUCTION

Veterinarians are often called upon to care for animals that have repeated seizures at very brief intervals. The repeated occurrence of seizures without intervening periods of consciousness is called status epilepticus. This is a serious condition which can lead to brain deterioration through laminar necrosis of the cerebral cortex and death through a rapid loss of homeostasis.

Frequent recurrent seizures with intervening periods of consciousness are probably less ominous, but still can be serious and may serve as a forewarning of impending status epilepticus. Either condition occurs in animals as a result of toxic agents, metabolic derangement, progressive brain disease, exacerbation of acquired or inherited epilepsy. Regardless of its cause, status epilepticus is an emergency of serious magnitude (1).

1.1.1. Seizures

Seizures are the clinical result of rapid, excessive neuronal discharge in the brain (2). Seizures can be initiated by four general mechanisms (3):

- altered neuronal membrane function that can lead to excessive depolarization (e.g., alteration of the Na^+/K^+ pump); permeability changes in the cell membrane (induced, for example by hypoxia, inflammation, or trauma);

- decreased inhibitory neurotransmitters, such as gamma amino-butyric acid (GABA), the potent inhibitory neurotransmitter in the central nervous system;
- increased excitatory neurotransmitters, such as glutamate;
- altered extracellular potassium and calcium concentration.

During a seizure, extracellular potassium increases and calcium decreases, increasing neuronal excitability and facilitating the initiation and spread of the seizure. Once initiated, the seizure discharge may synchronize with other neurons and propagate to surrounding areas in the brain (2).

1.1.2. Status epilepticus, cluster seizures

Status epilepticus refers to failure of a patient to recover to a normal alert between repeated tonic-clonic attacks that last more than 3 minutes or cluster episodes that last at least 30 minutes (4, 5). Cluster seizures are two or more isolated seizures observed within 24 hours. This is frequent in both cats and dogs and occasionally may lead to status epilepticus (6).

Convulsive or tonic-clonic status epilepticus is a medical emergency in which convulsive seizures must be terminated by treatment with anticonvulsant agents (6). The physiologic sequelae of frequent or continuous seizure activity leading to increased intracranial pressure and neuronal necrosis include systemic arterial hypertension, loss of cerebrovascular regulation, disruption of the blood-brain barrier,

and cerebral edema. If not treated appropriately, they may develop serious neurologic complications due to these events (7) and can lead to brain deterioration through laminar necrosis of the cerebral cortex and death through a rapid loss of homeostasis. The longer an epileptic seizure persists, the greater the incidence of mortality or morbidity (2).

1.1.3. Treatment of status epilepticus and cluster seizures

At this time, the treatment of choice for seizures in animals caused either by acquired or inherited epilepsy is anticonvulsant drug therapy (1). The time factor is very important for the suffering patient, and the advantage of using diazepam is that it has good anticonvulsant activity and crosses the blood-brain barrier more rapidly than do other anticonvulsant drugs.(8)

1.2. DIAZEPAM

Diazepam, an anticonvulsant drug, has been widely recognized as the drug of choice for the treatment of status epilepticus and for acute convulsive syndromes of nearly any type in dogs (9, 10, 11, 12, 4, 5, 13) and in cats (12, 6, 13). This benzodiazepine acts to prevent the spread of seizure activity, to elevate the seizure threshold, suggesting that it has a broad spectrum of activity (9). Diazepam enhances the inhibitory effects of GABA in both the brain and spinal cord (30). Thus it decreases seizure spread and also blocks arousal and centrally depresses spinal reflexes (2).

The rate of CNS distribution following IV administration of the anticonvulsant drug is important in a patient with status epilepticus. The drug must be sufficiently lipid-soluble to be rapidly distributed into the CNS so that therapeutic concentrations are reached.

Diazepam is greater than 90% protein bound (14); however, the lipid solubility is so great that distribution into the CNS is sufficiently rapid in patients with status epilepticus (8). After an IV bolus, this drug penetrates in the CNS and reaches its maximal concentration in less than one minute (15). The rate of entry from blood into CSF is P = 0.23/min for diazepam, and the corresponding values for desmethydiazepam range from 0.06 to more than 0.1/min (8).

Toxic effects have not been reported except for the possibility of respiratory depression, when large doses are administered intravenously. The manufacturers of diazepam warn against the occurrence of seizures if the drug is abruptly withdrawn from human patients after maintenance therapy.

Adverse effects (sedation, ataxia, increased appetite, and in some cases hyperactivity) are likely to occur if concentration reaches 500 ng/mL (2).

Although diazepam is used to control status epilepticus, it may occasionally induce or aggravate seizures in some patients with convulsive disorders. In high doses, it activates the drug metabolizing enzymes in the liver (16). Diazepam also possesses

dependence liability and may produce withdrawal symptoms, but has a wide margin of safety against poisoning (16).

The advantage of using diazepam is that it has good anticonvulsant activity and crosses the blood-brain barrier more rapidly than do other anticonvulsant drugs.(8)

1.2.1. Routes of diazepam administration during a neurological emergency

Oral administration of diazepam to a convulsing animal is not practical, and absorption is unpredictable. After oral administration, diazepam is rapidly adsorbed but, in consequence of extensive first-pass metabolism to desmethyldiazepam, the bioavailability of unchanged diazepam is only 1-3% (15).

Absorption after intra muscular (IM) administration of diazepam is characterized as variable and erratic, (17)(18) depending on the site of injection (19), and may induce necrosis at the injection site (20).

Thus it is generally accepted that diazepam should be administered intravenously IV for treatment of status epilepticus (21)or cluster seizures. Diazepam per rectum can be used as an at-home therapy to stop cluster seizure activity (22).

1.2.2. Diazepam metabolism and mechanism of action

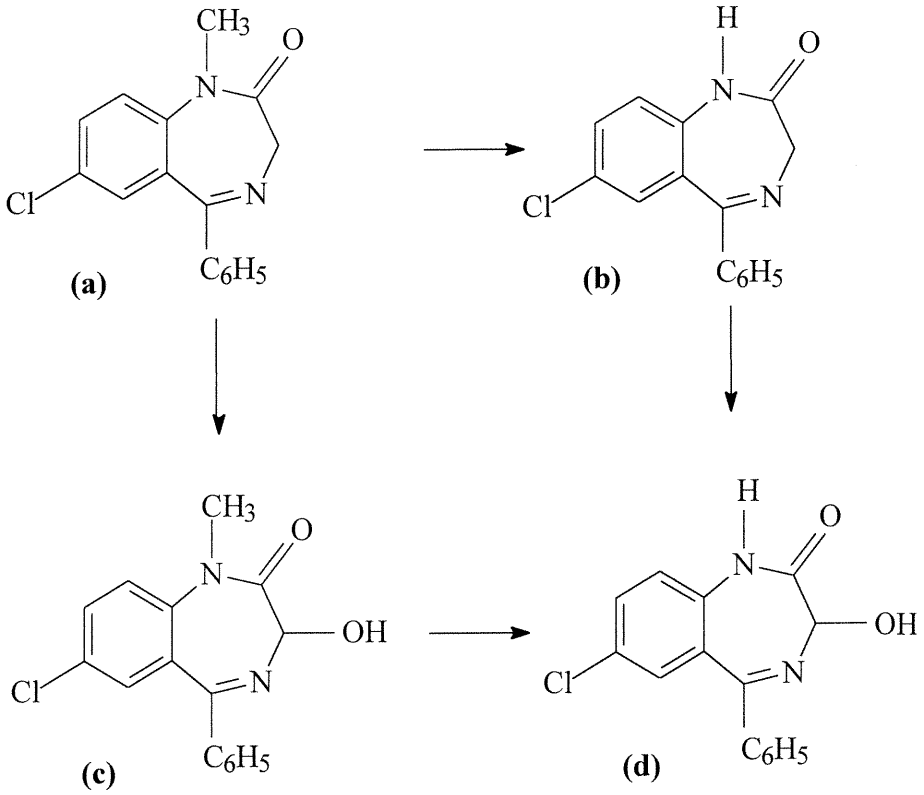


Fig 1.2.2 The phase I metabolism of diazepam (a) converted to N-desmethyldiazepam (b) and 3-OH diazepam (temazepam) (c). (b) and (c) are both converted to oxazepam (d).

Diazepam is the most lipid-soluble anticonvulsant and very rapidly distributes to the brain.

The probable sequence of diazepam metabolism in dogs is demethylation of diazepam to desmethyldiazepam, then hydroxylation of desmethyldiazepam to oxazepam (23) (Fig 1.2.2). Oxazepam is excreted in the urine, probably as oxazepam glucuronide.

Conversion of diazepam to temazepam (Fig 1.2.2) has not been recognized as an important pathway in dogs (24).

Diazepam and its metabolites are part of a larger class of drugs called benzodiazepines. The metabolites of diazepam, which have a longer half-life than the parent drug in dogs, have anticonvulsant activity that is equal to (23) or about a third of the anticonvulsant potency of unchanged diazepam (8, 15). As such it is likely that the metabolites contribute to the anticonvulsant activity of diazepam.

Benzodiazepines are drugs that increase inhibitory neurotransmission and hyperpolarize post-synaptic neuronal membranes, thus raising the seizure threshold of this cell. The outcome is the ability to prevent the spread of epileptic activity in the brain. The $GABA_A$ receptor is associated with a permeable chloride ion channel. Attachment of benzodiazepines to this receptor will result in increased chlorine permeability by facilitating chloride (Cl^-) passage into the cell, and subsequent membrane hyperpolarization (12).

1.2.3. Pharmacokinetic and Pharmacodynamic Properties

Several studies have been done on dogs following IV administration of diazepam. Löcher and Frey (8) gave dogs a dose of 2 mg/kg IV, and reported a triexponential decrease in diazepam concentrations for which the mean half-life of each of the 3 phases of the curve was 11.5 minutes, 43.8 minutes, and 3.15 hours. Papich and

Alcorn (9) reported that due probably to a lower dosage (0.5 mg/kg), or to an insufficiently sensitive assay, the three phases of the plasma concentration-time curve were not detected.

With the parenteral form, therapeutic peak blood levels are reached within 15 minutes after IV administration. The respective half-life is approximately 2-3 hours (16).
The plasma systemic clearance value reported for IV administrated diazepam (0.5 mg/kg) varies between 58 to 60 mL/kg/min (24). The same authors reported the half-lives between 0.236-0.258 h for DZ, 2.20-2.76 h for desmethyldiazepam (DMD) and 3.54-3.83 (h) for oxazepam. The volumes of distribution were 1.37 ± 0.57 L/kg for beagles (n=4) and 3.97 ± 5.18 L/kg for mixed-breed dogs.

Frey and Löcher (15) reported that DMD accumulates to very high concentrations within 15 min of injecting diazepam, and then exceeds the concentration of the parent drug by an order of magnitude; oxazepam reaches maximal concentrations between 1 and 5 hours after diazepam injections and its concentration remain intermediate between those of DMD and DZ.

The effective anticonvulsant plasma concentration of benzodiazepines in epileptic dogs is not precisely known. Many times the anticonvulsant drug concentrations found in humans are extrapolated to dogs and thus the reported therapeutic range varies between 100 to as high as 700 ng/mL. There is even less information about the therapeutic levels of benzodiazepines in cats. Different authors recommend doses from

0.25 mg/kg to 2 mg/kg diazepam as the effective IV doses for the treatment of the status epilepticus in dogs (2, 15, 22, 21), and 0.5 mg/kg to 1 mg/kg for cats (6, 25).

1.3. ANALYSIS OF DIAZEPAM AND METABOLITES

Methods for the determination of diazepam and its metabolites have been previously described in the literature. Some of these methods used gas chromatography (GC) technique (24, 26, 27) while others were using HPLC. The sensitivity achieved in previous methods was good (e.g. 5-10 ng/mL), but the volumes of biological fluid necessary for the extraction were quite large (28). Other methods described two steps liquid-liquid extraction (24), or additional acid clean-up steps (29), that would increase the time of sample preparation. Some of the organic solvents used for the extraction in different methods previously described, such as benzene (24) or chloroform (28) are considered relatively toxic.

Some authors used a more convenient technique, fluorescence polarization immunoassay (FPIA), but this assay is designed for nonspecific identification of benzodiazepines, and the manufacturer's product description suggests that the assay is only semi-quantitative. The FPIA analysis of the plasma samples by use of the TDx system was compared with the HPLC assay and showed a good correlation for diazepam, a weaker correlation for desmethyldiazepam, and no correlation for oxazepam (24).

1.4. STUDY OBJECTIVES

In the literature, most of the authors recommend the IV administration of diazepam for the effective treatment of status epilepticus and clustered seizures in dogs and cats. The bolus could be repeated if the convulsions are not controlled in ca. 10 min, or if new convulsions appear. Since diazepam is rapidly metabolized and although the metabolites have an anticonvulsant activity, their half-lives are only slightly longer than that of the parent in the dog, diazepam has a short anticonvulsant activity in the dog and further convulsions could appear.

The administration of an IV bolus could be repeated, but this would be done only if new convulsions are present, and it does not protect from new convulsions. Also the big fluctuations of plasma concentrations could induce other convulsions. An ideal treatment protocol for status epilepticus and cluster seizures requires a slow infusion of diazepam to allow the veterinarian to reach a therapeutic plasma concentration very fast, to be able to maintain this concentration stable for several hours, without exceeding it and finally to allow a slow and progressive withdrawal.

The first objective of this study was to demonstrate that the administration of an IV bolus followed by a constant IV infusion of diazepam is more appropriate than repeated IV bolus for the treatment of status epilepticus and cluster seizures in dogs and cats. The constant IV infusion should result in a constant concentration of diazepam in plasma and would also avoid the fluctuations in plasma concentrations.

L'Hôpital d'enseignement de la Faculté de Médecine Vétérinaire. (the Hospital for Small Animals of the Faculty of Veterinary Medicine at University of Montreal) uses a protocol of treatment consisting in the administration of a IV bolus of diazepam (0.5-1 mg/kg) followed by a constant IV infusion at a rate of 0.5-1 mg/kg/hour. If no other convulsion occurs in the first 6 hours, the animal is slowly weaned off the drug, decreasing the concentration by 25% every 4-6 hours. If the convulsions are still present, the diazepam concentration could be increased and/or phenobarbital can be added to the infusion at the rate of 0.5 to 1 mg/kg/hour.

This protocol proves to be very effective in most cases. However, in certain cases, the patient does not respond to this treatment. Among others, one of the factors responsible for this could be the fact that the plasma concentration of diazepam is below the therapeutic level.

Because successful anticonvulsant therapy depends on the maintenance of plasma drug concentrations within the therapeutic range, understanding the disposition of the drug is paramount to therapeutic success. Large fluctuations are generally undesirable because plasma drug concentrations are more likely to reach both toxic and subtherapeutic concentrations during the dosing interval.

Therefore the second objective of this study was to determine the pharmacokinetics of diazepam and its metabolites in dog and cat, that will allow to adapt the present

treatment protocols used at l'Hôpital d'enseignement de la Faculté de Médecine Vétérinaire. (the Hospital for Small Animals of the Faculty of Veterinary Medicine of University of Montreal).

The generation of active metabolites requires that all metabolites and the parent drug should be measured. In some of the previously published studies a rapid FPIA technique has been used, but this is a nonspecific assay for the identification of benzodiazepines.

Because of the importance of measuring specific metabolites, a high-performance liquid chromatography (HPLC) assay was developed that was capable of quantifying each metabolite as well as the parent drug in dog and cat serum. Validation tests to prove the robustness of this analytical method were performed before analyzing the dog and cat samples.

CHAPTER 2: MATERIALS AND METHODS

2.1. CLINICAL PROTOCOL

2.1.1. Animals

For this study the subjects were eight dogs and eight adult cats. All animals were submitted to complete physical and neurological, complete blood analysis (hematology and biochemical profile) and fecal analysis (for parasite egg detection) were done. Both dogs and cats were fed a commercial dry food for the respective animals during the study. The study was approved by the Deontology Committee of the Faculté de Médecine Vétérinaire which is in accordance with the principles published in the Canadian Council on Animal Care "Guide to the Care and Use of Experimental Animal". The weight of each animal is presented in the Table 2.1.1.

Table 2.1.1. Animal individual weight used to calculate the dose

	DOGS								CATS						
Subj. Nr	1	2	3	4	5	6	7	8	1	2	3	4	5	7	8
Weight (kg)	22	24	20	22	20	23	21	20	3.6	3.6	3.4	5.4	3.5	2.9	3.2

2.1.2. Materials and methods

For the administration of diazepam solution were used:

- PrecisionGlide® needles 22G1 from Becton Dickinson & Co, Buretrol®;

- Add-On Set (150 mL Valveless Burette Slide Clamp) from Baxter;

- Vented Basic Solution set(1.8 m lenght, 10 drops approx 1mL) from Baxter;

- Extension set (volume 5.0 mL, length 89 cm) from Baxter;

- Extension set (volume 0.45 mL, length 7'') MED-RX® from Benlan;

- I.V. catheter with wings (20GA 1.16 in; 1.1x30 mm; 60mL/min) Insite-W$_{TM}$ from Infusion Therapy Systems Inc.;

- Lactated Ringer's Injection USP (500 mL) from Baxter;

- Diazepam injection USP (5 mg/mL, IM-IV) from SABEX;

As an example for a dog of 20 kg, 4mL of diazepam were mixed with 83 mL of lactated Ringer's solution and administered for two hours at a rate of 43 mL/hour. The procedure was repeated twice with freshly prepared diazepam/lactated Ringer's solution.

2.1.3. Drug administration and sample collection for dogs

PHASE I: Repeated IV boluses of diazepam

Each dog received four IV boluses of 1 mg/kg diazepam, administrated at 0, 30, 60 and 90 minutes. Diazepam injections were made via a cephalic IV catheter that was flushed with heparinized saline solution. Blood samples (ca. 3 mL) were collected via a jugular catheter flushed with heparinized saline after each sampling. Some samples

were taken from a venipuncture (jugular catheter was not working) from any available vein (cephalic, jugular, saphenous, femoral).

The sampling times were as follows:

3, 5, 10, 15 and 30 minutes after the first bolus; 15 minutes after the second and third bolus and 0, 15, 30, 45, 60 minutes and 3, 6, 9, 12, and 15 hours after the last bolus.

PHASE II : One IV bolus followed by constant IV infusion

A 1 mg /kg IV bolus of diazepam was administrated via a cephalic IV catheter that was flushed with heparinized saline solution after the administration. A constant infusion was then started right after the administration of the IV bolus, at a rate of 0.5 mg/kg/hour and maintained for 6 hours at constant rate.

Three mL blood samples were collected using the same technique described for phase I at the following sampling times: 0, 15, 30, 45, 60 and 90 minutes, at 2, 4 and 6 hours, then every 15 minutes for one hour, every 30 minutes for another hour, and finally every 3 hours for the next 15 hours. The blood samples were collected in polypropylene tubes, centrifuged and the serum was stored in polypropylene tubes at below -15°C.

2.1.4. Drug administration and sample collection for cats

PHASE I: Repeated IV boluses of diazepam

Cats underwent the same procedure for the drug administration as dogs. Four IV boluses of 1 mg/kg diazepam were administrated at 0, 30, 60 and 90 minutes, for a total dose of 4 mg/kg diazepam to each cat. Since diazepam half-life in cats is longer than in dogs, a different sampling time scheme was adopted. The blood samples were collected at 3, 5, 10, 15 and 30 minutes after the first bolus; 15 minutes after the second and third bolus and 0, 15, 30, 45, 60 minutes and 2, 10, 18, 24, 36, 48, and 60 hours after the last bolus.

PHASE II : One IV bolus followed by constant IV infusion

After the administration of a 1 mg/kg IV bolus of diazepam, a constant infusion was started at a rate of 0.1 mg/kg/hour for 6 hours. Two mL blood samples were collected at 0, 15, 30, 45, and 60 minutes, at 2,4,6 hours, then at every 15 minutes for 1 hour, every 30 minutes for another hour, every 8 hours for 24 hours, and finally every 12 hours for the next 24 hours.

The first ml of blood (0.5 ml in cats) was discarded and the catheter was flushed with heparinized saline after each sampling.

2.1.5. Monitoring of animals

The body temperature, heart rate, respiratory frequency and mental status (sedation) were monitored during the infusion period and at each sampling time for 17 hours (50 hours for the cats) after the infusion was discontinued.

2.2. ANALYTICAL PROTOCOL

All chemicals and reagents used for samples analysis during this study were provided by ***LAB Pharmacological Research Int'l Inc****. The analytical study itself was conducted in the facilities of the above mentioned company which kindly facilitated the access to the necessary instrumentation, as well as the software used for the calculation of samples concentration.*

A rapid, robust and sensitive method was developed for the determination of diazepam and its 3 main metabolites desmethyldiazepam, temazepam and oxazepam in dog and cat serum. The main challenge was to find sensitive assay that would allow the use of a small volume of serum, since the available volume of blood is limited by the small volume of the animal, and sometimes by the difficulty in drawing the blood. Also another concern was to be able to use a liquid-liquid extraction that would give good recoveries without being a toxic solvent. For both dogs and cats, serum proved to have less endogenous interference than plasma with either Na EDTA or Na heparin as the anticoagulant. The mobile phase did not necessitate the adjustment of the pH and the

extraction consisted of a single liquid-liquid extraction in alkaline conditions, followed by fast evaporation due to the use of highly volatile solvents. For the reconstitution of the dry residue the same phosphate solution used to prepare the mobile phase was used. Although both mobile phase and the phosphate solution gave similar results when used to reconstitute the samples, the later was preferred because its degree of evaporation would be considerably lower compared with the mobile phase and injection of an aqueous solution reduced the potential for band broadening on the HPLC column. This was consequently allowing the analysis of a large number of samples over a long period of time, without having the composition of the sample changed.

2.2.1. Chemicals, reagents and materials

Dog serum was provided by University of Montreal, and cat serum was kindly donated by LAB Pharmacological Research Int'l Inc.

Diazepam, desmethyldiazepam, temazepam, oxazepam and midazolam (internal standard) were USP compounds and were handled according to the regulations regarding the controlled drugs stipulated by FDA. The following solvents and solutions were used:

- sodium hydroxide solution, 1N (Fisher);
- hexane (Caledon);
-ethyl acetate (Caledon);
- potassium phosphate monobasic (Fisher);

- acetonitrile,);

- methanol, HPLC grade (Caledon);

- deionized water (Fisher);

- 16x100 screw cap glass tubes;

- 16x100 culture glass tubes;

- polypropylene inserts (SPE).

2.2.2 HPLC instrumentation and operating conditions

The instruments and apparatus used are listed below:

- centrifuge: 103 NA from Western;

- evaporator: TorboVap® LV;

- column: Purospher, 125x4 mm, 5 microns from Merk;

- column heater: 250, Cera;

- pump: Hitachi and TSP;

- injector: Perkin-Elmer;

- UV detector: 2487 Dual Wvelenght formWaters and UV 150 from TSP;

- Data acquisition: Winner on Windows, TSP;

- Integrator: DataJet, TSP;

- Vortex Genie 2 TM from Fisher Scientific;

- automated pipettes (Eppendorf and Socorex).

The mobile phase consisted of a 60 : 35 : 5 mixture of 0.02M potassium phosphate dibasic : acetonitrile : methanol (V : V : V) and was delivered at a constant flow of 0.75 mL/min. This composition provided a good separation of the five compounds of interest from the endogenous serum components in a run time of ca. 17 minutes. The column was heated at 30°C, to ensure a constant retention time throughout the analysis of a whole batch of samples.

2.2.3. Calibration Standard and Quality Control samples preparation

Seven non-zero calibration standards (STD) and three quality control samples (QC) were prepared in drug free dog or cat serum, found free of endogenous interference. Spiking solutions containing all four drugs were prepared and added to the drug free serum to obtain the concentrations presented in table 2.2.3. Standard calibration samples and quality control samples were prepared from two different stock solutions of each compound. A stock check was performed in order to compare these stock solutions and differences below 5% were found for all four drugs.

Table 2.2.3: Calibration standard and quality control samples concentration

STD/***QC***	DIAZEPAM (ng/mL)	DMD (ng/mL)	OXAZEPAM (ng/mL)	TEMAZEPAM (ng/mL)
A	0.0	0.0	0.0	0.0
B	10.0	10.0	10.0	10.0
C	20.0	20.0	20.0	20.0
D	100.0	100.0	40.0	40.0
E	500.0	500.0	200.0	200.0
F	2000.0	2000.0	400.0	400.0
G	3000.0	3000.0	600.0	600.0
H	4000.0	4000.0	800.0	800.0
A	***25.0***	***25.0***	***25.0***	***25.0***
B	***1250.0***	***1250.0***	***250.0***	***250.0***
C	***2500.0***	***2500.0***	***500.0***	***500.0***

All STDs and QCs were aliquoted in polypropylene tubes and kept in freezer at -25°C. They were used for validation tests and/or for the analysis of the analytical samples.

2.2.4. Samples extraction

A volume of 200 μL of dog or cat serum was aliquoted in 16x100 screw cap glass tubes. To each tube were added 50 μL of internal standard (10.0 μg/mL midazolam in deionized water) and 10 mcL of a alkaline solution of sodium hydroxide, 1N. To the blank sample were added 50 mcL of deionized water instead of internal standard. After each step the samples were vortexed for ca. 5 seconds. Using an automatic dispenser, 6 mL of extraction solvent (hexane : ethyl acetate, 7 : 3, V ; V) were added and after the caps were placed, the samples were shaken in a overhead shaker for ca. 10 minutes, then centrifuged at 3000 rpm for 10 minutes. Using a acetone/dry ice bath, the aqueous bottom layer was frozen, the organic layer was transferred in clean glass culture tubes and dried in a evaporator under a gentle stream of nitrogen, at 30°C. The dry residue was reconstituted in 200 μL of 0.02 M potassium phosphate dibasic, and transferred in polypropylene injection vials. The injection volume was 50 μL.

2.2.5. Formulae Used in Validation:

Accuracy

$$\%\ Relative\ Error\ (\%\ RE) = \left[\frac{Concentration\ Found - Concentration\ Spiked}{Concentration\ Spiked} \right] \times 100$$

Precision

$$\%\ Coefficient\ of\ Variation\ (\%\ CV) = \left[\frac{Standard\ Deviation}{Mean} \right] \times 100$$

% Recovery of Drug and Internal Standard

$$\%\ Recovery = \left[\frac{Peak\ Height\ Extracted\ QCs}{Mean\ Peak\ Height\ Reference\ QCs} \right] \times 100$$

% Overall Recovery of Drug

$$\%\ Overall\ Recovery = \left[\frac{MRQC\ low + MRQC\ med + MRQC\ high}{3} \right]$$

$$\%\ Overall\ Precision = \left[\frac{\%\ CV\ QC\ low + \%\ CV\ QC\ med + \%\ CV\ QC\ high}{3} \right]$$

MR = Mean Recovery
RRT = Relative Retention Time

Calculations in this report are computerized. The last digit may vary from the original raw data or when trying to manually reproduce them.

2.3. PHARMACOKINETIC INTERPRETATION

Results were evaluated with the WinNonlin program using a noncompartmental model. An example of a ASCII file is presented in Appendix E.

CHAPTER 3: RESULTS AND DISCUSSION

3.1. VALIDATION RESULTS

Limit of Quantitation

The lower limit of quantitation (LLOQ) is defined as the lowest concentration that can be determined within the following limits: Intra-assay precision (% CV) ≤ 20% and accuracy (Mean % Relative Error) ≤ 20%. For Diazepam, desmethyldiazepam and temazepam and oxzepam in serum the LLOQs are 10.0 ng/mL, for each. (See Tables 3.1.7a - 3.1.7d.

Specificity

No endogenous interference was observed at the retention time of diazepam, desmethyldiazepam, temazepam, oxazepam and internal standard in the eight drug free dog serum samples tested.

Accuracy

The accuracy of the assay is indicated by the % relative error (% RE) which is the difference between the measured and the nominal concentration of quality control (QC) samples at each concentration level.

The % relative error for diazepam ranged between -2.8 and 2.4 for inter-assay and between 0.6 and 3.3 for intra-assay. For desmethyldiazepam, the % relative error

ranged between -2.8 and 1.9 for inter-assay and between -4.9 and -2.1 for intra-assay. For temazepam, it ranged between 0.9 and 1.9 for inter-assay and between -2.3 and -0.7 for intra-assay. For oxazepam, it ranged between -1.2 and 2.4 for inter-assay and between –5.7 and -0.5 for intra-assay.(See Tables 3.1.6a-d and 3.1.8a-d).

Precision

The precision of the assay is indicated by the % coefficient of variation (% CV) of the mean value of all QC sample results at each concentration level.

The % CV for diazepam ranged between 2.9 and 8.6 for inter-assay and between 1.1 and 5.1 for intra-assay. For desmethyldiazepam, the % CV ranged between 2.8 and 9.9 for inter-assay and between 3.4 and 4.2 for intra-assay. For temazepam, it ranged between 1.8 and 5.6 for inter-assay and between 1.4 and 3.3 for intra-assay. For oxazepam, it ranged between 1.6 and 6.1 for inter-assay and between 1.0 and 2.7 for intra-assay.(See Tables 3.1.6a-d and 3.1.8a-d).

