THE PHARMACOKINETICS OF DIMINAZENE ACETURATE AFTER INTRAMUSCULAR AND INTRAVENOUS ADMINISTRATION IN THE HEALTHY DOG

By
Dr. David B. Miller

Supervisor
Prof. G. E. Swan

Co-supervisors
Prof. R. G. Lobetti
Dr. L. S. Jacobson

Submitted in partial fulfillment for
the requirements of the degree

MMedVet (Med)

Department of Companion Animal Clinical Studies
Faculty of Veterinary Science
University of Pretoria,
0002

March 2003

DEDICATIONS

To my wife Sarah, thank you for putting up with my studies.
To my All Mighty GOD.
Thank You for the ability, knowledge and will to study the wonders of Thy creation.

ACKNOWLEDGEMENTS

The author wishes to thank the University of Pretoria for the opportunity to further his career and to do this research. I would also like to thank the Dean, Onderstepoort, Faculty of Veterinary Science, University of Pretoria and the South African Veterinary Foundation for the required funding to perform this work. Special thanks go to the South African Police Services for the loan of the dogs used in these studies.

I am indebted to a lot of people for the moral, physical and mental support given to me during the duration of the MMedVet (They appear in no specific order).

Mrs. Motjie Mulders and Dr. Linda Jacobson who taught me the “joys” of lab work and Mr. Jan du Preez who performed the HPLC tests. Proff. Gerry E. Swan and Remo Lobetti and Dr’s. Linda Jacobson, Rowan Milner, Deon van der Merwe, Ronette Gehring and Tarquin Vaughn-Scott, as well as Joseph Somo who helped ensure that all the blood samples were drawn exactly on time. Jacques Visser, Dreyer Schoeman, Petrus Scheepers and Lucie Runnals who took the dogs for daily walks and whose love of animals and empathy ensured that the study was a wonderful experience for all the dogs.

To Wayne Berry who inspired me to specialise in small animal medicine and to my co-promoters Professor Remo Lobetti and Dr. Linda Jacobson, you were excellent and Linda, thanks for the regular butt kicking.

Last but most definitely no least, I want to thank my Supervisor, Professor Gerry Swan, who put up with my warped sense of humour (needed to cope with pharmacokinetic analysis) and helped me through the Win–Nonlin mine-fields, ever instilling his enthusiasm into the project. Thank you very much Gerry.

RESUME

Diminazene is the therapy of choice for canine babesiosis in South Africa. Differences in the dosage described for diminazene usage and the occurrence of mortality at doses equal to or close to the recommended treatment dose for the treatment of canine babesiosis have been described. This has necessitated the need to more fully understand the absorption and disposition of diminazene in dogs.

An intravenous (i.v.) as well as an intramuscular (i.m.) pharmacokinetic study was conducted to determine the pharmacokinetics of diminazine in healthy dogs as well as to describe the binding characteristics of diminazine (in the blood) *in vivo* and *in vitro*.

Diminazene pharmacokinetics showed a large inter-individual variation after i.m. administration at 4.2 mg/kg (% CV 37 – 163) with a rapid absorption (K01-Hl - 6.6 $\pm$ 10.8 min resulting in a C_{max} of 1849 $\pm$ 268.7 ng/ml at T_{max} of 20 min. There was a rapid distribution phase ($T_{½\alpha}$ 21.6 $\pm$ 11.4 min) with the distribution into the peripheral compartment being more rapid than the distribution back in to the central compartment. A mean elimination half-life ($T_{½\beta}$ 5.31 $\pm$ 3.89 h) was derived. At 1 h after i.m. injection, 75 % of the diminazene in whole blood was in the plasma fraction. Compartmental analysis of the i.v. data after diminazene administration at 2 mg/kg revealed a C_{max} of 3725 $\pm$ 1672.8 ng/ml with a rapid distribution phase ($T_{½\alpha}$ 7.0 $\pm$ 6.2 min) with a long elimination half-life ($T_{½\beta}$ - 32.0 ± 28.8 h).

The distribution into the peripheral compartment was more rapid than the distribution back into the central compartment as measured by K_{12} and K_{21} (K_{12} - 8.78 $\pm$ 8.71;

K_{21} 0.32 ± 0.25). The i.v. pharmacokinetic results were very variable between the dogs with a % CV of 55.5 – 137.2.

We hypothesize that the rapid distribution phase is a result of diminazene being sequestered into the liver, followed by a slow terminal phase were diminazene is both redistributed to the peripheral tissues and renally excreted. The $T_{½\beta}$ of 32.0 ± 28.8 h in the i.v. study is considerably longer than the elimination half-life ($T_{½\beta}$ - 5.31 ± 3.89 h) found in the i.m. study. This is most likely due the 25 ng/ml limit of detection of the HPLC, detecting the i.v. tail but not the i.m. tail. This is not surprising as the C_{max} levels following i.v administration were more than 2 times higher than after i.m. administration.

Further pharmacokinetic studies with diminazene in dogs should take account of the rapid absorption of diminazene after i.m. administration and the low levels of diminazene in the terminal phases. The initial sequestration of diminazene in the liver and distribution to the peripheral compartment needs further clarification. With the knowledge gained of the pharmacokinetics of diminazene in healthy dogs, a population pharmacokinetic study in dogs with babesiosis is recommended. This will allow us to more fully appreciate alterations in the pharmacokinetics of diminazene in diseased populations and the potential covariants exerting an effect.

It is our current recommendation that diminazene given i.m. at 4.2 mg/kg not be repeated within a 21 day period.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

CHAPTER 1

INTRODUCTION

Babesia canis is a common tick-transmitted intra-erythrocytic protozoan parasite of dogs in South Africa[62]. The incidence of canine babesiosis at the outpatients clinic of the Onderstepoort Veterinary Academic Hospital [OVAH] over a six-year period was 11.7% (1253 of 10710 sick dogs presented per year)[55].

Diminazene is available in multiple formulations for the treatment of *B. canis*[58]. The value of the antibabesial market in South Africa (all species) has grown steadily over the last few years and was worth R7.293 million in the financial year 2002 (1997 – R5.56 million, 1998 – R6.82 million, 1999 – R7.11 million, 2000 – R7.039 million, 2001 – R6.77 million). This represents approximately 1 % of the South African veterinary drug market of R844.02 million in 2002 (Agriculture, Veterinary and Chemical Association of South Africa [AVCASA], PO Box 1995, Halfway House, South Africa).

Little pharmacokinetic work with diminazene aceturate has been done in dogs. Differences in the dosage described for diminazene usage and the occurrence of mortality at doses equal to or close to the recommended treatment dose for the treatment of canine babesiosis have been described[33, 42, 43, 49, 57]. This has necessitated the need to more fully understand the absorption and disposition of diminazene in dogs.

The objective of the current study was to determine the pharmacokinetics of diminazine in healthy dogs as well as to describe the binding characteristics of diminazine in the blood of these dogs.

This work was performed as part of the research theme, "Infectious tropical diseases of sub-Saharan Africa". The project was chosen due to the commonly fielded questions at the Faculty regarding dogs that have been treated correctly for canine babesiosis but still have clinical signs that can be attributed to the disease, with parasites still visible on their blood smears. By defining the "typical" pharmacokinetics of diminazene in healthy dogs, the groundwork has been laid for a study looking into the population pharmacokinetics for diminazene in a population of dogs with naturally occurring disease. These studies will hopefully shed light on why dogs with babesiosis appear to differ in their responses to chemotherapy with diminazene.

CHAPTER 2

LITERATURE REVIEW

2.1 DESCRIPTION OF DIMINAZENE

"Remarkable therapeutic success has been obtained during the last years in the treatment of protozoal diseases in domestic animals using a novel drug developed in the research laboratories of Fabwerke Hoechst A.G.". R. Fussgänger wrote this, in 1955, about his work on diminazene aceturate (Berenil), an aromatic diamidine compound discovered in 1944[18]. This aromatic diamidine was developed from a drug called "Congasin" and other aminoquinaldines that were found to be active against trypanosomes and other babesias'[19].

In South Africa, diminazene is sold as Berenil (Intervet), Dimisol (Virbac), Babezene (Milborrow–Bayer AH), Berenil RTU (Intervet), Crede–Bab–Minazene (Experto Vet), Dizene Cattle (Virbac) and Veriben (Sanofi AH)[58] and has historically been marketed as Azidine and Ganasang elsewhere in the world.

2.2 CHEMICAL AND PHYSICAL CHARACTERISTICS OF DIMINAZENE

2.2.1 Chemical structure

Chemically, diminazene is an aromatic diamidine. Despite the mode of action being similar to that of other diamidines[20] it has significant quantitative differences from these preparations, which is presumed to lie in the nature of the bridge which joins

the two benzamidine rings in the molecule[26]. The diaceturate salt found favour above dilactate because it was found to have a better solubility[20].

Diminazene aceturate is an N-acetyl glycine compound chemically described as 4-4'(diazoamino) dibenamidine diaceturate; 1,3–bis (p–amidinophenyl) triazene bis (N–acetylglycinate) diaceturate; 1,3 bis (4–guanylphenyl) triazene diaceturate; and 4–4'–diamidinodiazoaminobenzene diaceturate[37, 65].

2.2.2 Physicochemical characteristics

The diminazene products used to treat babesiosis in South Africa contain a combination of diminazene aceturate and antipyrine (1,2-dihydro-1,5-dimethyl-2-phenyl-3-h-pyrazole-3-one) mostly in a concentration of 45% m/m and 55% m/m, respectively[27]. Aqueous solutions of this preparation may remain stable at room temperature for 10–15 days. These solutions are required to conform to a pH range of 5.0 – 5.6 for a 10% m/v solution in water[27].

Diminazene has a molecular weight of 515.5 and decomposes at 217 ^{o}C. It is soluble in 14 parts of water at 20 ^{o}C, is slightly soluble in alcohol and only slightly soluble in ether or chloroform[37]. Due to the fact that diminazene aceturate consists of an organic base and an organic acid, once it is dissolved in water, it dissociates and each component has its own characteristics. Antipyrine is also an organic base[37].

2.3 GENERAL PHARMACOLOGICAL FEATURES OF DIMINAZENE

2.3.1 Mechanism of action of diminazene aceturate

Today, over 50 years after its development, the exact mechanism of action and *in vivo* behaviour of diminazene is still poorly understood. Its effect on the *Babesia* parasite appears to relate to interference with aerobic glycolysis, as well as with synthesis of DNA in the parasite[8, 10, 60].

Some of the exact actions have recently been elucidated[51]. Diminazene as an anti-trypanosomal agent binds to the AT-rich regions of nucleic acid duplexes. Binding occurs via complexation into the minor grooves of AT-rich domains of the DNA double helices. It can bind to DNA as well as RNA duplexes, while exhibiting properties characteristic of both intercalation and minor groove binding. This binding unwinds negative supercoils in plasmids and has also been found to interfere with the activities of the eukaryotic type II topo-isomerases enzymes[52]. A concentration-dependent inhibition of membrane Ca^{++}-ATPase activity, as well as significant secondary binding of diminazene within DNA corresponding to G+C rich sites have also been reported[8, 10].

2.3.2 Side effects and toxicity

Pharmacological studies showed that diminazene lowers the blood pressure for a few minutes (min) when given intravenously (i.v.) to dogs and cats. This was hypothesised to be mainly due to peripheral vasodilation[65]. A fall in blood pressure

was not observed after intramuscular (i.m.) injection[62]. Parenteral administration of diminazene occasionally resulted in acute clinical signs, with vomiting and diarrhoea seen far less often than seen with phenamidine usage[43].

Local tolerance of the drug was tested by intracutaneous and intramuscular injection in rabbits. Mild erythema was seen after 24 hours (h) but it cleared within 8 days[22]. During intramuscular treatment of over 1000 domestic animals with diminazene at 3.5 mg/kg, Bauer, very rarely, observed a mild transient swelling at the injection site[11, 12].

Losos[33] found mild intramuscular oedema at the site of injection 1-3 days after i.m. diminazene administration in dogs that died after natural infection with either babesiosis or trypanosomiasis. Bleeding and malacia in the mesencephalon and diencephalon was the predominant *post mortem* lesion seen. Losos then treated healthy dogs with 15 mg/kg of diminazene i.m. and found that the induced brain lesions mimicked the brain lesions seen in the dogs naturally infected with babesiosis[33].

