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A study of the population pharmacokinetics of diminazene in dogs naturally infected with *Babesia canis*

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For my parents, Willemien, Clara and Katrin.

Thank you.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	Description
α (h^{-1})	distribution constant
β (h^{-1})	elimination constant
A (µg/ml)	distribution phase intercept
AUC (h.µg/ml)	area under the concentration-time curve
AUMC	area under the concentration-time curve at first moment
B (µg/ml)	elimination phase intercept
BSA	body surface area
BW	body weight
C^0_p (µg/ml)	concentration at time zero
Cl (l/kg/h)	total body clearance
C_{max}	maximum plasma concentration
FDA	Food and Drug Administration
HPLC	high performance liquid chromatography
i.m.	intramuscular
i.v.	intravenous
K_{01} (h^{-1})	first order rate constant for absorption
$K_{01}^{half\ life}$ (h)	half life for absorption phase
K_{10} (h^{-1})	first order elimination rate constant
$K_{10}^{half\ life}$ (h)	half life for elimination phase
K_{12} (h^{-1})	rate constant for the distribution from central compartment
K_{21} (h^{-1})	rate constant for the distribution from peripheral compartment
K_{el} (h^{-1})	first order rate constant for elimination
MOF	minimum objective function
Mrt	mean residual time
MS	mental status
OVAH	Onderstepoort Veterinary Academic Hospital
PCV	packed cell volume
θ	fixed effect parameters
$t_{½}$	half-life
$t_{½}^{\beta}$ (h)	elimination half-life
$t_{½}^{\alpha}$ (h)	distribution half-life
$t_{½}^{el}$(h)	half life for elimination
T_{max}	time at maximum plasma concentration
V_c (L/kg)	volume of distribution of the central compartment
V^d_{β} (L/kg)	volume of distribution
VF	fractional volume of distribution
V_{ss} (L/kg)	volume of distribution at steady state
ΔMOF	delta minimum objective function
ε	intra-subject variability, or the residual error
η	random inter-subject variability due to non-identifiable effects
σ^2	population variance of ε
ω^2	population variance of η

SUMMARY

Diminazene is a drug that is commonly used in the treatment of canine babesiosis. Most of the pharmacokinetic work on diminazene has been undertaken in healthy individuals, while the influence of disease on diminazene pharmacokinetics has been investigated to a limited degree. Population pharmacokinetics allows for the investigation of factors (covariates) that influence pharmacokinetic parameters. The aim of this study was to provide a descriptive model of the population pharmacokinetics of intramuscularly administered diminazene in dogs naturally infected with *Babesia canis*. Thirty-nine dogs had 142 plasma samples collected. Another 56 samples from 8 healthy dogs, from a previous study, were added to the data set. Population pharmacokinetics was performed using WinNonMix® (Pharsight, Cary, NC). A one-compartment model was fitted to the data. Health status (presence or absence of babesiosis), packed cell volume (PCV), serum albumin concentrations, mental status (a marker for the severity of illness) and the presence of splenomegaly significantly influenced the population pharmacokinetics model. The PCV lost its significance when these covariates were modelled concurrently, due to its correlation to the health status. In the final model, the volume of distribution (health status and albumin) and K_{01} (health status) was significantly influenced by covariates.

The final estimates of the pharmacokinetic parameters (values are reported as mean ± standard deviation for normally distributed results and median (25%-75%) for non-normally distributed data) for healthy versus babesia-infected animals were:

Parameter	Healthy	Babesia-infected
VF (l/kg)	2.16 ± 0.32†	1.10 ± 0.35†
K_{01} (h^{-1})	16.07 (10.65 - 16.71)‡	23.34 (20.02 – 27.52)‡
K_{10} (h^{-1})	0.67 (0.66 – 0.69)	0.68 (0.64 – 0.75)
AUC (µg.h/ml)	2.76 (2.59 – 3.21)‡	5.66 (4.66 – 7.59)‡
Clearance (l/kg/h)	1.44 ± 0.27†	0.78 ± 0.25†
C_{max} (ug/ml)	1.66 (1.49 – 1.86)‡	3.54 (3.00 – 4.73)‡
T_{max} (h)	0.21 (0.20 – 0.30)‡	0.16 (0.14 – 0.17)‡

†: significant difference (t-test, $p < 0.01$); ‡: significant difference (Mann-Whitney Rank Sum Test, $p < 0.01$)

The results of this study are in agreement with the work by Anika and Onyeyilli on the effects of illness on the pharmacokinetic parameters. We further demonstrated that these effects are largely due to the effect of disease *per se*. Further work is required before this information can be applied clinically.

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

Babesiosis is an intra-erythrocytic protozoan disease of the red blood cells, known to infect man and animals.[57] Various strains of babesia, infecting different species, are known to exist.[57] Natural transmission occurs through the bite of ixodid ticks.[57] In South Africa, babesiosis of dogs is caused by *Babesia canis rossi* that is transmitted by the tick *Haemaphysalis leachi.*[57] *Babesia vogeli* has recently also been identified to cause canine babesiosis in South Africa.[36] It is transmitted by the tick *Rhipicephalus sanguineus*.[36] Babesiosis causes severe anaemia and death if not diagnosed and treated early. Various complications are known to exist.[2,13,21-24,30-32,41,42,57,58]

Babesiosis is an economically significant disease, costing the South African dog owning population over R20 million *per annum* in 1994.[13] At the OVAH (Onderstepoort Veterinary Academic Hospital) 11.7% (1253 cases out of 10710 per year) of ill dogs over a six-year period were diagnosed as having babesiosis.[50]

Diagnosis is made on thin blood smear, and provided the diagnosis is made early with no evidence of complications, prognosis is good. Babesiosis in dogs can be classified as complicated or uncomplicated.[22,24,31] Treatment is based on the severity of disease with mild to moderate cases requiring little more than anti-babesial treatment.[31] Severe uncomplicated cases require anti-babesials as well as blood transfusion, while all cases of complicated disease require additional therapies.

Numerous antiprotozoal drugs at varying doses have been used for the treatment of canine babesiosis.[31,57] Diminazene, imidocarb and trypan blue are commonly used.[31] Diminazene is the most frequently used drug at the OVAH. In a South African survey of canine babesia in private practice, diminazene was the preferred anti-babesial in 88% of respondents.[13] Forty-one percent of respondents noted side effects to diminazene (second to imidocarb). Clinical signs included nervous signs (71%), anaphylaxis (22%) and vomition (17%). Diminazene was the most incriminated drug in cases of suspected treatment failure or relapse.

Relapses following therapeutic dose administrations are known to occur.[8] Four reasons may explain this observation. Firstly, true resistance of *B. canis* to diminazene. Secondly, sub-therapeutic plasma concentrations of diminazene, despite appropriate dosing, due to altered pharmacokinetics. Thirdly, cure occurs, but then re-infection occurs which is interpreted as a relapse. Lastly, diminazene is

babesiostatic at the recommended dose of 4.2mg/kg.[42,44] Failure of the immune system to clear the erythrocytic parasite may result in the relapses.[8]

Diminazene has been shown to be safe and effective in treating dogs with *Babesia canis* infections at the recommended manufacturer's dose. Severe and lethal adverse effects are known to occur at higher doses.[44] However, occasional toxic effects are seen in animals treated at the therapeutic dose for no apparent reason.[42,48] Conversely, animals have been reported to survive apparent lethal doses with no side effects.[44]

Pharmacokinetic inter- and intra-subject variation in individual animals may explain high or low plasma drug concentrations. Dogs infected with trypanosomes have been shown to have altered diminazene pharmacokinetics.[7,46] Similarly, a study in cattle also showed significant differences between *Trypanosoma* infected and non-infected animals.[33] This study aimed at identifying factors (covariates) that may help predict the population pharmacokinetics of diminazene, in dogs infected with *Babesia canis.*

1.2 POPULATION PHARMACOKINETICS

> "Population pharmacokinetics can be defined as a study of the basic features of drug disposition in a population, accounting for the influence of diverse pathophysiological factors on the pharmacokinetics, and explicitly estimating the magnitude of the interindividual and intraindividual variability."[35]

1.2.1 Introduction

All drugs have inter-subject and intra-individual pharmacokinetic variability.[54] These may be insignificant and small or significantly large and clinically important. Population pharmacokinetics is the study of this variability, its source and magnitude in populations. This information is used to design dosage regimens that account for individual patient characteristics.[35] In drugs which are safe and which have a wide therapeutic index, variations in pharmacokinetics between individuals may not be important.[53] In contrast, drugs that have a narrow margin of safety and therapeutic index (with potentially fatal side effects if plasma concentrations are above the therapeutic range), small variations in the drug's pharmacokinetics play a significant role. Population pharmacokinetics

therefore seeks to identify and measure factors, and define the extent of their influence on the dose-concentration interaction.[56]

Dosage regimens have traditionally been determined based on detailed pharmacokinetic studies of a few, typically healthy, individuals. This dosage may therefore not be appropriate in the clinical use of a drug. Diseased animals and humans frequently have disturbed metabolic systems, which may alter drug absorption and disposition when compared to healthy individuals.[35] Flexible dosing may prove to be more appropriate.[65] Determining appropriate drug doses requires estimating the pharmacokinetic parameters (such as clearance and volume of distribution) as they relate to covariates or variables, including the precision of these estimates.[35,55]

The limitations of classical methodologies in investigating pharmacokinetic differences in populations led to the development of a novel pharmacokinetic approach.