Recovery

The recovery of diazepam, desmethyldiazepam and temazepam from spiked dog serum was calculated by direct comparison of the response of extracted QC's with the response of reference QC's of the same concentrations which were dissolved in the same solution as extracted QC's.

The overall recoveries for diazepam, desmethyldiazepam, temazepam and oxazepam were 87.9%, 94.7%, 94.9% and 91.1% respectively. Internal standard recovery was

84.0%. The overall precisions of recovery for diazepam, desmethyldiazepam, temazepam and oxazepam were 1.3%, 2.8% 2.1% and 1.3% respectively. Internal standard precision of recovery was 2.4%. (See Tables 3.1.5a-e).

Ruggedness

The chromatographic system handled a relatively large number of samples (ca. 400 injections) without peak shape deterioration or significant changes in relative retention times. A second column of the same type used for method validation gave similar chromatography for all four drugs and internal standard, when used for ruggedness tests.

3.1.1 Freeze-Thaw Stability

The influence of two freeze-thaw cycles at all QC concentration levels was examined in triplicate. Cycle 0 (never frozen) and cycle 1 were both tested against the same freshly spiked calibration curve.

Table 3.1.1a: Freeze-Thaw Stability Data for Diazepam Spiked in Dog Serum

Curve No.	Freeze-Thaw Cycles	Concentration Spiked (ng/mL)					
		25.0		1250.0		2500.0	
		Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE
CEY02	Cycle 0	26.4	5.6	1255.9	0.5	2627.0	5.1
		25.5	2.0	1269.4	1.6	2605.9	4.2
		25.1	0.4	1233.0	-1.4	2503.5	0.1
CEY02	Cycle 1	24.8	-0.8	1232.8	-1.4	2582.1	3.3
		25.7	2.8	1285.5	2.8	2620.8	4.8
		26.9	7.6	1275.5	2.0	2589.0	3.6
CEY03	Cycle 2	25.3	1.2	1237.1	-1.0	2594.5	3.8
		23.4	-6.4	1287.8	3.0	2641.1	5.6
		24.7	-1.2	1283.3	2.7	2583.9	3.4

Table 3.1.1b: Freeze-Thaw Stability Data for Desmethyldiazepam Spiked in Dog Serum

Curve No.	Freeze-Thaw Cycles	Concentration Spiked (ng/mL)					
		25.0		1250.0		2500.0	
		Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE
CEY02	Cycle 0	26.2	4.8	1240.4	-0.8	2508.2	0.3
		26.6	6.4	1238.0	-1.0	2547.5	1.9
		24.6	-1.6	1189.3	-4.9	2522.5	0.9
CEY02	Cycle 1	27.4	9.6	1226.4	-1.9	2558.3	2.3
		25.2	0.8	1236.0	-1.1	2526.7	1.1
		24.9	-0.4	1223.4	-2.1	2474.2	-1.0
CEY03	Cycle 2	26.0	4.0	1220.7	-2.3	2552.1	2.1
		24.4	-2.4	1255.6	0.4	2582.3	3.3
		24.5	-2.0	1263.8	1.1	2537.0	1.5

3.1.1. Freeze-Thaw Stability (Cont'd)

Table 3.1.1c: Freeze-Thaw Stability Data for Temazepam Spiked in Dog Serum

Curve No.	Freeze-Thaw Cycles	Concentration Spiked (ng/mL)					
		25.0		250.0		500.0	
		Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE
CEY02	Cycle 0	26.1	4.4	245.4	-1.8	497.3	-0.5
		27.6	10.4	249.2	-0.3	505.3	1.1
		26.4	5.6	244.5	-2.2	507.5	1.5
CEY02	Cycle 1	25.5	2.0	244.4	-2.2	505.3	1.1
		26.9	7.6	247.7	-0.9	503.8	0.8
		25.7	2.8	247.7	-0.9	500.3	0.1
CEY03	Cycle 2	26.9	7.6	245.8	-1.7	506.6	1.3
		24.0	-4.0	249.3	-0.3	507.7	1.5
		25.2	0.8	247.2	-1.1	502.3	0.5

Table 3.1.1d: Freeze-Thaw Stability Data for Oxazepam Spiked in Dog Serum

Curve No.	Freeze-Thaw Cycles	Concentration Spiked (ng/mL)					
		25.0		250.0		500.0	
		Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE
CEY02	Cycle 0	24.3	-2.8	246.7	-1.3	503.0	0.6
		25.2	0.8	247.9	-0.8	505.6	1.1
		24.9	-0.4	245.6	-1.8	509.8	2.0
CEY02	Cycle 1	24.7	-1.2	242.8	-2.9	504.9	1.0
		24.9	-0.4	248.0	-0.8	508.1	1.6
		25.7	2.8	246.0	-1.6	501.1	0.2
CEY03	Cycle 2	24.7	-1.2	243.4	-2.6	503.9	0.8
		25.0	0.0	247.9	-0.8	506.7	1.3
		25.6	2.4	249.3	-0.3	499.0	-0.2

Since % relative errors are within ± 15% and because no trend was observed between cycles, it was concluded that diazepam, desmethyldiazepam, temazepam and oxazepam in dog serum are stable over at least 2 freeze-thaw cycles.

3.1.2. In-Process Stability

Three sets of spiked quality control samples (all three levels) were thawed. After the addition of internal standard and the alkaline solution, one set was extracted immediately (0 h) while the other sets were extracted 1 hour and 2 hours after being left at room temperature.

Table 3.1.2a: In-Process Stability Data for Diazepam Spiked in Dog Serum

Time of Extraction	Concentration Spiked (ng/mL)					
	25.00		1250.00		2500.00	
	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE
0 h	24.6	-1.6	1317.1	5.4	2687.7	7.5
	24.4	-2.4	1288.3	3.1	2681.7	7.3
	25.0	0.0	1270.1	1.6	2568.1	2.7
1 h	28.3	13.2	1290.5	3.2	2579.0	3.2
	26.4	5.6	1291.9	3.4	2695.9	7.8
	27.4	9.6	1302.4	4.2	2625.1	5.0
2 h	28.2	12.8	1319.4	5.6	2713.0	8.5
	26.1	4.4	1287.9	3.0	2628.2	5.1
	27.8	11.2	1332.1	6.6	2722.2	8.9

Table 3.1.2b: In-Process Stability Data for Desmethyldiazepam Spiked in Dog Serum

Time of Extraction	Concentration Spiked (ng/mL)					
	25.00		1250.00		2500.00	
	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE
0 h	24.1	-3.6	1304.5	4.4	2638.8	5.6
	24.0	-4.0	1278.6	2.3	2642.6	5.7
	25.5	2.0	1238.4	-0.9	2537.5	1.5
1 h	29.6	18.4*	1286.2	2.9	2571.3	2.9
	26.7	6.8	1272.9	1.8	2724.2	9.0
	27.2	8.8	1261.2	0.9	2585.5	3.4
2 h	28.7	14.8	1319.4	5.6	2683.5	7.3
	26.0	4.0	1277.2	2.2	2594.5	3.8
	26.3	5.2	1342.4	7.4	2702.3	8.1

* Value outside acceptance range

3.1.2. In-Process Stability (Cont'd)

Table 3.1.2c: In-Process Stability Data for Temazepam Spiked in Dog Serum

Time of Extraction	Concentration Spiked (ng/mL)					
	25.0		250.0		500.0	
	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE
0 h	25.0	0.0	256.3	2.5	505.9	1.2
	27.7	10.8	247.9	-0.8	518.4	3.7
	23.5	-6.0	241.7	-3.3	499.0	-0.2
1 h	24.9	-0.4	255.6	2.2	506.2	1.2
	23.6	-5.6	252.9	1.2	527.0	5.4
	23.1	-7.6	247.1	-1.2	503.1	0.6
2 h	25.5	2.0	257.3	2.9	520.1	4.0
	22.7	-9.2	251.7	0.7	517.2	3.4
	23.1	-7.6	258.8	3.5	531.5	6.3

Table 3.1.2d: In-Process Stability Data for Oxazepam Spiked in Dog Serum

Time of Extraction	Concentration Spiked (ng/mL)					
	25.0		250.0		500.0	
	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE
0 h	23.7	-5.2	255.1	2.0	514.9	3.0
	23.4	-6.4	247.7	-0.9	512.1	2.4
	22.5	-10.0	244.0	-2.4	497.8	-0.4
1 h	25.5	2.0	257.4	3.0	505.3	1.1
	23.6	-5.6	253.2	1.3	519.6	3.9
	23.7	-5.2	247.8	-0.9	499.5	-0.1
2 h	25.8	3.2	255.1	2.0	522.4	4.5
	23.4	-6.4	250.3	0.1	511.5	2.3
	23.8	-4.8	257.1	2.8	524.8	5.0

Since only one value is outside the acceptance range (% RE within ± 15%) and because no trend was observed between samples extracted immediately and those left at room temperature, in alkaline conditions over the specified time periods, it was concluded that diazepam, desmethyldiazepam, temazepam and oxazepam in dog serum are stable for at least 3 hours under these conditions during sample processing.

3.1.3. Bench-Top Stability

Three sets of spiked quality control samples (all three levels) were thawed. One set was immediately extracted (0 h) while the other sets were extracted, 3 hours and 24 hours after being left on the bench at room temperature.

Table 3.1.3a: Bench-Top Stability Data for Diazepam Spiked in Dog Serum

Time of Extraction	Concentration Spiked (ng/mL)					
	25.00		1250.00		2500.00	
	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE
0 h	23.6	-5.6	1215.2	-2.8	2590.9	3.6
	23.2	-7.2	1253.3	0.3	2555	2.2
	25.7	2.8	1297.2	3.8	2607.6	4.3
3 h	24.4	-2.4	1285.6	2.8	2624.4	5.0
	24.2	-3.2	1283.8	2.7	2614.6	4.6
	26.1	4.4	1304.1	4.3	2617.5	4.7
24 h	24.5	-2.0	1294.8	3.6	2582.8	3.3
	26.4	5.6	1255.1	0.4	2617.4	4.7
	25.8	3.2	1261.6	0.9	2616.3	4.7

Table 3.1.3b: Bench-Top Stability Data for Desmethyldiazepam Spiked in Dog Serum

Time of Extraction	Concentration Spiked (ng/mL)					
	25.00		1250.00		2500.00	
	Conc. Found #REF!	% RE	Conc. Found #REF!	% RE	Conc. Found #REF!	% RE
0 h	25.4	1.6	1277.0	2.2	2713.1	8.5
	24.5	-2.0	1241.8	-0.7	2543.3	1.7
	24.2	-3.2	1239.1	-0.9	2503.1	0.1
3 h	26.6	6.4	1280.2	2.4	2615.2	4.6
	27.0	8.0	1244.7	-0.4	2541.0	1.6
	25.5	2.0	1234.6	-1.2	2501.0	0.0
24 h	26.3	5.2	1274.1	1.9	2601.3	4.1
	25.8	3.2	1212.8	-3.0	2532.3	1.3
	26.4	5.6	1212.9	-3.0	2509.6	0.4

3.1.3. Bench-Top Stability (Cont'd)

Table 3.1.3c: Bench-Top Stability Data for Temazepam Spiked in Dog Serum

Time of Extraction	Concentration Spiked (ng/mL)					
	25.0		250.0		500.0	
	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE
0 h	24.8	-0.8	249.0	-0.4	587.7	17.5*
	25.2	0.8	254.5	1.8	512.3	2.5
	24.6	-1.6	250.9	0.4	504.7	0.9
3 h	25.4	1.6	248.1	-0.8	509.9	2.0
	26.3	5.2	247.7	-0.9	500.2	0.0
	25.1	0.4	255.7	2.3	502.6	0.5
24 h	25.4	1.6	248.9	-0.4	507.5	1.5
	24.8	-0.8	242.6	-3.0	508.2	1.6
	25.4	1.6	243.7	-2.5	504.8	1.0

Table 3.1.3d: Bench-Top Stability Data for Oxazepam Spiked in Dog Serum

Time of Extraction	Concentration Spiked (ng/mL)					
	25.0		250.0		500.0	
	Conc. Found #REF!	% RE	Conc. Found #REF!	% RE	Conc. Found #REF!	% RE
0 h	25.4	1.6	251.9	0.8	625.9	25.2*
	25.3	1.2	254.4	1.8	511.0	2.2
	25.3	1.2	250.3	0.1	504.4	0.9
3 h	26.6	6.4	248.1	-0.8	506.1	1.2
	25.0	0.0	247.7	-0.9	505.0	1.0
	25.3	1.2	258.1	3.2	504.8	1.0
24 h	25.5	2.0	248.5	-0.6	509.1	1.8
	25.4	1.6	246.1	-1.6	507.7	1.5
	25.6	2.4	246.1	-1.6	504.4	0.9

Since only two values (one for temazepam and one for oxazepam) are outside the acceptance range (% RE within ± 15%) and because no trend was observed between samples extracted immediately and those left at room temperature over the specified time periods, it was concluded that diazepam, desmethyldiazepam, temazepam and oxazepam in dog serum are stable for at least 24 hours when left at room temperature.

3.1.4. Stability in Final Extract

Quality control samples were chromatographed immediately after extraction (0 h) and also at 134 and 232 hours after processing in order to establish the stability of extracted diazepam, desmethyldiazepam, temazepam, oxazepam and internal standard when kept in the autosampler at room temperature.

Table 3.1.4a: Stability Data for Extracted Diazepam

Time of Analysis	Concentration Spiked (ng/mL)					
	25.00		1250.00		2500.00	
	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE
0 h	29.4	17.6	1299.1	3.9	2536.7	1.5
	25.0	0.0	1268.4	1.5	2564.7	2.6
	26.1	4.4	1263.9	1.1	2564.5	2.6
134h	25.2	0.8	1295.5	3.6	2641.4	5.7
	25.7	2.8	1279.7	2.4	2562.0	2.5
	24.6	-1.6	1275.6	2.0	2652.4	6.1
232h	22.8	-8.8	1286.3	2.9	2553.6	2.1
	24.5	-2.0	1275.5	2.0	2613.2	4.5
	26.8	7.2	1291.8	3.3	2613.3	4.5

The peak height of the internal standard from all QCs used to demonstrate stability of extracted diazepam are tabulated to demonstrate the stability of extracted internal standard.

Table 3.1.4b: Stability Data for Extracted Internal Standard

Time of Analysis	Peak Height of Internal Standard (from QCs)			Mean n = 6	% Rel. Error
0 h	20865	20832	20721	20859.6	---
	20724	20904	21304		
	20437	20828	21121		
134 h	21971	21976	21446	21886.4	4.9
	22016	21477	22157		
	22503	22628	20804		
232 h	21809	21442	21399	21467.7	2.9
	21444	20749	22054		
	20983	22021	21308		

$$\% \text{ Rel. Error} = \frac{\text{Mean Peak Height (time)} - \text{Mean Peak Height (}}{\text{Mean Peak Height (0 h)}} \times 100$$

3.1.4. Stability in Final Extract (Cont'd)

Table 3.1.4c: Stability Data for Extracted Desmethyldiazepam

Time of Analysis	Concentration Spiked (ng/mL)					
	25.0		1250.0		2500.0	
	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE
0 h	26.9	7.6	1295.1	3.6	2548.2	1.9
	27.7	10.8	1277.8	2.2	2559.5	2.4
	27.0	8.0	1261.3	0.9	2537.2	1.5
134h	22.5	-10.0	1147.5	-8.2	2320.5	-7.2
	24.6	-1.6	1140.2	-8.8	2292.0	-8.3
	23.5	-6.0	1137.7	-9.0	2371.5	-5.1
232h	21.4	-14.4	1104.8	-11.6	2230.0	-10.8
	23.8	-4.8	1108.8	-11.3	2276.5	-8.9
	25.1	0.4	1118.3	-10.5	2282.3	-8.7

Table 3.1.4d: Stability Data for Extracted Temazepam

Time of Analysis	Concentration Spiked (ng/mL)					
	25.0		250.0		500.0	
	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE
0 h	24.6	-1.6	251.2	0.5	510.7	2.1
	23.0	-8.0	250.4	0.2	504.0	0.8
	24.8	-0.8	248.2	-0.7	503.6	0.7
134h	24.7	-1.2	250.6	0.2	507.8	1.6
	24.7	-1.2	250.5	0.2	498.0	-0.4
	23.5	-6.0	245.3	-1.9	511.5	2.3
232h	23.8	-4.8	246.0	-1.6	496.7	-0.7
	23.6	-5.6	245.8	-1.7	495.7	-0.9
	23.7	-5.2	240.6	-3.8	498.6	-0.3

3.1.4. Stability in Final Extract (Cont'd)

Table 3.1.4e: Stability Data for Extracted Oxazepam

Time of Analysis	Concentration Spiked (ng/mL)					
	25.0		250.0		500.0	
	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE	Conc. Found (ng/mL)	% RE
0 h	24.4	-2.4	251.7	0.7	509.1	1.8
	23.7	-5.2	250.9	0.4	502.1	0.4
	24.5	-2.0	248.6	-0.6	501.1	0.2
134h	25.0	0.0	255.1	2.0	514.5	2.9
	26.1	4.4	255.1	2.0	504.3	0.9
	25.1	0.4	248.7	-0.5	518.0	3.6
232h	25.6	2.4	253.8	1.5	512.7	2.5
	24.8	-0.8	255.7	2.3	508.5	1.7
	26.5	6.0	248.2	-0.7	511.9	2.4

Since % relative errors are within ± 15% and because no trend was observed between samples analyzed immediately after extraction and those analyzed over the specified time periods, it was concluded that extracted diazepam, desmethyldiazepam, temazepam, oxazepam and internal standard are stable for at least 134 hours in the autosampler at room temperature.

3.1.5. Recovery

Table 3.1.5a: Recovery of Diazepam from Dog Serum

Concentration Spiked (ng/mL)	Peak Height of Reference n = 3	Peak Height of Extracted QCs		Recovery (%)		Mean Recovery % CV
QC low	705	529	626	73.4	86.9	mean = 82.48
	735	575	605	79.8	84.0	% CV = 6.2
12.50	721	605	625	84.0	86.8	n = 6
	mean = 720.3					
QC med	23764	20030	21003	82.7	86.7	mean = 85.78
	24349	20971	21225	86.6	87.6	% CV = 2.1
120.00	24546	20515	20935	84.7	86.4	n = 6
	mean = 24219.7					
QC high	47825	46193	46105	94.0	93.8	mean = 95.52
	49593	47443	47470	96.6	96.6	% CV = 1.3
280.00	49992	47245	47135	96.2	95.9	n = 6
	mean = 49136.7					

% Overall recovery for Diazepam = 87.9
% Overall precision for Diazepam = 3.2

3.1.5. Recovery (Cont'd)

Table 3.1.5b: Recovery of Internal Standard from Dog Serum

Amount Added (mcg)	Peak Height of Reference n = 9	Peak Height of Extracted QCs		Recovery (%)		Mean Recovery % CV
Internal Standard (from QC) 0.200	24386	20783	21003	83.4	84.3	mean = 84.0
	24935	21029	21225	84.4	85.1	% CV = 2.4
	24544	21585	20935	86.6	84.0	n = 18
	24946	21002	19501	84.2	78.2	
	26089	21379	21211	85.8	85.1	
	24891	21250	21201	85.2	85.0	
	25068	20030	21362	80.3	85.7	
	25359	20971	21137	84.1	84.8	
	24145	20515	20807	82.3	83.5	
	mean = 24929.2					

Table 3.1.5c: Recovery of Desmethyldiazepam from Dog Serum

Concentration Spiked (ng/mL)	Peak Height of Reference n = 3	Peak Height of Extracted QCs		Recovery (%)		Mean Recovery % CV
QC low	848	728	802	85.0	93.6	mean = 89.30
	869	757	740	88.4	86.4	% CV = 4.0
25.0	853	799	763	93.3	89.1	n = 6
	mean = 856.7					
QC med	27933	28065	27524	98.9	97.0	mean = 98.30
	28513	28388	27283	100.0	96.1	% CV = 1.6
240.0	28699	27851	28283	98.1	99.7	n = 6
	mean = 28381.7					
QC high	57564	59096	55092	100.2	93.4	mean = 96.53
	59593	58365	56302	98.9	95.4	% CV = 2.7
560.0	59865	57085	55835	96.7	94.6	n = 6
	mean = 59007.3					

Overall recovery for Desmethyldiazepam = 94.7
Overall precision for Desmethyldiazepam = 2.8

3.1.5 Recovery (Cont'd)

Table 3.1.5d: Recovery of Temazepam from Dog Serum

Concentration Spiked (ng/mL)	Peak Height of Reference n = 3	Peak Height of Extracted QCs		Recovery (%)		Mean Recovery % CV
QC low	826	745	809	87.6	95.1	mean = 94.25
	860	791	805	93.0	94.6	% CV= 4.1
25.0	866	815	846	95.8	99.4	n = 6
	mean = 850.7					
QC med	7820	7357	7524	92.5	94.6	mean = 94.50
	7980	7535	7527	94.8	94.7	% CV= 1.4
240.0	8049	7676	7453	96.6	93.8	n = 6
	mean = 7949.7					
QC high	15639	15235	15303	95.3	95.7	mean = 96.00
	16119	15535	15318	97.2	95.8	% CV= 0.9
560.0	16197	15497	15200	96.9	95.1	n = 6
	mean = 15985.0					

Overall recovery for Temazepam = 94.9
Overall precision for Temazepam = 2.1

Table 3.1.5e: Recovery of Oxazepam from Dog Serum

Concentration Spiked 0	Peak Height of Reference n = 3	Peak Height of Extracted QCs		Recovery (%)		Mean Recovery % CV
QC low	999	872	915	86.3	90.5	mean = 88.50
	1025	908	877	89.8	86.7	% CV = 1.9
25.0	1009	905	892	89.5	88.2	n = 6
	mean = 1011.0					
QC med	9308	8444	8685	89.6	92.2	mean = 91.60
	9452	8633	8671	91.6	92.0	% CV = 1.3
240.0	9501	8759	8592	93.0	91.2	n = 6
	mean = 9420.3					
QC high	18547	17373	17527	91.9	92.7	mean = 93.05
	19017	17740	17651	93.9	93.4	% CV = 0.8
560.0	19137	17716	17525	93.7	92.7	n = 6
	mean = 18900.3					

Overall recovery for Oxazepam = 91.1
Overall precision for Oxazepam = 1.3

3.1.6. Intra-Assay Summary

Three sets of quality control samples at each concentration level (n=6) were extracted and analyzed with the same calibration curve to verify the intra-assay accuracy and precision. Six replicates of the LOQ were also analyzed with a single calibration curve and measured as samples (not part of the calibration curve).

Table 3.1.6a: Diazepam Intra-Assay Summary

Conc. Spiked (ng/mL)	Conc. Found (ng/mL)	Mean n = 6	Std Dev.	% CV (a)	% RE (b)
25.00	24.3 26.8 24.3 26.0 25.9 27.6	25.82	1.326	5.1	3.3
1250.00	1261.3 1265.5 1256.3 1231.5 1297.1 1242.2	1258.98	22.561	1.8	0.7
2500.00	2522.3 2491.2 2559.8 2519.8 2483.8 2517.2	2515.68	26.927	1.1	0.6

Table 3.1.6b: Desmethyldiazepam Intra-Assay Summary

Conc. Spiked (ng/mL)	Conc. Found (ng/mL)	Mean n = 6	Std Dev.	% CV (a)	% RE (b)
25.0	24.4 24.6 22.9 22.3 24.7 23.8	23.78	0.987	4.2	-4.9
1250.0	1279.8 1201.2 1212.4 1174.5 1254.0 1180.4	1217.05	41.784	3.4	-2.6
2500.0	2586.9 2385.8 2524.8 2395.2 2405.4 2389.7	2447.97	86.095	3.5	-2.1

a) Precision
b) Accuracy

3.1.6. Intra-Assay Summary (Cont'd)

Table 3.1.6c: Temazepam Intra-Assay Summary

Conc. Spiked (ng/mL)	Conc. Found (ng/mL)	Mean n=6	Std Dev.	% CV (a)	% RE (b)
25.0	24.3 24 23.7 24.2 24.4 26	24.43	0.807	3.3	-2.3
250.0	250.6 244.3 245.1 241.9 255.3 242.8	246.67	5.210	2.1	-1.3
500.0	502.1 499 506 490.7 491.7 489.9	496.57	6.754	1.4	-0.7

Table 3.1.6d: Oxazepam Intra-Assay Summary

Conc. Spiked (ng/mL)	Conc. Found (ng/mL)	Mean n=6	Std Dev.	% CV (a)	% RE (b)
25.0	24.7 23.5 23.5 22.7 23.4 23.6	23.57	0.644	2.7	-5.7
250.0	251.3 246.5 245.4 243.5 254.6 244.6	247.65	4.348	1.8	-0.9
500.0	500.5 499.7 505.1 494.3 491.4 493.8	497.47	5.152	1.0	-0.5

a) Precision
b) Accuracy

Acceptable intra-assay statistics (% CV and % RE within ± 15%) indicate that diazepam, desmethyldiazepam, temazepam and oxazepam in dog serum can be measured with adequate precision and accuracy at all three QC concentration levels.

3.1.7. LLOQ Summary

Table 3.1.7a: Diazepam LLOQ Summary

Conc. Spiked (ng/mL)	Conc. Found (ng/mL)	Mean n = 6	Std Dev.	% CV (a)	% RE (b)
LOQ 10.0	7.5 8.5 8.3 7.5 8.1 8.6	8.08	0.483	6.0	-19.2

Table 3.1.7b: Desmethyldiazepam LLOQ Summary

Conc. Spiked (ng/mL)	Conc. Found (ng/mL)	Mean n = 6	Std Dev.	% CV (a)	% RE (b)
LOQ 10.0	8.5 9.6 9.2 9.2 9.4 10.5	9.40	0.654	7.0	-6.0

Table 3.1.7c: Temazepam LLOQ Summary

Conc. Spiked (ng/mL)	Conc. Found (ng/mL)	Mean n=6	Std Dev.	% CV (a)	% RE (b)
LOQ 10.0	9.4 8.6 9.9 8.8 9.2 9.2	9.18	0.458	5.0	-8.2

Table 3.1.7d: Oxazepam LLOQ Summary

Conc. Spiked (ng/mL)	Conc. Found (ng/mL)	Mean n=6	Std Dev.	% CV (a)	% RE (b)
LOQ 10.0	9.4 9.0 10.0 8.5 9.0 8.4	9.05	0.592	6.5	-9.5

a) Precision
b) Accuracy

Acceptable intra-assay statistics (% CV and % RE within ± 20%) indicate that diazepam, desmethyldiazepam, temazepam and oxazepam in dog serum can be measured with adequate precision and accuracy at the LLOQ concentration level.

3.1.8. Inter-assay Summary

All control quality samples, including those that were not meeting the acceptance criteria, from five runs were tabulated for each drug and the statistics are reported in the tables below.

Table 3.1.8a: Inter-assay statistics for Diazepam and Desmethyldiazepam

	Diazepam			Desmethyldiazepam		
	Concentration Spiked (ng/mL)					
	25.0	1250.0	2500.0	25.0	1250.0	2500.0
	Concentration Found (ng/mL)					
Curve 1	25.1	1255.9	2627.0	26.2	1240.4	2508.2
	25.5	1269.4	2605.9	26.6	1238.0	2547.5
	26.4	1233.0	2503.5	24.6	1189.3	2522.5
Curve 2	24.1	1229.7	2494.3	26.7	1219.5	2470.7
	26.3	1273.1	2613.3	25.2	1243.5	2577.6
	25.3	1260.0	2642.8	24.7	1225.0	2622.8
Curve 3	24.6	1317.1	2687.7	24.1	1304.5	2638.8
	24.4	1288.3	2681.7	24.0	1278.6	2642.6
	25.0	1270.1	2568.1	25.5	1238.4	2537.5
Curve 4	17.1	1169.5	2345.1	16.4	1177.9	2378.8
	24.3	1212.5	2466.3	22.7	1195.9	2538.9
	23.8	1272.8	2429.5	24.0	1281.5	2455.1
Curve 5	23.6	1215.2	2590.9	25.4	1277.0	2713.1
	23.2	1253.3	2555.0	24.5	1241.8	2543.3
	25.7	1297.2	2607.6	24.2	1239.1	2503.1
Mean	24.3	1254.5	2561.2	24.3	1239.4	2546.7
SD	2.1	36.2	93.2	2.4	34.4	81.1
%CV	8.6	2.9	3.6	9.9	2.8	3.2
%RE	-2.8	0.4	2.4	-2.8	-0.8	1.9
n	15	15	15	15	15	15

3.1.8. Inter-assay Summary (Cont'd)

Table 3.1.8b: Inter-assay statistics for Temazepam and Oxazepam

	Temazepam			Oxazepam		
	Concentration Spiked (ng/mL)					
	25.0	250.0	500.0	25.0	250.0	500.0
	Concentration Found (ng/mL)					
Curve 1	26.1	245.4	497.3	24.3	246.7	503.0
	27.6	249.2	505.3	25.2	247.9	505.6
	26.4	244.5	507.5	24.9	245.6	509.8
Curve 2	25.2	244.8	500.1	24.4	246.9	501.1
	25.3	248.1	508.6	25.5	246.1	504.0
	25.5	244.4	509.4	25.5	244.3	508.1
Curve 3	25.0	256.3	505.9	23.7	255.1	514.9
	27.7	247.9	518.4	23.4	247.7	512.1
	23.5	241.7	499.0	22.5	244	497.8
Curve 4	25.8	244.9	487.7	25.1	247.7	491.2
	23.2	241.4	504.1	25.4	241.6	502.6
	22.8	254.2	491.9	24.7	253.6	491.8
Curve 5	24.8	249.0	587.7	25.4	251.9	625.9
	25.2	254.5	512.3	25.3	254.4	511.0
	24.6	250.9	504.7	25.3	250.3	504.4
Mean	25.2	247.8	509.3	24.7	248.3	512.2
SD	1.4	4.4	22.2	0.9	3.9	31.1
%CV	5.6	1.8	4.4	3.6	1.6	6.1
%RE	0.8	-0.9	1.9	-1.2	-0.7	2.4
n	15	15	15	15	15	15

Since inter-assay % CV and % RE are within ± 15% at all QC levels, it was concluded that diazepam, desmethyldiazepam, temazepam and oxazepam can be measured in dog serum with adequate precision and accuracy.