Bauer[11] reported that the greatest tolerated dose of diminazene in healthy dogs in his studies was 20 mg/kg i.m. and 12.5 mg/kg intravenously. Fussganger and Bauer[19] later reported that the main signs of acute diminazene toxicity were central nervous system signs. Tremor, nystagmus and ataxia were observed at lower doses, whilst higher doses resulted in spasms, uncoordinated movements, vomiting and eventually death in dogs 2–3 days after a dose of 30–35 mg/kg of diminazene intramuscularly. They also reported a study where diminazene's highest tolerated dose was 50 mg/kg i.m. daily for 5 days[11, 12, 18, 19, 20]. Enigk and Reusse[15] observed no sign of illness in healthy dogs given ten doses of diminazene within 25 days at 50 mg/kg

intramuscularly. However, diminazene is reported to have a low therapeutic index, with the highest tolerated dose in dogs being 20 mg/kg i.m. and 12.5 mg/kg i.v. in other studies[60]. The toxic dose varies between individuals and single doses at 4.2 mg/kg have been reported to cause clinical signs of mid-brain toxicity[33, 49]**.** Our clinical experience shows that these cases are rare and this is backed by the scarcity of these reports in the literature[49]. The histopathological changes in the brain due to diminazene toxicity (as described by Naude *et al*) can be impossible to differentiate from mid-brain lesions caused by cerebral babesiosis[43].

Healthy dogs given i.m. diminazene (multiple dosage regimes) showed severe clinical signs associated with damage to the central nervous system and then died[43]. Interestingly though, one dog was resistant to the toxic effects of diminazene despite repeated daily i.m. treatments at 3.5 mg/kg for 15 and 30 doses, whilst other dogs showed typical clinical signs after two doses. At necropsy the brain was oedematous and showed bilaterally symmetrical haemorrhages together with malacic lesions of the cerebellum, midbrain and thalamus[38, 43]. The incidence of this reaction is not known but it has been reported to occur due to overdose, as well as at therapy at the recommended dose[33, 43, 49].

In over 200 domestic animals treated at 3 mg/kg i.m., there was a very slight transient swelling seen at the injection site[11], this is an uncommon finding and could be caused by either the diminazene or antipyrine crystals[25]. A drop in blood pressure, diarrhoea and vomition has been described[41] but in the series of 200 animals, no parasympathetic signs were observed and Bauer stated that the product was safe in cardio-depressed animals. Vomiting and diarrhoea have been observed in clinically healthy dogs given large doses of diminazene[20].

Previous literature described a parasympathomimetic effect that was hypothesised to be mediated by acetylcholine esterase inhibition following i.v. diminazene administration[65]. Milner found that pseudo-choline esterase levels were not significantly changed 15 min after i.m. diminazene administration[41].

2.3.3 Effective Dose of Diminazene in Protozoan diseases

Fussganger and Bauer[19] found that for the treatment of *Trypanosoma congolense* in dogs and cattle, a single i.m. dose of 2.5 mg/kg resulted in complete cure whilst doses in the dog, ranging from 0.25–1 mg/kg resulted in clinical cure from *B. canis* infection with subsequent infectious premunity. Relapses after low dose diminazene were occasionally seen and all of these relapses recovered when treated with diminazene at higher doses. For complete recovery from *B. canis* infection, with elimination of all parasites, a dosage of 8–10 mg/kg was needed. This dose was also effective at sterilising *B. canis* infections in splenectomised dogs[19]. Enigk and Reusse[15] reported similar results from their studies. They recommended a dose of 2.5–3.5 mg/kg i.m. but found that recovery sometimes occurred with doses as low as 0.2 mg/kg. A dose of 4 mg/kg i.m. sometimes cleared the parasitaemia completely but a dose of 12 mg/kg was necessary for complete sterilisation of all the parasites in all cases studied[15]. Ryley[54] obtained similar results in splenectomised calves. These studies showed that the occurrence of relapse versus infectious premunity or complete sterilisation of protozoan infections has a dose relationship.

In the book, “Babesia of companion animals and man”[61], diminazene's dose rate is described as 3-5 mg/kg i.m. although the drug seems to be effective against *B.*

bigemina at much lower doses[61]. In a handout from the Hoechst Corporation, Bauer reported success after treating dogs with babesiosis at a dose rate of 0.25–1 mg/kg i.m[11]. He found a chemotherapeutic range of 1–6 mg/kg versus *Trypanosoma* and *Babesia canis*. He also referenced multiple articles where over 200 animals (cattle and dogs) treated for babesiosis at 3 mg/kg i.m. showed clinical remission but that a dose of 1 mg/kg was sufficient for clinical cure in animals with an acute form of the disease. Bauer found that at 10-12 mg/kg i.m. diminazene would sterilise *B. canis* infections[11].

The diminazene dose currently in use at the OVAH for treatment of canine babesiosis is 4.2 mg/kg intramuscularly.

2.4 FORMULATIONS

2.4.1 Types

The innovator drug, Berenil, is composed of 45 % m/m diminazene diaceturate and 55 % m/m antipyrin. Aqueous solutions (pH 7) of this preparation are prepared by adding 25 mλ sterile water to 2.36 g of Berenil granules containing 1.05 g of the active component diminazene aceturate. This results in a 42 mg/mλ of diminazene or a 4.2 % m/v solution. The solution is dosed at 1 mλ/10kg representing a dose rate of 4.2 mg/kg[27]. Ready–made or Ready–to–Use solutions [Dimisol (Virbac), Berenil RTU (Intervet), Dizene cattle (Virbac)] have recently been launched on the South African market.

2.4.2 Role of Antipyrine

In most formulations, antipyrin is added to diminazene at a concentration of 55 % m/v as a stabiliser, since diminazene is unstable in water. This represents a dose 5.24 mg/kg of antipyrine when diminazene is administered at its recommended dose[27]. This is 2-4 fold lower than the dose of antipyrine (10 - 20 mg/kg) given i.v. in pharmacokinetic studies[13].

Antipyrine is one of the most extensively used compounds to test the oxidative drug metabolising systems of the liver (cytochrome P-450 linked monooxygenase). Antipyrine is negligibly bound to tissue and plasma proteins[29].

2.5 DIMINAZENE ANALYTICAL METHODS

Through the years multiple analytical methods have been used to quantify concentrations of diminazene in plasma, blood or tissues of animals. These methods ranged from utilising the antibacterial effect of diminazene to inhibit bacterial growth of *Brucella sp* in culture[12], colorimetric analytical methods[32, 47, 48] read spectrophotometrically using a sensitive diphenylamine colour reaction[4], high performance liquid chromatographic (HPLC) methods[1, 25, 34], or through pre-labeling the diminazene with carbon-14 and determining the levels of radioactivity [22, 31].

In the current study, the HPLC method described by Gummow *et al*[24], originally derived from a method described by Aliu and Odegaard[2], was used. This method has a limit of quantification of 25 ng/mλ. The HPLC method was selected due to the fact that it is more specific and sensitive and the results obtained are more suitable for pharmacokinetic analysis.

2.6 PHARMACOKINETICS OF DIMINAZENE ACETURATE

Several studies on the pharmacokinetics of diminazene have been conducted in various species. These are summarized here and in Table 2.1 below.

Rabbits and Rats:

Gilbert[22], reported biphasic pharmacokinetics with maximum blood and interstitial fluid concentrations after 15 min in rabbits following an i.m. injection of 3.5 mg/kg. Using radiolabeled diminazene (Berenil®), the authors found a half-life for the first compartment of 1.3 h (similar to that found in rats by Raether as quoted by Gilbert) and 103 h for the second compartment.

Odika *et al*[45] found that in *T. b. brucei* infected rats, the concentration of diminazene after an i.m. injection of 3.1 mg/kg was significantly higher in the organs of infected compared to non-infected rats. Concurrent administration of lithium chloride with diminazene significantly increased the concentration of diminazene in the brain tissue of the rats.

Goats and Sheep:

In a study performed on the disposition and bioavailability of diminazene in dairy goats Alui and Olegaard[3] found 60-90% of the drug was bound to plasma proteins and reported a elimination half-life of 14 – 30 h.

After administration of diminazene to sheep, peak plasma concentrations of 6.3 – 7.57 ug/mλ at 20 – 45 min were reported[3]. In the same study plasma protein binding

of 65 – 85 % was reported. The systemic availability of the i.m. versus the i.v. dose was 95.10 ± 23.21 % and mean residence time averaged 14.16 ± 1.55 h when diminazene was administered to sheep at 3.5 mg/kg intramuscularly. The pharmacokinetics of diminazene administered i.v. was found to fit a 3-compartmental model.

Cattle:

Klatt and Hadju[32], using a colorimetric method of analysis, during investigation of the pharmacokinetics of a combination of diminazene and rolitetracycline, found a peak concentration of 3.23 μg/mλ of diminazene after 30 min and a second phase of elimination of diminazene with a half-life of 63 h. This long second phase of elimination was considerably shortened when diminazene was given in combination with rolitetracycline.

Kellner, Eckert and Volz[31] studied the disposition of diminazene in two calves. Radioactivity was determined in samples collected after i.m. injection of radiolabeled diminazene at 3.5 mg/kg intramuscularly. Peak blood concentrations of 4.6 and 4.7 ug/mλ occurred after 15 and 40 min and the decrease in concentration followed a biphasic process with half-lives of 2 h and 188 h.

The pharmacokinetics of diminazene in cows was described by Aliu *et al*[1]. Diminazene concentrations were determined using HPLC. Non-linear regression analysis of the i.v. and i.m. data indicated that the plasma disappearance curves were best described by tri-exponential equations. The i.v. bolus had a biphasic distribution with half-lives of 0.04 h and 0.58 h. Diminazene was rapidly absorbed following i.m. administration and peak plasma concentrations (C_{max}) of 4.68 ± 1.12

ug/mλ were attained in 10-15 min. The half–life of the terminal elimination phase was 145.48 h. *In vivo,* after 30 min, the diminazene was partitioned between plasma, whole blood and red blood cells at a ratio of 6.65 ± 0.06; 5.02 ± 0.27 and 1.93 ± 0.87 respectively. After 12 h the partition had changed to 1024 ± 0.08; 1.60 ± 0.07 and 1.99 ± 0.44 respectively. This showed that most of the diminazene was in the plasma 30 min after treatment but that after 12 h the majority of diminazene in the blood was bound to red blood cells. *In vitro,* diminazene was bound to bovine plasma albumin to the extent of 38.01–91.10%[1].

Mamman, Aliu and Peregrine[34] compared the pharmacokinetics of diminazene in non-infected and *T. congolense* infected cattle after a 3.5 mg/kg diminazene injection i.m. There were few significant pharmacokinetic differences between the cattle. However, the maximum concentration of the diminazene in plasma was significantly higher in animals with acute infection (8.25 ± 1.72 μg/mλ) versus animals with chronic infection (5.04 ± 0.26 ug/ml) and the non-infected cattle (4.76 ± 0.76 μg/mλ). Similarly the time to maximum concentration was significantly shorter in the acute infection versus chronic and non-infected cases (18 vs. 36 and 33.75 min)[34].

Gummow, Swan and du Preez[25] in a bioequivalence and pharmacokinetic evaluation of two diminazene aceturate formulations given i.m. in cattle found that a two-compartmental model best described the behaviour of diminazene in cattle. Peak concentrations of diminazene (3.24 ± 0.16 μg/mλ) were reached 49.8 ± 7.6 min after i.m. injection of 3.5 mg/kg of diminazene. They found a half life of absorbtion ($T_{½\alpha}$) of 1.93 ± 0.95 hours. Diminazene was slowly eliminated with a residence time of 13,27 days and a long elimination half life ($T_{½\beta}$ of 222 h).

Mdachi, Murilla, Omukuba and Cagnolati[39] repeatedly infected cows with *T. congolense* and then treated them with a different dose of diminazene each time. The results of their study indicate that that the level of parasitaemia and the degree of anaemia in the animal at the time of treatment affected the distribution, disposition and elimination of diminazene aceturate from the animal.