1.2.2 Pharmacokinetic variability in populations

Drug pharmacokinetic variability occurs within (intra-subject) and between (inter-subject) individuals. This variability can be attributed to fixed (identified) or random (non-identified or un-measurable) causes, that may also change over time.[53,54,56,65] More specifically, intra-subject variation refers to the (random) differences between an individual's observed pharmacokinetic parameters and that of the individual's predicted model. Inter-subject variability refers to the difference of the individual's kinetic model and that of another distinct individual in the population.[53]

Inter-subject variability can be attributed to fixed effects (variables or covariates that are identifiable and can be quantified). Fixed effects are the population's average values of the pharmacokinetic parameters.[65] These parameters (θ) may be expressed as functions of measurable patient covariates (such as plasma creatinine concentrations) and hence refer to variations between individuals. Inter-subject variability can also be due to non-identifiable random effects (η) that are not described by fixed patient covariates (such as sex, age, body weight, breed, metabolism and illnesses indexes).[35,53,65]

Intra-subject variability (ε), or the residual error, is attributed to random biological changes that occur from day to day (inter-occasional), or due to experimental error (noise). When individuals are sampled on a single occasion, the inter-occasional variation will be reflected in the inter-subject parameter variability, η. This may lead to poor therapeutic dose adjustments, using feedback mechanisms.

Additionally, the increased variability may result in kineticists seeking covariant-parameter interactions when none exist.[56]

True population pharmacokinetic models need to ascertain the degree of influence that random effects have on the models. Repeated measurements of the intra- (ε) and inter-subject (η) variation in a group of individuals would allow for an estimation of the population variance, σ^2 and ω^2, respectively. Depending on the distribution of σ^2 and ω^2, parametric or non-parametric methods would need to be employed during the modelling.[35,53] Models, through their design, may define these distributions to be one or the other.[55] In the case of parametric models, the population means of both ε and η would be zero. These assumptions should be critically assessed during pharmacokinetic modelling as they may not always hold true.

By combining a regression model based on fixed effects with a variance model using σ^2 and ω^2, a probability distribution can be employed to give the most likely outcome of a measurable variable – such as plasma drug concentration, based on the characteristics of the population. The size of the random variability is important as it may determine the efficacy and safety of a drug.[60] The latter is determined by the drug's safety margin and the former by the therapeutic window. Large unexplained variation necessitates a larger safety margin to accommodate the large unexplained variation in drug plasma concentrations.

1.2.3 Evaluation of population kinetics prior to population pharmacokinetic models

The "standard two-stage" and "naïve pooled method" are two non-population-model approaches used to determine population estimates.[35,40,60] Results are less general and require large sampling data per individual.[35] In the two stage approach, classical pharmacokinetic methods (i.e. using compartment and non-compartment models) are used to determine an individual's pharmacokinetic parameters. Population means and standard deviations are calculated on the pharmacokinetic parameters using classical statistical techniques.[53,56,65] Finally these results are compared to covariates by regression statistics. The measurement of the population's variance therefore includes both inter- and intra-subject variability.[35] Studies may need to be repeated in different sub-populations to determine the full range of pharmacokinetic variability in the greater clinical population. An example would be doing a digoxin pharmacokinetic study in dogs with advanced renal failure. There are numerous disadvantages associated with this approach: [35,53,55,65]

1. The sub-population to be investigated needs to be defined, and is by necessity small, homogenous and limited (e.g. 10 male dogs with advanced renal failure). Inter-subject variability is kept to a minimum. Since multiple patient categories will exist (e.g. dogs with

liver but not renal failure), many studies will need to be performed to get the full spectrum of pharmacokinetic data in the greater patient population.

2. Using classical pharmacokinetic studies, large amounts of data points are required. This introduces an ethical component, as frequent blood sampling is required in ill patients. Additionally, frequent sample collection may be detrimental to critically ill patients and excluding them from a trial will introduce bias.
3. Research of this nature is usually expensive.
4. Results are described in terms of pharmacokinetic parameter means and standard deviations. Predictions of an individual's pharmacokinetics cannot be made with probability values, but only with confidence levels of an individual's pharmacokinetic model fitting into the population's range.
5. Rigid experimental designs are needed which cannot combine data sources from different studies.
6. Poor individual pharmacokinetic parameter estimates will result in biased population pharmacokinetic estimates during the second stage.
7. They poorly characterise random effects that influence a population's pharmacokinetic profile.
8. In simulation studies, the traditional approach has been shown to have upward bias of inter-individual variability in comparison with a true population pharmacokinetic model.

The main advantage of this approach is that the research is usually straightforward, prospective and experimental in nature which could hold to better statistical scrutiny when designed properly.[35,53] Given large experimental pharmacokinetic drug trial data, this method may be adequate.[56]

1.2.4 Population pharmacokinetic models – mixed effects approaches

Population pharmacokinetic models allow for an alternative approach in which the clinical trial has been replaced by a modelling method. Here, the emphasis is placed on the individual rather than the population variability with the individual's data being the unit of analysis. As a result of the limitations associated with the traditional population methodologies, Beal and Sheiner developed a single stage hierarchical, nonlinear, mixed effect model used to estimate an individual's response at a particular point.[1,56] The data of all individuals are analysed simultaneously with concurrent inclusion of fixed and random covariates or effects (hence the term "mixed effects").[40]

Population pharmacokinetic models describe drug pharmacokinetics in terms of mean population pharmacokinetics, inter-subject variability and residual (intra-subject) variability.[29] Different models exist and depending on which is used, covariates and random effects are defined as being parametric or non-parametric.[35] There are numerous advantages to using population pharmacokinetic models:[29,35,40,54-56,61,65]

1. The whole population is investigated as a unit, rather than the individual as is done in classical pharmacokinetic studies. This approach also allows sampling of representative treatment populations, as opposed to the small and homogenous study populations usually seen in traditional studies.
2. Data collected may be analysed prospectively or retrospectively, and is frequently done alongside other drug studies. Additionally, the use of "routine" data collected during normal patient care, such as during therapeutic drug monitoring, is cost effective. This data, though, needs to be of a superior quality for it to have meaningful use. Routine patient care covers the cost of sample analysis and these studies consequently become cheaper. Ethical questions are also less concerning, as the sample collection is required for other primary clinical reasons, such as therapeutic monitoring.
3. Population pharmacokinetic studies, by their very nature, allow analyses of sparse data obtained with less stringent study designs, provided that the data is accurate and of good quality. Less data points per subject are required. This must be balanced though by investigating a greater number of individuals. One to 6 data points per individual are required. Dense data sets can also be used.
4. The methods employed do not rely on data being collected from strict experimental designs as population models were designed to deal with observational type data. Population studies allow data from previous studies to be integrated, each adding its strength to the analysis. This feature enables multi-centre data studies.
5. The results are more representative of the true pharmacokinetics of populations.

The most attractive features of population pharmacokinetic studies are that they can quantitatively determine the influence that clinical features (i.e. covariates) have on the population pharmacokinetic parameters.[35] Secondly, they can provide quantitative information about the effect of inter- and intra-subject random variability in estimations of pharmacokinetic parameters. This information enables pharmacokinetic predictions to be made in an individual, relative to the population's model, with a certain degree of probability. Numerous population model programs have been developed, with NONMEM being the first and probably the best known and most widely used.[1] Despite their seemingly magical ability, population models make numerous assumptions about the data that must be valid for the results to be meaningful.[26]

1.2.5 Data requirements

Covariant data required for population pharmacokinetics can be described as 1) pharmacokinetic and 2) demographic.[65]

Pharmacokinetic data can be divided into that which describes or reflects the dosage regimens associated with a plasma concentration or, secondly, concentration-time data which will yield information on the different pharmacokinetic parameters.

Demographic data in turn reflects patient covariates that may affect inter-subject variability and needs to factor in changes of covariates over time. Using sparse (1-6) data points usually precludes the calculation of an individual's pharmacokinetic profile. This problem is overcome by using data from larger numbers of individuals (50-500) and analysing these simultaneously to obtain estimates of the population's pharmacokinetic parameters.[35] Despite the ability to analyse sparse data points collected routinely, emphasis needs to be placed on accuracy, reliability and consistency of data, which may vary with time, due to the nature by which they are collected, and introduce bias.[55]

1.2.6 Study design of population pharmacokinetic studies

Baseline pharmacokinetic parameters and models should be known and the major elimination pathways should have been investigated.[56] The amount of information gleaned from these population pharmacokinetic trials depends on the study design and data available.[56,65] With increasing yields of information, studies can be classified into two broad groups, as either a trough screen or full pharmacokinetic screen.[56]

Trough studies, by their nature require a multiple dosing drug regime in a steady state.[56,65] In a single trough screen, one sample is taken at the trough plasma concentration. Measuring peak plasma concentrations is not advised. This data is presented as a frequency distribution of plasma drug concentration, which will indicate the variability of the drug's trough concentration. Covariates are then regressed against these results. The limitations are that only qualitative information on fixed effects will be gained. Pharmacokinetic information is limited to clearance only. No quantitative information is gained and no other pharmacokinetic parameters can be estimated. Random effects cannot be separated into inter- and intra- subject variability. Strict compliance with dosing prior to collecting plasma is required. Sample times and dosing regimes need to be identical between individuals. These

limitations require fairly rigid designs, else operator induced variability will cloud true random fluctuation and overestimate fixed variability.

Multiple trough screens involve two or more blood samples taken from patients close to trough or steady state levels.[56] Similar disadvantages as the above method applies. However, due to greater individual sampling, fewer individuals are required and precision is increased. Random effects can now be separated into the inter-and intra- individual components.

A full pharmacokinetic screen requires multiple blood samples to be taken from individuals at various time points over the entire concentration time profile.[56,65] Using this approach, estimations of pharmacokinetic parameters are possible with explanations of the observed variability.