3.2. ABSORPTION OF DIAZEPAM IN PLASTIC MATERIALS

3.2.1. Experimental

In order to test the absorption of the diazepam solution in plastic tubing and burette, the conditions in which the perfusion was administrated to the feline subjects were reproduced in the laboratory, using the same type of tubing and burette. Moreover additional tests were performed to compare the absorption of diazepam on different plastic tubing, and containers.

The example of a cat weighing 3.6 kg was considered, with the perfusion administrated at a rate of 0.1 mg/kg/hour, 15 mL/hour. A solution with a concentration of 25.0 μg/mL was prepared by adding 1.25 mL diazepam solution (5mg/mL) to a 250.0 mL volumetric flask containing Ringer solution. After successive dilution, a calibration curve was prepared at the following concentrations: 25.0, 20.0, 15.0, 10.0, 5.00, 1.00 and 0.500 μg/mL. Also quality control samples were prepared at the concentrations: 17.5, 7.50 and 2.00 mcg/mL. Desmethyldiazepam was used as the internal standard to minimize the errors due to the injector. A solution of internal standard was added to all samples in the same proportion, before samples were injected in the HPLC.

Every 2 hours, 30 mL of the same solution of 25.0 μg/mL diazepam in Ringer solution was added to the burette. Samples were collected at the end of the tubing at the times

indicated in the table 2.4.2, over a period of 6 hours, the same time period as in the clinical experiment. Also diazepam solution was collected from the burette, at time 0, 30, 60, 90, 120 min from the first 30 mL (burette 1). After the second 30 mL diazepam solution was added to the burette, new samples were collected at 120, 150, 180, 210 and 240 minutes (burette 2). The major plastic material in contact with fluids in the burette is polyvinyl chloride.

Diazepam absorption was tested also in two different containers:

1) PAB® mixing container, 150 mL from McGraw Inc. (a rigid polyolefine sac);

2) IntraViaTM container, 150 mL from Baxter Healthcare Corporation (a PVC sac).

In parallel, tests were performed using a different type of plastic tubing over a period of 1.5 hours:

- low sorbing tubing set, 103 inches approx., priming volume of 17 mL approx., model 72923, distributed by IVAC Corporation.

The slope and intercept of the calibration curve was obtained by weighted linear regression (1/c) of the peak height ratios of diazepam / internal standard versus the concentration of diazepam. In the table 3.2.1 are presented the results of the calibration curve and quality control samples used for the calculation of the diazepam concentration in test samples.

Table 3.2.1: Calibration curve and quality control samples used for the calculation of diazepam concentration in test samples.

Conc. Spiked (ng/mL)	Conc. Found (ng/mL)	%RE
STD		
25000	26551	6.2
20000	18855	-5.7
15000	15155	1.0
10000	9474	-5.3
5000	4947	-1.1
1000	987	-1.3
500	531	6.1
QC		
17500	17680	1.0
17500	16304	-6.8
7500	7226	-3.7
7500	6978	-7.0
2000	1926	-3.7
2000	1865	-6.8

3.2.2. Results

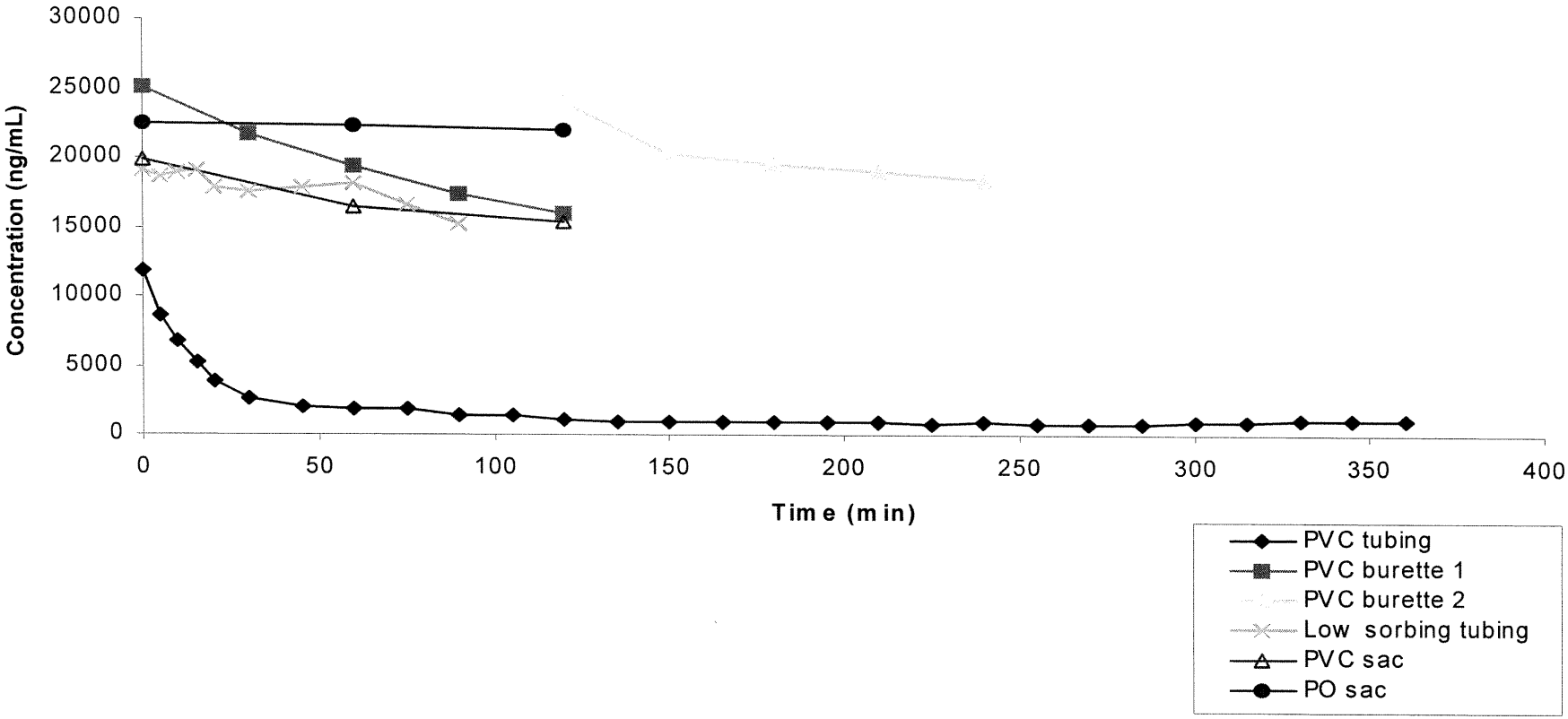
The above calibration curve was then used to interpolate the concentration of diazepam in test samples from their calculated peak height ratios.

The concentrations found in the test samples are represented in the table 3.2.2 and in the graph (Fig 3.2.2).

Table 3.2.2: Concentration of diazepam in samples collected from different plastic tubing and containers

Time (min)	Concentration Found (ng/mL)					
	burette 1	burette 2	burette + PVC tubing	burette + low sorbing tubing	PVC sac	PO sac
0	25098		11885	19128	19842	22432
5			8667	18660		
10			6775	18856		
15			5179	19008		
20			3867	17770		
30	21616		2661	17580		
45			1980	17914		
60	19320		1850	18141	16443	22293
75			1804	16578		
90	17441		1422	15207		
105			1313			
120	16035	24002	1091		15314	22051
135			976			
150		20331	907			
165			891			
180		19589	853			
195			849			
210		19110	847			
225			815			
240		18393	850			
255			842			
270			752			
285			728			
300			858			
315			870			
330			1002			
345			1042			
360			1118			

Fig 3.2.2: Concentration of diazepam in samples collected from different plastic tubing and containers



3.2.3. Previous tests

Two other tests were made before the test presented above. Due to lack of experience in manipulating the IV administration sets, some errors occurred during the experiment. Nevertheless the results are significant in demonstrating that, at two different concentrations of the diazepam in the Ringer solution, the percentage of diazepam loss is similar.

First solution was prepared considering as example a dog weighing 20 kg and having a solution of 240 μg/mL diazepam in lactated Ringer solution administrated with a speed of 42 mL/h to achieve an infusion at a constant rate of ca. 0.5 mg/kg. A second solution was prepared as described at 3.2.1, obtaining a final concentration of 25.0 μg/mL diazepam.

During the test at higher concentration, at around 80 minutes an important volume of solution was purged through the delivery set. Thus an increase in concentration could be observed for about 40 minutes, followed by an important decrease. The samples from the test at higher concentration were diluted six times by adding 200 μL of Ringer solution to 40 μL of each sample.

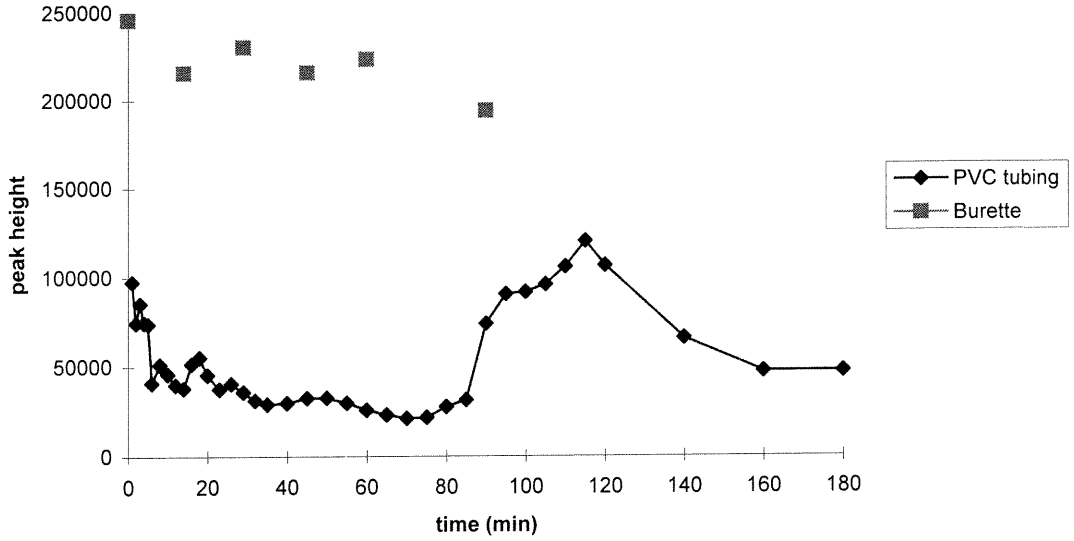
Samples were injected in a HPLC system in similar conditions as those employed for the analysis of clinical samples, except for the mobile phase which contained a higher

percentage of acetonitrile in order to decrease the run time per sample. The results are presented in the Table 3.2.3 and as a graph representation in Fig. 3.2.3a and 3.2.3b.

Table 3.2.3: Diazepam loss in plastic IV delivery set

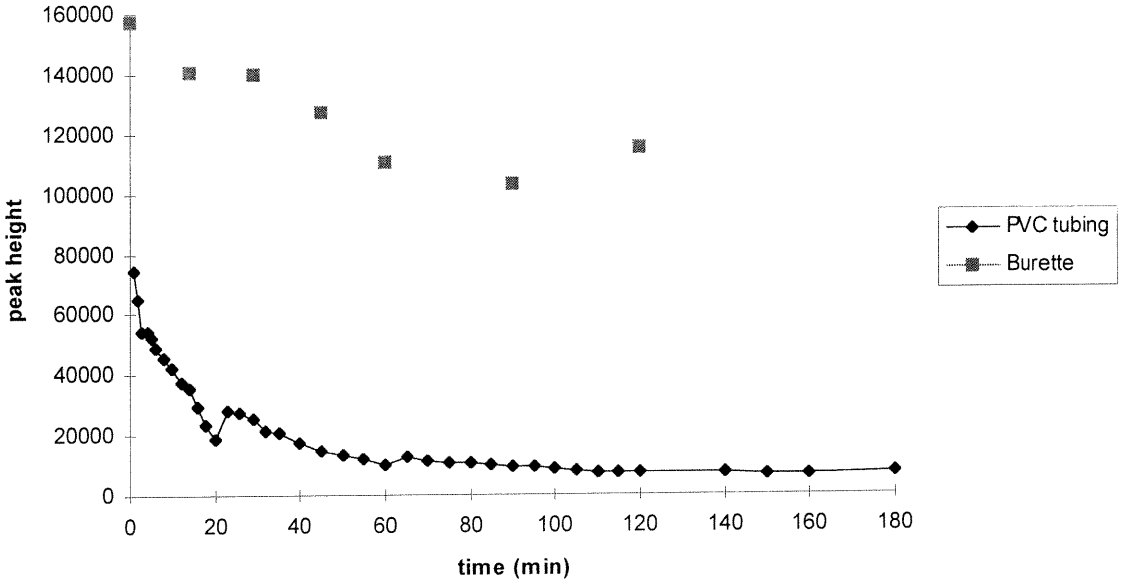
Diazepam sol'n (240 ug/mL)			Diazepam sol'n (25.0 ug/mL)		
	Peak Height			Peak Height	
Time (min)	PVC tubing	Burette	Time (min)	PVC	Burette
0		245827	0		157119
1	97475		1	74084	
2	74580		2	64805	
3	85349		3	54181	
4	74775		4	54513	
5	73895		5	52409	
6	41021		6	48613	
8	51268		8	45772	
10	46021		10	41989	
12	39948		12	37176	
14	38175	215691	14	35316	140547
16	51643		16	29371	
18	55245		18	23752	
20	45519		20	18513	
23	37571		23	28229	
26	40704		26	27355	
29	36065	230380	29	25352	139667
32	31257		32	21448	
35	29120		35	20680	
40	29844		40	17563	
45	32680	215953	45	14992	127341
50	32733		50	13180	
55	29924		55	12228	
60	25875	223357	60	10271	110245
65	23225		65	12419	
70	21267		70	11652	
75	21801		75	11004	
80	27693		80	10621	
85	31605		85	10041	
90	73967	193972	90	9529	102837
95	90623		95	9063	
100	91600		100	8671	
105	95951		105	7831	
110	105843		110	7524	
115	120311		115	7623	
120	106673		120	7061	115143
140	66004		140	7192	
160	47431		150	6599	
180	47329		160	6685	
			180	7405	

Fig 3.2.3a: Diazepam absorption on plastic materials at high concentration



Note: at ca. 80 minutes the tubing was purged with the diazepam solution

Fig 3.2.3b Diazepam absorption on plastic materials at low concentration



3.3. BENZODIAZEPINES SERUM CONCENTRATION

The clinical samples were analysed using the analytical method described in section 2.2.4. A seven point calibration curve as well as a blank, a Std 0 (drug free sample with internal standard) and three concentration levels of QCs (low medium and high) are analyzed with each batch. The slope and the intercept of the calibration curves are obtained by weighted linear regression (1/c) of the peak height ratios of the diazepam, desmethyldiazepam, temazepam or oxazepam / internal standard versus the concentration of the drug (internal standard method). These calibration curves are then used to interpolate the concentration of the four drugs in serum from the peak height ratios.

Benzodiazepines serum concentrations are presented in Appendices A1 - A8 for dogs and B1 - B7 for cats. Serum profiles of the four drugs for each animal are presented in Appendices C1 - C8 for dogs and D1 - D7 for cats.

Cat #6 died after phase one of the study (repeated IV bolus) and samples were not analyzed. The real sampling times are reported in Appendices A1 - B8, thus any deviation from the original protocol being indicated.

3.4. PHARMACOKINETIC ANALYSIS

Plasma drug concentration-time course data were analyzed in terms of conventional noncompartmental analysis (30) which included the rate constant (lambda_z), the terminal half-life time ($t_{1/2}$_lambda_z), the area under the curve from zero to the last measurable concentration or the last sampling time (AUC_t), the area under the curve from zero to infinity (AUC_∞), the volume of distribution based on the terminal phase (Vd) and the total body clearance (CL).

The results were analyzed using WinNonlin Professional Edition, Version 1.5. The method used to calculate AUC is the linear trapezoidal rule. An example of the ASCII file produced by WinNonlin is presented in Appendix E. A summary of the diazepam pharmacokinetic parameters for phase I of the study (four IV bolus) is presented in Tables 3.4a and 3.4d for dogs and cats respectively.

The above mentioned parameters were determined for phase I of the study, while for phase II (IV bolus + infusion) only the lambda_z, $t_{1/2}$_lambda_z, AUC_t and AUC_∞ are reported in tables 3.4b and 3.4e, for dogs and cats respectively. Since the real diazepam dose that was actually administrated to the animals during the infusion is unknown due the absorption by the buretrol and the PVC tubing, distribution based on the terminal phase (Vd) and the total body clearance (CL) were not calculated.

Table 3.4a: Pharmacokinetic Parameters of Diazepam Following four IV Bolus Administration in Dogs

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6	Dog 7	Dog 8	mean	SD
Lambda_z	0.1012	0.0950	0.1316	0.2827	0.1660	0.2005	0.1138	0.1660	0.1571	0.062477
$T_{1/2}$ (h)	6.9	7.3	5.1	2.5	4.2	3.5	6.1	4.2	4.98	1.69
$AUC_{0\text{-}16.5}$	2207	3307	2287	2123	1773	4267	3227	2590	2722.7	820.9
AUC_{inf}	2426	3586	2433	2148	1829	4493	3442	2730	2885.9	885.1
Vz_{obs} (L)	358.5	281.9	241.2	144.9	263.4	102.1	214.5	176.5	222.88	81.72
Vz_{obs} (L/kg)	16.3	11.7	12.1	6.6	13.2	4.4	10.2	8.8	10.42	3.77
$Cl_{(obs)}$	36.3	26.8	32.9	40.9	43.7	20.4	24.4	29.3	31.84	8.12

Table 3.4b: Pharmacokinetic Parameters of Diazepam Following one IV Bolus + Infusion in Dogs

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6	Dog 7	Dog 8	mean	SD
Lambda_z	0.1879	0.1055	0.1254	0.1977	0.1570	0.1078	0.1756	0.1146	0.14644	0.03767
$T_{1/2}$ (h)	3.7	6.6	5.5	3.5	4.4	6.4	3.9	6.0	5.00	1.27
$AUC_{0\text{-}23}$	1093	1604	1310	684	1181	4465	1367	1481	1648.1	1171.9
AUC_{inf}	1132	1666	1366	762	1246	4578	1420	1577	1718.4	1188.9

Table 3.4c: Estimate of Percent Difference Due to Absorption of Diazepam on the Tubing During Infusion in Dogs

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6	Dog 7	Dog 8	mean	SD
Inf / IV %	28.9	28.6	41.5	14.0	57.5	102.5	21.7	43.7	42.29	27.91

$$\text{Inf / IV \%} = \frac{(AUC_{inf} - AUC_{IV}/4)}{3/4\ AUC_{IV}}$$

Table 3.4d: Pharmacokinetic Parameters of Diazepam Following four IV Bolus Administration in Cats

	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5	Cat 7	Cat 8	mean	SD
Lambda_z	0.0292	0.0339	0.0645	0.0241	0.2374	0.0674	0.2722	0.10410	0.10479
$T_{1/2}$ (h)	23.8	20.4	10.7	11.4	2.9	10.3	2.5	11.71	8.03
AUC_{0-56}	12283	12444	11864	17179	6069	11258	7295	11198.9	3662.5
AUC_{inf}	13764	13028	13063	22251	6142	11413	7345	12429.4	5250.7
Vz_{obs} (L)	35.9	32.6	16.1	40.3	9.6	15.1	6.4	22.29	13.66
Vz_{obs} (L/kg)	10.0	9.1	4.7	7.5	2.7	5.2	2.0	5.88	3.05
$Cl_{(obs)}$	1.0	1.1	1.0	1.0	2.3	1.0	1.7	1.30	0.51

Table 3.4e: Pharmacokinetic Parameters of Diazepam Following one IV Bolus + Infusion in Cats

	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5	Cat 7	Cat 8	mean	SD
Lambda_z	0.0380	0.0476	0.0716	0.0388	0.3391	0.0867	0.1843	0.11516	0.11104
$T_{1/2}$ (h)	18.2	14.6	9.7	17.9	2.0	8.0	3.8	10.60	6.52
$AUC_{0-61.5}$	4086	2920	1796	3658	1603	3402	2140	2800.7	969.8
AUC_{inf}	4412	3142	1924	4027	1697	3557	2193	2993.1	1071.2

Table 3.4f: Estimate of Percent Difference Due to Absorption of Diazepam on the Tubing During Infusion in Cats

	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5	Cat 7	Cat 8	mean	SD
Inf / IV %	47.0	-5.9	-68.5	-46.0	17.5	41.1	32.4	2.53	44.82

$$\text{Inf / IV \%} = \frac{(AUC_{inf} - AUC_{IV}/4)}{3/4\ AUC_{IV}} \times 3 / 0.6$$

Table 3.4g: Pharmacokinetic Parameters of Desmethyldiazepam Following Four IV Bolus Administration in Dogs

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6	Dog 7	Dog 8	mean	SD
Lambda_z	0.0726	0.0478	0.0843	0.1018	0.1733	0.0323	0.0482	0.1069	0.0834	0.04505216
$T_{1/2}$ (h)	9.5	14.5	8.2	6.8	4.0	21.5	14.4	6.5	10.68	5.73
$AUC_{0\text{-}16.5}$	11563	17542	8734	18391	8163	15193	20726	8726	13629.8	4970.6
AUC_{inf}	16906	30450	11757	22963	8673	37447	38898	10700	22224.3	12143.5

Table 3.4h: Pharmacokinetic Parameters of Desmethyldiazepam Following One IV Bolus + Infusion in Dogs

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6	Dog 7	Dog 8	mean	SD
Lambda_z	0.1563	0.1343	0.1279	0.2860	0.2291	0.0706	0.1196	0.1058	0.15	0.07
$T_{1/2}$ (h)	4.4	5.2	5.4	2.4	3.0	9.8	5.8	6.6	5.33	2.29
$AUC_{0\text{-}23}$	2493	3284	3647	3105	2086	6084	5491	2146	3542.0	1497.5
AUC_{inf}	2568	3457	3877	3212	2142	7561	5870	2285	3871.5	1905.6

Table 3.4i: Pharmacokinetic Parameters of Desmethyldiazepam Following four IV Bolus Administration in Cats

	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5	Cat 7	Cat 8	mean	SD
Lambda_z	0.0038	0.0105	0.0144	0.0025	0.4180	0.0227	0.0354	0.0724714	0.15278852
$T_{1/2}$ (h)	183.5	65.9	48.2	276.9	16.6	30.5	19.6	91.60	99.89
AUC_{0-56}	22348	23350	21469	34419	27475	18611	67582	30750.5	17027.6
AUC_{inf}	114437	50800	42403	325292	30465	25233	77466	95156.4	106043.9

Table 3.4j: Pharmacokinetic Parameters of Desmethyldiazepam Following one IV Bolus + Infusion in Cats

	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5	Cat 7	Cat 8	mean	SD
Lambda_z	0.0072	0.0124	0.0187	0.0108	0.0441	0.0393	0.0422	0.02496	0.01624
$T_{1/2}$ (h)	96.6	56.0	37.1	64.3	15.7	17.6	16.4	43.39	30.63
$AUC_{0-61.5}$	8376	7205	5942	9948	10225	5828	14907	8918.7	3169.4
AUC_{inf}	26380	15601	9427	23253	11248	6652	16525	15583.7	7219.7

The parameter AUC_{inf} for dogs was 2885.9 ± 885.1 for phase I and only 1718.4 ± 1188.9 for phase II of the study. For cats AUC_{inf} was 12429.4 ± 5250.7 for phase I and only 2993.1 ± 1071.2 for phase II of the study.

For dogs, both treatments started with a bolus of diazepam administrated IV. Then during phase I, three consecutive 1 mg/kg IV boluses were administered at 30 minutes interval each, while for phase II, an infusion of 0.5 mg/kg/h for a total of six hours was performed. Thus, during both administration procedures, dogs received theoretically the same final dose of diazepam (4 mg/kg).

The total dose of diazepam administrated to the cats was 4 mg/mL during the phase I and 1.6 mg/mL during phase II.

As the tests on the absorption of diazepam presented in section 3.2 showed, the burette and especially the PVC tubing used to infuse diazepam retained an important fraction of diazepam. The difference between AUC_{inf} of the phase I and phase II is explained by the absorption of diazepam in the delivery set.

From the moment when diazepam in Ringer solution was added to the burette until the first fraction of solution was collected at the end of the tubing, as much as 53% of the drug was retained. During the next 30 minutes, the concentration dropped again by 78% representing only ca. 10% of the initial concentration.

The extensive absorption tests were done using the diazepam solution at the concentration used for cats (25 mcg/mL). The first tests regarding diazepam absorption, were performed using diazepam solution at both concentrations, for dogs and cats. As presented in table 3.2.3, after one hour, only 10.5% and 6.5% of the initial amount of diazepam was present in the infusion solution for dogs and cats, respectively. Although the concentration of diazepam solution for dogs was almost 10 times higher than that of the solution for cats, diazepam loss was very severe in both cases, this suggesting that increasing the concentration in diazepam will not substantially decrease the absorption. Also diazepam is gradually retained in time, and any delays between the moment when the infusion was prepared and the moment when it was actually administrated could translate in important variations of the amount infused.

The terminal slopes of the diazepam and desmethyldiazepam serum concentration curve were analyzed to calculate the half-life of these drugs.

The results presented in tables 3.4a and 3.4b, show a inter-subject variability for dogs not mentioned in previous studies. Diazepam half-life time in dogs ranged between 2.5 and 7.3 hours for phase I, and between 3.5 and 6.6 for phase II. This could explain the observations made at the Hospital for small animals that some animals do not respond to this treatment for status epilepticus. The patients with a higher elimination rate need higher doses of diazepam to reach and maintain the therapeutic levels.

Diazepam pharmacokinetic parameters presented in tables 3.4d and 3.4e show an important inter-subject variability for cats too. While for two subjects, diazepam half-life time was low (between 2.0 and 3.9 hours), for other two subjects was within 8.0 and 10.7 hours, and for the last three subjects it ranged between 11.4 and 23.8 hours. Similarly, AUC_{inf} is lower for the cats with a higher elimination rate. As previously mentioned for dogs, this is an important factor to be considered when treating cats for status epilepticus.

Also in the Compendium of Pharmaceuticals and Specialties (16), it is mentioned that diazepam solution concentration for infusion should be maximum 80 mg/L. Diazepam prepared in lactated Ringer solution, and infused to the dogs, had a concentration four times higher than this limit. Although the solution seemed clear, since 80 mg/L is indicated as the solubility limit, it is expected that a certain fraction of diazepam was not delivered to the animals.

As much as 94% of the diazepam could be sorbed when administered through the PVC sets, as the tests presented in chapter 3.2 indicates. The decrease in concentration was explained in the literature as an absorption phenomenon (the drug tends to enter into the polymer matrix and to cross it). Diazepam is a very lipophilic drug and its lipophilicity appears to be the explanation for the sorption of diazepam on PVC surfaces (31). This test was done in the laboratory when the ambient temperature was around 30°C. Previous findings state that diazepam's absorption is directly related with temperature (32). The actual phase II of the clinical experiment for the feline subjects, was done in the summer, in conditions with extremely high ambient temperature, over 30°C, while the

experiment involving the dogs was done at lower temperatures. These conditions could explain why, according to the results presented in table 3.4c and 3.4f, the percent difference due to absorption of diazepam on the tubing during infusion is negative for some of the cats. Also, the percent of absorbed diazepam is higher from solutions with lower drug concentration.