Mamman and co-workers[35, 36] looked at the pharmacokinetics of diminazene in the plasma and CSF as well as in the plasma and lymph of goats following a 3.5 mg/kg i.m. injection of diminazene. A peak concentration of 4.31 μg/mλ was found in the plasma in the CSF study and the diminazene concentrations in the cerebro-spinal fluid were 3-4 times lower than in the plasma. A median peak plasma concentration of 4.30 μg/mλ was detected in the lymph study. Diminazene could be detected (concentrations not given) in all the plasma samples collected from the goats for 5 weeks (35 days).

Canine studies:

Bauer[12] used serum diminazene concentrations to inhibit growth of *Brucella sp.* in culture as compared to control concentrations of diminazene, thus using the bactericidal action of the drug to biologically determine the blood concentrations after diminazene injection. Concentrations of diminazene as low as 1 μg/mλ could be determined. In these studies peak serum concentrations of diminazene occurred at 3 h (3 μg/mλ) and all traces of diminazene were absent by 24 h. He concluded that diminazene was excreted via the kidneys within 24 hours.

Onyeyili and Anika[47, 48] used a colorimetric method to determine the influence of *T. congolense* on the disposition of diminazene in the dog using each dog as its own control. They reported that drug elimination followed a biphasic process, irrespective of infection but that infection significantly shortened the $T_{½\alpha}$ of diminazene from 0.17 h to 0.12 h, although the urinary excretion of the drug remained constant [47] They also gave 3.5 mg/kg diminazene i.m. to both healthy dogs and dogs with trypanosomiasis. Dogs were autopsied at 48, 72, 120, 168 and 240 h after injection. Mean plasma concentrations were reported as 0.2 ± 0.008 µg/ml, but no peak concentrations were given. No diminazene was found in the plasma after 48 hours. Higher plasma concentrations were found in dogs infected with trypanosomes, and higher tissue concentrations were present in healthy dogs. In all tissues sampled at 48 h, the highest concentrations of diminazene were found in the kidneys and liver in both groups and low diminazene concentrations were found in the brain. Diminazene persisted in the tissues for more than 10 days[48]. A further publication by the same authors, regarding the same study, reported a $T_{½\beta}$ of 9.87 h in healthy dogs and 12.51 h. in *T.b.brucei* infected dogs. The $T_{½\alpha}$ was significantly decreased in dogs after trypanosome infection (0.14 h vs. 0.2 h)[47].

Blood products:

Alvi *et al.*[4] incubated diminazene with blood products and found that binding to plasma and serum was 50% and 35% respectively. On examination of the red blood cells they found that 70% of diminazene was bound to purified haemoglobin and that red blood cell membranes did not show any binding. They concluded that diminazene binds to a number of blood proteins and could cross the red cell membrane to bind to haemoglobin.

2.7 CONCLUSION AND RESEARCH QUESTION

Diminazene is the most frequently used anti-babesial for canine babesiosis at the Onderstepoort Veterinary Academic Hospital. The fact that diminazene toxicity in individual animals is unpredictable and there are some anecdotal reports of the drug not exerting the expected antiparasitic action as well as reported relapses within 3 days to 2 weeks of therapy, dictates the need for further study. Understanding the pharmacokinetics of diminazene will enable us to understand the cause for the variation in the clinical responses seen. As little data on the pharmacokinetics of diminazene in dogs is known we felt that this baseline data was needed. The data is essential to explain the differences in efficacy as well as safety profiles. Pharmacokinetic data will also act as a control when population pharmacokinetic work is done and studies are performed to see if anaemia has the same effect on C_{max} in dogs with babesiosis as was described in cattle infected with *T. b. brucei.*

Table 2.1 Summary of the diminazene pharmacokinetic literature

Animal	n	Dosage		Pharmacokinetic parameter										Reference
		Dose mg/kg	Route	A (µg/mλ)	B (µg/mλ)	α (1/h)	β (1/h)	$T_{½\alpha}$ (h)	$T_{½\beta}$ (h)	Cλ (mλ/kg/min)	Vd (λ/kg)	C_{max} (µg/mλ)	T_{max} (min)	
Dogs	4	7	i.m.									3	180	Bauer 1957
	.	7	i.m.									2-7	60-180	Dibbern1956
	5	3.5	i.v.	4.42	1.55	4.09	0.062	0.17	11.57	140	2.39			Onyeyili 1989
	5	3.5	i.v.	4.0	1.82	3.56	0.072	0.2	9.87	140	0.62 V_{cc} 1.94 Vd			Onyeyili 1989
Cattle	12	2.5	i.m.					3	63		0.6	3.23	30	Klatt/Hadju'76
	2	3.5	i.m.					2	188			4.65	15-45	Kellner et al'85
	5	3.5	i.v					0.04	31.71	1.74	1.91±0.42			Aliu '93
	5	3.5	i.m.	16.5±7.8						1.59	1.84	4.68	14±2.24	Aliu '93
	5	3.5	i.m							144±0.15	1.37±0.17	4.76±0.76	36±8.2	Peregrine '93
	10	3.5	i.m.	2.35	1.65	0.357	0.0031	1.93±0.95	222.14±91		0.92±0.11	3.24±0.16	49.8±7.8	Gummow '94
Rabbit	.	3.5	i.m.					1.3	103			1.1	15	Gilbert 1983
Goats	3	2.0	i.v.	8.41	2.16	0.54	0.068			0.656	0.393			Aliu 1984
	3	3.5	i.m.	2.49	1.45	0.318	0.0355		14-30	0.624	0.9	3.9-4.5	48	Aliu 1984
	5	3.5	i.m.							1.16±0.02	1.14±0.11	4.31±0.22	24.6±17.4	Mamman et al '94
	6	3.5	i.m.							0.63	2.57	4.30		Mamman et al '96
Sheep	4	3.5	i.m.					3.8-5.6		0.89±0.14	0.76±0.18	6.3-7.57		Aliu *et al* '85
	4	2.0	i.v.					0.42	9.3	1.1±0.09	0.56±0.04		20-45	Aliu '85

n	Number of animals in study	α	Distribution constant	β	Elimination constant
A	Distribution phase intercept	Vd	volume of distribution	C_{max}	Peak plasma concentration
B	Elimination phase intercept	$T_{½\alpha}$	Distribution half-life	T_{max}	Time of peak plasma level
$T_{½\beta}$	Elimination half-life	Cλ	Total body clearance		

CHAPTER 3

MATERIALS AND METHODS

3.1 STUDY DESIGN

The pharmacokinetics of diminazene aceturate after intramuscular and intravenous administration was examined in healthy, male, German shepherd dogs in a single group, two-phase study. Intramuscular treatments were administered during Phase I and intravenous treatments during Phase II, following a washout period of 11 days. Only half of the animals used in Phase I were included in Phase II. The binding of diminazene to plasma and red blood cell contents were examined both *in vitro* and on *ex vivo* samples.

The study was approved by the Animal Use and Care Committee and the Research Committee of the Faculty of Veterinary Science, University of Pretoria (No. 36.5.274).

3.2 ANIMALS

Twelve male German shepherd Dogs of the same age (18 months) and weight range (30 $\pm$ 2.5 kg) were used. The dogs were obtained from the Roodeplaat dog breeding station, of the South African Police Service (SAPS). The dogs were returned to the dog breeding station, to continue their normal duties, once the study was completed.

Dogs were included as prospective study animals if they were fully vaccinated, were found to be clinically healthy and were easy to handle. Further selection procedures are detailed below (see Section 3.4)

Dogs that had been treated for *B. canis* in the preceding 3 months, received any chemotherapy within a 3 week period, or had been dipped for ectoparasite control within a 2 - week period prior to the start of the study, were excluded.

3.3 HOUSING AND MANAGEMENT

Two weeks before the study, all the dogs used in the study and the standby dogs were treated with permethrin 2% m/v (Defendog®, Virbac) for ectoparasitic control. The eight dogs selected for the intramuscular study were housed at the Onderstepoort Veterinary Academic Research Unit for four days before the study and for the duration of the study.

The dogs were kept in cages that were clearly marked with the dog's name and a unique number. Collars were fitted to each dog and identified in the same way. Unique markings, as well as the dogs' names, weights and descriptions, were noted and recorded, as a backup in the event that identification problems had arisen.

The hair overlying each dog's cephalic, saphenous and jugular veins was clipped and the dogs were conditioned to people working on their legs and necks. This was

achieved by placing the dogs on a table and performing sham blood collection twice a day.

All dogs were fed the same diet, *viz.* Masterfoods, Pedigree chunks, twice daily at 07h30 and at 16h30 and received the same drinking water *ad libitum*.

3.4 SELECTION OF STUDY ANIMALS

A group of 12 dogs, conforming to the initial inclusion and exclusion criteria were chosen to undergo the selection procedure. All the dogs underwent a full clinical examination, urine analysis, a complete haematology, total serum protein, albumin, globulin, alkaline phosphatase, alanine aminotransferase, urea and creatinine tests to rule out any underlying disease that might affect diminazene pharmacokinetics. In accordance with the inclusion criteria all the test results mentioned above had to be within normal limits[#].

3.5 TREATMENTS

Multiple bottles of diminazene (Berenil)[ϕ], from the same batch, R8469 (08 2002) were weighed to ensure that they contained the correct amount of content. The drug was repackaged by Kyron[θ] laboratories specifically for the study. A part of the contents of each of the bottles was kept to analyse the drug concentration after dilution. The

[#] Reference range for the Section of Clinical Pathology, OVAH.

[ϕ] Hoechst Roussel Vet (Pty) Ltd, PO Box 6065 Halfway House, 1685

[θ] Kyron Laboratories (9Pty) Ltd, POBox 27329 Benrose, 2011

mixture was prepared by reconstituting the 1.05 g diminazene with 25 mλ sterile water to give a resultant volume of 25 mλ and a concentration of 42 mg/mλ.

3.5.1 Intramuscular

Eight dogs were chosen for the i.m. study and three additional dogs that passed the selection criteria were kept on standby at Roodeplaat dog breeding station. The dogs were injected with freshly diluted diminazene at a dose of 4.2 mg/kg. Feed was withdrawn from all the dogs 12 h before treatment and they were fed after the 4 h sample.

Intramuscular injections were performed in the *M. biceps femoris,* midway between the hip and the stifle joints. Dogs were injected at two-minute intervals.

3.5.2 Intravenous study

Pre-study testing, housing, feeding and sample collection were performed exactly the same as in the i.m. study, except that only four of the dogs from the i.m. study were used. The dogs were treated with freshly reconstituted diminazene from the same batch as the i.m. study at 2 mg/kg i.v. via the cephalic vein. This dose was used as it is the reported dose in the majority of i.v. studies reported in the literature and summarised in table 1.

All the dogs were weighed two days prior to the start of the study, following a 12 h period of food withdrawal and after the dogs had been taken out to urinate and

defaecate. The dose of diminazene for each dog was calculated and recorded according to the fasted body weight.

3.6 COLLECTION AND HANDLING OF BLOOD SAMPLES

Blood samples from the dogs treated i.m. were collected 1-2 min pretreatment (Time 0), and over a period of 21 days at 0.33, 0.66, 1, 2, 3, 4, 8, 12, 18, 24, 36, 48, 72, 120, 168, 240, 336 and 504 h post treatment. In dogs treated i.v. samples were collected pre-treatment (Time 0) and over 4 days at 0.08, 0.17, 0.25, 0.5, 0.7, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 18, 24, 36, 48,72 and 96 h post treatment. Sample timing was based on available diminazene pharmacokinetic data, with specific attention given to C_{max} and $T_{½ e\lambda}$, reported in the diminazene literature.

A “dummy run” was performed the day before the i.m. study to familiarise everyone participating with their responsibilities. A schedule was drawn up with the exact time for each sample collection and who would be responsible for drawing the sample. A designated clock-watcher ensured that the schedule was followed to the second. Dogs were injected at two-minute intervals and blood samples were collected at the allotted times. From 0 – 36 h, the blood samples were drawn exactly on time. From, and including, the 48 h sample, the bleeding was performed over a 15 min period within 1 min of the allotted time.