1.2.7 Data collection

Data collected must reflect the research questions asked.[65] From the above it can be seen that studies, which seek to identify and quantify fixed and random effects on drug pharmacokinetic parameters, require a full pharmacokinetic screen with concentration-time data. The advantage that allows population pharmacokinetic models to analyse a small number of data points per individual (i.e. <3 plasma concentrations), does not however, imply that classical pharmacokinetic models are identified and pharmacokinetic parameters precisely estimated.[61] The basic pharmacokinetic model should therefore already be described prior to undertaking the study.[60] Inaccuracies of timing in sample collection with single trough studies (i.e. single trough screen) will misreport the inter-subject variability. These experimental or sampling errors will be reflected in the variability accounted by inter-subject factors, rather than intra-subject variability.[60] Avoidance of this pitfall can be ensured by taking multiple samples from a subset of patients in any single trough screen.[60] Applying computer data analyses need to be scrutinised. Models make certain assumptions of about the normality of data and also the distribution of the pharmacokinetic parameters, which may not always have unimodal distributions in a population.[65]

1.2.8 The role of population pharmacokinetics in practice

Population pharmacokinetics describe the influence of variables on pharmacokinetic parameters and as such, they provide the framework required for variable drug dosing regimens[53] and goal orientated

individualised drug dosing[25], the success of which depends on the populations pharmacokinetic parameter's quality and accuracy.[35,65] Information concerning drug residues in production animals can be ascertained[35] and post marketing drug vigilance can be done using routine clinical data.[66]

Population pharmacokinetic studies are encouraged to, and do, play an ever-increasing important role during development and registration of new drugs.[60,61] The Food and Drug Administration (FDA) of the United States is encouraging population pharmacokinetics during drug development and registration.[56,60,66] In 83% (of 47) drug applications to the FDA, population pharmacokinetics impacted on the drug labeling.[56]

1.3 DIMINAZENE

1.3.1 Physical and chemical properties of diminazene

Diminazene is a yellow odourless powder which is poorly soluble in organic solvents, but soluble in water (1:14) and decomposes at 217°C. Its chemical formula is $C_{22}H_{29}N_9O_6$ and it has a molecular weight of 515.53.[12]

1.3.2 Chemotherapeutic properties

Diminazene aceturate (e.g. Berenil®) belongs to the diamidine group of chemotherapeutics. These have been shown to have antitrypanosomal, antiprotozoal, antifungal and antibacterial effects.[11,20]

1.3.3 Absorption and distribution

After intramuscular (i.m.) administration, diminazene is rapidly absorbed. Five minutes after i.m. injection of diminazene, Kellner measured plasma concentrations of 2.7 and 1.6 µg/ml in cattle (maximum plasma concentration, C_{max}, was noted as around twice these values).[27] The half-life ($t_{½}$) of absorption was given as 0.6 hours. In 7 out of 8 dogs given i.m. diminazene, C_{max} was found to be the first plasma sample collected 20 minutes after i.m. administration.[38] It is quite reasonable to assume that maximum plasma concentration was attained at some time with-in the 20 minute period post

administration, although plasma concentrations may still have risen and then dropped to lower concentrations by the time the second samples were taken at 40 minutes. Other studies in different species have shown similar rapid absorption rates. Maximum plasma concentrations are consistently reached in less than one hour.

The absolute bioavailability of i.m. compared to intravenous (i.v.) administration was given as 59% in a canine model, suggesting that absorption from intra-muscular injection sites is not complete over the period measured.[39] Absorption following intra-muscular injections appears to be complete in cattle[3] and sheep. In a study on goats absorption was not complete.[5]

Volume of distribution was larger than calculated body water in a study of cattle.[3] The interstitial fluid concentration reached a peak after 3 hours (±0.2µg/ml) in rabbits given 3.5 mg/kg diminazene intra-muscularly.[17]

1.3.4 Metabolism

In a study of 2 calves, diminazene was excreted unchanged of the urine in one animal while in the other, 74% of excreted diminazene was unchanged.[27] No diminazene metabolites were found on chromatography in the liver of 2 calves. Mdachi *et al* claim from unpublished data that there is little metabolism and breakdown of ^{14}C -radiolabeled drug.[37] No metabolic breakdown product could be found in the plasma and urine of rabbits.[17]

1.3.5 Elimination

Healthy dogs have been shown to have an elimination half-life of between 9.87 and 32.02 hours (Table 1.1). In an intravenous study the elimination half-life ($t_{½}^{el}$) was reported as 207 hours.[39] This was attributed to a long tail being measured. The author comments that non-compartment models could serve better for estimations of washout periods.

Diminazene is largely excreted in the urine and is probably a function (in cattle[3]) of glomerular filtration and tubular reabsorption processes as the renal clearance was less than inulin clearance.[7,17,27] Twenty eight percent and 27% of diminazene was recovered in the urine 72 hours

after intravenous administration in healthy dogs.[7,46] Clearance (Cl) in the dog is reported to range from 0.14 to 0.7 l/kg/h (Table 1.1).

The first 24 hour urine excretion accounted for an average of 28% of total urine excreted diminazene in a study on 2 calves.[27] Another study demonstrated that only 7 - 9% of diminazene was eliminated in the urine following intra-muscular injection in 5 cattle after 24 hrs.[3] After 7 days, 47.1% of diminazene had been eliminated via the urine and 7.1% in the faeces in the study of 2 calves.[27] At 20 days these values were 72.2% and 10.3%, respectively. Elimination in both faeces and urine followed a bi-phasic process. In rabbits 40-50% of diminazene was recovered in urine after 7 days, whereas faecal elimination accounted for 8-10%.[17] Urinary excretion was almost linear.

Intra-subject elimination of diminazene appears to vary considerably. In a study of 3 cows, which were each given repeated diminazene treatments whilst pregnant and lactating, the elimination half-life varied within a cow by up to 3 times in 2 of the cows.[37] However, the experimental design was poor and these results need to be interpreted cautiously as each cow was in a different reproductive stage when they received their diminazene administrations. Additionally, treatment times between cows were not standardised with the reproductive cycle.

1.3.6 Pharmacokinetic models

In an intravenous study of diminazene in dogs, data was fitted best to an open two-compartment model.[39] In one of the three dogs, a three-compartment model was found to be adequate as well. Data was fitted both to compartmental and non-compartmental models. When intra-muscular data was analysed a two-compartment model was again best fitted to the data. A non-compartment pharmacokinetic model was also used to describe the pharmacokinetics (Table 1.1 for details). In two further studies on dogs, two-compartmental models were also fitted (Table 1.1 for details).[7,46]

Miller argues that the non-compartment pharmacokinetic model describes the pharmacokinetics of diminazene better.[39] A long tail in the time-concentration curve probably reflects a gamma phase that is not reflected in the intravenous compartmental model. Naidoo subsequently modelled a gamma phase and attributed it to enterohepatic circulation. (Dr V. Naidoo, personal communication, University of Pretoria, 2006, unpublished data)

As far as the author is aware no population pharmacokinetic model of diminazene in any species has been reported in the literature.

1.3.7 Effects of illness

In two different studies of ill dogs treated with intravenous diminazene, significant pharmacokinetic differences were seen between healthy dogs and dogs infected with *Trypanosoma brucei brucei*[7] and *T. congolense*.[46] In both studies the significant differences were similar between infected animals (distribution phase intercept – increased; concentration at zero time – increased; distribution volume of the central compartment – decreased; distribution half-life – decreased; rate constant for distribution from central compartment – increased). Decreased renal clearance was noticed in one study and was postulated to result from trypanosomal nephrosis, impaired circulatory function and decreased liver or kidney uptake of diminazene in sick animals.[7] Unfortunately, no comparisons were made with haematological and biochemical variables. In a study of pregnant cows, high levels of trypanosome parasites, indicated by anaemia, increased the elimination half-life.[37] Criticisms of this study have been noted earlier.

Significant findings are also noted in cattle infected with *T. congolense*.[33] Cattle with acute infections had the highest C_{max} followed by chronic and then non-infected cattle after i.m. administration of diminazene (8.25, 5.04 and 4.7 µg/ml, respectively). Similar findings were found when time at maximum plasma concentration (T_{max}) was compared (18, 33 and 36 minutes, respectively). Significant differences did not exist between chronic and non-infected cattle for both parameters. Interestingly, the haematocrit was not significantly different between acutely infected and non-infected cattle while this was the case for chronic and non-infected cattle. This might suggest that anaemia, *per se*, does not play a role in peak concentrations and T_{max}. Body temperature was however significantly different. At eight hours post injection, the time-concentration curves were similar and clearance did not differ significantly between the 3 groups. The rate constant for the distribution from the peripheral compartment (k_{21}) was significantly slower between acute and non-infected infected cattle. The rapid distribution and elimination phase showed no difference. AUC and AUMC (area under the concentration-time curve and area under the concentration-time curve at first moment) showed no significant difference, while volume of distribution at steady state was significantly different between the acute infected group and the other two groups, while that of the central compartment was not.