The absorption tests show that polyolefine bags do not retain diazepam, and also low sorbing tubing deliver diazepam solution at a concentration ca. 10 times higher than the PVC tubing which contains di-2-ethylhexyl phthalate (DEHP) as platicizer. It was estimated that drug loss into a PVC administration set occurs through partition and diffusion of the drug to DEHP used as a plasticizer of PVC (33). Therefore the main change to be considered for the administration of diazepam in infusion is the use of a delivery set with the following properties:

- a rigid container made of a crystalline plastic material (polypropylene, polyethylene or polyolefine) without plasticizers;
- low sorbing tubing composed of several layers with polyethylene coating on the inside or polyolefine type tubing;
- the shortest possible tubing set with a small diameter in order to decrease the surface area of the plastic to volume of solution ratio.

A therapeutic level of diazepam in serum could also be maintained by increasing the infusion speed (consistent with safe clinical use), while remaining within the limits of diazepam solubility (80 mg/L).

CHAPTER 4: GENERAL CONCLUSIONS

The first objective of this study was to demonstrate that the administration of a IV bolus followed by a constant IV infusion of diazepam is more appropriate than repeated IV bolus for the treatment of status epilepticus and clustered seizures in dogs and cats.

Due to extensive uptake of diazepam by PVC administration sets used for the infusion of the drug, a final conclusion whether the first method of treatment is better could not be drawn, based on the PK results. PVC is a high sorbing material, thus only a small percentage of the dose was actually administrated to the animals in the majority of the cases..

Nevertheless the second objective of this study was achieved by describing the pharmacokinetics of diazepam after a four IV bolus treatment for both dogs and cats. This information could be used by the clinician when adopting a treatment scheme.

Also for further studies an improved clinical protocol will allow to better describe the pharmacokinetics of diazepam after an IV bolus followed by a constant IV infusion thus permitting the clinician to choose the appropriate treatment of clustered seizures and SE treatment with diazepam. Results of the present study are strongly indicative to adopt the following procedure:

1. The maximum concentration permitted by solubility of diazepam in lactated Ringer solution is 80 mg/l, therefore concentration of the solution prepared for infusion should not exceed this limit.

2. A rigid container or bag made of a crystalline plastic material should replace the PVC burette, since this type of polymer does not present an affinity for diazepam.

3. PVC tubing should be replaced by short, low sorbing tubing in order to minimize diazepam loss during infusion.

Thus, using a low sorbing tubing such as a polyolefine-type tubing coupled with a polyolefine bag, or a container made of mutilayer material for the administration of diazepam will allow a greater fraction of the intended dose to actually be delivered to the patients. Increasing the infusion rate will compensate for the limited solubility of diazepam in lactated Ringer solution.

A very sensitive , selective, reliable and robust analytical method for the determination of diazepam and its three metabolites was developed and validated for the purpose of this study. Since only a very small volume of serum is required (e.g. 200 mcL), a greater number of reduced blood volumes could be collected in further studies, this allowing for a better investigation of the pharmacokinetics of diazepam and its metabolites.

The literature does not offer precise information about the therapeutic concentration level of diazepam for the treatment of status epilepticus and clustered seizures in dogs and cats thus studies in this area should be considered.

REFERENCES

1. Holliday AT. Seisure Disorders. Vet Clin North Am Small Anim Pract 1980;10:3-29.

2. Boothe DM. Anticonvulsant Therapy in Small Animals. Vet Clin North Am Small Anim Pract 1998; 28:411-448.

3. McNamara JD. Drugs effective in the therapy of epilepsies. Goodman and Gilman's The Pharmacological Basis of Therapeutics. New York, McGraw-Hill, 1996; 461-485.

4. Indrieri RJ. Status Epilepticus. Probl. Vet Med 1989;1:606-618.

5. Lane SB, Bunch SE. Medical Management of recurrent seizures in dogs and cats. J Vet Intern Med 1990; 4:26-39.

6. Parent JML, Quesnel AD. Seizures in cats. Vet Clin North Am Small Anim Pract 1996; 26:811-825.

7. Delgado-Escueta AV, Wasterlain CG, Treiman DM, et al. Status Epilepticus: Mechanisms of Brain Damage and Treatment. New York, Raven Press, 1983.

8. Löscher W, Frey H-H. Pharmacokinetics of diazepam in the dog. Arch Int Pharmacodyn Ther 1981; 254:180-195.

9. Brown SA. A nticonvulsant therapy in small animals. Vet Clin North Am Small Anim Pract 1988; 18:1197-1216.

10. Farnbach GC. Seisures in the dog. Part II. Control. Compend Contin Educ Pract Vet 1985; 7:505-509.

11. Boothe DM. Anticonvulsant drugs and analeptic drugs. In Adams R (ed): Veterinary Pharmacology and Therapeutics. Ames, IA: Iowa University Press, 1996; 372-392.

12. Podell M. Antiepileptic Drug Therapy. Clin Tech in Small Anim Pract. 1998;13:185-192

13. Parent JM. Clinical management of canine seizures. Vet Clin North Am Small Anim Pract 18:947-964, 1998

14. Klotz U, Antonin K-H, Bieck PR. Pharmacokinetics and plasma binding of diazepam in man, dog, rabbit, guinea pig and rat. J Pharmacol Exp Ther 1976;199:67-73.

15. Frey H-H, Löscher W. Pharmacokinetics of anti-epileptic drugs in the dog: a review. J Vet Pharmacol Therap 1985;8:219-233.

16. CPS Compendium of Pharmaceuticals and Specialities, 29th ed, Canadian Pharmaceutical Association, Ottawa, Ontario, Canada ; ed. Carmen M. E. Krogh, 1994; 368-369, 1393.

17. Greenblatt DJ, Koch-Weser J. Intramuscular injection of drugs. N Engl J Med 1976;295:542-546.

18. Kortilla K, Linnoila M. Absorption and sedative effects of diazepam after oral administration and intramuscular administration into vastus lateralis muscle and the deltoid muscle. Br J Anaesth 1975;47:857-862.

19. Greenblatt DJ, Shader RI, Abernethy DR. Current status of benzodiazepines (2parts). N Engl J Med 1983;309:354-358,410-416.

20. Magnussen I, Oxlund HRW, Alsbrink KE, et al. Absorption of diazepam in man following rectal and parenteral administration. Acta Pharmacol Toxicol 1979;45:87-90.

21. Papich MG. Table of common drugs: approximate dosages. In: Bonagura JD, ed. Current veterinary therapy XII. Philadelphia: WB Saunders Co, 1995;1233-1249.

22. Podell M. Seizures in dogs. Vet Clin North Am Small Anim Pract 1996;26:779-809.

23. Randall LO, Scheckel CL, Banziger RF. Pharmacology of the metabolites of chlordiazepoxide and diazepam. Curr Ther Res 1965;149:423-435.

24. Papich, MG, Alcorn, J. Adsorption of diazepam after its rectal administration in dogs. Am J Vet Res 1995;12:1629-1636.

25. Schvartz-Porsche D, Kaiser E Feline epilepsy. Probl Vet Med 1989;1:628-649.

26. Schütz H. Benzodiazepines. A hand book. New York: Springer-Verlag, 1982;209-218.

27. Löscher W. Rapid gas chromatografic measurement of diazepam and its metabolites desmethyldiazepam, oxazepam, and 3-hydroxydiazepam (temazepam) in small samples of plasma. Ther Drug Monit 1982; 4(3):315-8.

28. MacKichan JJ, Jusko WJ, Duffner PK, Cohen ME. Liquid-chromatographic assay of diazepam and its major metabolites in plasma. Clin Chem, 1979;25(6):856-9.

29. Stebler T, Geentert TW. Determination of diazepam and nordiazepam in milk and plasma in the presence of oxazepam and temazepam. J Chromatogr, 1991564 (1): 330-7.

30. Gibaldi M, Perrier D. Pharmacokinetics. 2nd ed. New York: Marcel Dekker Inc, 1982;45-111.

31. Tchiakpe L., Airaudo C.B., Abdelmalik O.M., Gayte-Sorbier A., Verdier M., Guerri, J. Stedium 6 and Clearflex, two new multilayer materials for infusion containers. Comparative study of their compatibility with five drugs versus glass flasks and polyvinyl chloride bags. J. Biomater. Sci.,Polym. Ed. (1995), 7(3): 199-206.

32. Kowaluk E.A., Roberts M.S., Polack A.E. Factors affecting the availability of diazepam stored in plastic bags and administered through intravenous sets. Am. J. Hosp. Pharm., 1983, 40: 417-423.

33. Kawano K., Matsunaga A., Nakazawa K., Komatsu R., Nakajima S. The effect of pH in the solutions on the sorption of midazolam into a plastic container or a administration set. Hosp. Pharm., Yamanashi Med. Coll., Yamanashi, 409-38, Japan.

APPENDIX A1: BENZODIAZEPINES SERUM CONCENTRATION IN DOG 1

DOG 1
Phase One: Repeated IV Bolus

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.05	1864.0	0.05	187.0	0.05	0.0	0.05	7.8
0.083	998.3	0.083	363.5	0.083	0.0	0.083	28.2
0.17	606.5	0.17	434.1	0.17	0.0	0.17	39.2
0.25	411.4	0.25	477.3	0.25	0.0	0.25	46.5
0.5	178.3	0.5	446.0	0.5	0.0	0.5	59.9
0.75	579.6	0.75	814.6	0.75	0.0	0.75	91.4
1.25	682.7	1.25	1066.0	1.25	0.0	1.25	125.9
1.5	368.1	1.5	1094.3	1.5	0.0	1.5	137.0
1.75	823.5	1.75	1453.7	1.75	0.0	1.75	166.0
2	424.9	2	1398.9	2	0.0	2	172.3
2.25	250.7	2.25	1151.5	2.25	0.0	2.25	159.0
2.5	215.8	2.5	1143.0	2.5	0.0	2.5	159.9
4.5	73.9	4.5	933.6	4.5	0.0	4.5	190.4
7.5	51.0	7.5	724.5	7.5	0.0	7.5	147.9
10.5	50.3	10.5	507.1	10.5	0.0	10.5	86.8
13.5	27.4	13.5	475.4	13.5	0.0	13.5	66.0
16.5	22.1	16.5	387.9	16.5	0.0	16.5	42.2

DOG 1
Phase Two: IV Bolus + Infusion

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.25	555.7	0.25	409.3	0.25	0.0	0.25	56.8
0.5	237.3	0.5	373.6	0.5	0.0	0.5	64.6
0.75	174.9	0.75	350.3	0.75	0.0	0.75	69.0
1	124.8	1	317.0	1	0.0	1	65.4
1.5	108.2	1.5	299.4	1.5	0.0	1.5	68.5
2	87.7	2	247.5	2	0.0	2	61.6
4	96.4	4	190.4	4	0.0	4	52.3
6	107.2	6	197.4	6	0.0	6	54.5
6.25	61.0	6.25	203.9	6.25	0.0	6.25	54.2
6.5	44.2	6.5	175.9	6.5	0.0	6.5	50.9
6.75	37.9	6.75	178.7	6.75	0.0	6.75	48.5
7	30.7	7	142.9	7	0.0	7	41.6
7.5	29.4	7.5	141.0	7.5	0.0	7.5	41.4
8	23.4	8	114.8	8	0.0	8	34.3
11	14.9	11	70.9	11	0.0	11	26.0
14.5	7.4	14.5	43.4	14.5	0.0	14.5	34.1
17	0.0	17	25.5	17	0.0	17	21.6
20	0.0	20	19.8	20	0.0	20	13.5
23	0.0	23	11.8	23	0.0	23	0.0

Appendix A2: BENZODIAZEPINES SERUM CONCENTRATION IN DOG 2

DOG 2
Phase One: Repeated IV Bolus

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.05	2683.4	0.05	171.2	0.05	0.0	0.05	0.0
0.083	1928.6	0.083	370.0	0.083	0.0	0.083	16.0
0.17	1099.1	0.17	562.1	0.17	0.0	0.17	29.6
0.25	716.8	0.25	664.9	0.25	10.5	0.25	44.0
0.5	251.6	0.5	666.5	0.5	0.0	0.5	59.4
0.75	881.1	0.75	1341.3	0.75	0.0	0.75	99.7
1.25	1031.5	1.25	1866.7	1.25	0.0	1.25	127.1
1.5	536.8	1.5	1911.1	1.5	0.0	1.5	146.0
1.75	1527.4	1.75	2275.5	1.75	15.1	1.75	164.9
2	838.9	2	2352.8	2	0.0	2	173.4
2.25	561.3	2.25	2212.1	2.25	0.0	2.25	172.1
2.5	382.0	2.5	1996.5	2.5	0.0	2.5	163.5
4.5	90.0	4.5	1192.9	4.5	0.0	4.5	113.0
7.5	57.5	7.5	927.3	7.5	0.0	7.5	104.0
10.5	43.6	10.5	793.4	10.5	0.0	10.5	91.3
13.5	38.4	13.5	826.2	13.5	0.0	13.5	93.5
16.5	26.5	16.5	617.0	16.5	0.0	16.5	66.7

DOG 2
Phase Two: IV Bolus + Infusion

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	10.6	0	0.0	0	0.0	0	0.0
0.25	685.0	0.25	503.6	0.25	12.0	0.25	69.5
0.5	449.5	0.5	476.9	0.5	0.0	0.5	80.8
0.75	318.2	0.75	419.9	0.75	0.0	0.75	81.1
1	263.7	1	410.6	1	0.0	1	84.6
1.5	229.0	1.5	339.0	1.5	0.0	1.5	80.0
2	119.9	2	271.7	2	10.0	2	68.8
4	144.9	4	246.3	4	0.0	4	61.4
6	128.3	6	246.8	6	0.0	6	65.0
6.25	76.3	6.25	252.2	6.25	0.0	6.25	65.9
6.5	61.1	6.5	230.5	6.5	0.0	6.5	64.1
6.75	42.6	6.75	200.7	6.75	0.0	6.75	56.3
7	47.5	7	203.5	7	0.0	7	56.9
7.5	39.9	7.5	158.6	7.5	0.0	7.5	47.7
8	36.5	8	156.6	8	0.0	8	45.0
11	22.6	11	114.8	11	0.0	11	49.9
14	13.9	14	78.7	14	0.0	14	41.4
17	8.6	17	40.7	17	0.0	17	24.6
20	7.1	20	32.0	20	0.0	20	16.7
23	6.5	23	23.2	23	0.0	23	27.2

Appendix A3: BENZODIAZEPINES SERUM CONCENTRATION IN DOG 3

DOG 3
Phase One: Repeated IV Bolus

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.05	1581.4	0.05	290.0	0.05	0.0	0.05	25.7
0.083	1180.5	0.083	484.4	0.083	0.0	0.083	57.0
0.17	616.4	0.17	417.9	0.17	0.0	0.17	74.7
0.25	498.5	0.25	575.5	0.25	0.0	0.25	102.1
0.5	205.8	0.5	458.0	0.5	0.0	0.5	111.7
0.75	518.9	0.75	730.6	0.75	0.0	0.75	156.8
1.25	790.6	1.25	1081.1	1.25	0.0	1.25	227.7
1.5	454.4	1.5	941.3	1.5	0.0	1.5	234.1
1.75	727.5	1.75	1187.2	1.75	0.0	1.75	264.6
2	430.1	2	1230.7	2	0.0	2	283.5
2.25	274.7	2.25	1032.6	2.25	0.0	2.25	254.2
2.5	151.8	2.5	663.4	2.5	0.0	2.5	188.9
4.5	100.7	4.5	697.2	4.5	0.0	4.5	231.4
7.4	74.9	7.4	549.1	7.4	0.0	7.4	175.9
10.4	57.6	10.4	404.0	10.4	0.0	10.4	145.4
13.6	32.2	13.6	326.6	13.6	0.0	13.6	126.6
16.4	19.9	16.4	255.0	16.4	0.0	16.4	93.8

DOG 3
Phase Two: IV Bolus + Infusion

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.25	699.9	0.25	479.8	0.25	0.0	0.25	66.8
0.5	344.2	0.5	483.3	0.5	0.0	0.5	76.5
0.75	325.8	0.75	458.6	0.75	0.0	0.75	86.0
1	152.5	1	395.2	1	0.0	1	86.0
1.5	100.8	1.5	350.5	1.5	0.0	1.5	81.5
2	112.8	2	307.3	2	0.0	2	80.3
4	103.4	4	241.4	4	0.0	4	76.6
6	133.4	6	275.7	6	0.0	6	89.8
6.25	69.7	6.25	294.3	6.25	0.0	6.25	98.7
6.5	50.3	6.5	260.7	6.5	0.0	6.5	93.8
6.75	36.5	6.75	249.8	6.75	0.0	6.75	96.8
7	29.7	7	207.2	7	0.0	7	86.5
7.5	23.8	7.5	188.6	7.5	0.0	7.5	84.0
8	20.5	8	179.8	8	0.0	8	81.2
11.1	15.2	11.1	145.1	11.1	0.0	11.1	74.7
14	9.7	14	93.0	14	0.0	14	61.7
17	7.1	17	58.9	17	0.0	17	45.1
20.2	0.0	20.2	33.6	20.2	0.0	20.2	27.7
23	0.0	23	29.4	23	0.0	23	22.6

Appendix A4: BENZODIAZEPINES SERUM CONCENTRATION IN DOG 4

DOG 4
Phase One: Repeated IV Bolus

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.05	1450.1	0.05	130.7	0.05	0.0	0.05	0.0
0.083	1001.8	0.083	228.7	0.083	0.0	0.083	12.3
0.17	637.7	0.17	458.5	0.17	0.0	0.17	28.4
0.25	474.1	0.25	476.2	0.25	0.0	0.25	37.2
0.5	178.8	0.5	511.4	0.5	0.0	0.5	50.6
0.75	597.7	0.75	1008.3	0.75	0.0	0.75	79.0
1.25	800.7	1.25	1477.3	1.25	0.0	1.25	109.8
1.5	395.6	1.5	1609.9	1.5	0.0	1.5	124.6
1.75	963.8	1.75	1781.9	1.75	11.1	1.75	136.7
2	560.7	2	1864.1	2	0.0	2	147.7
2.25	434.1	2.25	2104.0	2.25	10.1	2.25	216.0
2.5	264.1	2.5	1878.9	2.5	0.0	2.5	152.9
4.5	84.2	4.5	1595.2	4.5	0.0	4.5	117.6
7.5	33.1	7.5	1244.4	7.5	0.0	7.5	104.0
10.4	11.1	10.4	919.0	10.4	0.0	10.4	100.9
13.5	7.2	13.5	677.7	13.5	0.0	13.5	57.1
16.6	0.0	16.6	465.3	16.6	0.0	16.6	45.1

DOG 4
Phase Two: IV Bolus + Infusion

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.25	407.9	0.25	457.1	0.25	0.0	0.25	53.9
0.5	186.1	0.5	435.2	0.5	0.0	0.5	62.5
0.75	122.6	0.75	416.6	0.75	0.0	0.75	68.4
1	102.3	1	415.6	1	0.0	1	63.4
1.5	69.9	1.5	394.2	1.5	0.0	1.5	65.8
2	55.6	2	326.7	2	0.0	2	62.1
4	61.7	4	263.6	4	0.0	4	57.2
6	82.8	6	288.0	6	0.0	6	51.7
6.25	40.6	6.25	285.9	6.25	0.0	6.25	50.4
6.5	28.7	6.5	252.8	6.5	0.0	6.5	47.6
6.75	23.2	6.75	253.9	6.75	0.0	6.75	44.5
7	17.0	7	216.8	7	0.0	7	45.0
7.5	15.4	7.5	193.1	7.5	0.0	7.5	46.8
8	0.0	8	196.2	8	0.0	8	51.0
14	0.0	14	30.5	14	0.0	14	15.6
17	0.0	17	0.0	17	0.0	17	0.0
20	0.0	20	0.0	20	0.0	20	0.0
23	0.0	23	0.0	23	0.0	23	0.0

Appendix A5: BENZODIAZEPINES SERUM CONCENTRATION IN DOG 5

DOG 5
Phase One: Repeated IV Bolus

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.05	1518.3	0.05	299.7	0.05	0.0	0.05	25.7
0.083	1001.7	0.083	407.2	0.083	0.0	0.083	37.5
0.17	482.1	0.17	451.2	0.17	0.0	0.17	70.8
0.25	344.0	0.25	520.2	0.25	0.0	0.25	79.4
0.5	108.0	0.5	364.8	0.5	0.0	0.5	84.7
0.75	461.5	0.75	927.6	0.75	0.0	0.75	146.9
1.25	581.3	1.25	1344.4	1.25	0.0	1.25	199.9
1.5	263.8	1.5	1159.6	1.5	0.0	1.5	188.5
1.75	691.6	1.75	1696.5	1.75	11.0	1.75	239.9
2	299.2	2	1602.6	2	0.0	2	238.2
2.25	178.7	2.25	1295.7	2.25	0.0	2.25	227.8
2.5	129.5	2.5	1140.0	2.5	0.0	2.5	213.1
4.5	50.5	4.5	646.4	4.5	0.0	4.5	139.1
7.5	65.1	7.5	489.5	7.5	0.0	7.5	112.5
10.5	61.6	10.5	226.6	10.5	0.0	10.5	118.3
13.5	12.9	13.5	144.9	13.5	0.0	13.5	146.1
16.5	9.4	16.5	88.3	16.5	0.0	16.5	70.4

DOG 5
Phase Two: IV Bolus + Infusion

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.25	468.7	0.25	521.0	0.25	0.0	0.25	73.1
0.5	195.4	0.5	455.9	0.5	0.0	0.5	89.7
0.75	122.3	0.75	389.4	0.75	0.0	0.75	86.7
1	107.8	1	358.2	1	0.0	1	82.1
1.5	75.3	1.5	270.8	1.5	0.0	1.5	73.6
2	260.7	2	221.7	2	0.0	2	64.2
4	80.6	4	173.4	4	0.0	4	59.2
6	85.6	6	151.5	6	0.0	6	59.3
6.25	46.2	6.25	167.6	6.25	0.0	6.25	54.8
6.5	32.7	6.5	132.7	6.5	0.0	6.5	47.5
6.75	27.5	6.75	126.2	6.75	0.0	6.75	44.5
7	25.9	7	120.9	7	0.0	7	54.9
7.5	23.3	7.5	107.1	7.5	0.0	7.5	42.3
8	28.3	8	108.2	8	0.0	8	43.5
11	13.5	11	40.1	11	0.0	11	16.0
14	9.6	14	23.6	14	0.0	14	0.0
17	10.2	17	13.0	17	0.0	17	10.0
20	0.0	20	0.0	20	0.0	20	0.0
23	0.0	23	0.0	23	0.0	23	0.0

Appendix A6: BENZODIAZEPINES SERUM CONCENTRATION IN DOG 6

DOG 6
Phase One: Repeated IV Bolus

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.05	2506.4	0.05	167.8	0.05	0.0	0.05	0.0
0.083	1807.4	0.083	325.5	0.083	0.0	0.083	0.0
0.17	798.0	0.17	505.7	0.17	0.0	0.17	24.5
0.25	802.7	0.25	522.9	0.25	0.0	0.25	29.8
0.5	369.5	0.5	540.2	0.5	0.0	0.5	37.7
0.75	1277.9	0.75	950.4	0.75	0.0	0.75	64.7
1.25	1709.2	1.25	1307.3	1.25	0.0	1.25	89.5
1.5	804.2	1.5	1408.5	1.5	0.0	1.5	100.0
1.75	1342.4	1.75	1540.1	1.75	12.0	1.75	122.0
2	914.2	2	1624.5	2	10.4	2	129.2
2.25	623.0	2.25	1489.8	2.25	0.0	2.25	126.7
2.5	515.7	2.5	1414.5	2.5	0.0	2.5	126.4
4.5	158.4	4.5	1054.3	4.5	0.0	4.5	109.1
7.4	91.0	7.4	887.9	7.4	0.0	7.4	103.8
10.5	47.6	10.5	620.9	10.5	0.0	10.5	70.2
13.5	55.0	13.5	722.8	13.5	0.0	13.5	81.6
16.5	45.3	16.5	718.4	16.5	0.0	16.5	82.1

DOG 6
Phase Two: IV Bolus + Infusion

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.25	731.0	0.25	684.5	0.25	0.0	0.25	42.7
0.5	491.7	0.5	640.9	0.5	0.0	0.5	47.2
0.75	517.9	0.75	582.7	0.75	0.0	0.75	48.5
1	249.8	1	559.7	1	0.0	1	43.6
1.5	1046.6	1.5	480.7	1.5	0.0	1.5	53.0
2	988.4	2	388.5	2	0.0	2	49.7
4	752.6	4	399.6	4	0.0	4	54.2
6	199.6	6	462.7	6	0.0	6	58.8
6.25	86.0	6.25	426.0	6.25	0.0	6.25	60.4
6.5	61.2	6.5	427.8	6.5	0.0	6.5	64.4
6.75	37.0	6.75	342.8	6.75	0.0	6.75	50.5
7	37.6	7	334.9	7	0.0	7	55.0
7.5	20.2	7.5	249.0	7.5	0.0	7.5	54.7
8	20.2	8	233.6	8	0.0	8	43.1
11	21.7	11	225.9	11	0.0	11	49.6
14	21.2	14	226.0	14	0.0	14	50.1
17	10.6	17	135.7	17	0.0	17	39.5
20	9.3	20	127.5	20	0.0	20	29.5
23	12.1	23	104.3	23	0.0	23	163.7

Appendix A7: BENZODIAZEPINES SERUM CONCENTRATION IN DOG 7

DOG 7
Phase One: Repeated IV Bolus

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.05	1579.4	0.05	562.1	0.05	0.0	0.05	38.2
0.083	1277.6	0.083	549.9	0.083	0.0	0.083	37.3
0.17	901.8	0.17	502.5	0.17	0.0	0.17	56.3
0.25	626.5	0.25	597.0	0.25	0.0	0.25	50.1
0.5	260.9	0.5	508.4	0.5	0.0	0.5	64.0
0.75	913.8	0.75	1105.3	0.75	0.0	0.75	110.0
1.25	1131.9	1.25	1425.7	1.25	0.0	1.25	151.7
1.5	633.8	1.5	1550.0	1.5	0.0	1.5	152.3
1.75	1173.5	1.75	1872.5	1.75	15.2	1.75	179.6
2	732.6	2	1950.3	2	12.4	2	189.4
2.25	473.8	2.25	1834.2	2.25	0.0	2.25	198.1
2.5	393.9	2.5	1748.7	2.5	0.0	2.5	202.3
4.5	134.3	4.5	1528.9	4.5	0.0	4.5	189.4
7.5	70.3	7.5	1389.7	7.5	0.0	7.5	158.0
10.5	44.7	10.5	1067.1	10.5	0.0	10.5	142.8
13.5	35.2	13.5	1054.2	13.5	0.0	13.5	139.6
16.5	24.4	16.5	876.1	16.5	0.0	16.5	116.9

DOG 7
Phase Two: IV Bolus + Infusion

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.25	688.9	0.25	500.1	0.25	0.0	0.25	63.7
0.5	357.3	0.5	540.2	0.5	0.0	0.5	81.2
0.75	204.0	0.75	457.5	0.75	0.0	0.75	86.4
1	161.4	1	477.5	1	0.0	1	77.5
1.5	123.0	1.5	442.4	1.5	0.0	1.5	85.2
2	137.0	2	479.3	2	0.0	2	93.4
4	121.9	4	422.2	4	0.0	4	111.3
6	123.4	6	391.3	6	0.0	6	98.7
6.25	64.9	6.25	400.8	6.25	0.0	6.25	92.8
6.5	41.8	6.5	354.4	6.5	0.0	6.5	93.8
6.75	32.7	6.75	400.6	6.75	0.0	6.75	96.2
7	35.2	7	373.3	7	0.0	7	104.9
7.5	30.8	7.5	329.0	7.5	0.0	7.5	88.2
8	26.6	8	282.8	8	0.0	8	88.4
11	20.0	11	213.4	11	0.0	11	87.2
14	9.4	14	140.1	14	0.0	14	46.1
17	9.4	17	110.3	17	0.0	17	46.8
20	0.0	20	74.1	20	0.0	20	33.9
23	0.0	23	45.4	23	0.0	23	16.8

Appendix A8: BENZODIAZEPINES SERUM CONCENTRATION IN DOG 8

DOG 8
Phase One: Repeated IV Bolus

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.05	1636.4	0.05	294.0	0.05	0.0	0.05	31.0
0.083	1272.9	0.083	347.4	0.083	0.0	0.083	44.6
0.17	688.9	0.17	410.2	0.17	0.0	0.17	69.6
0.25	489.9	0.25	439.3	0.25	0.0	0.25	86.0
0.5	217.7	0.5	412.4	0.5	0.0	0.5	102.8
0.75	785.1	0.75	884.1	0.75	0.0	0.75	167.8
1.25	945.8	1.25	1286.0	1.25	10.3	1.25	252.6
1.5	424.4	1.5	1077.3	1.5	0.0	1.5	225.1
1.75	802.4	1.75	1341.9	1.75	13.7	1.75	282.2
2	549.3	2	1259.3	2	11.1	2	273.7
2.25	360.3	2.25	1178.5	2.25	0.0	2.25	271.2
2.5	271.1	2.5	1066.5	2.5	0.0	2.5	262.9
4.5	111.2	4.5	747.2	4.5	0.0	4.5	237.5
7.5	63.4	7.5	454.6	7.5	0.0	7.5	170.7
10.5	43.3	10.5	336.0	10.5	0.0	10.5	158.6
13.5	24.0	13.5	230.6	13.5	0.0	13.5	98.4
16.5	23.2	16.5	211.0	16.5	0.0	16.5	91.3