One sample was drawn at each time point except for Time 0 and the one and two hour samples, when two tubes each were drawn. All samples, except the extra 0, 1 and 2 h samples, were drawn in 5 mλ heparin tubes. The extra 1 h sample was a 10

mλ heparin sample and the 0 and 2 h samples were drawn in 5 mλ serum tubes. The 1 h sample was used to examine the blood partitioning of diminazene, whereas the 0 and 2 h serum samples were stored for future examination of any influence that diminazene might have on inflammatory mediators.

The i.v. sample collections were made at the precise allotted times and handled as described for the i.m. study.

The samples were drawn into evacuated, uniquely identified heparinized tubes (vacutainer)• from either the cephalic or jugular veins. The heparinized blood was stored on ice until it was centrifuged.

The tube was centrifuged (Bromma LKB, 2161, Midispin R) at 3000 rpm for 15 min within 30 min of being drawn (except for the extra 1 h sample from the i.m. study, which was processed immediately). The separated plasma was stored at – 20^0C in pre-marked polypropylene tubes. The samples were processed and initially stored at the Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, South Africa.

After completion of collection of all the samples, the samples were transported to Potchefstroom University on dry ice for drug analysis. The samples were transported 1 week after the i.v. study was completed and analysed within the next 2 weeks. Plasma samples from a given animal were all processed on a single day.

3.7 DRUG PARTITIONING IN BLOOD

The eight extra 1 h samples were separated into packed RBC and plasma. The packed RBC were washed 3 times and pooled. The plasma was micro-centrifuged, as described later, and the resulting 8 groups of two fractions (water fraction and filtrate) were pooled separately. The 3 separate pooled fractions (washed red blood cells, plasma and filtered water fraction) were each split into as many 1 ml aliquots as the available volumes allowed. The aliquots were sent for drug analysis, except for 1 backup set which was retained at the Department of Paraclinical Sciences, Faculty of Veterinary Science. The samples were all processed on the same day and 5 replicates of each pooled group were analysed and the average concentration for each fraction determined.

3.8 *IN VITRO* STUDY

Prior to the study, 425 mλ of blood, collected in acid-citrate dextrose "ACD", was drawn from a dog chosen following the same selection criteria as used for the dogs in the pharmacokinetic studies. Three 50 mλ bags of the blood were fortified to 3 different concentrations of diminazene (Berenil®) and the rest of the blood was discarded. These concentrations were prepared by initially adding 10 mg diminazene to 50 mλ water, to make up a stock solution of 10 000 µg/50 mλ or 200 µg/mλ. This was diluted as shown in Table 3.1. The three blood bags were placed in a refrigerator at 4^0C for 24 h to allow drug to red blood cell binding to occur. They were turned every 4h to ensure mixing of the diminazene and blood.

• BD Vacutainer Systems, Preanalytical Solutions, Belliver Industrial Estate, Plymouth. PL6 7BP, UK.

Table 3.1: *In vitro*, diminazene sample preparation

Bag	Blood (mλ)	Volume of stock added (µλ)	µg/50mλ	Final concentration (µg/mλ)
1	50	125	25	0.5
2	50	375	75	1.5
3	50	750	150	3.0

After 24 h, two 10 mλ samples of blood were drawn from each bag, centrifuged and divided into plasma and packed RBC. The packed RBC from each individual bag was washed 3 times using physiological saline and then pooled. Half of the plasma was microcentrifuged through a 10 000 micropore filter at 10000 rpm for 30min (Beckman♦ CS-15R centrifuge with Beckman F1010 head, radius 8 cm). This procedure left three samples of (1) washed packed RBC; (2) plasma and (3) filtered plasma (water fraction). Five replicates of each sample were prepared and frozen in polypropylene tubes. All samples for the *in vitro* study were processed on the same day and 6 replicates were performed for each fraction. These samples also acted as the *in vitro* quality control to test for the repeatability of the analyses.

3.9 DETERMINATION OF DIMINAZENE CONTENT

Paired-ion extraction and high performance liquid chromatographic (HPLC) determination, with a limit of quantification of 25 ng/mλ of diminazene, was performed by the University of Potchefstroom using the technique as described by Gummow, du Preez and Swan[24].

♦ Beckman Coulter PTY LTD, Midrand, South Africa

3.9.1 Apparatus and reagents

Materials and reagents:

A HP 1050 series HPLC, equipped with a HP1050 quanternary pump, HP1050 auto-sampler, HP 1050–diode array detector and Chemstation (Rev.A.04.02) with data acquisition and analysis software, was used. All chemicals used were of analytical grade, except the acetonitrile, which was of HPLC grade. Water was deionised and treated with Milli-Q water treatment system to obtain water suitable for HPLC. A reverse phase column (Luna C18, 150 x 4.6 mm, 5 micron, Phenomenex, Torrance, CA) was used. The washing solvent was 10 % methanol/water and the sample diluent, 0.05 M disodium edetate in water. Sample elution was performed with, 90/10 acetonitrile/0.025 M octane sulphonic acid sodium salt in water containing 2 % glacial acetic acid, as the mobile phase and a flow rate of 1 mλ/min. and injection volume of 100 μλ. A UV detector was used to detect diminazene at 370 nm and the imidocarb internal standard at 254 nm (wavelength switching between the two peaks). Retention time was ± 4.5 min and 7.5 min for diminazene and imidocarb, respectively. The test characteristics of the HPLC test for the assay for diminazene are given in Table 3.2.

3.9.2 Sample preparation

To 1 mλ of plasma, 50 µλ internal standard (stock imidocarb 2.5 µg/mλ) and 1 mλ sample diluent (0.05M disodium edetate in water), were added. The solution was vortexed for 20 seconds and applied to a solid phase extraction column (LC-18 SPE tubes, 100 mg, 1 mλ, Supelco, Bellefonte, PA) that had been prepared by passing 3 mλ methanol through them, followed by 2 mλ water. The samples were passed through the columns at 1 mλ/min or less. The columns were vacuum dried for 10 min and eluted using a 2 mλ elution solution (90/10 acetonitrile/0.025 M octane sulphonic acid and 2% glacial acetic acid in water) into 5 mλ glass tubes. These were evaporated to dryness under a stream of nitrogen in a water-bath at 60^0C and re-dissolved in 250 µλ of the mobile phase. They were then vortexed for 20 seconds, added to 250 µλ microvials and injected onto the HPLC.

With some of the samples, there was less than 1 mλ of plasma available. In these cases, the volume of plasma was measured with a 100 µλ syringe. The volume was noted and the end result adjusted accordingly.

The RBC samples were first homogenised with a Heidolph DIAX600 disperser[ϕ] equipped with a type 6 g disperser tool. The disperser was operated at 9500 rpm for 30 seconds. The sample was then centrifuged at 14000 rpm in an Eppendorf 5415C centrifuge[θ].

Table 3.2 HPLC test characteristics for the assay of diminazene

TEST	RESULT
Specificity	Complies
Range	25 to 2000 ng/mλ

ϕ Heidolph Elektro Gmbh and CO KG, Kelheim, Germany
θ Eppendorf, Hamburg, Germany

Linearity	**R^2 = 0.9987 over range**
Accuracy	**96 % to 101.6 %**
Precision	**RSD 1.3 to 9.4 % over range**
Ruggedness	**Complies**

The accuracy and precision were determined by analysing six sets of spiked samples of known concentration against a set of standards that were prepared separately. Over the concentration range of 25 ng/mλ to 2000 ng/mλ, the method yielded an accuracy of 96 - 101.6 % and precision (%RSD) of 1.3 – 9.4 %. Repeatability was measured by inter-day and intra-day repeatability. The ruggedness of the samples in the mobile phase was such that it allowed multiple analysis by auto-injection to be done. No interference was found from the reagents, as plasma samples from untreated dogs were all negative.

3.10 PHARMACOKINETIC ANALYSIS

Non-linear compartmental analysis of the diminazene plasma concentration versus time data was performed by means of PC Nonlin Version 4.2 (Statistical Consultants, Inc., New York, USA) computer programme using the Nelder-Mead algorithm[44]. Initial pharmacokinetic parameter estimates, used for the non-linear analysis, were derived automatically by initial linear analysis performed by the programme. Akaike's information criterion[66], based upon the mean values of the final estimates of the associated pharmacokinetic parameters and lack of systematic deviations around the fitted disposition curve, was used to determine the number of exponential terms that best described the data.

Primary pharmacokinetic parameters in the intravenous study were derived from a two-compartmental analysis with IV-push input, and first order output, using macro-

constants as primary parameters (Model 8), yielding the micro constants (K10, K12 and K21), the partial exponents (α and ß) and the coefficients (A and B). Secondary disposition parameters, including AUC, $T_{½\alpha}$, $T_{½el}$, elimination constant half-life (K10-HL), total body clearance (Cλ), volume of the central compartment (Vc), apparent volume of distribution at steady state (Vss), AUMC and MRT, were derived from the primary parameters. Total plasma concentration of diminazene at pseudoequilibrium (Cp^0) was calculated as the sum of the coefficients (A+B).

Primary pharmacokinetic parameters for the i.m. study were derived by two-compartmental analysis with first order input, first order output and lag time (Model 13) of the plasma concentration-time data for each individual animal yielding the microconstants K01 and K10. Secondary disposition parameters, including AUC, K01 half-life (K01-HL) and K10 half-life (K10-HL) were derived from the primary parameters utilising standard procedures[21].

Non-compartmental analysis of the plasma concentration versus time data for the i.m. study was also performed. The area under the plasma concentration versus time curve (AUC, zero-moment) and the first non-normalized moment (AUMC) were calculated according to the trapezoidal method from time zero to the last sample time[21]. Extrapolation of AUC to infinity (AUC_{inf}) was performed using the slope of the terminal phase (ß). Since AUMC to infinity could not be determined in some animals these were not reported. The mean residence time (MRT, first moment) was derived from AUC/AUMC. Maximum plasma concentration (C_{max}) of diminazene and time to C_{max} (T_{max}) were read directly from the individual plasma concentrations.

The pharmacokinetic analysis on the i.m. data was truncated at the 72 h sample as diminazene plasma concentrations of 6 of the 8 eight dogs were below the level of detection at this stage and the remaining 2 dogs had very low levels that fluctuated widely .

CHAPTER 4

RESULTS

4.1 CLINICAL EXAMINATION AND CHEMICAL PATHOLOGY

All the dogs examined for use in the study were found to be within normal limits as regards their clinical examination, urine and faecal examination and all serum chemistry tests (Addendum 1). The eight dogs with the best temperaments were chosen for the study. The blood used in the *in vitro* work was drawn from one of the dogs which passed all the selection criteria but was a fear biter.

4.2 ADMINISTRATION OF MEDICATION

Analysis of the reconstituted solution confirmed a concentration of 4.2 % m/v.

4.2.1 Intramuscular study

None of the dogs showed any pain reaction at the time of injection and no muscle swelling was seen at the injection site when the dogs were subjected to their daily clinical examination. Dogs 2, 4 and 8 developed diarrhoea within 20 min of diminazene administration. Dog 8 had diarrhoea again after 45 min while Dog 5 had diarrhoea after 1 h and Dog 7 after 1 h 15 min The diarrhoea was of a watery consistency. All the dogs ate when they were offered food after the 4 h sample had been drawn and none of the dogs exhibited diarrhoea again for the duration of the i.m. study.

4.2.2 Intravenous study

Two of the dogs collapsed within 1-2 min of the i.v. injection. They showed depression and typical parasympathetic signs, namely diarrhoea, salivation and vomiting. The episode lasted for 2-3 min and the dogs were completely normal 5 min after the injection. The other two dogs showed no adverse clinical effects.

4.3 DEVIATIONS FROM MATERIALS AND METHODS

Dog 3, was kicked by a horse whilst being taken for exercise and had the symphysis of his jaw broken 1.5 h before the 120 h sampling time period. The sample was collected at the correct time, where after the dog was operated on. The dog remained in the study.