In a study of diminazene tissue residue concentrations, plasma concentrations where higher in dogs infected with *T. congolense* and *T. b. brucei* than healthy dogs 24 hours after receiving diminazene (3.5 mg/kg i.m.).[47] Highest tissue concentrations were found in the kidneys and liver in all three groups. Brain, skeletal muscle and heart tissue diminazene concentrations were markedly lower. When comparing drug concentrations, healthy dogs had higher concentrations of diminazene in the

liver, kidney, heart and skeletal muscle than infected dogs. This was in contrast to brain concentrations, which were higher in infected animals. The authors hypothesise that inflammatory mediators were responsible for these changes by decreasing tissue perfusion, increasing permeability of the blood brain barrier. Tissue damage caused by the trypanosomiasis was additionally thought to be responsible for the changes seen.[47] In rats given diminazene, with an osmotic agent to increase the permeability of the blood brain barrier, diminazene concentrations were found to be significantly higher in organs (kidney, liver, brain and spleen) of trypanosomal infected rats compared to uninfected rats.[45]

Illness resulted in an increase in elimination half life (9.87 versus 12.51 and 11.57 versus 15.5 h), but the change was not significant[7,46]. Anika and Onyeyilli[7] recovered more than 26% of diminazene in the urine after 72 hours following a single i.v. injection in dogs. There was no significant difference between healthy dogs (28% recovered) and those with *T. b. brucei* (26% recovered). A further study with *T. congolense* showed similar results.[46]

1.3.8 Diminazene binding – body tissues

In rabbits 35 - 50% of the administered dose was present in the liver 7 days after administering diminazene intra-muscularly at 3.5 mg/kg.[17] In the study of two calves, large amounts of unchanged diminazene were sequestrated in the liver.[27] Twenty two percent and 4.4% of the administered diminazene dose was present in the liver and kidneys after 7 days. After 20 days this was 15% and 1% respectively. Diminazene tissue concentrations in the two calves were highest in liver tissue, followed by kidneys, brain and skeletal muscle. When the calves' livers were fed to rats, only 23% of the diminazene was bio-available. In contrast to the two calves, the tissue concentrations were highest in the rats' kidneys, while the liver concentrations was 50% of that.

In rats, kidney concentrations are higher than liver concentrations 6 hours post treatment.[45] Total diminazene content may still be higher in the liver due to it being a larger organ.

In calves fed milk with diminazene residues (from lactating cows injected with diminazene), greater amounts of the drug was excreted via the faeces than the urine, indicating the strong binding force of diminazene to milk as oral diminazene is completely absorbed.[37]

In pregnant cows highest diminazene concentrations are seen in the kidneys, followed by liver and heart muscle concentration.[37]

1.3.9 Diminazene binding – plasma proteins

Diminazene is highly protein bound.[6] When diminazene (20 µg) was incubated with 1ml heparinised rabbit blood only 7% of the diminazene could be recovered. When repeated with serum (1ml), 65% diminazene was recovered. Ghost red blood cells showed no absorption, while purified haemoglobin from 1ml blood bound 70% of the diminazene. These results suggest that diminazene is not inhibited by the RBC membrane and most likely crosses biological membranes readily.

An *in vitro* study using cattle albumin showed diminazene to be bound to albumin in proportion to the diminazene concentration.[3] The albumin binding increased from 38% of the diminazene content at a 0.5 µg/ml concentration to 91% at a 5 µg/ml concentration. This then decreased to 85.4% when the diminazene concentration increased to 10 µg/ml. One possible explanation is that the saturation level of albumin is reached between 5 and 10 µg/ml diminazene.

In sheep plasma, the *in vivo* protein bound fraction of diminazene varied between 65-85%. The bound fraction was inversely related to time from treatment and hence the total blood concentration, suggesting that plasma free diminazene is eliminated faster than the protein bound fraction.[4] When calculated from data presented by Aliu *et al*, the total quantity of plasma bound diminazene drops after 30 hours to 0,08 of the original diminazene quantity per ml plasma, while the free quantity of diminazene drops to 0,02.[4]

1.3.10 Partition of diminazene between red blood cells and plasma

Aliu *et al* showed in sheep[4] and cattle[3] that plasma concentration of diminazene was initially higher than that of whole blood. Plasma concentration then decreased at a faster rate relative to whole blood and was lower than the whole blood concentration after 12 hours in cattle[3] and 24 hour in sheep[4]. In cattle, red blood cell concentrations[3] did not change with time, while in sheep[4] the concentration did decrease with time. This might suggest that binding to haemoglobin varies in species. In both cases the C_{rbc}/C_p (the red blood cell to plasma drug ratio) did increase with time. Keller showed similar results – C_{max} of plasma was higher than that of whole blood in 2 calves.[27] Plasma concentrations remained on average 20 - 30% higher for the duration of the study. The plasma and blood half-lives were similar.

Calculations from the data presented by Aliu *et al* shows that half-an-hour after i.v. injection, red blood cells held 13% of whole blood diminazene content.[3] After 12 hours this was 45%. Similar results were seen in dogs.[39] The red blood cell fraction one-hour post injection was 18.5%[39] as compared to 16.6% in the cattle study.[3] Seventy-five percent of whole blood diminazene was found in the plasma fraction. An *in vitro* blood binding analysis in which diminazene was incubated with canine blood for 24 hours showed that 85-94.5% of whole blood diminazene was found in the plasma fraction.[39] Seventeen – twenty four percent of the plasma diminazene was found in the free water fraction.[39]

Taken together it seems that diminazene concentration is cleared first from the plasma and then from the RBC fraction at a slower rate. The diminazene blood partitioning would also seem to suggest that degrees of anaemia may play a lesser role than blood protein concentrations in influencing the pharmacokinetics.

1.3.11 Blood brain barrier

Diminazene is a large molecule that does not easily cross the blood brain barrier. Tissue concentrations 6 hours post treatment were 29 times lower in rat brain compared with the liver and 34 times lower than the kidney.[45] Lithium chloride, an agent that opens the blood brain barrier, was shown to increase diminazene brain tissue concentrations in trypanosomal infected rats by four times.[45] In a study of goats, cerebrospinal fluid concentrations were 34 time lower than plasma concentrations.[34]

1.3.12 Pregnancy and lactation

No transplacental diffusion of diminazene could be demonstrated in pregnant cows.[37] However, the poor experimental design prevented conclusions on whether pregnancy played a role in diminazene disposition.

1.3.13 Adverse effects and toxicity

Pain has been noted after intra-muscular injections.[3] Transient subcutaneous swelling have been noted in cattle given subcutaneous injections.[16] In cattle, muscle necrosis has been noticed at the site of i.m. injections.[16] Transient muscle tremors have been noticed in cattle injected with high doses of diminazene.[16]

Therapeutic doses injected intravenously have resulted in collapse, depression, salivation, diarrhoea and vomiting. These effects seem to be transient and not permanent.[39,43] Transient diarrhoea was also noted after intra-muscular injections.[39,43] Similar signs are seen in cattle, including muscle tremors, following intravenous administration.[3]

Toxicity is most likely related primarily to peak plasma concentrations as this drops rapidly after reaching its C_{max}. Individual variation in susceptibility to diminazene toxicity seems to exist.[44] In a toxicity experiment individual dogs were very resistant to toxicity effects when given high doses of diminazene.[44] Unfortunately no plasma concentrations were measured to correlate toxicity to plasma concentrations.

Table 1.1: Pharmacokinetic parameters for dogs injected with diminazene

Parameter	A - normal[46] (n=5) mean±S.E.M	B – normal[7] (n=5) mean±S.E.M.	A - infected[46] (n=5) mean±S.E.M	B - infected[7] (n=5) mean±S.E.M	C - normal[39] (n=8) mean± SD	C - normal[39] (n=8) mean± SD	C - normal[39] (n=3) mean± SD	C - normal[39] (n=3) mean± SD
Administration	i.v.	i.v.	i.v.	i.v.	i.m.	i.m.	i.v.	i.v.
Kinetic model	Compartment	Compartment	Compartment	Compartment	Non-compartment	Compartment	Non-compartment	compartment
A (µg/ml)	4.42 ±0.27[a]	4.00 ±0.47[b]	6.13 ±0.46[a]	5.70 ±0.32[b]		7.38±8.35		10.44±8.48
B (µg/ml)	1.55 ±0.25	1.82 ±0.22	1.68 ±0.14	1.90 ±0.30		0.53±0.20		0.27±0.22
C^0_p (µg/ml)	5.97 ±0.45[a]	5.83 ±0.61[b]	7.89 ±0.30[a]	7.60 ±0.59[b]	1.85±0.27			
AUC (h. µg/ml)	25.85 ±2.87[a]	26.50 ±2.40	35.72 ±2.49[a]	33.00 ±2.15	5.09±2.18		8.669±0.665	8.14±4.42
Cl (L/kg/h)	0.14 ±0.02	0.14 ±0.01[b]	0.10 ±0.01	0.11 ±0.01[b]				0.7±0.4
V^d_β (L/kg)	2.39 ±0.41	1.94 ±0.21	2.09 ±0.18	1.96 ±0.38				
V_c (L/kg)	0.60 ±0.05[a]	0.62 ±0.05[b]	0.45 ±0.02[a]	0.47 ±0.04[b]				0.7±0.7
$t_{½}^{\beta}$ (h)	11.57 ±1.06	9.87 ±0.78	15.15 ±2.39	12.51 ±1.43		5.31±3.89		32.02±28.77
$t_{½}^{\alpha}$ (h)	0.17 ±0.01[a]	0.20 ±0.02[b]	0.12 ±0.01[a]	0.14 ±0.01[b]		0.36±0.19		0.12±.010
$t_{½}^{el}$(h)					27.5±24.96			
K_{21} (h^{-1})	1.09 ±0.15	1.15 ±0.10	1.33 ±0.13	1.31 ±0.17		0.52±0.29		0.32±0.25
K_{el} (h^{-1})	0.24 ±0.01	0.22 ±0.003	0.21 ±0.02	0.22 ±0.01	0.07±0.07	0.89±0.36		1.78±1.37
K_{12} (h^{-1})	2.82 ±0.15[a]	2.26 ±0.29[b]	4.27 ±0.49[a]	3.61 ±0.20[b]		1.13±0.52		8.78±8.71
K_{01} (h^{-1})						42.9±35.21		
K_{10} $^{\text{half life}}$ (h)						0.95±0.45		1.29±1.77
K_{01} $^{\text{half life}}$ (h)						0.11±0.18		
α (h^{-1})	4.09 ±0.21	3.56 ±0.35[b]	5.76 ±0.52	5.08 ±0.35[b]		2.3±0.88		10.80±9.48
β (h^{-1})	0.062 ±0.01	0.072 ±0.005[b]	0.05 ±0.01	0.058 ±0.01[b]		0.16±0.12		0.11±0.09
V_{ss} (L/kg)								17.41±12.12
MRT					10.32±5.44			42.05±39.40
T_{max} (h)					0.37±0.12			
Sensitivity	250ng/ml	250ng/ml	250ng/ml	250ng/ml	25 ng/ml	25 ng/ml		25 ng/ml
Analysis methods	Colorimetric	Colorimetric	Colorimetric	Colorimetric	Ion-paired HPLC	Ion-paired HPLC	Ion-paired HPLC	Ion-paired HPLC