DOG 8
Phase Two: IV Bolus + Infusion

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.25	666.6	0.25	429.4	0.25	0.0	0.25	104.6
0.5	338.1	0.5	372.9	0.5	0.0	0.5	119.8
0.75	251.0	0.75	324.5	0.75	0.0	0.75	110.3
1	163.9	1	293.7	1	0.0	1	110.0
1.5	150.8	1.5	245.5	1.5	0.0	1.5	116.0
2	147.3	2	215.2	2	0.0	2	96.2
4	144.2	4	166.2	4	0.0	4	88.4
6	112.1	6	145.4	6	0.0	6	87.4
6.25	64.6	6.25	130.0	6.25	0.0	6.25	84.9
6.5	46.2	6.5	110.3	6.5	0.0	6.5	85.4
6.75	42.9	6.75	115.9	6.75	0.0	6.75	81.8
7	35.0	7	100.1	7	0.0	7	77.4
7.5	30.8	7.5	84.4	7.5	0.0	7.5	85.5
8	28.6	8	82.5	8	0.0	8	65.2
11	20.0	11	53.3	11	0.0	11	58.7
14	14.4	14	47.5	14	0.0	14	54.0
17	10.3	17	28.0	17	0.0	17	44.5
20	11.0	20	27.8	20	0.0	20	27.0
23	0.0	23	14.7	23	0.0	23	23.1

Appendix B1: BENZODIAZEPINES SERUM CONCENTRATION IN CAT 1

CAT 1
Phase One: Repeated IV Bolus

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0.05	---	0.05	---	0.05	---	0.05	---
0.083	1127.9	0.083	18.6	0.083	29.6	0.083	0.0
0.17	853.8	0.17	28.4	0.17	37.3	0.17	0.0
0.25	769.7	0.25	36.9	0.25	36.6	0.25	0.0
0.5	602.6	0.5	61.4	0.5	43.4	0.5	0.0
0.75	1180.4	0.75	98.2	0.75	75.4	0.75	0.0
1.25	1503.2	1.25	174.6	1.25	122.7	1.25	0.0
1.5	1228.6	1.5	177.3	1.5	123.2	1.5	0.0
1.75	1614.4	1.75	234.9	1.75	149.2	1.75	0.0
2	1464.1	2	256.4	2	159.6	2	10.2
2.25	1142.1	2.25	248.6	2.25	153.2	2.25	11.7
2.5	1051.6	2.5	288.3	2.5	152.7	2.5	12.8
3.4	665.4	3.4	317.0	3.4	137.2	3.4	19.9
11.75	223.6	11.75	370.2	11.75	130.9	11.75	67.8
19.4	155.8	19.4	408.3	19.4	61.3	19.4	86.6
24.4	117.8	24.4	387.3	24.4	88.2	24.4	97.7
37.4	72.4	37.4	393.1	37.4	40.1	37.4	126.0
49.5	59.0	49.5	350.3	49.5	24.7	49.5	101.0
61.75	43.2	61.75	347.8	61.75	32.8	61.75	129.7

CAT 1
Phase Two: IV Bolus + Infusion

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.25	1008.9	0.25	75.3	0.25	42.9	0.25	0.0
0.5	709.7	0.5	104.9	0.5	48.9	0.5	0.0
0.75	532.9	0.75	116.0	0.75	46.1	0.75	0.0
1	470.7	1	98.9	1	47.2	1	0.0
2.4	210.4	2.4	119.3	2.4	37.6	2.4	0.0
4	135.7	4	121.3	4	28.1	4	0.0
6	101.1	6	112.2	6	21.0	6	11.7
6.25	93.4	6.25	117.7	6.25	24.3	6.25	13.1
6.5	93.5	6.5	126.1	6.5	23.4	6.5	13.8
6.75	101.1	6.75	134.9	6.75	23.2	6.75	14.6
7	95.3	7	116.1	7	19.5	7	11.8
7.5	86.2	7.5	128.9	7.5	21.3	7.5	16.0
8.1	87.6	8.1	128.0	8.1	24.1	8.1	14.9
17.5	68.2	17.5	172.9	17.5	23.0	17.5	31.7
24	52.5	24	168.1	24	15.7	24	36.5
32	35.7	32	163.7	32	10.1	32	39.4
46	25.9	46	149.9	46	0.0	46	40.4
56	12.4	56	129.2	56	0.0	56	37.8

Appendix B2: BENZODIAZEPINES SERUM CONCENTRATION IN CAT 2

CAT 2
Phase One: Repeated IV Bolus

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0.05	1766.7	0.05	29.1	0.05	31.0	0.05	0.0
0.083	1985.0	0.083	21.4	0.083	30.4	0.083	0.0
0.2	1106.3	0.2	73.6	0.2	51.3	0.2	0.0
0.25	954.4	0.25	77.4	0.25	60.5	0.25	0.0
0.5	833.5	0.5	76.0	0.5	63.3	0.5	0.0
0.75	1554.7	0.75	159.5	0.75	117.0	0.75	0.0
1.25	1948.9	1.25	203.6	1.25	157.8	1.25	0.0
1.5	1617.9	1.5	238.5	1.5	175.9	1.5	11.0
1.75	1926.6	1.75	264.9	1.75	210.0	1.75	14.5
2	1808.3	2	295.9	2	209.5	2	14.6
2.25	1455.8	2.25	324.1	2.25	219.2	2.25	18.5
2.5	1205.5	2.5	364.1	2.5	222.7	2.5	22.5
3.5	709.6	3.5	381.8	3.5	176.6	3.5	29.9
11.5	173.3	11.5	369.1	11.5	82.9	11.5	78.7
19.4	133.7	19.4	480.7	19.4	159.3	19.4	160.1
25.4	100.4	25.4	421.0	25.4	77.0	25.4	126.4
37.4	72.1	37.4	388.2	37.4	46.0	37.4	125.8
49.4	47.5	49.4	368.7	49.4	22.3	49.4	115.3
61.6	19.8	61.6	288.9	61.6	11.7	61.6	104.1

CAT 2
Phase Two: IV Bolus + Infusion

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.25	911.5	0.25	54.6	0.25	31.7	0.25	0.0
0.5	608.3	0.5	76.0	0.5	42.9	0.5	0.0
0.75	537.8	0.75	108.3	0.75	53.7	0.75	0.0
1	431.8	1	100.9	1	51.8	1	0.0
2	203.2	2	111.7	2	43.3	2	0.0
4	93.3	4	110.1	4	28.8	4	12.9
6.1	71.8	6.1	107.5	6.1	27.3	6.1	18.6
6.25	74.0	6.25	109.9	6.25	31.4	6.25	22.8
6.5	64.0	6.5	108.0	6.5	25.5	6.5	19.3
6.75	68.7	6.75	111.4	6.75	26.9	6.75	20.1
7	62.5	7	107.1	7	23.9	7	18.3
7.5	56.7	7.5	107.1	7.5	23.6	7.5	20.6
8.1	57.3	8.1	111.4	8.1	24.8	8.1	22.2
16.6	45.2	16.6	147.1	16.6	23.4	16.6	44.5
24	35.5	24	162.1	24	17.9	24	52.3
32	17.3	32	131.5	32	13.9	32	51.1
45.2	12.5	45.2	124.5	45.2	0.0	45.2	72.2
56.1	10.6	56.1	103.9	56.1	0.0	56.1	65.2

Appendix B3: BENZODIAZEPINES SERUM CONCENTRATION IN CAT 3

CAT 3
Phase One: Repeated IV Bolus

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0.05	---	0.05	---	0.05	---	0.05	---
0.083	1551.6	0.083	35.3	0.083	43.5	0.083	0.0
0.17	---	0.17	---	0.17	---	0.17	---
0.25	1166.3	0.25	57.3	0.25	67.2	0.25	0.0
0.5	638.2	0.5	91.9	0.5	78.5	0.5	0.0
0.75	1478.3	0.75	127.6	0.75	119.4	0.75	0.0
1.25	1847.8	1.25	237.6	1.25	198.7	1.25	0.0
1.5	1489.7	1.5	267.7	1.5	212.7	1.5	0.0
1.75	2142.4	1.75	304.6	1.75	246.7	1.75	0.0
2	1623.5	2	357.5	2	265.1	2	13.8
2.25	1453.3	2.25	363.9	2.25	259.5	2.25	16.1
2.5	1197.9	2.5	394.3	2.5	260.8	2.5	18.9
3.5	619.8	3.5	404.7	3.5	205.0	3.5	29.0
11.75	209.3	11.75	589.1	11.75	122.6	11.75	114.9
19.6	123.4	19.6	436.9	19.6	110.8	19.6	118.4
25.5	92.5	25.5	445.1	25.5	70.5	25.5	114.5
37.6	51.2	37.6	434.4	37.6	34.9	37.6	154.5
49.75	16.2	49.75	301.3	49.75	12.2	49.75	88.6
61.9	77.4	61.9	625.0	61.9	16.9	61.9	81.4

CAT 3
Phase Two: IV Bolus + Infusion

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.25	631.2	0.25	64.3	0.25	33.8	0.25	0.0
0.5	509.3	0.5	90.6	0.5	45.4	0.5	0.0
0.75	385.8	0.75	107.6	0.75	46.1	0.75	0.0
1	291.6	1	111.2	1	47.7	1	0.0
2	149.7	2	119.7	2	38.7	2	0.0
4.1	74.1	4.1	116.2	4.1	27.2	4.1	0.0
6	64.2	6	127.5	6	23.6	6	11.1
6.25	57.4	6.25	129.5	6.25	23.9	6.25	11.7
6.5	58.5	6.5	126.3	6.5	23.6	6.5	12.4
6.75	60.2	6.75	146.7	6.75	23.1	6.75	13.5
7	55.2	7	130.2	7	23.2	7	12.2
7.6	52.3	7.6	142.9	7.6	24.6	7.6	15.9
8	47.1	8	133.5	8	21.7	8	15.2
15.4	29.0	15.4	132.8	15.4	14.9	15.4	24.1
24	18.4	24	124.9	24	0.0	24	34.0
31	9.2	31	106.5	31	0.0	31	32.3
46	0.0	46	77.0	46	0.0	46	28.9
56	0.0	56	65.1	56	0.0	56	21.8

Appendix B4: BENZODIAZEPINES SERUM CONCENTRATION IN CAT 4

CAT 4
Phase One: Repeated IV Bolus

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0.05	2089.1	0.05	141.6	0.05	31.9	0.05	0.0
0.083	1859.1	0.083	160.4	0.083	35.9	0.083	0.0
0.2	999.8	0.2	160.3	0.2	46.5	0.2	0.0
0.25	1198.3	0.25	152.7	0.25	42.8	0.25	0.0
0.5	703.2	0.5	157.1	0.5	46.3	0.5	0.0
0.75	1854.3	0.75	266.2	0.75	79.8	0.75	0.0
1.25	2351.8	1.25	333.7	1.25	113.1	1.25	0.0
1.5	1935.6	1.5	475.3	1.5	125.8	1.5	0.0
1.75	3272.9	1.75	482.9	1.75	149.2	1.75	0.0
2	2384.6	2	453.1	2	149.6	2	0.0
2.25	1621.5	2.25	504.9	2.25	133.3	2.25	12.1
2.5	1180.8	2.5	449.9	2.5	110.2	2.5	0.0
3.5	501.0	3.5	407.6	3.5	72.0	3.5	13.6
11.4	314.0	11.4	606.9	11.4	49.4	11.4	45.0
19.5	265.5	19.5	812.5	19.5	54.6	19.5	57.8
25.5	245.5	25.5	761.7	25.5	35.5	25.5	55.2
37.75	188.5	37.75	809.4	37.75	34.3	37.75	60.6
49.5	122.1	49.5	728.0	49.5	22.3	49.5	66.8
63.2	0.0	63.2	0.0	63.2	0.0	63.2	0.0

CAT 4
Phase Two: IV Bolus + Infusion

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.25	1154.0	0.25	109.8	0.25	56.7	0.25	0.0
0.5	984.3	0.5	156.8	0.5	66.2	0.5	0.0
0.75	651.8	0.75	183.0	0.75	67.7	0.75	0.0
1	517.4	1	189.2	1	63.6	1	0.0
2	230.6	2	162.2	2	45.3	2	0.0
4	111.3	4	152.3	4	34.6	4	13.1
6	88.0	6	163.1	6	32.0	6	18.1
6.25	89.0	6.25	167.5	6.25	31.8	6.25	20.1
6.5	90.6	6.5	174.5	6.5	31.8	6.5	18.8
6.75	80.1	6.75	163.5	6.75	28.3	6.75	18.8
7	83.2	7	165.3	7	27.6	7	18.9
7.5	74.7	7.5	157.5	7.5	22.6	7.5	18.3
8	77.2	8	168.4	8	25.5	8	21.8
15.5	58.9	15.5	184.9	15.5	20.9	15.5	31.4
24	38.6	24	216.8	24	14.1	24	47.8
30	28.0	30	182.8	30	10.8	30	46.3
44	19.9	44	189.1	44	0.0	44	57.5
55	14.3	55	143.4	55	0.0	55	58.0

Appendix B5: BENZODIAZEPINES SERUM CONCENTRATION IN CAT 5

CAT 5
Phase One: Repeated IV Bolus

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0.05	1801.2	0.05	45.4	0.05	30.2	0.05	0.0
0.083	1195.5	0.083	73.2	0.083	56.3	0.083	0.0
0.17	1045.9	0.17	76.3	0.17	69.7	0.17	0.0
0.25	809.5	0.25	95.3	0.25	73.8	0.25	0.0
0.5	582.2	0.5	113.6	0.5	80.0	0.5	0.0
0.75	1195.9	0.75	206.7	0.75	141.7	0.75	0.0
1.25	1329.8	1.25	293.4	1.25	201.8	1.25	0.0
1.5	1045.8	1.5	357.4	1.5	205.4	1.5	11.4
1.75	1444.9	1.75	384.2	1.75	227.2	1.75	15.1
2	1052.1	2	457.0	2	225.1	2	17.8
2.25	963	2.25	452.8	2.25	212.8	2.25	20.3
2.5	813.2	2.5	494.9	2.5	204.1	2.5	27.3
3.5	467.2	3.5	618.3	3.5	156.0	3.5	41.8
11.6	87.2	11.6	1020.6	11.6	95.2	11.6	128.4
19.5	17.1	19.5	669.7	19.5	49.8	19.5	106.1
25.5	0.0	25.5	539.0	25.5	35.6	25.5	110.2
37.8	0.0	37.8	227.2	37.8	0.0	37.8	57.0
49.5	0.0	49.5	208.1	49.5	0.0	49.5	47.5
61.5	0.0	61.5	125.1	61.5	0.0	61.5	39.9

CAT 5
Phase Two: IV Bolus + Infusion

DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.25	865.1	0.25	129.9	0.25	61.9	0.25	0.0
0.5	682.2	0.5	164.6	0.5	76.4	0.5	0.0
0.75	497.7	0.75	185.1	0.75	77.4	0.75	0.0
1	408.8	1	214.0	1	78.2	1	0.0
2	234.6	2	311.3	2	69.4	2	11.4
4	104.6	4	353.4	4	37.3	4	21.3
6	56.0	6	378.5	6	27.2	6	33.1
6.25	51.2	6.25	405.7	6.25	24.1	6.25	33.6
6.5	43.3	6.5	365.1	6.5	23.7	6.5	32.0
6.75	44.6	6.75	440.3	6.75	25.0	6.75	35.6
7	42.1	7	387.5	7	19.5	7	34.9
7.5	31.9	7.5	375.1	7.5	18.3	7.5	32.8
8	32.2	8	382.5	8	17.8	8	38.8
15.4	0.0	15.4	263.5	15.4	0.0	15.4	52.2
24	0.0	24	185.4	24	0.0	24	45.4
32	0.0	32	134.8	32	0.0	32	38.1
47	0.0	47	67.4	47	0.0	47	32.8
56	0.0	56	45.1	56	0.0	56	12.4

Appendix B6: BENZODIAZEPINES SERUM CONCENTRATION IN CAT 7

CAT 7 Phase One: Repeated IV Bolus							
DIAZEPAM		**DMD**		**TEMAZEPAM**		**OXAZEPAM**	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0.05	1337.5	0.05	34.7	0.05	35.1	0.05	0.0
0.083	1301.5	0.083	30.8	0.083	38.8	0.083	0.0
0.25	1050.7	0.25	40.6	0.25	51.5	0.25	0.0
0.3	1002.7	0.3	44.9	0.3	56.2	0.3	0.0
0.6	700.7	0.6	56.8	0.6	61.6	0.6	0.0
0.7	1232.7	0.7	122.3	0.7	108.2	0.7	0.0
1.25	1704.7	1.25	220.3	1.25	180.2	1.25	0.0
1.5	1568.9	1.5	223.6	1.5	187.7	1.5	12.1
1.75	1829.5	1.75	268.9	1.75	214.2	1.75	16.3
2	1432.1	2	306.1	2	225.4	2	18.2
2.25	1287.3	2.25	318.5	2.25	224.4	2.25	19.7
2.5	1158.5	2.5	334.3	2.5	229.0	2.5	23.8
3.5	601.8	3.5	324.2	3.5	172.9	3.5	33.6
11.5	253.2	11.5	424.1	11.5	96.2	11.5	102.3
19.5	177.1	19.5	510.1	19.5	73.0	19.5	142.6
25.7	69.4	25.7	335.5	25.7	31.6	25.7	98.0
37.5	35.1	37.5	267.9	37.5	14.3	37.5	81.0
49.5	14.6	49.5	197.1	49.5	0.0	49.5	53.5
61.6	10.4	61.6	150.3	61.6	0.0	61.6	48.2

CAT 7 Phase Two: IV Bolus + Infusion							
DIAZEPAM		**DMD**		**TEMAZEPAM**		**OXAZEPAM**	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.25	1131.0	0.25	94.0	0.25	76.5	0.25	0.0
0.5	827.2	0.5	109.8	0.5	85.5	0.5	0.0
0.75	662.0	0.75	96.5	0.75	81.0	0.75	0.0
1	559.8	1	118.7	1	80.7	1	0.0
2	313.8	2	137.3	2	62.8	2	0.0
4	179.5	4	145.8	4	47.5	4	18.0
6	117.7	6	157.1	6	35.1	6	24.0
6.25	116.0	6.25	154.6	6.25	33.2	6.25	24.0
6.5	122.8	6.5	163.8	6.5	34.5	6.5	27.1
6.75	108.4	6.75	163.3	6.75	33.2	6.75	25.0
7	117.5	7	170.1	7	35.8	7	29.3
7.5	116.8	7.5	177.8	7.5	33.3	7.5	34.3
8	101.0	8	174.5	8	31.6	8	30.0
16	46.3	16	148.4	16	22.2	16	48.1
24	24.8	24	135.7	24	15.0	24	54.7
32	13.5	32	83.0	32	0.0	32	44.6
45	0.0	45	58.4	45	0.0	45	40.7
55	0.0	55	32.4	55	0.0	55	20.5

Appendix B7: BENZODIAZEPINES SERUM CONCENTRATION IN CAT 8

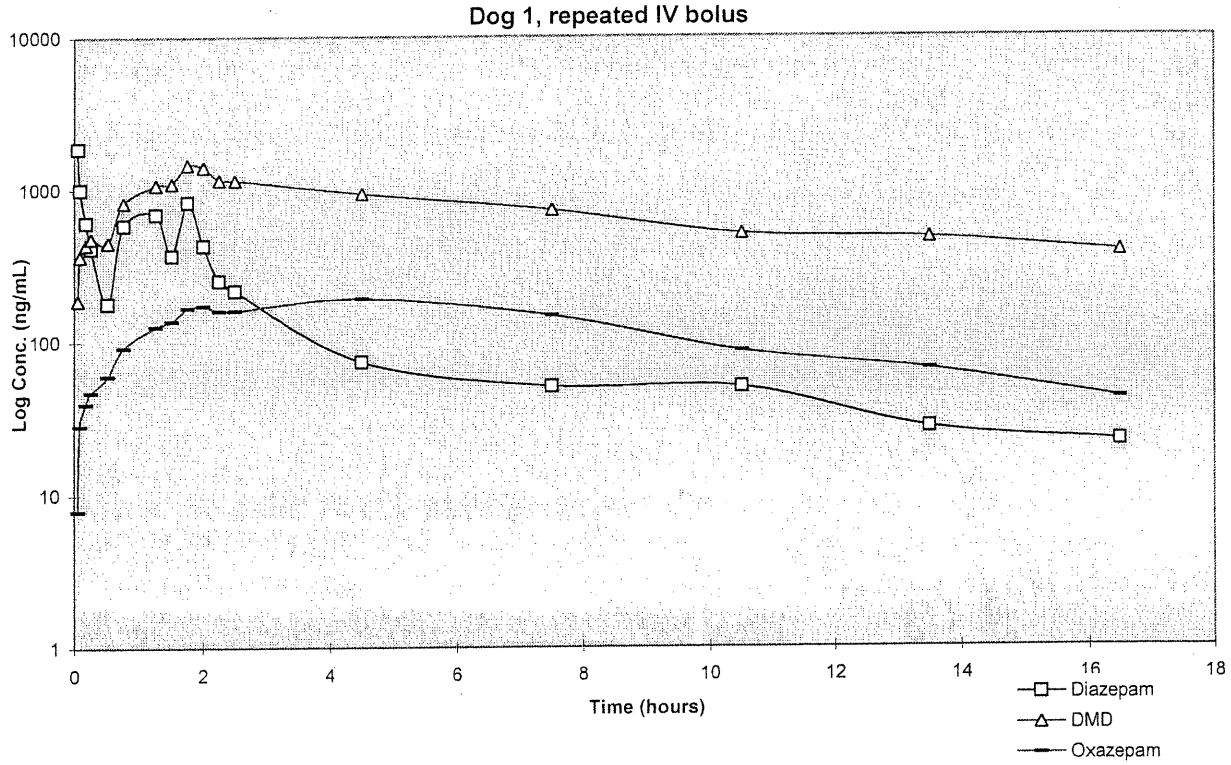
CAT 8
Phase One: Repeated IV Bolus

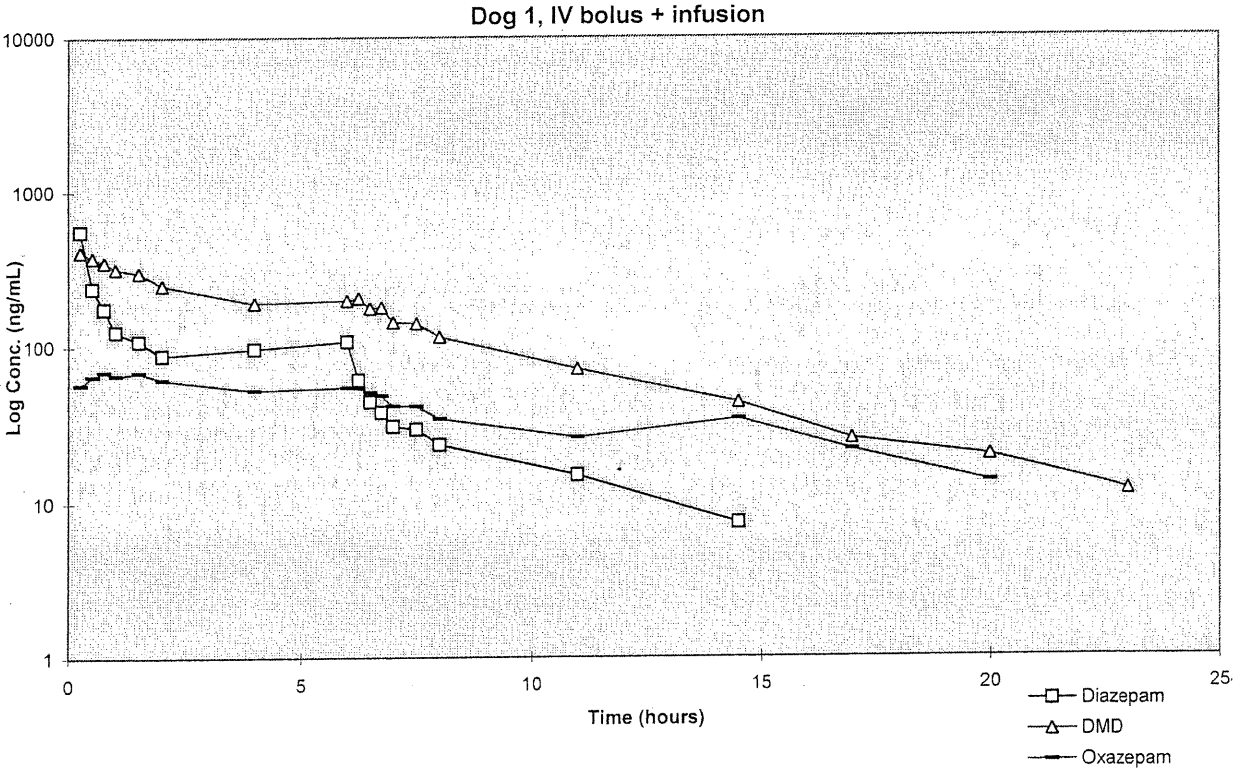
DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0.05	1578.9	0.05	66.6	0.05	27.7	0.05	0.0
0.083	1304.8	0.083	48.9	0.083	32.3	0.083	0.0
0.17	1092.8	0.17	112.0	0.17	46.5	0.17	0.0
0.25	834.3	0.25	118.8	0.25	48.4	0.25	0.0
0.5	685	0.5	201.2	0.5	59.8	0.5	0.0
0.75	1451	0.75	319.0	0.75	94.9	0.75	0.0
1.25	1873.1	1.25	542.6	1.25	142.0	1.25	0.0
1.5	1399.7	1.5	701.9	1.5	158.5	1.5	0.0
1.75	1904.2	1.75	750.5	1.75	179.2	1.75	13.8
2	1481.5	2	991.3	2	207.4	2	17.0
2.25	1302.6	2.25	1017.8	2.25	199.9	2.25	17.9
2.5	1085	2.5	1161.9	2.5	204.5	2.5	22.8
3.5	623.3	3.5	1285.0	3.5	176.0	3.5	34.3
11.5	50.4	11.5	1692.8	11.5	44.6	11.5	94.8
19.2	13.4	19.2	1765.1	19.2	14.0	19.2	137.6
25.8	0.0	25.8	1290.2	25.8	9.1	25.8	120.2
37.5	0.0	37.5	961.1	37.5	0.0	37.5	131.8
49.5	0.0	49.5	707.6	49.5	12.0	49.5	133.9
61.7	0.0	61.7	349.5	61.7	12.6	61.7	96.6