4.4 ANALYSIS OF MACROMOLECULAR BINDING CHARACTERISTICS OF DIMINAZENE

The macromolecular binding characteristics of diminazene are summarised in Table 4.1. Seventy five percent of the diminazene in whole blood was present in the plasma 1 h after i.m. injection. Twenty four hours after the *in vitro* blood/diminazene admixture, 85 – 94.5 % of the diminazene was extracted from the plasma fraction. The percentage of diminazene in the filtrate (water fraction) was 21 %, 11 % and 19 % of the total plasma diminazene, respectively for the 3 concentrations of diminazene in the *in vitro* study and 24 % for the 1 h samples collected in the i.m. study.

The haematocrit of the 3 groups of washed packed red blood cells in the *in vitro* study was 0.81, 0.84 and 0.87% respectively. The plasma concentration of diminazene from the *in vivo* study, summarised in Table 4.1, was corrected for a haematocrit of 0.5% so that the equations reflected the actual volume of plasma per mℓ of blood rather than the diminazene concentrations per mℓ of plasma. Seventy six percent of the diminazene was extracted from the plasma fraction and the percentage of diminazene bound to the red blood cells was 18.5% of the total plasma diminazene.

Table 4.1: Macromolecular binding of diminazene in canine blood examined *in vitro* and *in vivo.*

SAMPLE	DIMINAZENE CONCENTRATIONS (ng/mλ)			BINDING (%)	
	PLASMA	FILTRATE	RBC's	PLASMA	RBC's
In Vitro					
500 ng/mλ	330	69	48	85	4.8
1500 ng/mλ	1152	127	49	94.5	1.6
3000 ng/mλ	2138	410	148	93.1	4.4
In Vivo					
1 h sample*	913 ± 408**	222**	339**	75.7*	18.5*

* = mean of all dogs.

** = mean of two animals

4.5 PHARMACOKINETIC ANALYSIS OF THE INTRAMUSCULAR STUDY

A semilogarithmic plot of the mean concentrations of the diminazene concentrations versus time after i.m. administration was constructed (Fig 4.1). The derived compartmental and non-compartmental data are summarised in Tables 4.2 and 4.3.

An open, two-compartmental model best described the diminazene plasma concentration versus time profile following i.m. administration in dogs. A secondary

diminazene plasma concentration peak can be seen on the semi-logarithmic plot (Fig. 4.1). This peak resulted from an increase in the plasma concentrations measured at 120 h after treatment in Dog 2 (Annexure II). Similar prominent secondary peaks were not found in the other dogs.

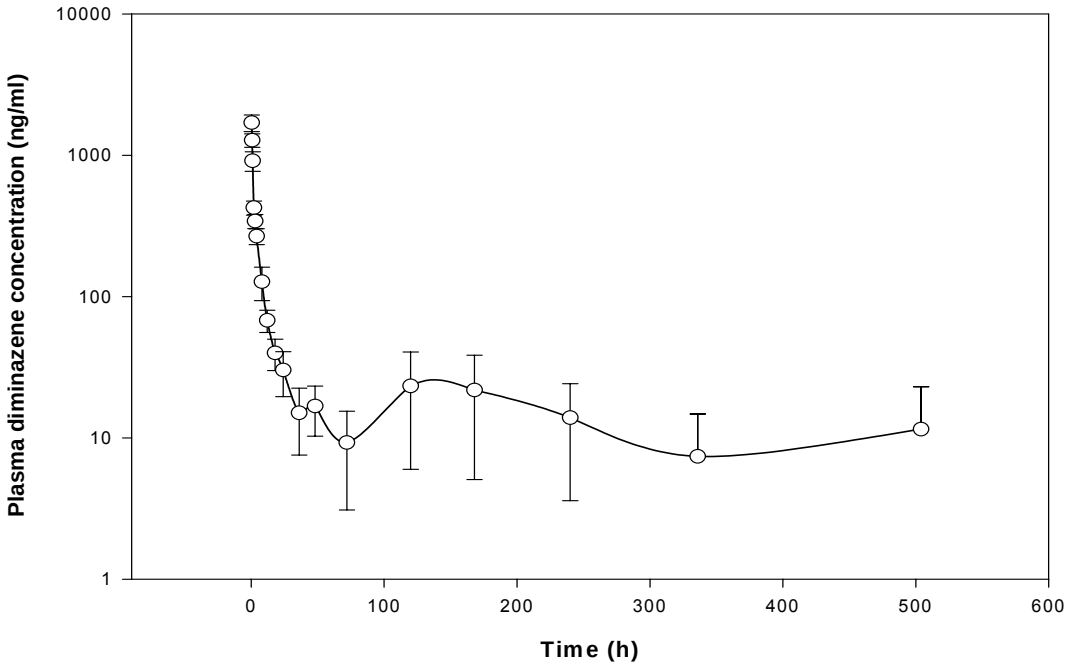

Figure 4.1: Natural logarithm of diminazene plasma concentration (mean± SD) versus time profile in dogs (n=8) following intramuscular administration

Compartmental pharmacokinetic analysis revealed a rapid rate of absorption as measured by the half-life of absorption (K01-HL) 6.6 ± 10.8 min and rapid distribution half-life ($T_{½\alpha}$) 21.6 ± 11.4 min (Table 4.2). The distribution into the peripheral compartment was more rapid than the distribution back into the central compartment.

A mean elimination half-life ($T_{½ß}$) of 5.31 ± 3.89 h was derived. A large inter-subject variation in the pharmacokinetic results occurred (% CV 37 – 163).

Table 4.2: Pharmacokinetic results following intramuscular administration in dogs (n=8) derived by two-compartmental analysis

Pharmaco -kinetic variable	Individual animal results								MEAN ± SD	%CV
	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6	Dog 7	Dog 8		
A (ng/mλ)	25063	3156	4219	2646	2435	2479	15315	3718	7379±83478	113.1
B (ng/mλ)	393	546	566	525	709	802	144	556	530±199	37.5
K01	3.50	5.21	89.34	50.00	50.01	67.97	1.31	75.91	42.9±35.2	82.0
α (h^{-1})	2.87	1.00	3.03	1.76	2.76	2.59	1.13	3.27	2.30±0.88	38.4
β (h^{-1})	0.0840	0.0926	0.1958	0.2019	0.0283	0.3711	0.0536	0.2740	0.163±0.119	73.3
K10	0.80	0.37	1.10	0.76	0.90	1.37	0.50	1.32	0.89±0.36	40.5
K12	1.85	0.47	1.59	0.73	1.28	1.00	0.56	1.55	1.13±0.52	46.0
K21	0.3015	0.2502	0.5410	0.4658	0.8638	0.9283	0.1210	0.6784	0.519±0.291	56.1
K10-HL (h)	0.87	1.87	0.63	0.91	0.77	0.67	1.38	0.52	0.95±0.45	47.4
K01-HL (h)	0.20	0.13	0.01	0.01	0.01	0.01	0.53	0.01	0.11±0.18	163.6
$T_{½\,\alpha}$ (h)	0.24	0.69	0.23	0.40	0.25	0.27	0.61	0.21	0.36±0.19	52.8
$T_{½\,\beta}$ (h)	8.25	7.49	3.54	3.43	2.45	1.87	12.92	2.53	5.31±3.89	73.326

A – distribution phase intercept (initial serum drug concentration), B – elimination phase intercept, K01 – rapid absorption phase, α - distribution constant, β - elimination constant, K10 – elimination constant, K12 – rate constant for drug removal/distribution from central compartment, K21 – Rate constant for distribution from peripheral compartment, K10-HL – terminal elimination phase half life, K01–HL – elimination half-life, $T_{½\,\alpha}$ – half– life of absorption, $T_{½\beta}$ - elimination half– life.

Peak plasma concentrations (C_{max}) of 1849.9 ± 268.7 ng/mλ occurred at 22.3 ± 7 min (T_{max}) after intramuscular administration. An elimination half-live ($T_{½el}$) of 27.5±24.96 h and MRT of 10.32 ± 5.44 h were observed following non-compartmental analysis.

TABLE 4.3: Pharmacokinetic results following intramuscular administration in dogs (n=8) derived by non-compartmental analysis

Pharmacokinetic Variable	Individual animal results								MEAN	SD	%CV
	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6	Dog 7	Dog 8			
Tmax (h)	0.33	0.33	0.33	0.33	0.33	0.33	0.66	0.33	0.37	0.12	31.42
Cmax (ng/mλ)	1998	2188	2083	1983	1632	1779	1361	1775	1850	269	14.52
K_{el}	0.0574	0.0165	0.0224	0.0139	0.0098	0.1854	0.1388	0.1196	0.071	0.068	96.73
$T½_{el}$ (h)	12.07	41.93	31.01	49.77	70.69	3.74	4.99	5.80	27.5	25.0	90.76
AUC_{all} (ng.h/mλ)	6283	9746	4935	4996	4929	3041	3769	3013	5089	2184	42.91
AUC_{inf} (ng.h/mλ)	6510	12348	5517	5930	8091	3111	3863	3122	6062	3078	50.78
Vc/f (λ/kg)	11.2	20.6	34.1	50.9	52.9	7.3	7.8	11.2	24.5	19.1	77.76
Cl/f (mλ/kg/h)	0.6	0.3	0.8	0.7	0.5	1.3	1.1	1.3	0.83	0.37	45.13
MRT (h)	10.01	14.03	14.06	13.37	18.06	3.21	5.42	4.38	10.3	5.4	52.71

Tmax – time when peak plasma drug level is attained, Cmax – peak plasma drug level, K_{el} – rate constant for elimination, $T½_{el}$ – elimination half life, AUC_{all} . area under the plasma concentration curve to the last measurable plasma concentration, AUC_{inf} . projected AUC to infinity, Vc/f – fractional distribution volume of the central compartment, Cl/f – fractional clearance due to product being given i.m, MRT – mean residence time

4.6 INTRAVENOUS STUDY PHARMACOKINETICS

A semi–logarithmic plot of the mean diminazene plasma concentration versus time data of Dogs 2, 3 and 4 following intravenous administration was constructed (Fig 4.2). The derived compartmental pharmacokinetic data are summarised in Table 4.4. The individual diminazene plasma concentration versus time data are given in Appendix III. The data of Dog 1 was not used since the plasma versus time concentration profile was not typical of i.v. administration and it appeared that some of the drug could have been deposited subcutaneously.

The diminazene plasma concentration versus time profile best fitted an open 2-compartmental model for all dogs. In one dog a 3-compartmental model was also found to be adequate. A secondary diminazene plasma concentration peak was observed 12 –18 h after intravenous administration in all dogs.