[a] and [b] are significantly different ($p<0.05$)

Study A[46]: dogs not infected and infected with *Trypanosoma congolense*

Study B[7]: dogs not infected and infected with *Trypanosoma brucei brucei*

Study C[39]: normal dogs

i.m. - Intra-muscular

i.v. - Intravenous

A (µg/ml)	Distribution phase intercept
B (µg/ml)	Elimination phase intercept
C^0_p (µg/ml)	Concentration at time zero
AUC (h. µg/ml)	Area under the concentration-time curve
Cl (L/kg/h)	Total body clearance
V^d_β (L/kg)	Volume of distribution at pseudo-equilibrium
V_c (L/kg)	Volume of distribution of the central compartment
$t_{½}^{\beta}$ (h)	Elimination half-life
$t_{½}^{\alpha}$ (h)	Distribution half-life
K_{21} (h^{-1})	Rate constant for the distribution from peripheral compartment
K_{el} (h^{-1})	Rate constant for elimination
K_{12} (h^{-1})	Rate constant for the distribution from central compartment
α (h^{-1})	Distribution constant
β (h^{-1})	Elimination constant
V_{ss} (L/kg)	Volume of distribution at steady state
T_{max}	Time at maximum plasma concentration
MRT	Mean residual time
K_{01} (h^{-1})	Rate constant for absorption
$K_{01}^{\text{half life}}$ (h)	Half life for absorption phase
$K_{10}^{\text{half life}}$ (h)	Half life for elimination phase
$t_{½}^{el}$(h)	Half life for elimination

CHAPTER 2: RESEARCH QUESTIONS, HYPOTHESIS AND OBJECTIVES

2.1 RESEARCH QUESTIONS

What covariates (clinical, pathological or otherwise) affect the population pharmacokinetics of diminazene in dogs with naturally acquired uncomplicated *Babesia canis* infections requiring only outpatient treatment with diminazene?

2.2 RESEARCH HYPOTHESES

The pharmacokinetic parameters of diminazene in dogs with mild *Babesia canis* infections are influenced by measurable covariates.

2.3 RESEARCH OBJECTIVES

2.3.1 Primary objective

Describing the population pharmacokinetics of diminazene in dogs suffering from mild, uncomplicated babesiosis in comparison to healthy dogs treated with diminazene.

2.3.2 Secondary objectives

Identifying patient co-variants in dogs with *Babesia canis* that affected the pharmacokinetic parameters of diminazene.

CHAPTER 3: MATERIALS AND METHODS

3.1 STUDY DESIGN

The population pharmacokinetics of intramuscular administered diminazene aceturate was studied in a population of client owned dogs naturally infected with *Babesia canis* in an open, non-randomised, stratified prospective clinical population pharmacokinetic trial.

This study was approved by the Animal Use and Care Committee and the Research Committee of the Faculty of Veterinary Science, University of Pretoria (No. 36.5.522).

3.2 ANIMALS

Dogs, diagnosed with *Babesia canis* at the Outpatient Clinic of the Onderstepoort Veterinary Academic Hospital (OVAH), University of Pretoria were used as the study population. Samples from 39 animals were collected. In addition, data from 8 healthy animals, published previously, was added to this study's data. [38]

3.3 PATIENT SELECTION

3.3.1 Inclusion criteria:

1. Dogs diagnosed with babesiosis requiring only outpatient treatment with diminazene, with no further care needed.
2. The diagnosis of babesiosis was made by identification of *Babesia canis* on thin blood-film evaluation.
3. Dogs having concurrent illnesses or diseases (not attributed to the babesiosis) were not excluded, as these form part of the normal infected population. Records were kept for analysis purposes.

3.3.2 Exclusion criteria:

1. Owners not giving consent, or withdrawing consent, prior to collection of any data points.
2. Dogs having received diminazene aceturate (or phenamidine or other aromatic diamidine) during the previous 2 months.
3. Dogs that had baseline blood samples for co-variant analysis taken after diminazene treatment.
4. Any form of severe or complicated babesiosis (defined as illness attributed to babesiosis requiring treatment other than diminazene aceturate administration).
5. Dogs that were too small to safely collect the required blood volume (less than 2.5 kg) or where the packed cell volume (PCV) was less than 0.16 l/l).

3.4 PROCEDURES AND MANAGEMENT

Owners of dogs eligible for inclusion in the study were given an information sheet (Appendix 1). A signed written owner consent form (Appendix 2) was obtained prior to admitting the patient into the trial. Owners were interviewed prior to diminazene treatment and co-variable data recorded. Blood for haematological and biochemical analysis was collected after diagnosis and before administration of treatment. Rectal body temperature was taken immediately prior to administration of diminazene.

Admitted dogs were housed in the Outpatient day-ward of the Onderstepoort Veterinary Academic Hospital for the duration and purposes of data collection. Animals were discharged after collection of the last blood sample.

3.5 DIMINAZENE PREPARATION

Berenil® granules (Intervet South Africa; Lot number: 01W027, Expiratory date: July 2007) containing diminazene aceturate 44.5% m/m were used to treat the dogs. Two hundred and thirty-six milligrams of Berenil® were sterilely packaged (Kyron Laboratories Pty Ltd, South Africa) into glass bottles for reconstitution with 2.5ml of sterile water for injection (Kyron Laboratories Pty Ltd; South Africa). Each

bottle contained 105mg diminazene aceturate. Dogs were treated at a dose of 1ml per 10 kg (4.2 mg/kg) of the reconstituted Berenil® powder.

A 2.5 ml syringe was used to measure the volume of water for reconstitution. The exact amount of water used to reconstitute diminazene aceturate powder in each bottle was calculated by weighing each bottle with an electronic scale (Mettler P1200 electronic scale, Switzerland), in grams to 3 decimal points, before and after the addition of water. The concentration calculated was used to determine the actual dose of diminazene aceturate administered to each dog.

Reconstituted bottles of Berenil® were stored at 5℃ and used for no more than 24 hours before being discarded.

3.6 DIMINAZENE ADMINISTRATION

Diminazene was administered via intra-muscular injection in the *M. biceps femoris*, (midway between the stifle and the hip joint).

3.7 BLOOD COLLECTION AND SAMPLE HANDLING

All blood samples were collected from the jugular vein.

3.7.1 Blood collection for covariant data

Blood samples were collected at admission, prior to treatment, in 3 ml Na-EDTA (BD Vacutainer Systems; Becton, Dickinson and Company; UK) and 5 ml plain serum evacuated blood tubes (BD Vacutainer Systems; Becton, Dickinson and Company; UK). These blood samples were used for the full blood count and biochemistry (total serum proteins, albumin, globulin, and creatinine). Patients between 2,5 and 5 kg had blood drawn using a syringe and needle and samples were placed in a 0.5 ml paediatric EDTA (BD Microtainer; Becton, Dickinson and Company; UK) and 1 ml paediatric serum gel tubes (BD Microtainer; Becton, Dickinson and Company; UK) to prevent anaemia being exacerbated through blood collection.

Blood samples were processed for analysis via the normal hospital operating procedure.

Full blood counts on samples collected after-hours was performed by the investigator within 1 hour of blood collection. Blood smears were made at the same time for analysis by the staff in the Section of Clinical Pathology the next working day.

Serum samples collected after hours were spun down, separated and stored at -20°C in labelled polypropylene tubes. Analysis was done the following working day via normal hospital operating procedure.

3.7.2 Blood collection for plasma diminazene quantification

Blood for determination of diminazene plasma concentration was collected in 5 ml heparinised evacuated blood tubes (BD Vacutainer Systems; Becton, Dickinson and Company; UK). Blood samples were centrifuged at 3000 rpm for 15 minutes. The separated plasma was stored at -80°C in labelled 4 ml polypropylene tubes (Cryvials; Pastro Scientific Pty Ltd; South Africa). In all cases, plasma processing was completed within one hour after collection.

Four samples per animal were taken over a 6-hour period for diminazene plasma concentration determination. Dogs were be randomly assigned (Appendix 3) to one of three groups: A, B or C. Blood was collected at the following times according to the corresponding group.

Group A: 5 minutes, 20 minutes, 3 hours and 6 hours;

Group B: 10 minutes, 40 minutes, 2 hours and 5 hours; or

Group C: 15 minutes, 60 minutes, 1.5 hours and 4 hours

Sample times were recorded as the time, in seconds, from immediately after diminazene administration, to immediately after blood was collected.