CAT 8
Phase Two: IV Bolus + Infusion

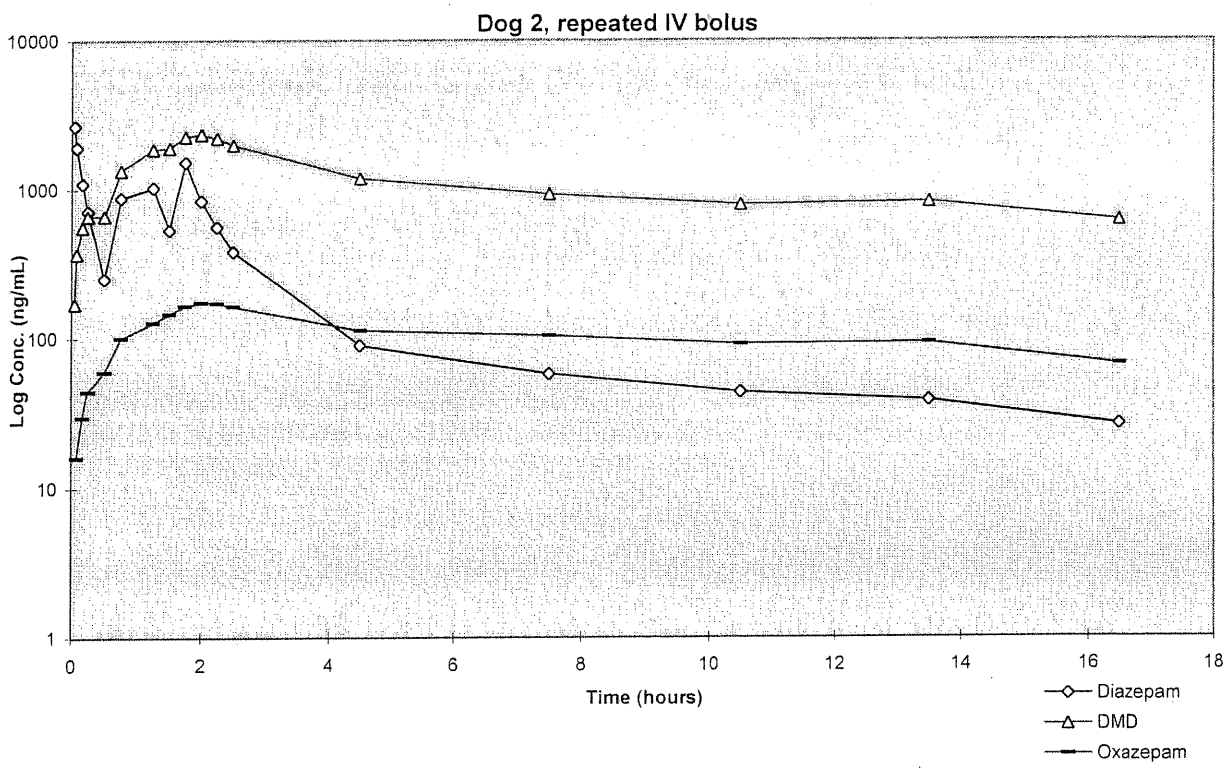
DIAZEPAM		DMD		TEMAZEPAM		OXAZEPAM	
Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)	Time (hours)	Conc. (ng/mL)
0	0.0	0	0.0	0	0.0	0	0.0
0.25	1029.0	0.25	169.7	0.25	60.6	0.25	0.0
0.5	914.0	0.5	199.6	0.5	75.6	0.5	0.0
0.75	687.7	0.75	299.4	0.75	90.5	0.75	0.0
1	546.2	1	322.8	1	92.9	1	0.0
2	287.8	2	409.2	2	77.8	2	19.4
4	107.1	4	451.4	4	44.4	4	20.5
6	58.2	6	469.8	6	35.3	6	27.3
6.25	61.4	6.25	477.3	6.25	39.3	6.25	28.2
6.5	63.2	6.5	485.6	6.5	38.1	6.5	30.0
6.75	46.0	6.75	516.9	6.75	33.9	6.75	31.7
7	50.0	7	474.8	7	34.3	7	30.6
7.5	39.5	7.5	480.7	7.5	27.3	7.5	31.8
8	40.3	8	511.5	8	38.5	8	32.0
15.7	9.9	15.7	381.2	15.7	7.5	15.7	42.7
24.1	0.0	24.1	325.8	24.1	10.8	24.1	48.0
31	0.0	31	206.4	31	8.6	31	42.0
44	0.0	44	141.8	44	0.0	44	30.0
54	0.0	54	68.2	54	0.0	54	13.8