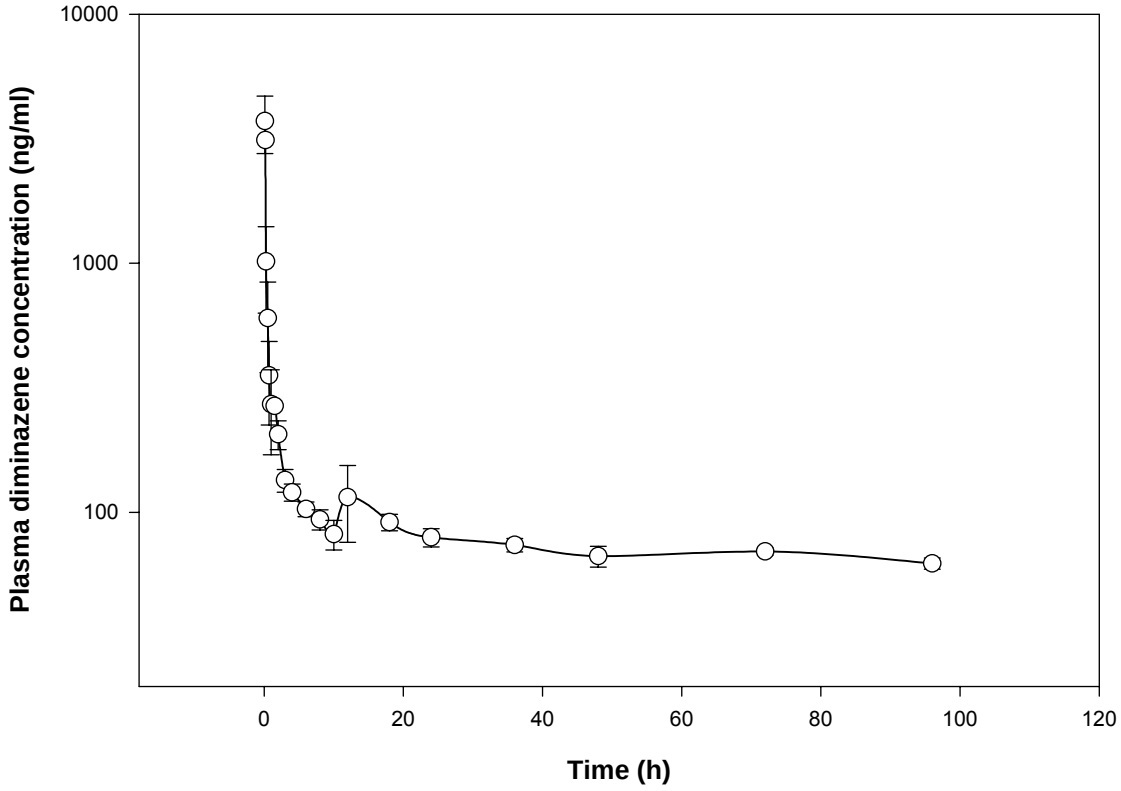

Figure 4.2: Natural logarithm of diminazene plasma concentration (mean±SD) versus time profile in dogs (n=3) following intravenous administration

Compartmental analysis of the i.v. data (Table 4.2) revealed a very rapid $T_{½\alpha}$ (7.0 ± 6.2 min) and a slow $T_{½\beta}$ (32.0 ± 28.8 h). The distribution into the peripheral compartment was more rapid than the distribution back into the central compartment as measured by K_{12} and K_{21}. A large V_{ss} of 8.7 ± 6.1 λ/kg and a slow clearance rate of 0.7 λ/kg/h were observed. The pharmacokinetic results were very variable between the different dogs with a %CV of 55.5 – 137.2.

.

Table 4.4: Pharmacokinetic results following intravenous administration in dogs (n=3) derived by two-compartmental analysis

Pharmacokinetic Variable	Individual animal results			Mean ± SD	% CV
	Dog 2	Dog 3	Dog 4		
A (ng/mλ)	2510.0	9421.3	19381.4	10438 ± 8482	81.26
B (ng/mλ)	135.3	518.8	155	270 ± 216	80.05
α (1/h)	3.00	8.05	21.36	10.8 ± 9.5	87.70
β (1/h)	0.1138	0.2058	0.0218	0.114 ± 0.092	80.84
AUC (ng.h/mλ)	12726	3690	8016	8144 ± 4519	55.49
K10-HL (h)	3.33	0.26	0.28	1.29 ± 1.77	137.2
$T_{½\alpha}$ (h)	0.2312	0.0861	0.0325	0.12 ± 0.10	88
$T_{½\beta}$ (h)	60.91	3.37	31.80	32.0 ± 28.8	90
K10	0.2077	2.6932	2.4371	1.78 ± 1.37	77
K12	2.64	4.95	18.75	8.78 ± 8.71	99
K21	0.1642	0.6153	0.1910	0.32 ± 0.25	78
Cl (λ/kg/h)	0.15	0.55	0.25	0.35 ± 0.2	57
V_c (λ/kg)	0.8	0.2	0.1	0.35 ± 0.35	100
MRT $_{last}$	82.1123	3.3582	40.6884	42.1 ± 39.4	93.68
V_{ss} (λ/kg)	13.55	1.91	10.66	8.7 ± 6.1	70
% Corr	98.6	99.4	99.1	99.0 ± 0.4	0.4

A – distribution phase intercept (initial serum drug concentration), B – elimination phase intercept, α - distribution constant, β - elimination constant, AUC - area under the plasma concentration curve, K10-HL -– terminal elimination phase half life, $T_{½\alpha}$ - distribution half life, $T_{½\beta}$ - elimination half life, K10 – terminal elimination phase, K12 – rate constant for drug removal/distribution from central compartment, K21 – rate constant for drug removal/distribution from peripheral compartment, Cl – total body clearance, V_c – volume of distribution (quantitative estimate of the extent of drug distribution), MRt – mean residence time, V_{ss} –volume of distribution at steady state, % Corr – percentage curve fit for a 2 compartmental model, %CV – percentage coefficient of variance

CHAPTER 5

DISCUSSION

5.1 INTRAVENOUS STUDY

The general pharmacokinetic features of diminazene following i.v. administration observed in the current study were similar to those previously reported. The plasma concentration versus time curve was characterised by a two-compartmental model with a rapid distribution half-life ($T_{½\alpha}$ = 0.12 ± 0.10 h), long elimination half-life ($T_{½\beta}$ = 32.02 ± 28.8 h), large volume of distribution (Vd_{ss} = 8.7 ± 1 ℓ/kg) and an apparent rapid total body clearance (Cℓ = 5.8 mℓ/kg/min). A large intra-subject variation in the disposition of diminazene was observed.

A shorter $T_{½\beta}$, smaller Cℓ and smaller Vd were noted in previous studies[39, 40]. Most of these differences could be ascribed to the more sensitive and specific HPLC analytical method used for the determination of diminazene in plasma in the current study. Diminazene plasma concentrations of 62.3 ± 5.5 ng/mℓ were still present at 96 h following an i.v. dose of 2.0 mg/kg. In the earlier studies diminazene plasma concentrations could only be measured up to 36 h[46, 48] despite a larger i.v. dose (3.5 mg/kg). The colorimetric method used in earlier studies had a sensitivity of 250 ng/mℓ compared to the limit of quantification of 25 ng/mℓ achieved with the HPLC method used in this study. The methods used in the calculation of the pharmacokinetic variables in these studies e.g. Vd and Vd_{ss} further explain the differences in the pharmacokinetic results reported.

In the compartmental i.v. analysis, it was found that the rate of elimination (K10-HL) was relatively rapid while the $T_{½\beta}$ was in excess of 32 h showing a very rapid distribution of the drug from the central compartment, leading to a much slower total body clearance. The large V_{ss} of 8.7 ℓ/kg explains the long half-life as most of the drug was retained in the peripheral compartment and slowly returned to the central compartment. This is confirmed by the K12:K21 ratio of 34:1.

5.2 INTRAMUSCULAR STUDY

This is the first comprehensive study of the pharmacokinetics of diminazene following i.m. administration in dogs and there is therefore little/no comparable published data. A rapid rate of absorption of diminazene was observed following i.m. administration at 4.2 mg/kg. Maximum plasma concentrations (C_{max} - 1850 ± 269 ng/mℓ) were measured at 22.2 ± 6min (T_{max}). The K01–HL was very rapid in most dogs (0.11 ± 0.18 h). It is most likely that the C_{max} was under estimated since the peak plasma concentrations were already measured in the first blood samples collected, at 20 min after treatment, in 7 out of the 8 dogs.

The apparent shorter elimination half-life after i.m. administration as compared to the i.v. study ($T_{½\beta}$ - 5.31 ± 3.89 h vs. 32.02 ± 28.8 h i.v.) is most likely due to the lower diminazene blood concentrations measured in animals after i.m. treatment. It was therefore not possible to determine the terminal half-life accurately. Diminazene plasma concentrations were already below the level of quantification between 12 and 72 h after i.m. treatment in 6 of the 8 dogs. The two other dogs had very low fluctuating diminazene plasma concentrations from 12 – 504 h after treatment.

The absolute bioavailability of diminazene after i.m. administration was 37.7 % after dose correction in the i.v. study. A precipitous drop in the initial plasma concentrations was apparent and can be ascribed to the rapid distribution of diminazene into the peripheral compartment ($T_{½\alpha}$ = 0.12 ± 0.10 h). It seems likely that the liver serves as an initial sump for diminazene. Onyeyili and Anika[47] found that 7 kg dogs given 3.5 mg/kg of diminazene had 81 µg/g of diminazene in their livers 48 h after diminazene administration. This accounts for 78.8 % of the total drug if one takes the liver weight at 3.4 % of body mass[23]. We thus concluded that diminazene is first sequestered in the liver, then is slowly released back into the central compartment and redistributed into less well perfused peripheral tissues, such as the muscle before finally being eliminated. The slower redistribution to and from these peripheral tissues is in our opinion mainly responsible for the long elimination half-life of diminazene. In addition, retention of a portion of the dose at the site of injection could have contributed to the apparent low bioavailablility. Similar retention has been reported in cattle[25]. Further absorption of drug retained at the site of administration is presumed to be slow and prolonged. This, coupled with the rapid peripheral distribution of diminazene could have contributed to the fact that we could not detect the terminal phase of distribution/elimination due to the presence of non-quantifiable plasma concentrations of diminazene. The fact that we may have missed the C_{max} in some of the dogs would also have resulted in a smaller AUC measured after i.m. administration and therefore added to the apparent low bioavailability.

The secondary peak at 30 min in the i.m. study was due to one dog and was thus not considered representative. The peak is not considered to be due to an enterohepatic circulation as the drug is predominantly renally excreted[5, 48]but is probably due to the redistribution of diminazene from the liver[47]. Return of diminazene sequestered in the

muscle is also possible, but due to much lower concentrations within the muscle[47], it is less likely.

Gummow *et al* found that injection site reactions occurred in cattle[25] and reasoned that this could result in secondary peaks. It is possible that the same reaction is responsible for the biphasic absorption seen in our i.m. study but as the distribution phase is faster than renal excretion (normal creatinine clearance rates 2.8 ± 0.96 ml/min/kg[17] to 4.09 ± 0.52 ml/min/kg[50]) we are of the opinion that the sequestration within the liver is the more likely hypothesis. This would also explain the differences in the rate of distribution to and from the peripheral compartment (K12 versus K21).

5.3 TOXICITY

Collapse, salivation and diarrhoea have been described in clinically healthy dogs given large doses of diminazene i.v.[43] but have not previously been reported following i.m. administration. In the investigators clinical experience, vomiting and diarrhoea are rare findings after i.m. injection of diminazene. Furthermore, it would be difficult to ascribe these clinical signs to diminazene in diseased dogs as they could be as a result of the babesiosis, the condition for which the drug is being used. The fact that five of the eight dogs in our trial displayed bouts of diarrhoea after i.m. diminazene administration is worth taking note of. The signs of collapse seen in two dogs in the i.v. study, were the classic described signs for intravenous diminazene. These clinical signs have been ascribed to choline-esterase inhibition, alpha–receptor antagonism, bradykinin and histamine effects[6, 7, 9, 16, 56], although none have been conclusively confirmed. Since it appears unlikely that the signs are caused by

choline-esterase inhibition [41], we hypothesise that the diminazene either acts directly on the parasympathetic receptors or via another messenger system.

5.4 Clinical Implications in Dogs with Babesiosis

Babesiosis may significantly affect the pharmacokinetics of diminazene in the dog. Higher plasma concentrations of diminazene were found in dogs infected with trypanosomes compared to the tissue concentrations present in healthy dogs and the $T_{½\beta}$[5] was found to be 9.87 h in healthy dogs compared to 12.51 h in infected dogs. The total body clearance was significantly lower in healthy dogs than infected dogs, and the distribution half-life was significantly decreased in dogs after infection with *T. brucei*[5]. Higher diminazene[47] residues were also found in the tissues of healthy dogs than in dogs infected with *T. congolense* in all tissues tested except, interestingly enough, in the brain. This is an interesting finding as there is a widely held belief that cerebral diminazene toxicity is more commonly seen in dogs that do not have babesiosis but are treated with diminazene as no other cause for the clinical signs can be found. The distribution half–life was significantly reduced after infection (0.12 h to 0.17 h) and infection increased the rate at which diminazene was distributed to the body[46, 47, 48]. Mamman *et al*[34], in a study of healthy cattle and those with acute and chronic *T. congolense* infection found that C_{max} was significantly greater and that T_{max} was significantly shorter in the acute infection group than in the other 2 groups. Similar studies have not been reported in babesia - infected dogs or cattle but these factors must e considered when trying to determine the influence of disease on the pharmacokinetics.