3.8 DETERMINING OF DIMINAZENE PLASMA CONCENTRATIONS

A paired-ion extraction and high performance liquid chromatographic technique as described by Gummow was used to determine plasma diminazene concentrations by the Section of Pharmacology and Toxicology, Faculty of Veterinary Science, Onderstepoort. [18,19]

3.9 COVARIATES INVESTIGATED

Data from the full blood count and biochemistry analysis were used for covariant analysis. Demographic covariant data were collected concurrently using patient records and a questionnaire and included: breed, age, body weight, gender, neutered, reproductive cycle status for non neutered bitches, mental status, mucus membrane colour, capillary refill time, icterus, in-saline-agglutination, heart rate, respiratory rate, thoracic auscultation, vomition, diarrhoea, body score, splenomegaly, hepatomegaly, concurrent illnesses / diseases and medication administration prior to presentation (i.e. home medication by owners). Adverse reactions associated with the administration of diminazene aceturate within 6 hours of injecting the drug were recorded. These included: swelling at injection site, muscle pain at injection site, diarrhoea, salivation, vomiting, muscle twitching, collapse, hyperaesthesia, or any other possible reaction. Appendix 4 provides details of the various scoring systems used.

3.10 POPULATION PHARMACOKINETIC ANALYSIS

Population pharmacokinetics was performed using WinNonMix® (Version 2.0.1, Pharsight Corporation, California, USA). The program assumes parametric distributions of residual error values. In addition, detailed pharmacokinetic experimental data of diminazene administered intramuscularly to healthy dogs, were added to this study's data set from another study.[39] Eight healthy adult German shepherd dogs of both genders were injected at 4.2 mg/kg in the same site as in this study. Diminazene plasma concentrations from samples collected at 0.33, 0.66, 1, 2, 3, 4, and 8 hours were used.

3.10.1 Model building

Model building proceeded in a stepwise manner.[52] Initially, data analysis involved the exploratory graphical and statistical evaluation of covariates and their correlations. Thereafter, the base pharmacokinetic model was determined using WinNonMix with no covariates. The inter-individual and residual error specification was investigated on the base model. The initial pharmacokinetic parameters used for the modelling were based on work by Miller[39], Onyeyilli and Anika[46] and Anika and Onyeyilli.[7]

An initial investigation of the individual parameter – covariant relationship was done by means of graphical visualization and statistics using the parameter estimates from the base model.[14]

The selection of covariates for inclusion into the model was based not only on an exposed parameter – covariant relationship, but also on the scientific (i.e. pharmacokinetic) plausibility and practicality. All investigated covariates were singularly and sequentially added individually to the model. The covariant – parameter modelling and intra-individual random effect was additive to minimise computational time. A non-linear covariant – parameter relationship was investigated for covariates with significant effects or where covariates were non-linear (e.g. dose in mg/m^2). The following criteria were used for identifying significant covariates:[15,51,63] 1) reasonability of results; 2) graphical visualisation of the results; 3) unity between predicted versus actual diminazene concentration; 4) mechanistic plausibility of the results; 5) precision of the parameter estimates (i.e. coefficients of variation and means); 6) repeatability of results with varying initial values and limits; 7) clinical importance of the covariate; and 8) statistically significant improvements in the models.

The significance level was set at $p = 0.05$. This equates to a difference (delta: Δ) of the minimum objective function (MOF) of 3.84 between two models with one added covariant, using 1 degree of freedom.[63] The MOF has an approximate X^2 distribution. Models with the highest ΔMOF were not automatically selected, depending on the additional evaluation criteria above. However, selected models needed to show a significant improvement (i.e. ΔMOF of > 3.84).

The final model building was done by sequentially adding the covariates identified above from most significant to the least significant to the final model. Thereafter, the final model was evaluated by removing each covariant, one at a time, and increasing the significance level to $p = 0.001$, which required a difference in MOF between the full and reduced model of >10.83.[63] The covariate was retained if its removal worsened the model significantly.

The final model was investigated for its validity by investigating the fulfilment of modelling assumptions: normal distribution around zero of inter- and intra-individual residual errors; and

normality around the means of the estimated pharmacokinetic parameters. Bias was investigated by looking at the graphical display of the weighted residuals.

3.11 STATISTICAL ANALYSIS

Sigma Stat (Version 2.0; Jandel Corporation; USA) and Sigma plot (Version 4.0.1; SPSS Corporation; USA) were used for descriptive statistical analysis of covariant data, covariant – parameter relationships and investigating colinearity between parameters. The power was set at 0.8 and significance level (p) at 0.05. Data were not log transformed for statistical analysis or population pharmacokinetic modelling.

CHAPTER 4: RESULTS

4.1 COVARIANT DATA

Thirty-nine dogs were admitted to the trial. Appendix 6 provides a summary of the covariate data for each patient. Covariate data that were normally distributed were reported using means. Where normality was not present, the median was given. Data were not recorded for some of the assessed covariates / parameters for various reasons, mainly due to lack of numbers (e.g. neutering status). There were 17, 10 and 12 dogs in groups A, B and C, respectively.

4.1.1 Dog breeds

Eighteen dogs breeds were represented by the dogs sampled (Figure 4.1). There were 3 Fox terrier crosses and 1 Husky crossbreed that were included in these two respective breed categories.

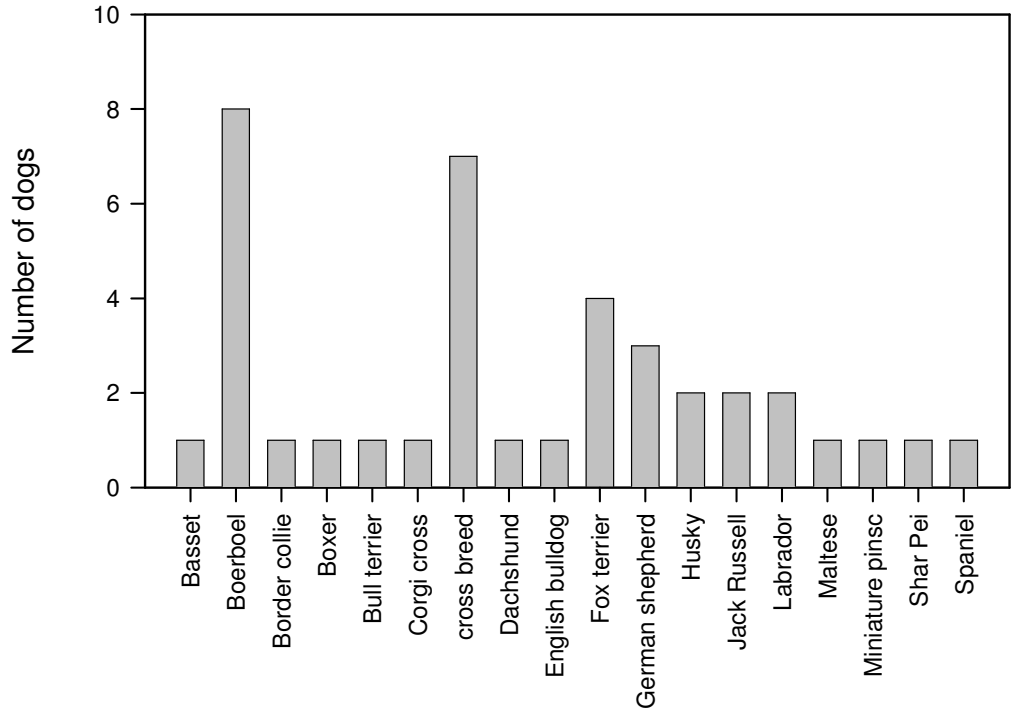


Figure 4.1: Bar chart of the various dog breeds in the sampled population.

4.1.2 Age

The age ranged from 3.2 months to 9 years, with a median of 1.5 years (Figure 4.2).

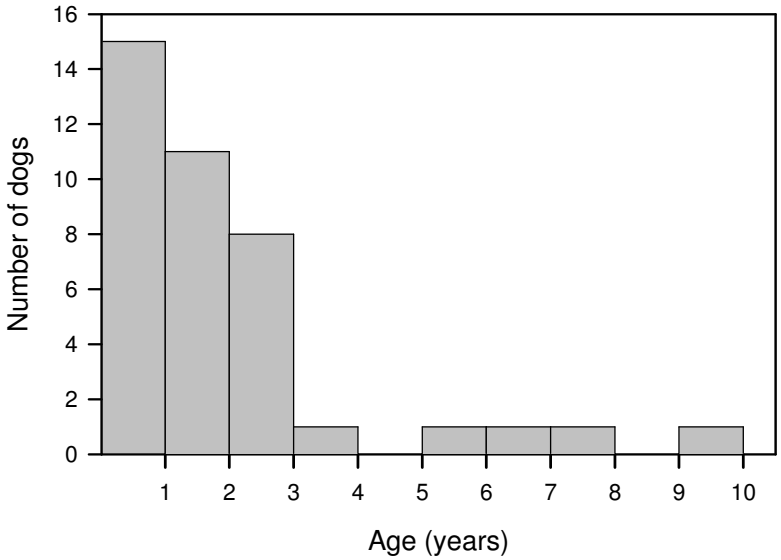


Figure 4.2: Age distribution of the dogs in the population sampled.

4.1.3 Body weight and surface area

The median body weight was 16.6 kg, with the lightest dog weighing 3.12 kg and the heaviest 60.28 kg (Figure 4.3). Body surface area (BSA) was calculated from the body weight (BW) in kg using:

$$BSA = 0.101 \times BW^{(2/3)}$$

The body surface area ranged form 0.217 to 1.553 m^2 with a mean of 0.705 m^2.