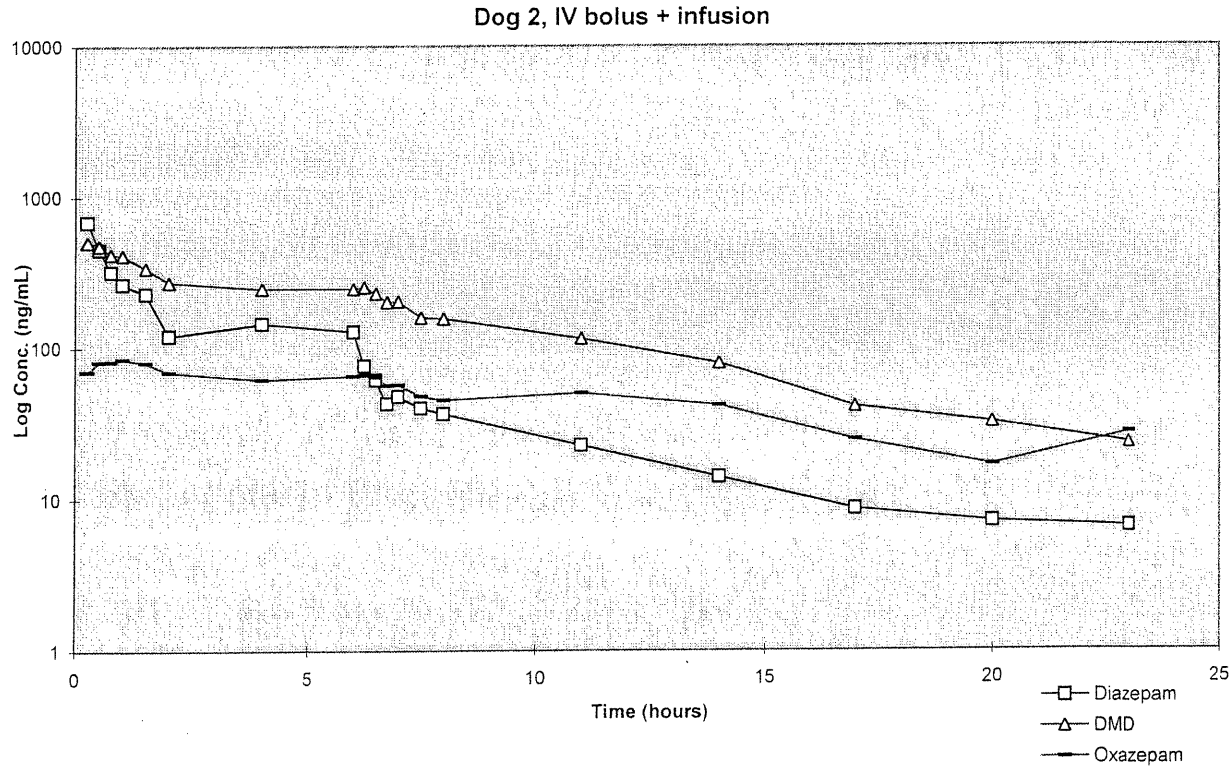
APPENDIX C1: PROFILE OF DOG 1



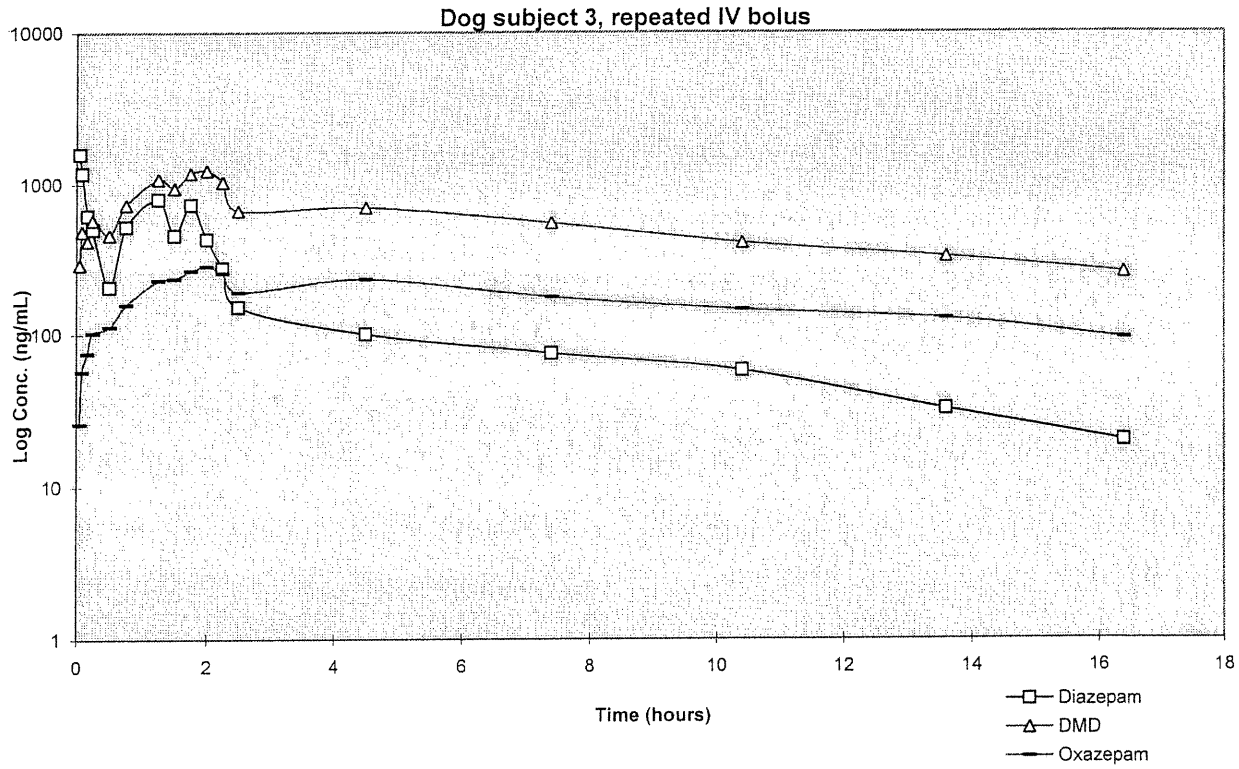


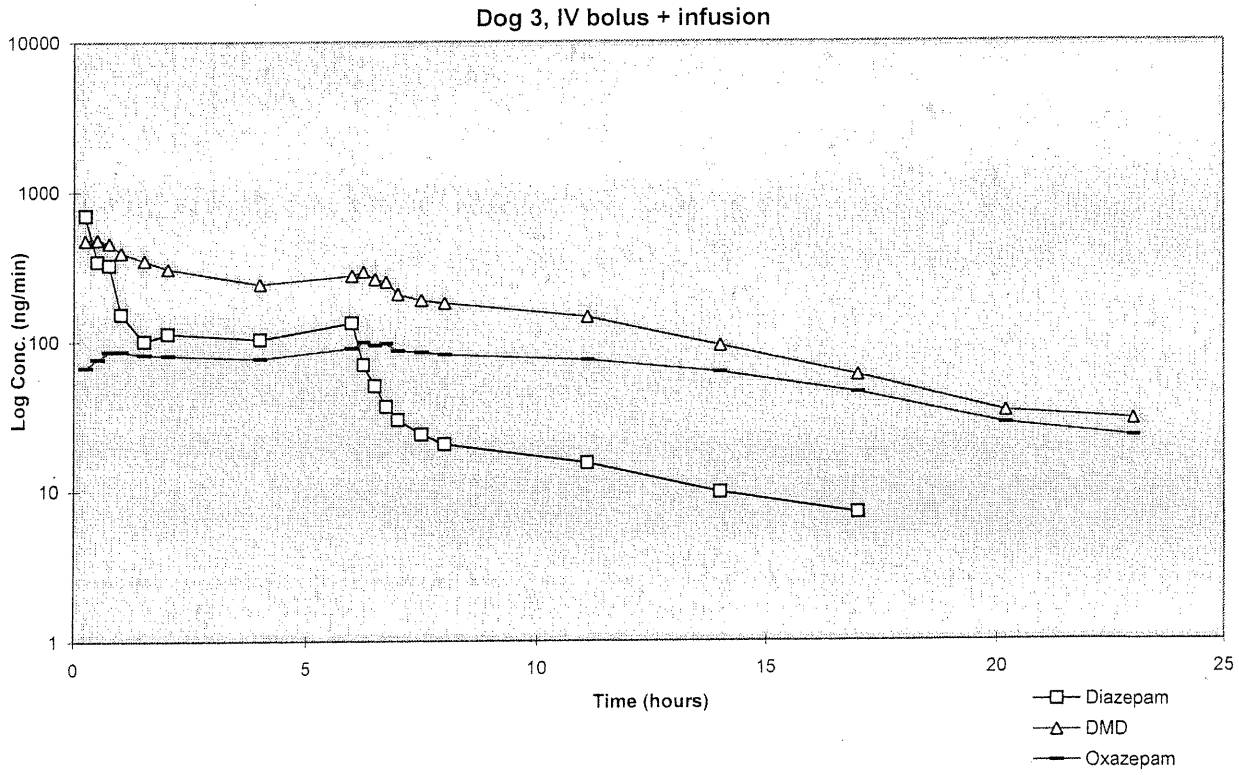
APPENDIX C2: PROFILE OF DOG 2



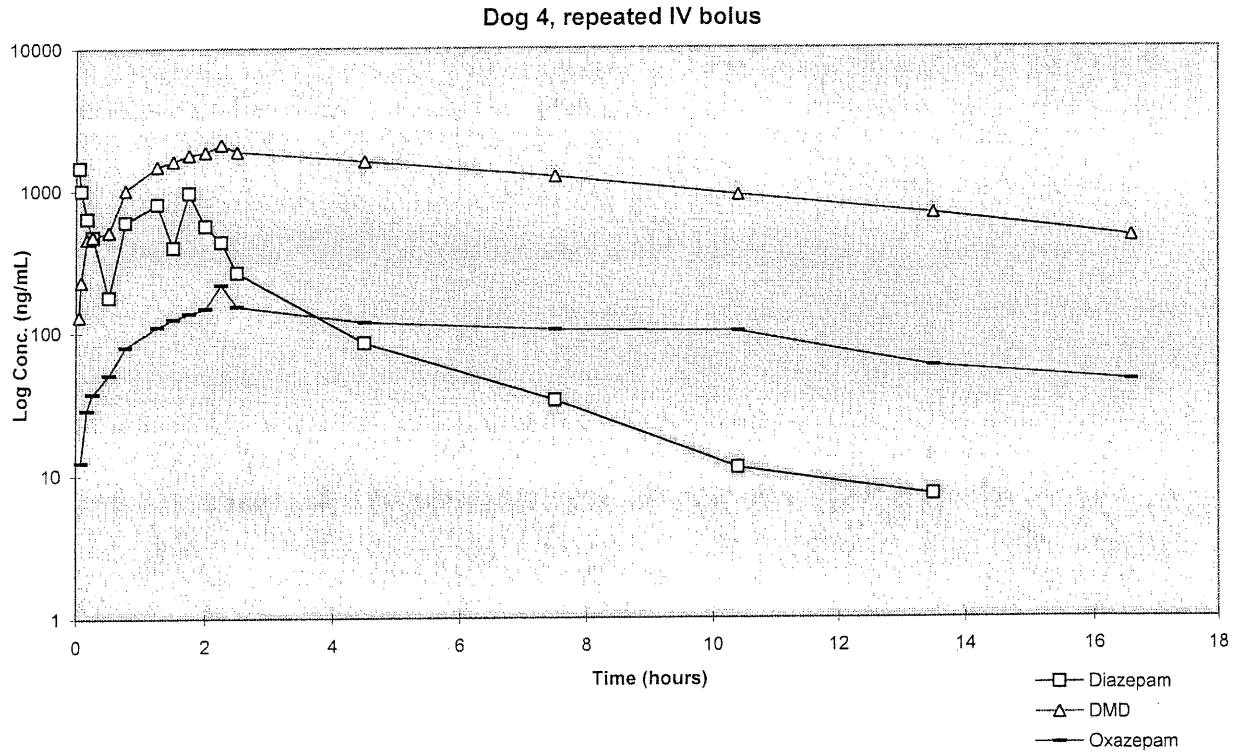


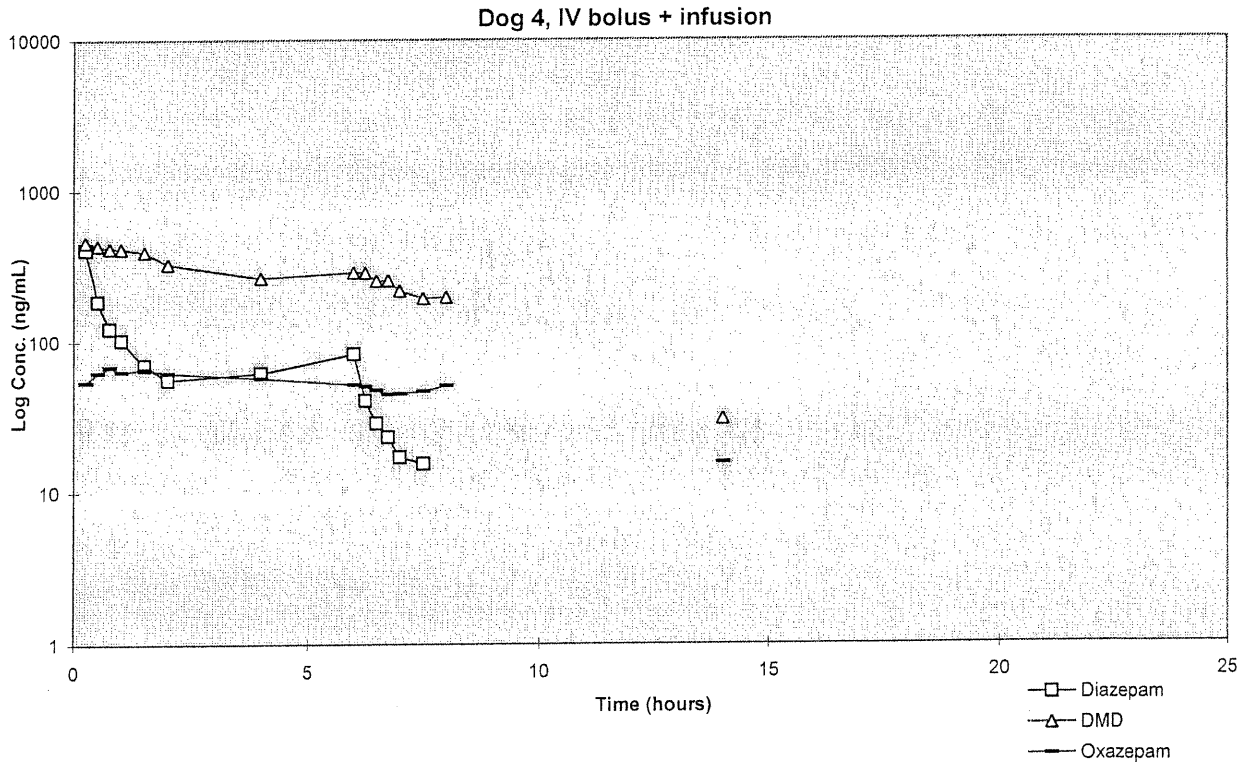
APPENDIX C3: PROFILE OF DOG 3



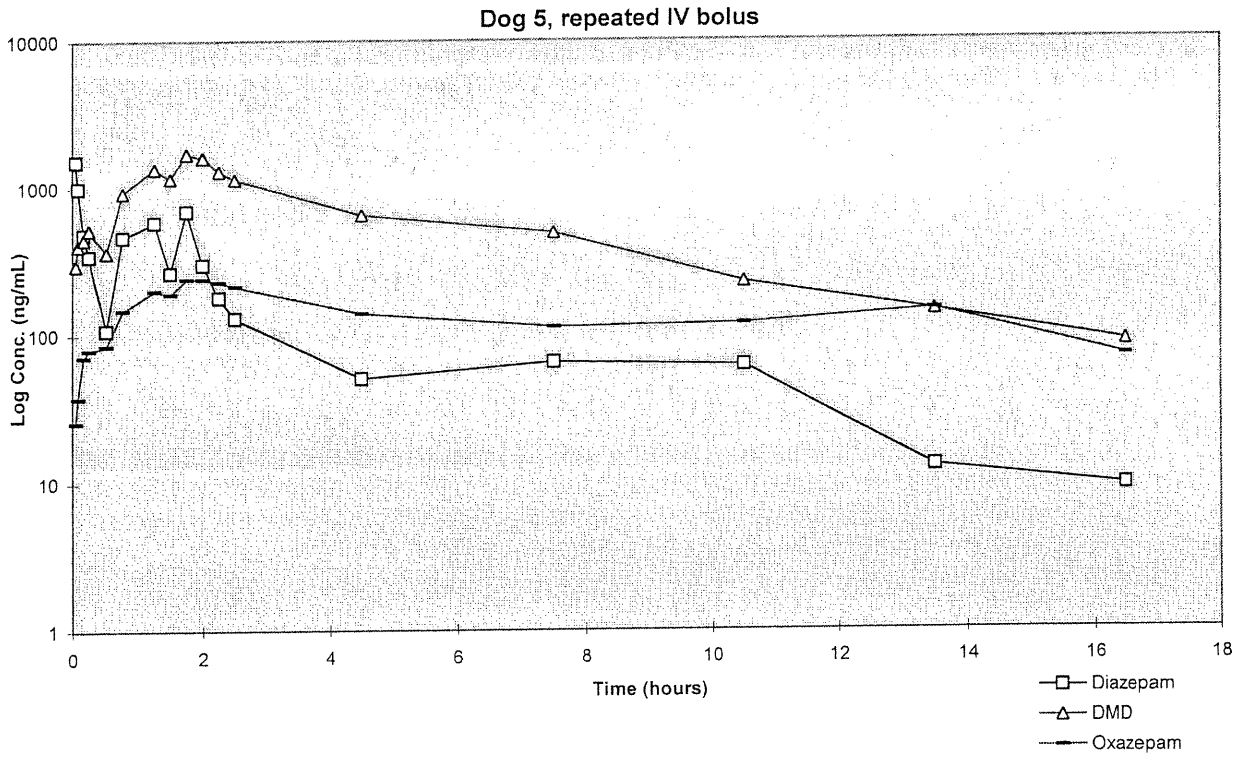


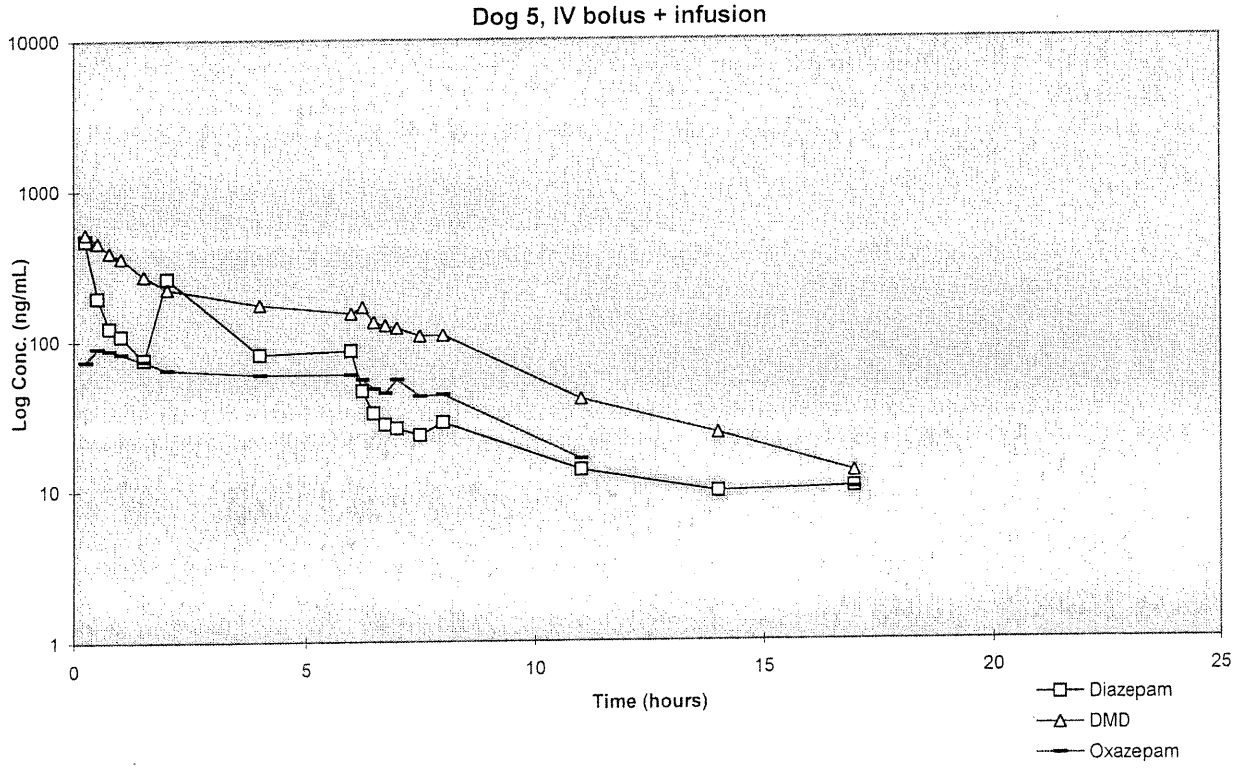
APPENDIX C4: PROFILE OF DOG 4



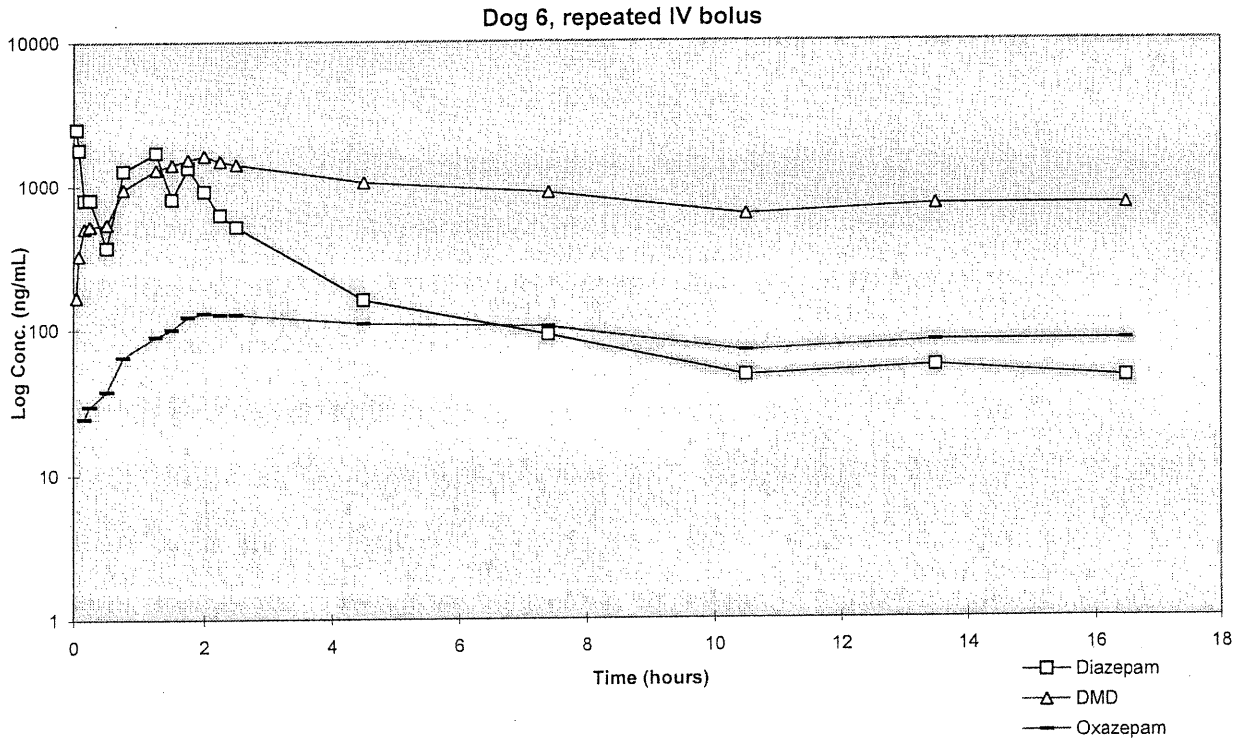


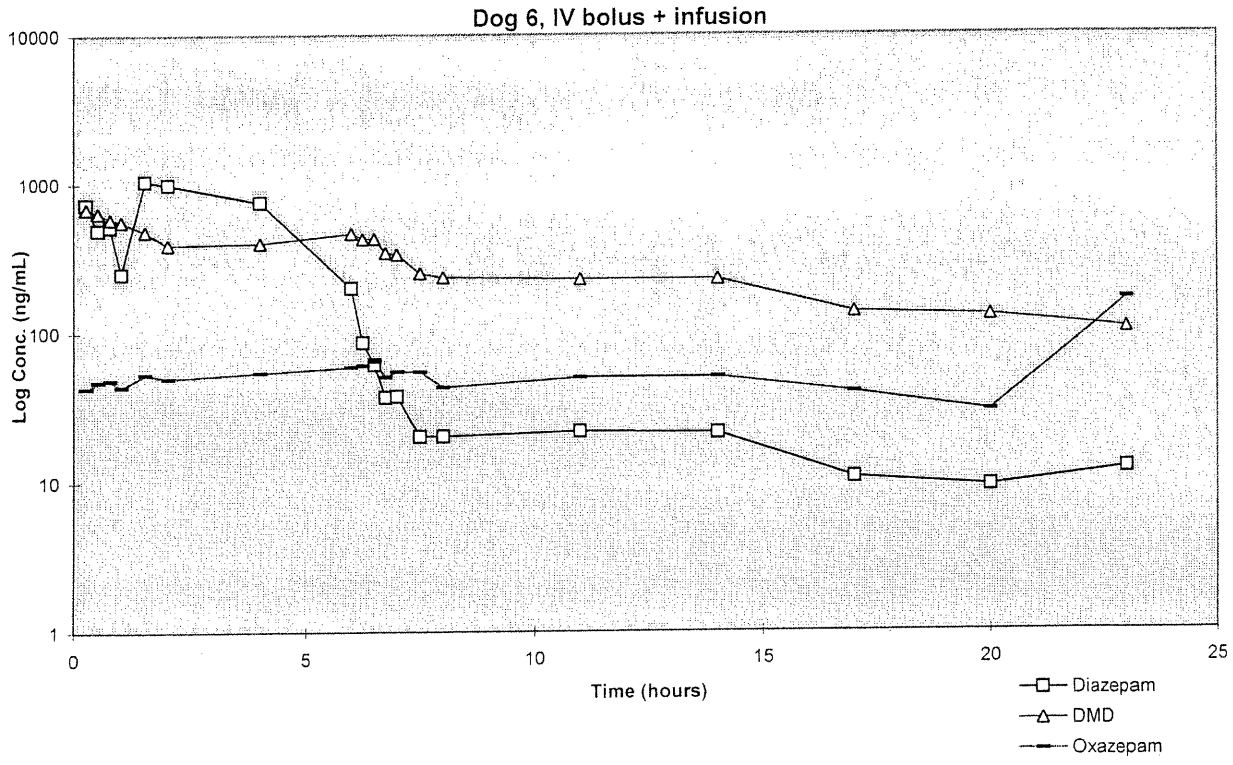
APPENDIX C5: PROFILE OF DOG 5



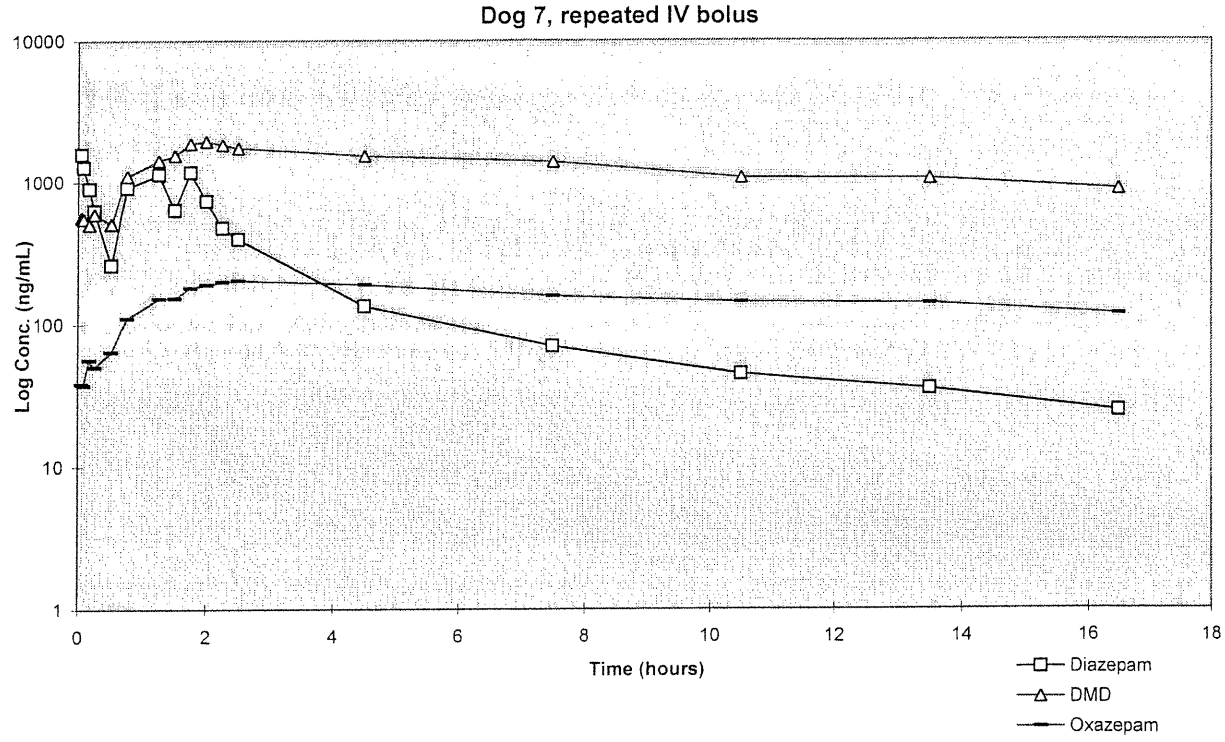


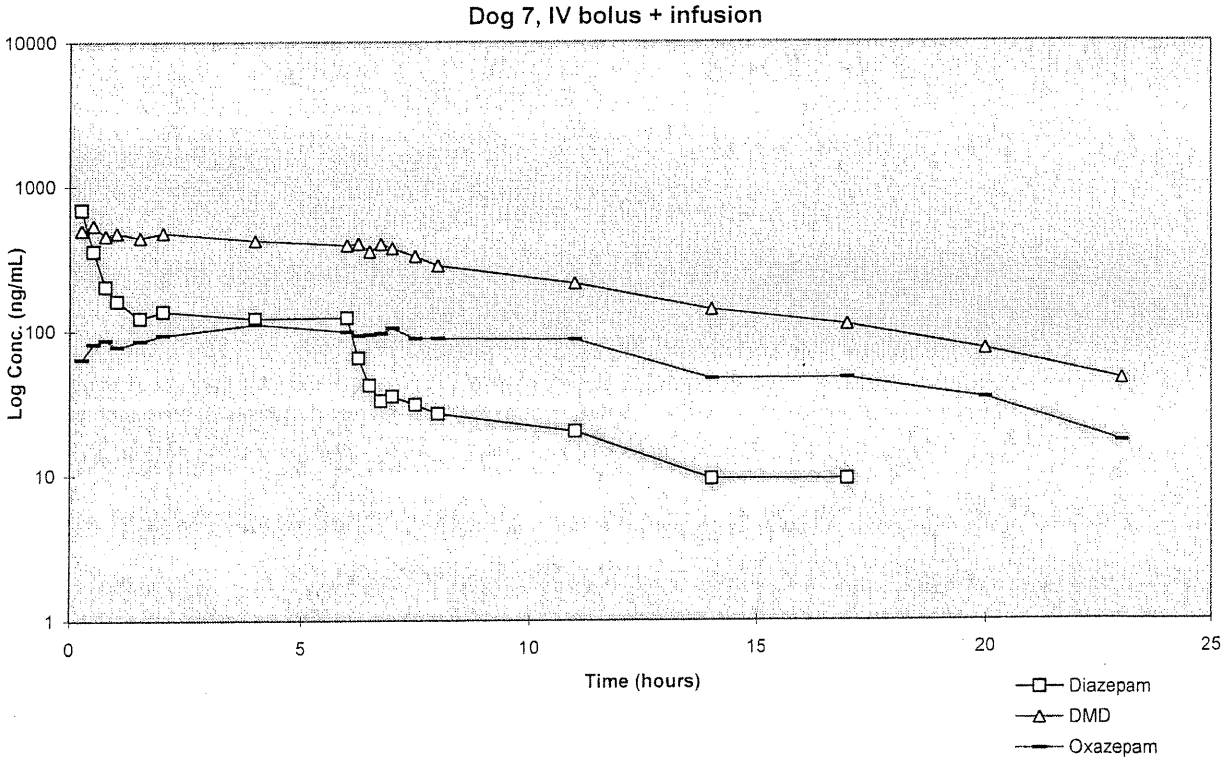
APPENDIX C6: PROFILE OF DOG 6



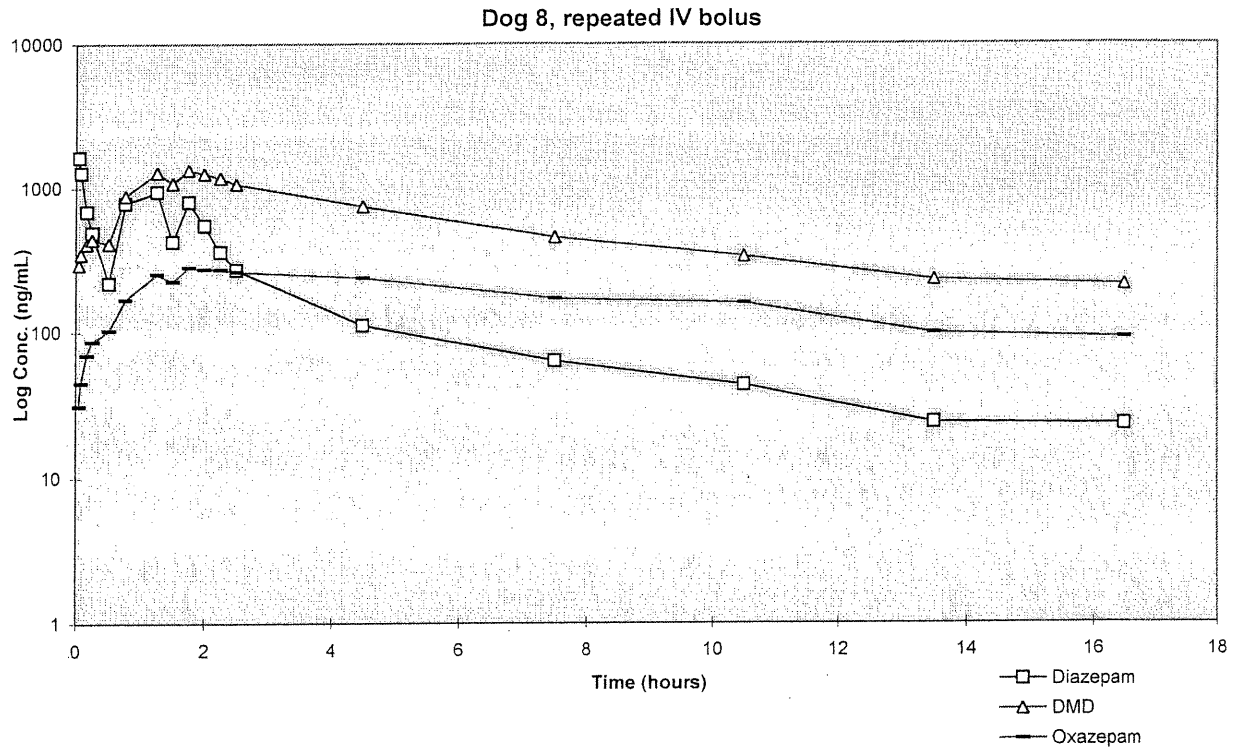


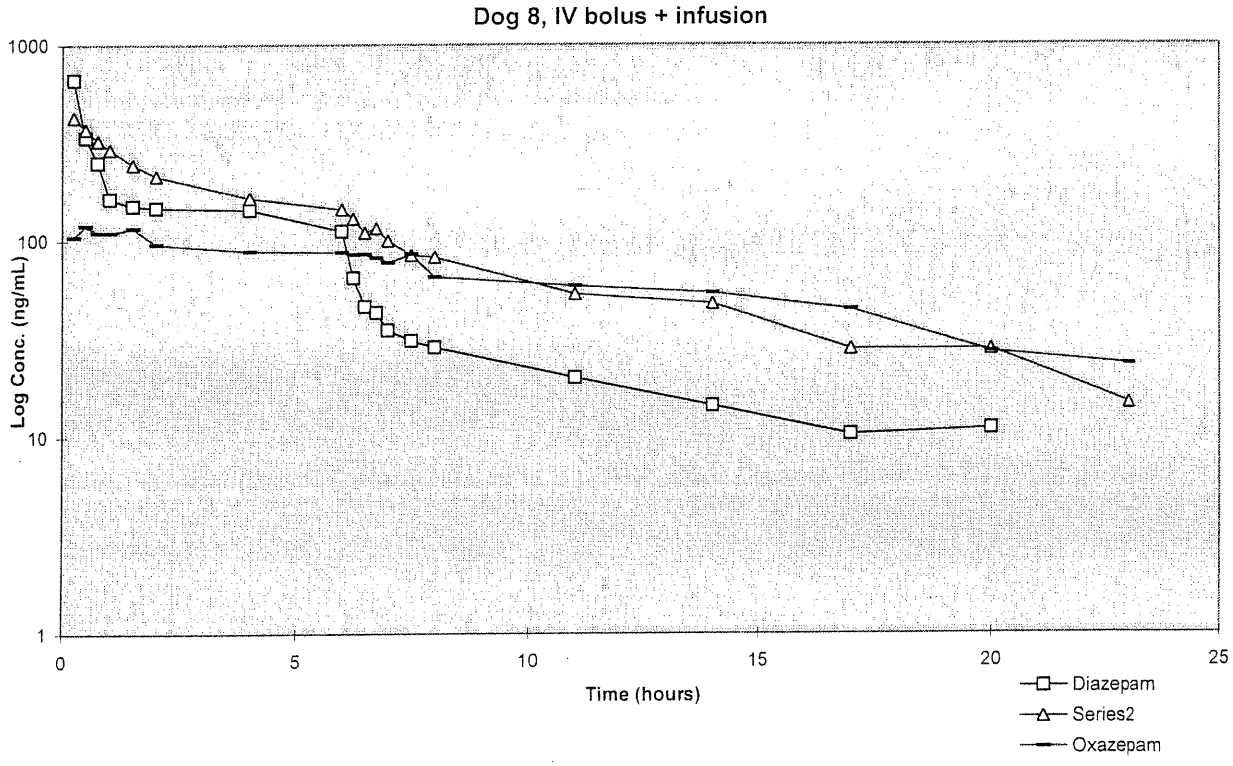
APPENDIX C7: PROFILE OF DOG 7



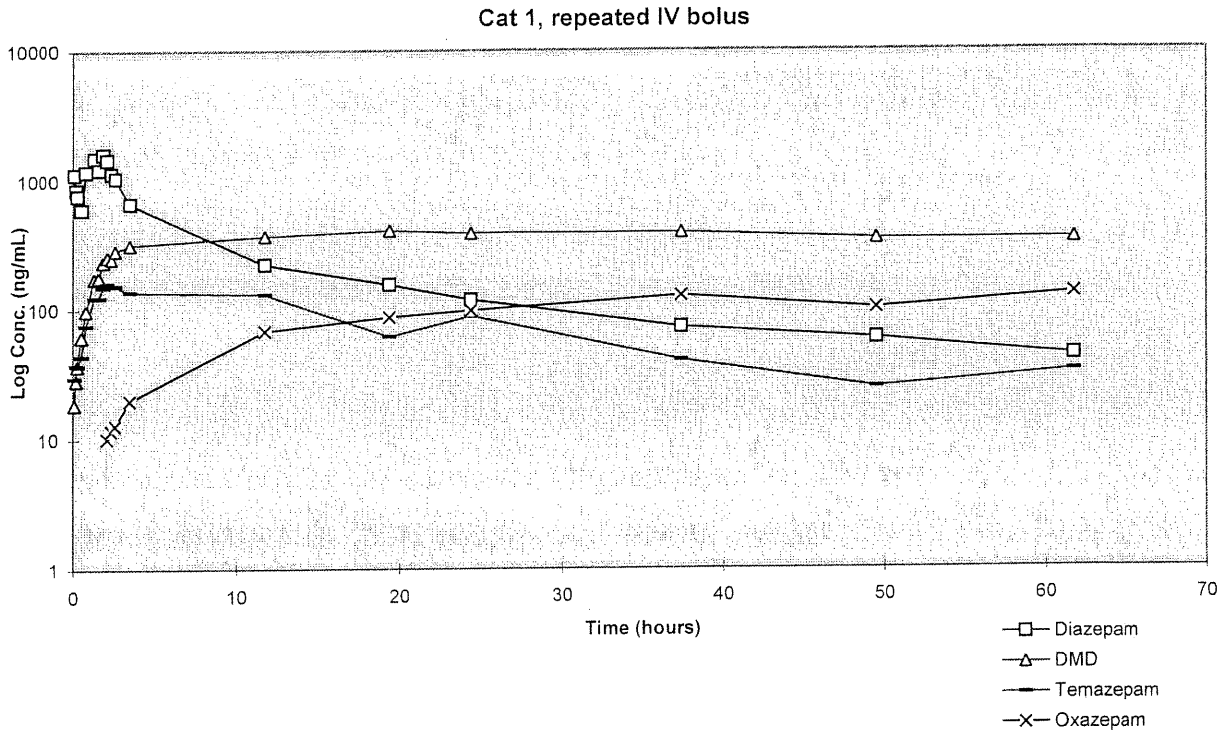


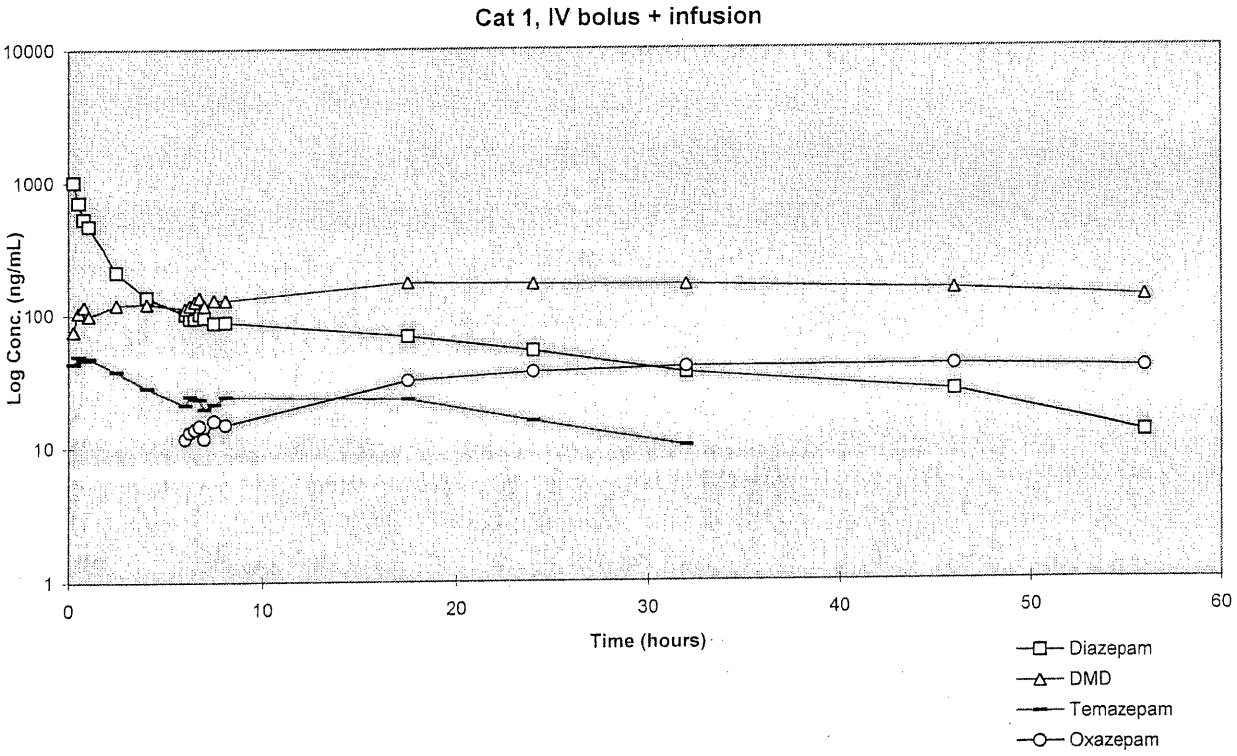
APPENDIX C8: PROFILE OF DOG 8



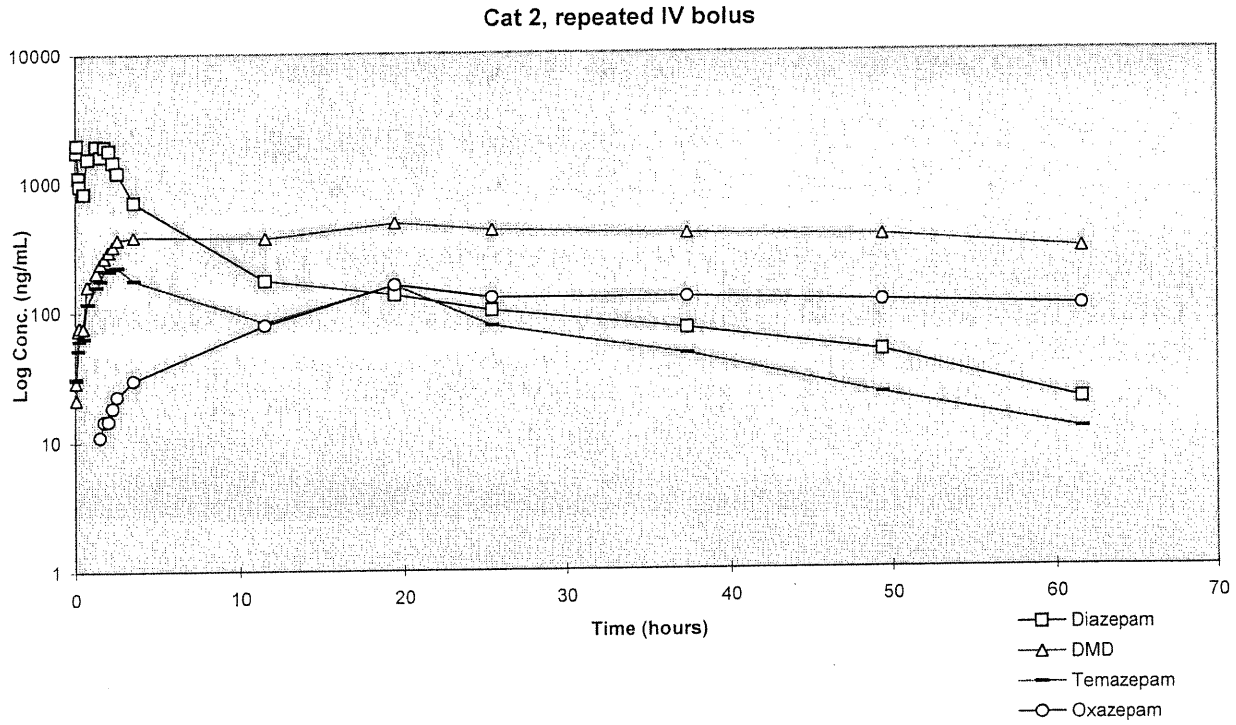


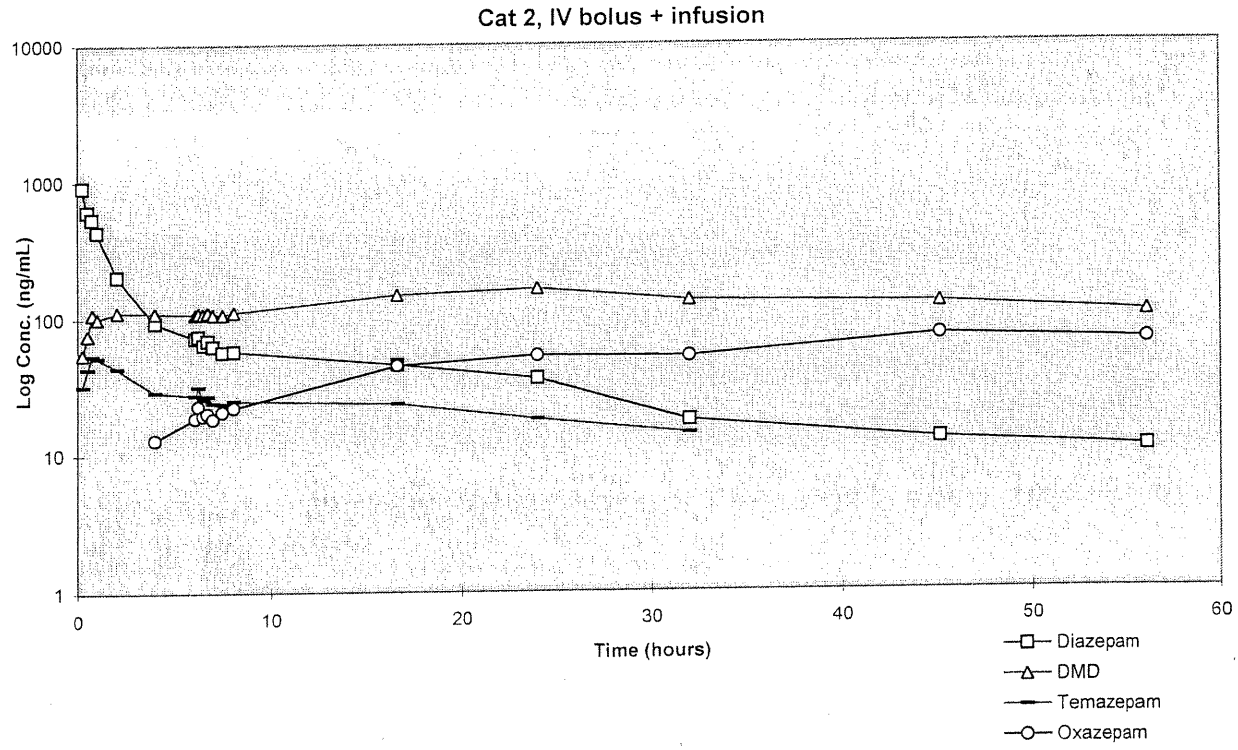
APPENDIX D1: PROFILE OF CAT 1



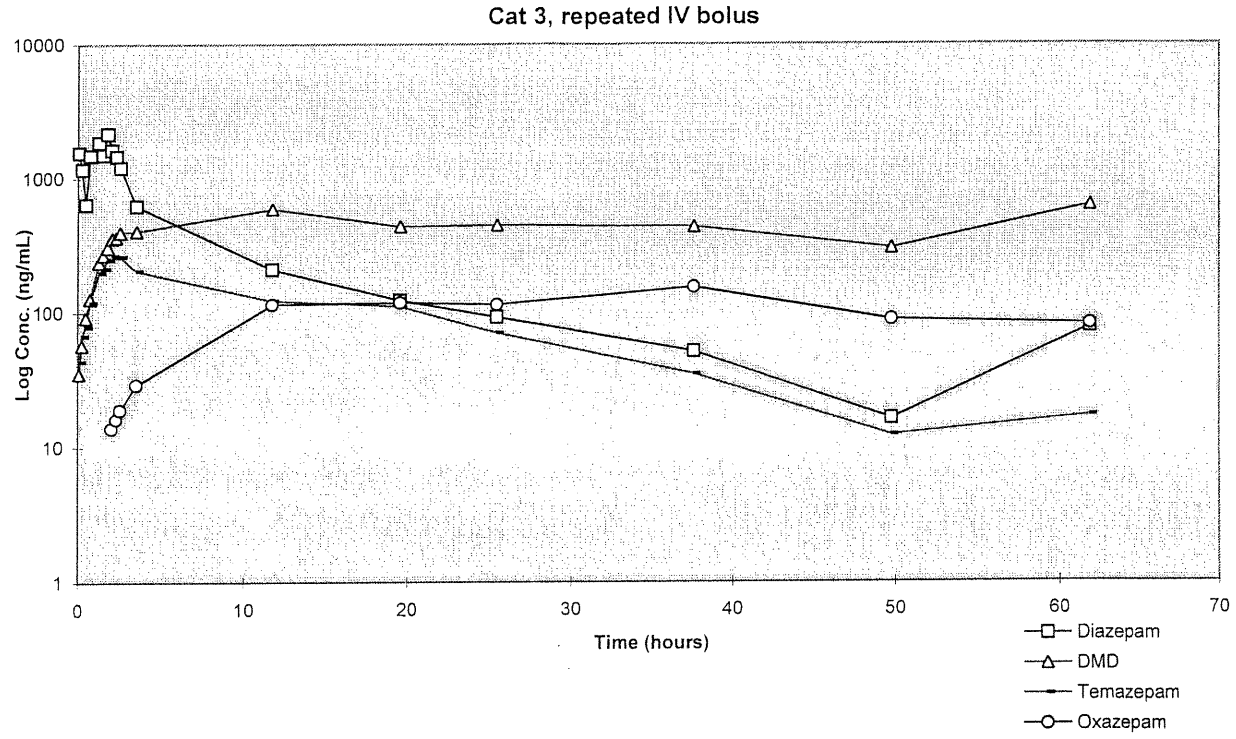


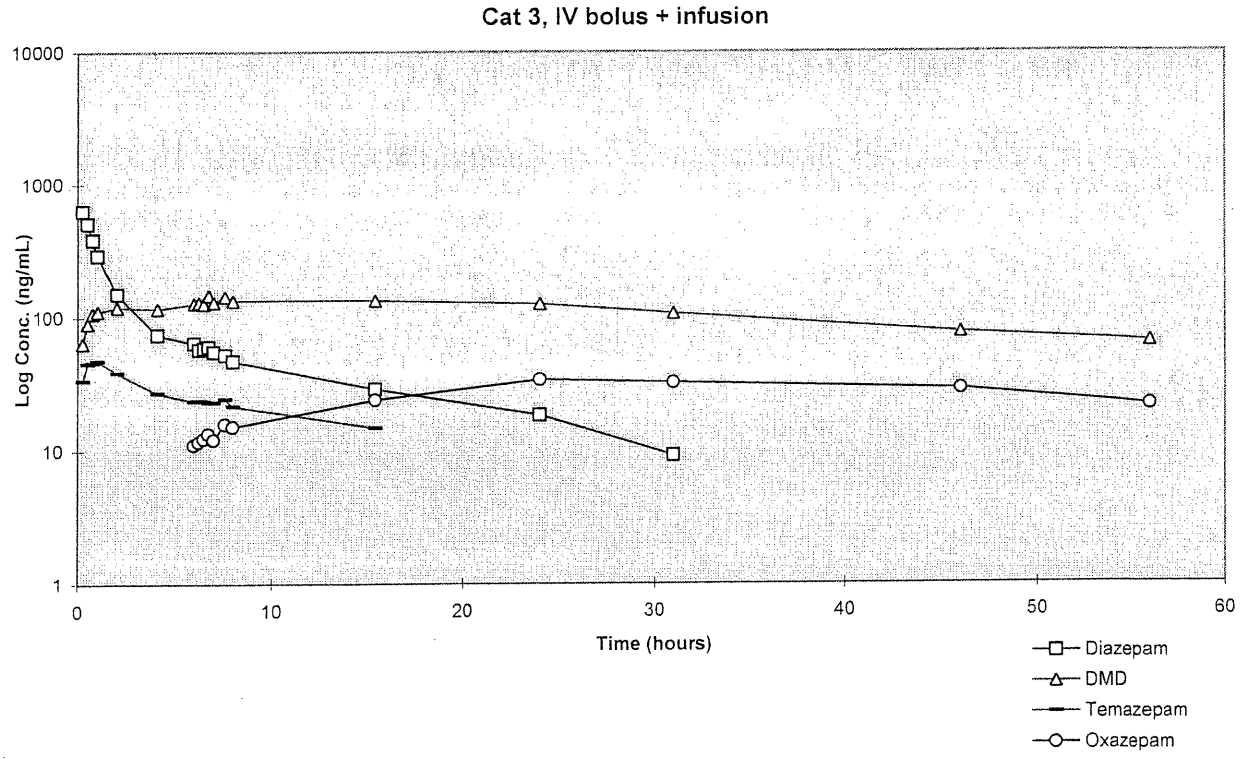
APPENDIX D2: PROFILE OF CAT 2



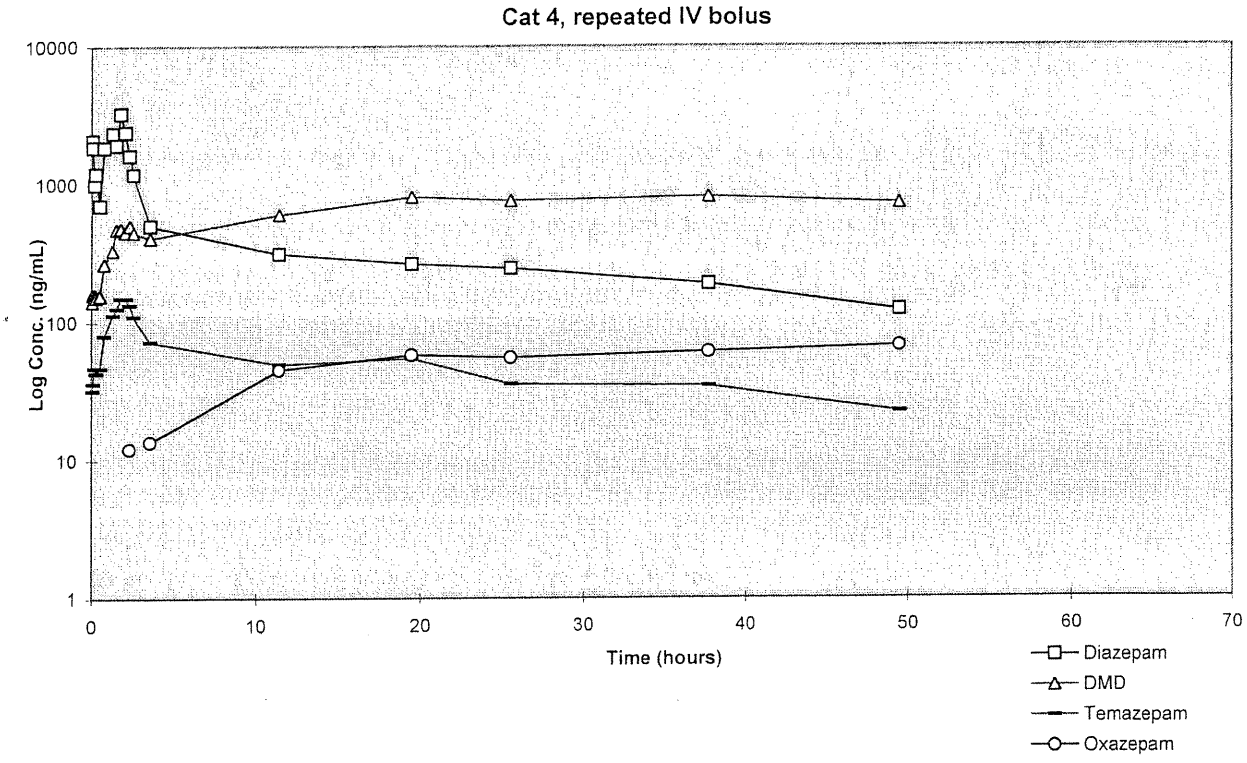


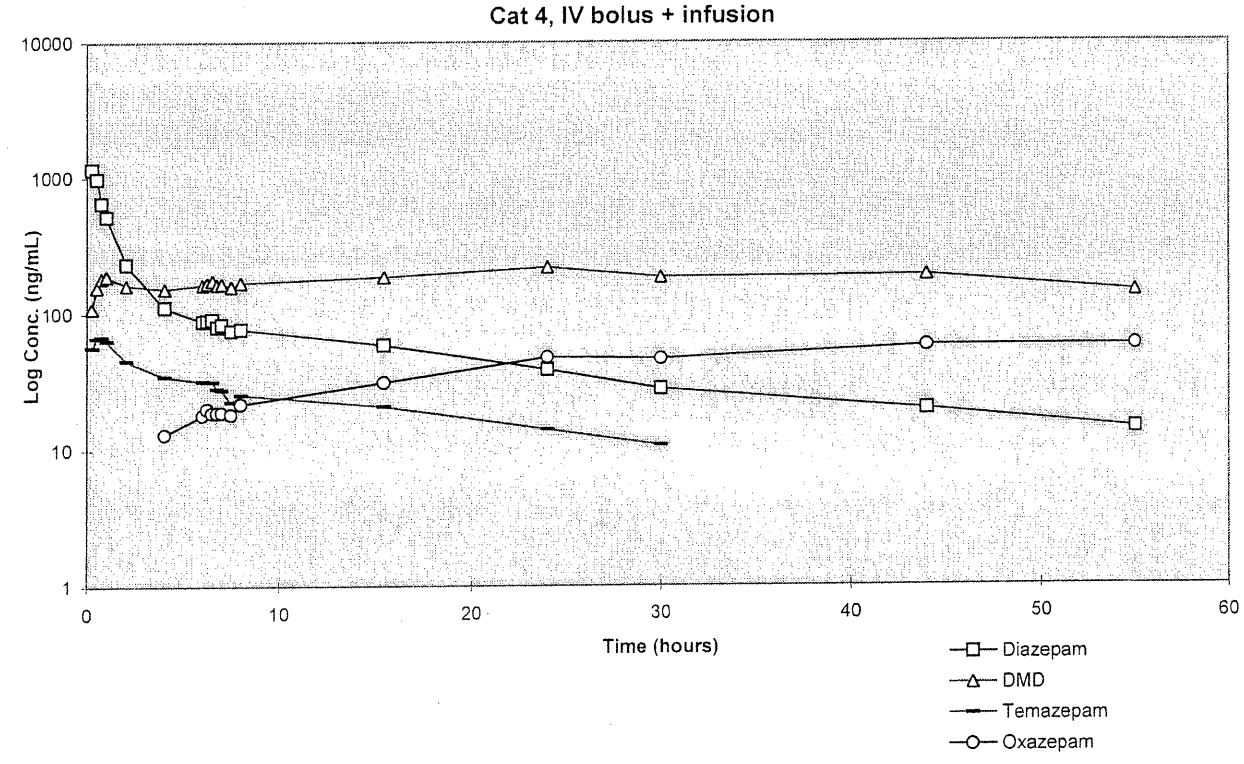
APPENDIX D3: PROFILE OF CAT 3



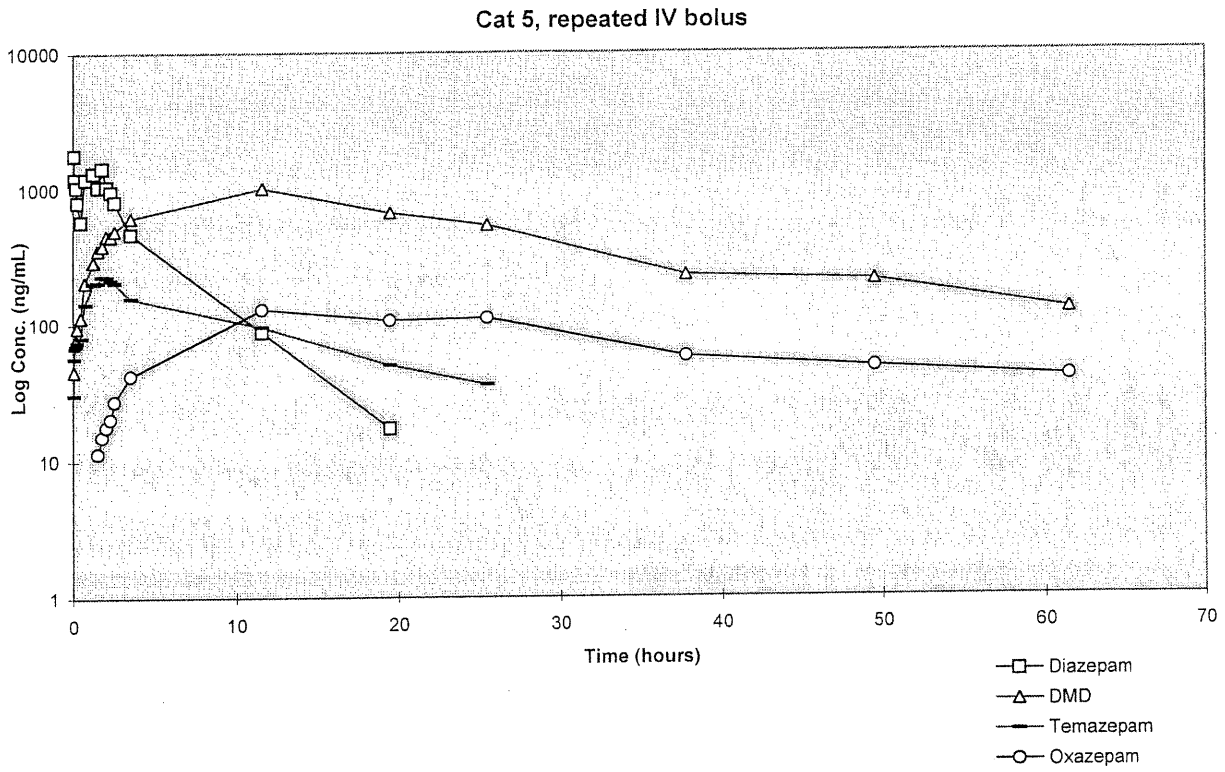


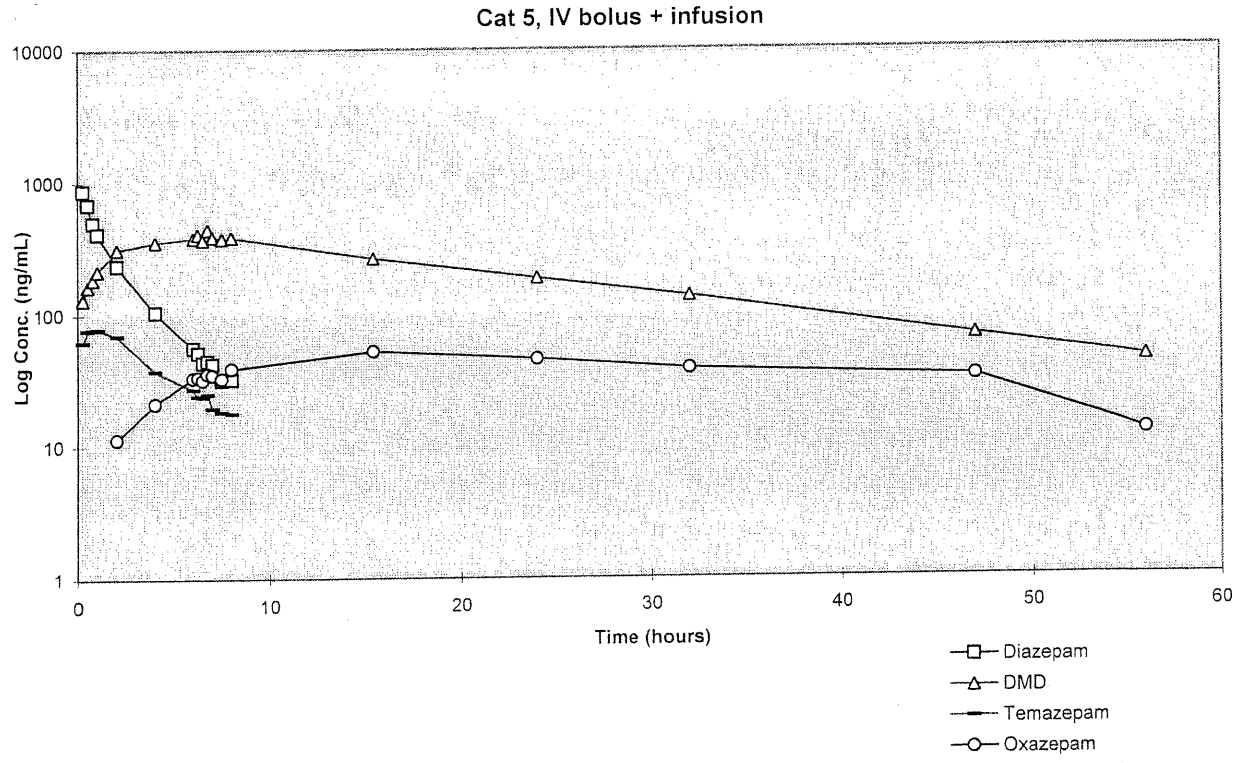
APPENDIX D4: PROFILE OF CAT 4



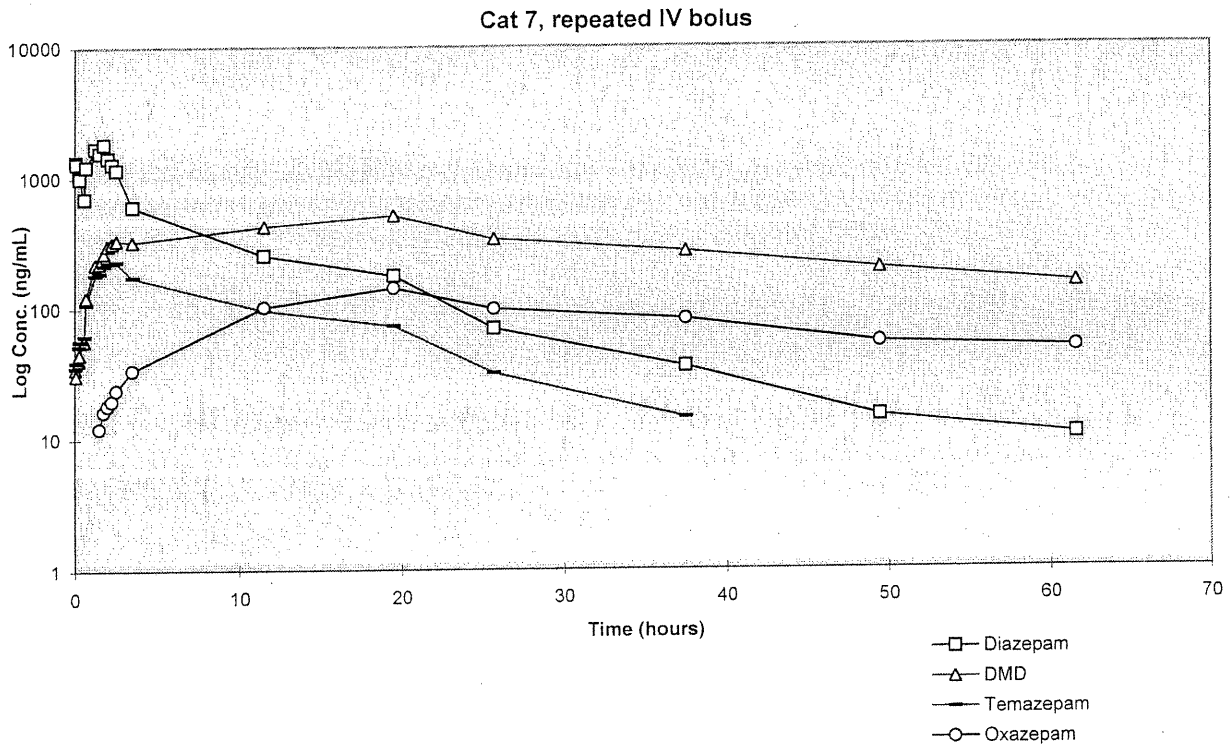


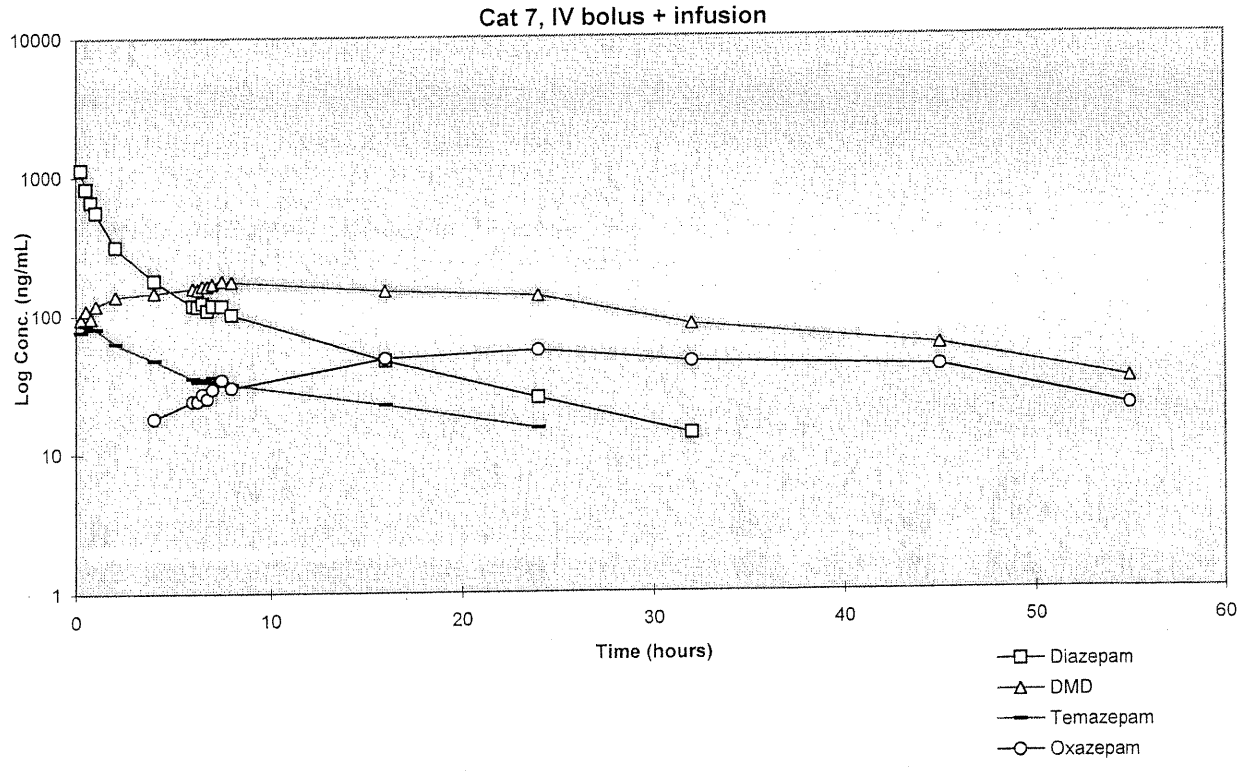
APPENDIX D5: PROFILE OF CAT 5



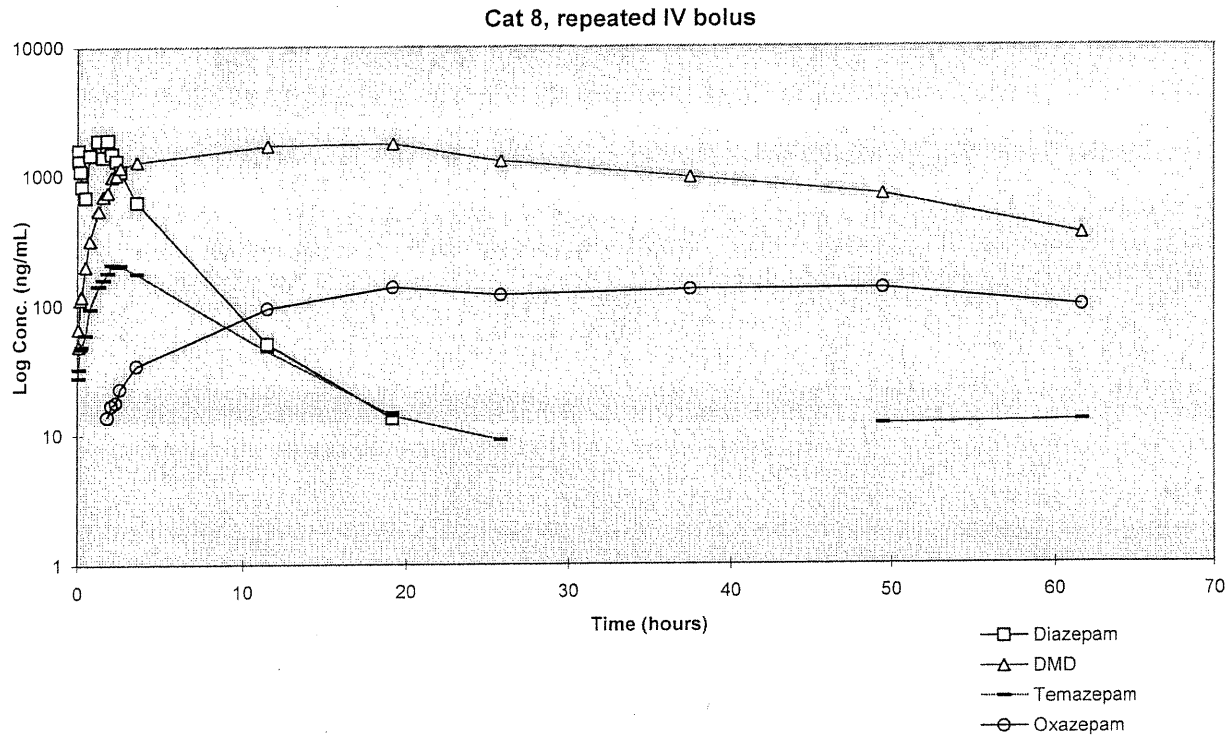


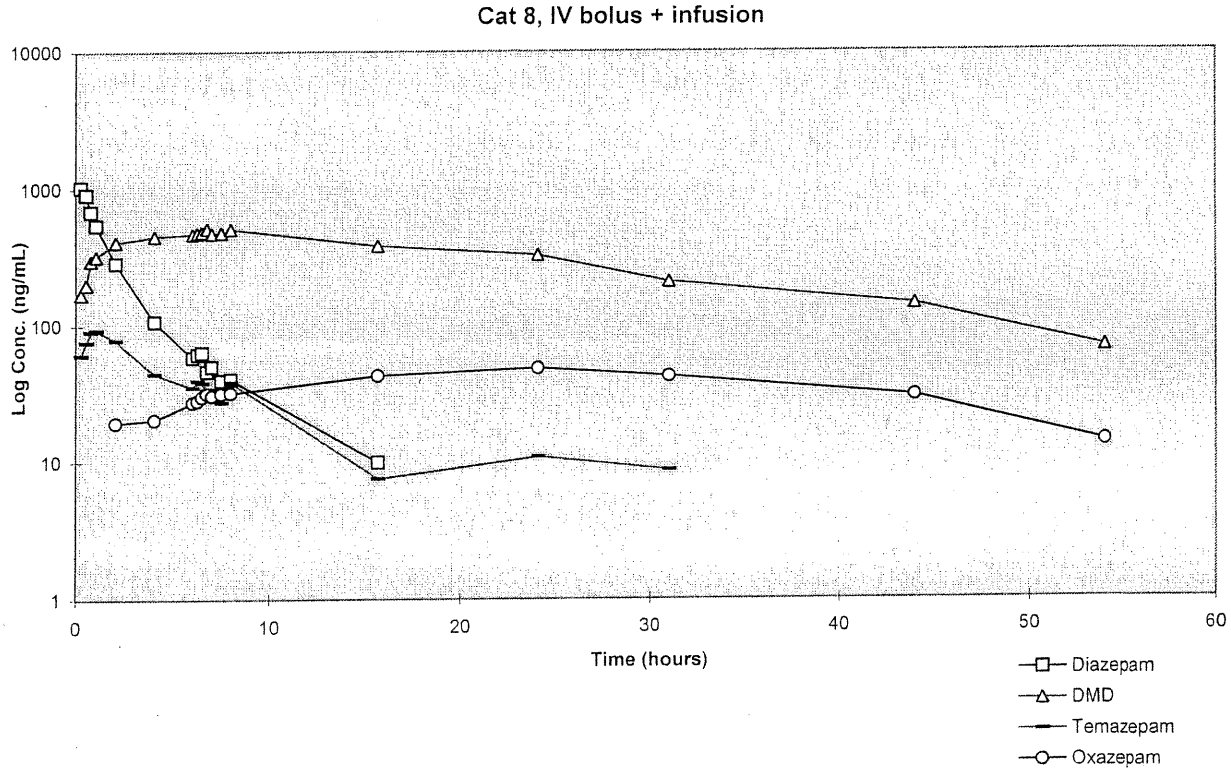
APPENDIX D6: PROFILE OF CAT 7





APPENDIX D7: PROFILE OF CAT 8





APPENDIX E: Example of WinNonlin ASCII File

Dog 1 phase 1

Input File: Data - [Untitled.1]

Start Time: 11:47:45 11-12-1999
End Time: 11:47:45 11-12-1999

WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM (V01.5A)
Core Version 14Jul97

Listing of input commands

```
MODEL 201
NVARIABLES 2
NPOINTS 100
XNUMBER 1
YNUMBER 2
DTIME 0