In dogs with babesiosis factors such as hypotension, anaemia, acidosis, changes in albumin concentrations and altered endothelial integrity due to a systemic inflammatory response syndrome (SIRS)[14, 64], as well as possible altered drug absorption from the injection site may alter the pharmacokinetics of diminazene. Alterations in hepatic and renal perfusion may have a large influence on diminazene distribution and thus may influence plasma concentrations of diminazene and play a role in either potentiating or decreasing the chances of toxicity. Furthermore a definite diagnosis of diminazene toxicity is complicated by the difficulty in distinguishing this from cerebral babesiosis, as well as from the clinical signs of hypoglycaemia in severe babesiosis[30].

Cerebral diminazene toxicity is a rare finding at the OVAH. Cerebral diminazene toxicity has been experimentally induced resulting in central nervous signs, seen secondary to midbrain or thalamic lesion[43]. The cause of the typical brain lesions of diminazene toxicity is not known, however, it was shown that CNS toxicity was dose related[15, 19]. Bauer[11] reported that repeated doses of diminazene could also exert CNS toxicity due to accumulative effects. Our i.v. study results show that diminazene is extensively distributed and has a long elimination half-life, but we do not know how this translates into tissue concentrations within the brain or the effect it may have on transport mechanisms in the blood brain barrier[40, 63]. From the results in our study a washout time of 6 days (five ½ lives) would appear to be sufficient[53]. Following i.m. treatment, an even shorter period would have therefore been adequate. Despite these findings we recommend a washout period of 21 days. Our conservative approach is based on the fact that most of the drug is distributed to the peripheral compartment and the fact that the level of detection was too low to determine the terminal phase and the AUC correctly in the i.m. study. This is further based on the

possibility that the disease process may alter diminazene pharmacokinetics and due to the fact that in one canine study, diminazene persisted in the tissues for over 10 days[48].

In a study by Welzl *et al*[64], it was reported that bile acid levels in severely ill animals with babesiosis were raised above 20 units in 30 of 92 dogs (32.6 %). Jacobson *et al*[28] showed that hypotension is a common phenomenon in clinical babesiosis cases. Hypotension is usually associated with splanchnic vasoconstriction to ensure cerebral blood flow. Apparent differences in the susceptibility of dogs to diminazene toxicity have been implied in South Africa[59] and by the fact that some dogs are resistant to 20 mg/kg diminazene i.m. repeatedly while other dogs have show signs of diminazene toxicity at the recommended dosage[11, 12, 18, 19, 20]. The role of the blood-brain barrier extrusion pumps in the occurrence of ivermectin toxicity in certain breeds[40] raises the question whether this may also be applicable with diminazene toxicity.

These factors are of significance taking into consideration the distribution pharmacokinetics of diminazene, in particular the role of the liver and the increased distribution of diminazene to the brain in *Trypanasoma* infected dogs[47].

5.5 BINDING IN THE BLOOD

Alvi *et al* found that up to 70 % of diminazene was bound to red blood cells as well as to purified haemoglobin. Only 50 % and 30 % was shown to be bound to plasma and serum, respectively. They concluded that diminazene bound to a number of blood proteins and could cross the red cell membrane to bind to haemoglobin[4]. Our *in vitro* work showed diminazene binding to plasma ranged from 85 – 95 % (depending on

the diminazene concentration) after 24 h incubation, whilst the red cell binding was only 1.5–4.8 %. Our pooled one-hour *in vivo* samples, following i.m. injection, showed 75 % plasma binding and 18.5 % red blood cell binding. Most (80 - 89 %) of the diminazene in the plasma was bound in the protein fraction and not in the filtrate. We did not analyse a second sample after 12 or 24 h so we do not know if the red blood cell binding would have increased with time *in vivo*. In our opinion, the binding of diminazene to plasma and or red blood cells does not play an important role in the $T_{½\beta}$.

CONCLUSION AND RECOMMENDATIONS

Diminazene pharmacokinetics has a large inter-individual variation in healthy dogs. A very rapid absorption of diminazene occurred after i.m. administration. After both i.v. and i.m. administration there was a rapid distribution phase, our hypothesis being that the diminazene is first sequestered in the liver, followed by a slow terminal phase where diminazene is both redistributed to the peripheral tissues and renally excreted. The diarrhoea seen after i.m. injection is previously unreported and is worth noting as a potential side effect to warn owners about. A withdrawal period of 21 days is recommended despite the fact that 5 elimination half lives is less than 30 h for the compartmental analysis and less than 6 days for the non-compartmental analysis. We erred on the side of caution as we felt the tail should be better defined before we recommended a shorter withdrawal time. The role of the haematocrit in diminazene blood levels and pharmacokinetics does not seem to play a large role at all, but the role of the liver in the pharmacokinetics of diminazene has potential far-reaching effects as regards toxicity and blood levels and may help to explain why some dog breeds are felt to be more sensitive to the effects of diminazene.

Further pharmacokinetic studies with diminazene in dogs should take account of the very rapid absorbtion and a more sensitive analytical method for the determination of diminazene would be preferable to enable more accurate description of the terminal phase of the plasma concentration versus time profile. The initial sequestration of the diminazene in the liver and distribution to the peripheral compartment needs further clarification as does the role that disease, systemic inflammation and hypotension have on the diminazine pharmacokinetics.

With the knowledge gained of the pharmacokinetics of diminazene in healthy dogs, a population pharmacokinetic study in dogs with babesiosis is recommended. This will allow us to more fully appreciate alterations in pharmacokinetics of diminazene and the potential covariants having an effect. This may lead to the possible contributing causes of diminazene toxicity. These studies should attempt to link diminazene concentrations in the blood to the liver mass and /or function of the patient.

BIBLIOGRAPHY

1. Aliu Y O, Mamman M, Peregrine A S 1993 Pharmacokinetics of diminazene in female Boran (Bos indicus) cattle. *Journal of Veterinary Pharmacology and Therapeutics* 16: 291-300.

2. Aliu Y O, Odegaard S 1983 Paired-ion extraction and high-performance liquid chromatographic determination of diminazene in plasma. *Journal of Chromatography* 276: 218-223.

3. Aliu Y O, Odegaard S, Sognen E 1984 Diminazene/Berenil: bioavailability and disposition in dairy goats. *Acta Veterinaria Scandinavia* 25: 593-596.

4. Alvi N K, Haqqi T M, Hadi S M 1985 Irreversible binding of Berenil, a trypanocidal drug to blood proteins. *Indian Journal of Experimental Biology* 23: 172-173.

5. Anika S M, Onyeyili P A 1989 Effects of trypanosomal infections on the pharmacokinetics of diminazene aceturate in dogs. *Tropical Medicine and Parasitology* 40: 419-421.

6. Arawola R O A, Adepolu F O 1981 The interaction of Berenil with Histamine on isolated mammalian tissues. *Transactions of the Royal Society of Tropical Medicine and Hygeine* 75: 302-3.

7. Arawola R O A, Eyre P 1984 Pharmacological interaction of Three trypanasomal drugs with selected autonomic and autocoid mediator substances. *Tropical Veterinarian* 2: 68-75.

8. Ariyibi A, Odunuga O, Olorunsogo O 2001 Concentration-dependant inhibition of sheep erythrocyte ghost plasma membrane Ca^{2+}-ATPase activity by Berenil [1,3-bis (4'-amidinophenyl) triazene] *in vitro*. *Journal of Veterinary Pharmacology and Therapeutics* 24: 233-236.

9. Bamgbose S O A 1973 Antagonism by Tubocurarine. *Navnyn-scheimdeberg's Archive of Pharmacology* 280: 103-6.

10. Barcelo F, Oritz-Lombardia M, Portugal J 2001 Heterogeneous DNA binding modes of Berenil. *Biochimica et Biophysica Acta, Gene Structure and Expression* 1519: 175-184.

11. Bauer F 1967 Treatment of Babesiasis with Berenil. *Die Blauen Hefte fur die Tierartz* Hoechst Heft 13.

12. Bauer F R, Ergebnisse der klinischen Prufung von Berenil: Hoechst.

13. Boothe D M, Cullen J M, Calvin J A 1994 Antipyrine and caffeine dispositions in clinically normal dogs and dogs with progressive liver disease. *American Journal of Veterinary Research* 55: 254-261.

14. Brady C A, Otto C M 2001 Systemic inflammatory response syndrome, sepsis and multiple organ dysfunction. *Veterinary Clinics of North America Small Animal Practice* 31: 1147-62.

15. Enigk K, Reusse U 1955 Berenil eun neus Heilmettel fur die babesiosen der Haustiere. *Z. Tropenmed. U. Parasit.* 6: 141-150.

16. Eyre P 1972 Effects of some antiprotozoal diamidines on voluntary muscle. *Archives Internationales de Pharmacodynamie et de Therapie* 198: 245-8.

17. Finco D R, Tabaru H, Brown S A, Barsanti J A 1993 Endogenous creatinine clearance measurement of glomerular filtration rate in dogs. *American Journal of Veterinary Research* 54: 1575-1578.

18. Fussgänger R, 1995. Berenil in veterinary medicine: Report from the chemotherapeurical institute Institute of Faberwerke Hoechst, AG.

19. Fussgänger R, Bauer F, 1962. Berenil, a new chemotherapeutic agent in veterinary medicine: The Chemotherapeutic and the Parasitological laboratory of Farbwerke Hoechst, 504-531.

20. Fussgänger R, Bauer F, 1962. Berenil® ein neues Chemotherapeuticum in der Veterinärmedizin. Frankfurt: Faberwerke Hoechst AG.

21. Gibaldi M, Perrier D 1982 *Pharmacokinetics*. Marcel dekker Inc.,New York

22. Gilbert R J 1983 Studies in rabbits on the disposition and trypanocidal activity of the anti-trypanosomal drug, diminazene aceturate (Berenil). *British Journal of Pharmacology* 80: 133-139.

23. Guilford C, Strombeck, Williams, Meyer 1996 *Strombeck's Small Animal Gastroenterology*. W B Saunders,Philadelphia

24. Gummow B, Du Preez J L, Swan G E 1995 Paired-ion extraction and high-performance liquid chromatographic determination of diminazene in cattle plasma: a modified method. *Onderstepoort Journal of Veterinary Research* 62: 1-4.

25. Gummow B, Swan G E, Du Preez J L 1994 A bioequivalence and pharmacokinetic evaluation of two commercial diminazene aceturate formulations administered intramuscularly to cattle. *Onderstepoort Journal of Veterinary Research* 61: 317-326.

26. Hawking F, 1956. Berenil. *Conference on the use of drugs for the control of animal trypanosomiasis*. London.

27. Hoechst. *Berenil (package insert)*. Frankfurt.

28. Jacobson L S, Lobetti R G, Vaughan-Scott T 2000 Blood pressure changes in dogs with babesiosis. *Journal of the South African Veterinary Association* 71: 14-20.

29. Janus K, Antoszek J 1999 The effect of sex on antipyrine metabolism in cattle at different ages. *Journal of Veterinary Pharmacology and Therapeutics* 22: 163-169.

30. Keller N, Jacobson L S, Nel M, Schoeman J P, 2002. Hypoglycaemia in Babesiosis: Common, Catastrophic and Curable. Schoeman J P, ed. *South African Veterinary and Paraveterinary Congress*. Sandton, South Africa: Document Transformation Technologies.

31. Kellner H M, Eckert H G, Volz H M 1985 Studies in cattle on the disposition of the anti-trypanosomal drug diminazene diaceturate (Berenil). *Tropical Medicine and Parasitology* 36: 199-204.

32. Klatt P, Hajdu P 1976 Pharmacokinetic investigations on diminazene and rolitetracycline in comparison to a combination of both. *Veterinary Record* 99: 372-374.

33. Losos G J, Crockett E 1969 Toxicity of Berenil in the Dog. *Veterinary Record* 85: 196.

34. Mamman M, Aliu Y O, Peregrine A S 1993 Comparative pharmacokinetics of Diminazene in noninfected boran (Bos indicus) cattle and boran cattle infected with Trypanosoma congolense. *Antimicrobial Agents and Chemotherapy* 37: 1050-1055.

35. Mamman M, McKeever D J, Aliu Y O, Peregrine A S 1996 Pharmacokinetics of diminazene in plasma and lymph of goats. *American Journal of Veterinary Research* 57: 710-714.