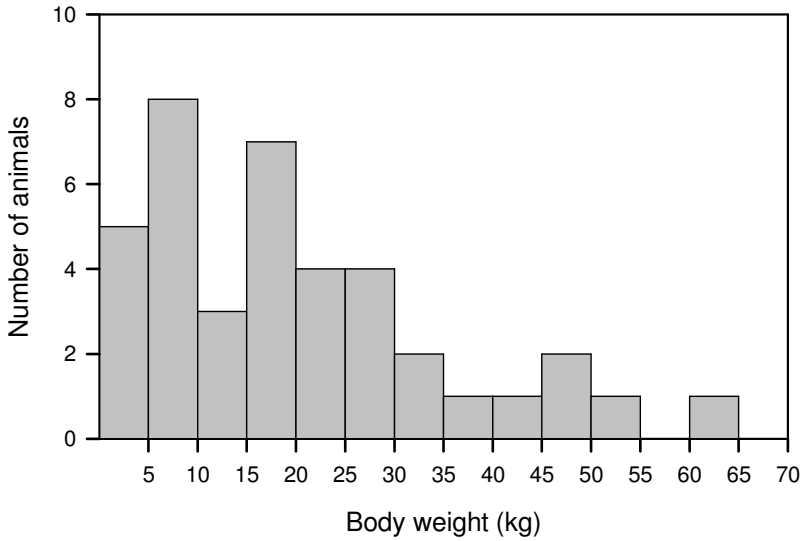


Figure 4.3: Frequency distribution of body weight.

4.1.4 Gender

There were 15 female dogs, of which 10 were neutered. Only 4 of the 24 male dogs were castrated.

4.1.5 Mucus membranes

Twelve dogs were subjectively assessed as having normal mucus membrane colour, while 23 were regarded as having pale mucus membranes and 3 as very pale (Figure 4.4). There were no dogs that were considered to have congested or white mucus membranes. There was a large overlap between the subjective mucus membrane colour estimates and the PCV measured.

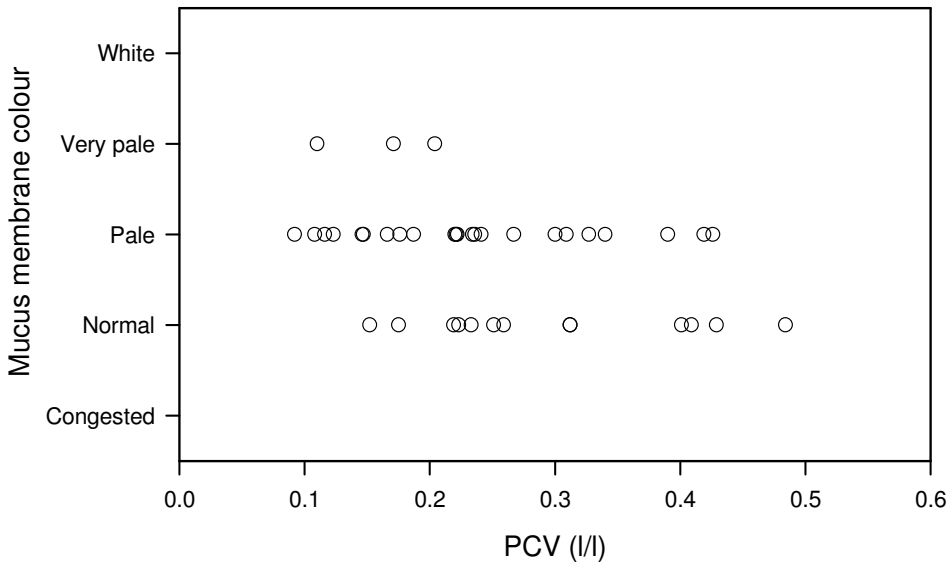


Figure 4.4: Mucus membrane colour plotted against the PCV.

4.1.6 Capillary refill time

The capillary refill time was subjectively accessed (Figure 4.5). Half of the dogs had capillary refill times that were outside of the normal range (1-2 seconds).

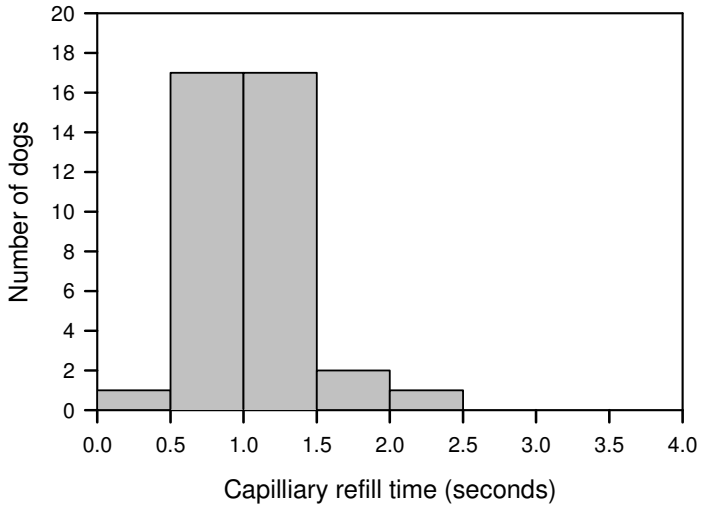


Figure 4.5: Frequency distribution of capillary refill time.

4.1.7 Heart rate

Heart rates ranged from 72 to 200, with a mean of 136 beats per minute. The degree of anaemia seemed to have little influence on the heart rate (Figure 4.6).

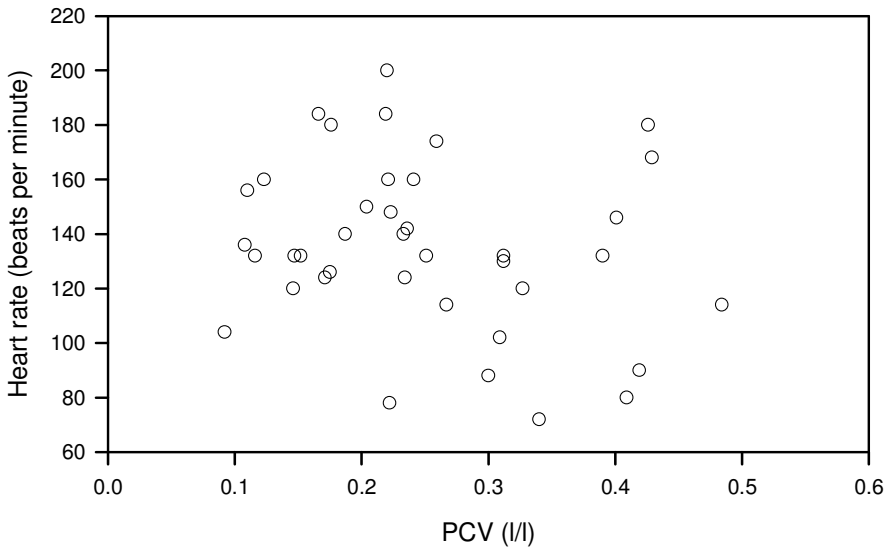


Figure 4.6: Heart rate plotted against the PCV.

4.1.8 Respiratory rate

Respiratory rate was recorded for 22 animals. Sixteen additional dogs were panting at the time of examination. The degree of anaemia seemed to have little influence on the respiratory rate (Figure 4.7).

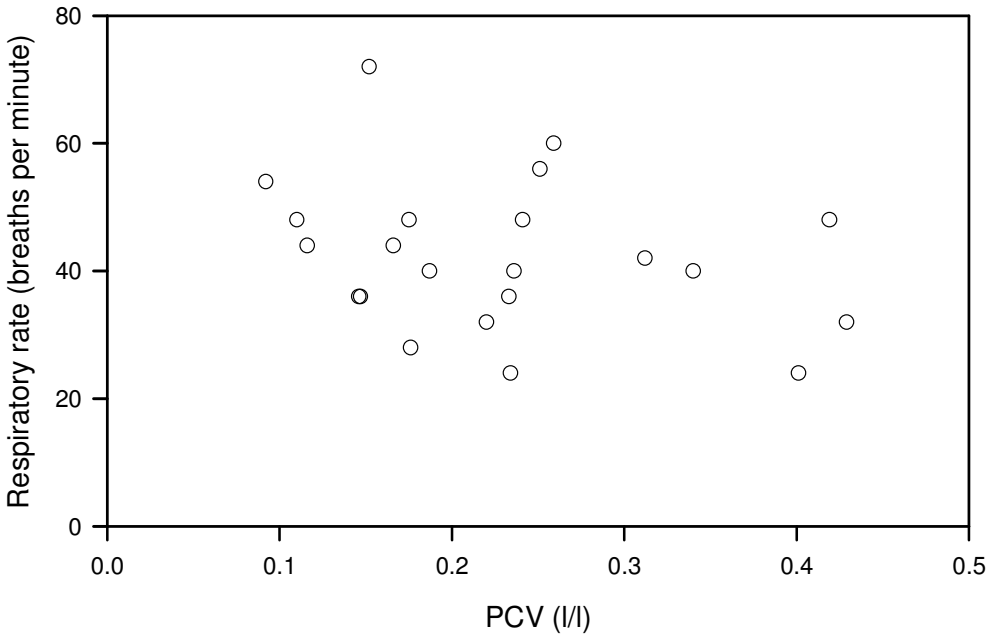


Figure 4.7: Respiratory rate plotted against the PCV.

4.1.9 Mental status

The mental status and the number of dogs in each category are shown in Figure 4.8.