NCONSTANTS 1
CONSTANTS 88000
METHOD 2  'Linear trapezoidal
BTIME 4.5,16.5
MISSING 'Missing'
NOBSERVATIONS 17
DATA 'WINNLIN.DAT'
BEGIN
```

WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM

Noncompartmental Analysis for Bolus IV Administration

Linear Trapezoidal Rule Used to Compute AUC, AUMC

X		Y	Pred.	Res.	AUC	AUMC	WEIGHT
.0000	@	4801.			.0000	.0000	
.5000E-01		1864.			166.6	2.330	
.8300E-01		998.3			213.9	5.235	
.1700		606.5			283.7	13.32	
.2500		411.4			324.4	21.56	
.5000		178.3			398.1	45.56	
.7500		579.6			492.8	111.0	
1.250		682.7			808.4	433.1	
1.500		368.1			939.8	608.8	
1.750		823.5			1089.	857.9	
2.000		424.9			1245.	1144.	
2.250		250.7			1329.	1321.	
2.500		215.8			1388.	1459.	
4.500	*	73.90	75.10	-1.202	1677.	2331.	1.000
7.500	*	51.00	55.44	-4.439	1865.	3404.	1.000
10.50	*	50.30	40.92	9.375	2017.	4770.	1.000
13.50	*	27.40	30.21	-2.810	2133.	6117.	1.000

APPENDIX E: Example of WinNonlin ASCII File (Cont'd)

Dog 1 phase 1 (Cont'd)

16.50 * 22.10 22.30 -.2008 2207. 7218. 1.000

---

*) Starred values were included in estimation of Lambda_z.

@) Note - the concentration at time zero (DTIME) was added for extrapolation purposes

Dosing_time	.0000
Rsq	.9395
Rsq(adjusted)	.9193
Corr(x:y)	-.9693
Tmax	.0000
Cmax	4801.0035
No._points_Lambda_z	5
Tlast	16.5000
Clast	22.1000
AUClast	2207.3153
Lambda_z	.1012
Lambda_z_lower	4.5000
Lambda_z_upper	16.5000
t1/2_Lambda_z	6.8503
AUCall	2207.3153
AUCINF(observed)	2425.7269
AUCINF(observed)/D	.0276
AUC_%Extrap(obs.)	9.0040
Vz(observed)	358.5289
Cl(observed)	36.2778
AUCINF(predicted)	2427.7113
AUCINF(predicted)/D	.0276
AUC_%Back_Ext(obs.)	6.8691
AUC_%Back_Ext(pred.)	6.8635
AUC_%Extrap(pred.)	9.0783
Vz(predicted)	358.2359
Cl(predicted)	36.2481
AUMClast	7218.4595
AUMCINF(observed)	12980.7860
AUMC_%Extrap(obs.)	44.3912
AUMCINF(predicted)	13033.1404
AUMC_%Extrap(pred.)	44.6146
MRTlast	3.2702
MRTINF(observed)	5.3513
Vss(observed)	194.1332
MRTINF(predicted)	5.3685
Vss(predicted)	194.5977

NORMAL ENDING