36. Mamman M, Peregrine A S 1994 Pharmacokinetics of diminazene of plasma and cerebrospinal fluid of goats. *Research in Veterinary Science* 57: 253-255.

37. Martindale 1989. Reynolds J E F, Parfitt K, Parsons A V, Sweetman S C, eds. *The Extra Pharmacopoeia*. The Pharmaceutical Press, London: 34-35.

38. McDonald L R, Roberson E L 1988 Antiprotozoan Drugs. Booth N H, McDonald L E, eds. *Veterinary Pharmacology and Therapeutics*. Iowa State University Press, Ames, Iowa: 963.

39. Mdachi R E, Murilla G A, Omukuba J N, Cagnolati V 1995 Disposition of diminazene aceturate (Berenil) in trypanosome-infected pregnant and lactating cows. *Veterinary Parasitology* 58: 215-225.

40. Mealey K L, Bentjen S A, Gray J M, Cantor G H 2001 Ivermectin sensitivity in collies associated with a deletion mutation of the mdr1 gene. *Pharmacogenetics* 11: 727-33.

41. Milner R J, Reyers F, Taylor J H, van den Berg J S 1997 The effect of diminazene aceturate on cholinesterase activity in dogs with canine babesiosis. *Journal of the South African Veterinary Association* 68: 111-3.

42. Moore D J 1979 Therapeutic Implications of □Babesia canis□ infections in dogs. *Journal of the South African Veterinary Association* 50: 346-352.

43. Naude T W, Basson P A, Pienaar J G 1970 Experimental diamidine poisoning due to commonly used babesiocides. *Onderstepoort Journal of Veterinary Research* 37: 173-184.

44. Nelder M, Mead R 1965 A simplex method for function minimization. Computing Journal: 308-313.

45. Odika I E, Asuzu I U, Anika S M 1995 The effects of hyperosmolar agents lithium chloride and sucrose on the brain concentration of diminazene aceturate in rats. *Acta Tropica* 60: 119-125.

46. Onyeyili P A, 1982. The absorption, distribution and excretion of diazoaminobenzene diaceturate (Diminazene) (Ganasang) in goats.

47. Onyeyili P A, Anika S M 1991 Diminazene aceturate residues in the tissues of healthy, *Trypanosoma congolense* and *Trypanosoma brucei brucei* infected dogs. *British Veterinary Journal* 147: 155-162.

48. Onyeyili P A, Anika S M 1989 The influence of *Trypanosoma congolense* infection on the disposition kinetics of diminazene aceturate in the dog. *Veterinary Research Communications* 13: 231-236.

49. Oppong E N W 1969 Toxicity of Berenil in the Dog. *Veterinary Record* 85: 370.

50. Osborne C A, Finco D R 1995 *Canine and feline nephrology and urology*. Williams and wilkins,Philadelphia

51. Pilch D S, Kirolos M A, Liu X, Plum G E, Breslaver K J 1995 Berenil [1,3-bis(4-amidinophenyl) triazene] binding to DNA duplexes and to a RNA duplex: evidence for both intercalative and minor groove binding properties. *Biochemistry* 34: 9962-9976.

52. Portugal J 1994 Berenil acts as a poison of topoisomerase II. *FEBS Letters* 344: 136-138.

53. Ritchell W A 1980 *Handbook of basic pharmakokinetics*. Illinois: Hamilton Drug Intelligence Publications Inc.,Illinois

54. Ryley J F 1957 The chemotherapy of babesia infections. *Annals of Tropical Medicine and Parasitology* 51: 38-49.

55. Shakespeare A S 1995 The incidence of canine babesiosis amongst sick dogs presented to the Onderstepoort Veterinary Academic Hospital. *Journal of the South African Veterinary Association* 66: 247-250.

56. Steinman V, Estler C J, Dann O 1987 Adrenoceptor-blocking properties of some trypanocidal diamidines. *Archives Internationales de Pharmacodynamie et de Therapie.* 290: 207 - 214.

57. Stewart C G 1983 A comparison of the efficacy of isometamidium, amicarbalide, and diminazene against Babesia canis in dogs and the effect on subsequent immunity. *JSAVA* 54: 47-51.

58. Swan G 2002 *Speciality Index Mims, IVS*. Johnnic Publications / Mims,Johannesburg

59. Swan G, 2000. Guidline for the use of Diminazene in dogs. Berea Printers: South African Veterinary Council, 1.

60. Swan G E 1995 Antibabesial drugs in dogs and cats. Lewis B D, Jacobson L S, eds. *Proceedings of a symposium on Canine Babesiosis*. University of Pretoria, Pretoria: 64-68.

61. Taboada J, Merchant S 1991 Babesiosis of Companion Animals and Man. *Veterinary Clinics of North America: Small Animal Practice* 21: 103-122.

62. Van Heerden J 1995 Problems in canine Babesiosis. Lewis B D, Jacobson L S, eds. *Proceedings of a symposium on Canine Babesiosis*. University of Pretoria, Pretoria: 1-7.

63. Webb A A, Muir G D 2000 The blood-brain barrier and its role in inflammation. *Journal of Veterinary Internal Medicine* 14: 399-411.

64. Welzl C, Leisewitz A L, Jacobson L S, Vaughan-Scott T, Myburgh E 2001 Systemic inflammatory response syndrome and multiple organ damage/dysfunction in complicated babesiosis. *Journal of Veterinary Pharmacology and Therapeutics* 72: 158-62.

65. Wien R 1943 The pharmacological actions of certain aromatic diamidines possessing trypanocidal activity. *Annals of Tropical Medicine and Parasitology* 37: 1-18.

66. Yamaoka K, Nakagawa T, Uno T 1978 Statistical moments in pharmacokinetics. *Journal of Pharmacokinetics and Biopharmaceutics*: 547-558.

ADDENDA

Addendum I: Clinical Pathology of the 8 dogs as well as the 9th dog used for the *in vivo* study.

Dog No & Name	*1. Rex*	*2. Wolf*	*3. Rufus*	*4. Gerra*	*5. Xcel*	*6. Gina*	*7. Buddy*	*8. Xist*	*9. Jessie*	*Normal Values*
Tests:	**Clinical Chemistry**									
TSP	56.6	79.1 HIGH	64.4	57.2	55.9	63.6	63.7	65.8	59.9	53 – 75 g/λ
ALB	33.9	32.8	31.5	35.8	35.2HIGH	31.2	31.7	39.9 HIGH	35.7HIGH	27 – 35 g/λ
GLOB	22.7	36.3 HIGH	32.9	21.4	20.7	32.4	32.0	25.9	24.2	20 – 37 g/λ
A/G	1.49	0.71	0.96	1.67	1.70 HIGH	0.96	0.99	1.54 HIGH	1.48 HIGH	0.6 – 1.2
ALT	26	8	20	41	29	26	32	35	20	5 – 40 U/λ 25°C
ALP	18	26 LOW	37 LOW	39	63	22 LOW	25 LOW	No result because of haemolysis	32 LOW	40 - 190 U/λ25°C
Urea	7.7	5.7	6.8	9.3	6.4	5.1	7.7	5.6	7.8	3.6 – 8.9 mmol/λ
Creat	104	113	87	117	9	97	107	95	106	40 – 133 μmol/λ
	Haematology									
Hb	163	189	147	164	152	159	149	165	172	
RCC	7.96	8.83 HIGH	6.53	7.49	6.53	6.61	6.98	7.23	7.58	5.5 – 8.5 x10^{12}/λ
HT	0.477	0.554HIGH	0.428	0.477	0.434	0.456	0.425	0.472	0.497	0.37 – 0.55 λ/λ
MCV	60.0	62.8	65.5	63.7	66.5	68.9	60.9	65.3	65.6	60 – 77 ƒλ
MCHC	34.0	34.1	34.5	34.4	34.9	34.8	35.0	35.0	34.6	32 – 36 g/dλ cells
RDW	18.1	16.3	15.7	17.1	14.9	15.5	15.2	15.2	15.7	%
WCC	10.4	17.0 HIGH	11.2	14.3	10.8	15.3 HIGH	10.0	14.9	15.9 HIGH	6.0 – 15.0 x10^{9}/λ
AbNmat	5.82	9.52	7.39	9.58	5.08	10.10	7.70	9.54	10.02	3.0 – 11.5 x10^{9}/λ
AbNimm	0.00	0.17	0.11	0.00	0.00	0.00	0.00	0.15	0.00	0.0 – 0.5 x10^{9}/λ
AbLymph	2.50	4.93 HIGH	1.57	2.57	4.54	1.99	0.80 LOW	2.68	1.59	1.0 – 4.8 x10^{9}/λ
AbMono	0.73	1.02	0.90	0.86	0.65	1.07	0.60	0.60	2.39 HIGH	0.15 – 1.35x10^{9}/λ
AbEos	1.35	1.36 HIGH	1.23	1.29HIGH	0.54	1.99 HIGH	0.90	1.94 HIGH	1.75 HIGH	0.10 – 1.25x10^{9}/λ
AbBaso	0.00	0.00	0.00	0.00	0.00	0.15 HIGH	0.00	0.00	0.16 HIGH	0.00 – 0.10x10^{9}/λ
Thr C	195.0	217.0	204.0	244.0	277.0	273.0	354.0	353.0	235.0	200 – 500 x10^{9}/λ

Addendum II: Diminazene plasma concentrations following intramuscular administration in dogs.

TIME (h)	Diminazene plasma concentrations (ng/mλ)								MEAN ± SD
	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6	Dog 7	Dog 8	
0.33	1998	2188	2083	1983	1632	1779	159	1775	1699.6 ± 648.6
0.667	1925	1816	1050	1255	949	994	1361	864	1276.7 ± 401.3
1	736	1891	696	924	732	848	869	608	913 ± 408.3
2	397	736	344	412	399	322	480	311	425.1 ± 137.1
3	375	590	342	314	300	283	301	226	341.3 ± 109.4
4	342	468	290	229	216	192	210	192	267.3 ± 96.4
8	148	327	72	102	84	31	194	61	127.3 ± 95.6
12	142	93	66	39	39	61	54	49	67.8 ± 34.6
18	59	95	27	25	34	bld	48	31	39.8 ± 28.2
24	69	74	31	27	40	bld	bld	bld	30.1 ± 29.9
36	42	46	bld	32	bld	bld	bld	bld	15 ± 21.1
48	bld	32	36	26	40	bld	bld	bld	16.7 ± 18.3
72	bld	43	bld	bld	31	bld	bld	bld	9.2 ± 17.4
120	bld	136	50	bld	bld	bld	bld	bld	23.2 ± 48.8
168	bld	133	41	bld	bld	bld	bld	bld	21.7 ± 47.1
240	bld	81	30	bld	bld	bld	bld	bld	13.8 ± 29.1
336	bld	59	bld	bld	bld	bld	bld	bld	7.3 ± 20.9
504	bld	92	bld	bld	bld	bld	bld	bld	11.5 ± 32.5

Bld – below level of detection

Addendum III: Diminazene plasma concentrations following intravenous administration in dogs.

Time (h)	Diminazene plasma concentrations (ng/mλ)			Mean ± SD
	Dog 2	Dog 3	Dog 4	
0.08	2083	5427	3665	3725 ± 1672.8
0.17	Missing	3121	missing	*3121 ± *
0.25	1412	1392	245	1016.3 ± 668.1
0.5	584	1023	198	601.7 ± 412.8
0.7	405	552	108	355 ± 226.2
1	278	445	93	272 ± 176.1
1.5	273	269	260	267.3 ± 6.7
2	151	234	232	205.7 ± 47.4
3	113	130	161	134.7 ± 24.3
4	113	139	109	120.3 ± 16.3
6	89	110	110	103 ± 12.1
8	111	86	84	93.7 ± 15.0
10	99	85	61	81.7 ± 19.2
12	163	67	missing	**115 ± **
18	80	104	90	91.3 ± 12.1
24	79	91	68	79.3 ± 11.5
36	74	82	66	74 ± 8
48	79	63	58	66.7 ± 11.0
72	72	65	72	69.7 ± 4.0
96	66	65	56	62.3 ± 5.5

* representing a single animal

** mean of two animals