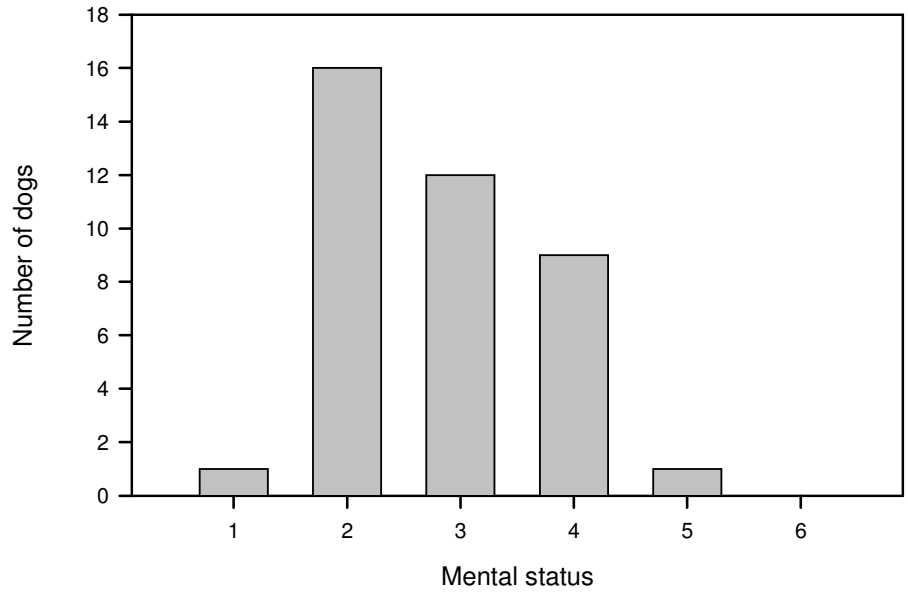


Figure 4.8: Box chart showing the number of dogs in each mental status category (as defined in Appendix 4).

4.1.10 Body temperature

The mean body temperature was 40.0 ℃ with a range of 38.3 - 41.2 ℃. Almost a quarter of the dogs had a normal body temperature despite being infected with *Babesia canis* (Figure 4.9).

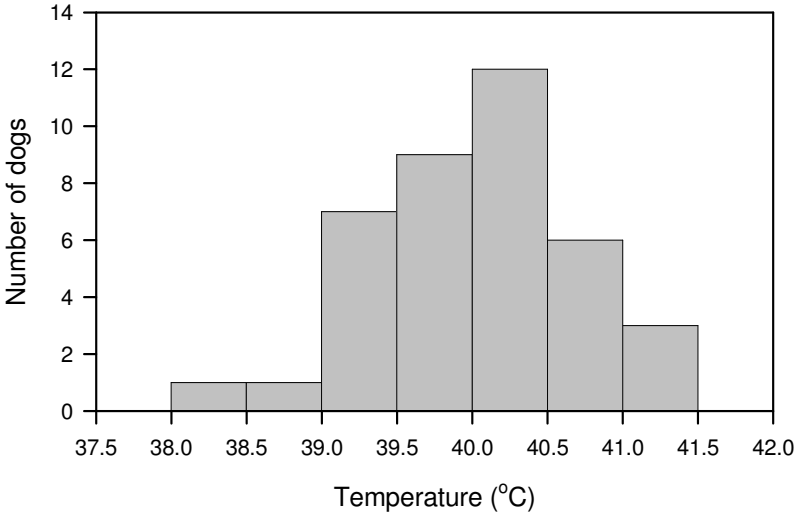


Figure 4.9: Frequency distribution of the body temperature.

4.1.11 In-saline agglutination

Only one dog was positive for in-saline agglutination. This patient was treated with prednisolone after collection of the final blood sample.

4.1.12 Full blood count

The minimum and maximum with mean or median for the full blood count results are shown in Table 4.1. Despite all dogs having the same parasitic disease, there was a large range in the red cell count, from severe anaemia to normal. Seven dogs had PCVs in the reference range. Seven dogs had a MCHC above the reference range, indicating haemolysis. There were 11 dogs with neutropenia. Eleven dogs had a degenerative left shift. Lymphopenia was seen in 15 dogs and monocytopenia in two dogs, while monocytosis was seen in 3 dogs. All dogs had thrombocyte counts below the reference range, an expected finding.[28]

Table 4.1: Full blood count results.

Parameter	Reference range*	Units	Mean	Median	Range
Haemoglobin	120-180	g/l	88		36 – 176
Red cell count	5.5 – 8.5	$x10^{12}$/l	3.7		1.2 – 7.0
Packed cell volume	0.37-0.55	l/l	0.25		0.09 – 0.48
Mean cell volume	60-77	Fl	67		58 – 75
Mean cell haemoglobin concentration	32 – 36	g/dl		35.0	31.5 – 69
Red cell distribution width	15.5 -19.5	%		16.1	13.4 – 28.2
White cell count	6.0– 15.0	$x10^{9}$/l	7.0		3.2 – 15.1
Neutrophils (mature)	3.0 – 11.5	$x10^{9}$/l		3.7	1.19 – 10.4
Neutrophils (bands)	0 – 0.5	$x10^{9}$/l		0.2	0 – 2.8
Lymphocytes	1.0 – 4.8	$x10^{9}$/l		1.2	0.2 – 4.4
Monocytes	0.15 – 1.35	$x10^{9}$/l		0.56	0 – 3.31
Eosinophils	0.10 – 1.25	$x10^{9}$/l		0.01	0 –0.47
Basophils	0 – 0.1	$x10^{9}$/l		0	0 –0.04
Thrombocytes	200-500	$x10^{9}$/l		4.4	0 - 145

* Reference range from the Section of Clinical Pathology at the Onderstepoort Veterinary Academic Hospital, University of Pretoria

4.1.13 Biochemistry results

The minimum and maximum with mean or median for the biochemistry results are shown in Table 4.2. All dogs, save the one dog that died, had creatinine concentrations within the normal range.

Table 4.2: Serum biochemistry results.

Parameter	Units	Reference range*	Mean	Median	Range
Total serum proteins	g/l	53 - 75		53.6	38.5 - 85.4
Albumin	g/l	27 – 35	25.4		17.3 - 35.7
Globulin	g/l	20 - 37		30.5	18.8 – 65.4
Creatinine	µmol/l	40 – 133	63.1		15 - 168

* Reference range from the Section of Clinical Pathology at the Onderstepoort Veterinary Academic Hospital, University of Pretoria

4.1.14 Concurrent illnesses and other medication administered

Of the 39 dogs, none were suffering from concurrent chronic diseases. One dog was being treated for concurrent illness (acute gastro-enteritis - “garbage disease”) and was receiving antibiotics and corticosteroids. Three patients had been receiving vitamin or ”liver tonic” supplements, and one was also receiving a laxative. Three patients had aspirin administered to them by their owners. Two dogs were administered paracetamol. One dog was administered a “pain tablet”. The owners could not recall the drug’s name.

4.1.15 Mortalities

One dog (patient 4) died acutely after collection of the third sample. Necropsy (Appendix 6) revealed typical pathology associated with fatal babesiosis. No other causes for the sudden death were noted. The initial blood diminazene concentration was markedly lower than for all other animals (Figure 4.16, Figure 4.17).

4.1.16 Side effects

Seventeen dogs showed adverse effects temporally associated with diminazene administration. These included: excessive bleeding from the venepuncture site (2 dogs), death (1 dog), diarrhoea (4 dogs), pain on diminazene administration (7 dogs), hiccups (1 dog), vomiting (3 dogs), salivation (1 dog) and body tremors (1 dog).

4.1.17 Body condition score

Body condition scores ranged from 4-8 (Figure 4.10).

4.1.18 Organomegaly

Of the 39 dogs, organomegaly was recorded for 34. Twenty-five and 2, of the 34 dogs, had splenomegaly and hepatomegaly, respectively. Only 1 dog had both splenomegaly and hepatomegaly.

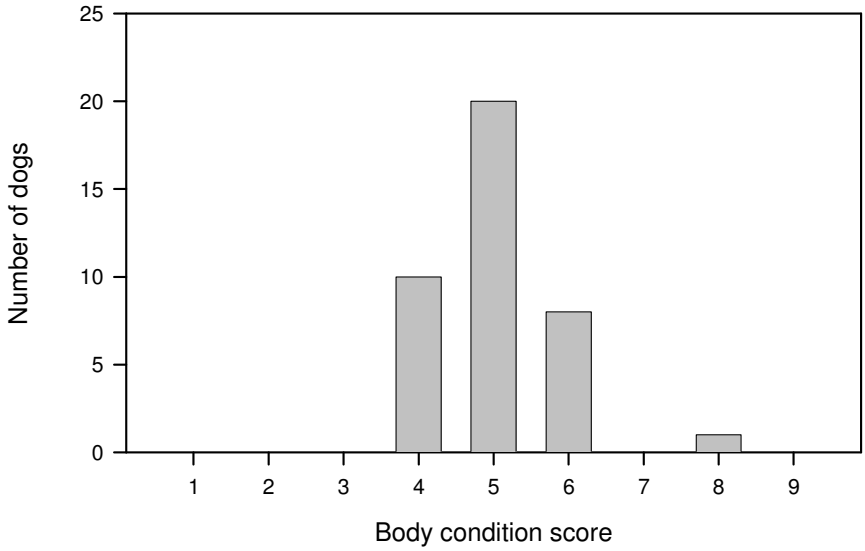


Figure 4.10: Bar chart of the number of dogs in each body condition score (as defined in Appendix 4).

4.2 COLINEARITY

4.2.1 Health status and PCV

There was a significant difference in the PCV between the healthy and babesia infected dogs (Mann-Whitney Rank Sum Test, $p < 0.001$; median: 0.46 and 0.23, respectively). Figure 4.11and Figure 4.12 shows the PCV for both sick and healthy dogs plotted against mental status and splenomegaly, respectively.

4.2.2 Health status and albumin

There was a statistical difference between the albumin concentrations of healthy dogs and babesia infected dogs (t-test, $p < 0.001$; mean ± SD: 34.0 ± 2.9 and 25.4 ± 4.3 respectively). Figure 4.13 and Figure 4.14 show the albumin concentrations for both sick and healthy animals.

4.2.3 PCV and mental status

There was insufficient power (power = 0.06) to detect a statistical difference in the PCV between those babesia dogs with different mental status scores (one-way ANOVA, $p = 0.37$). Figure 4.11.

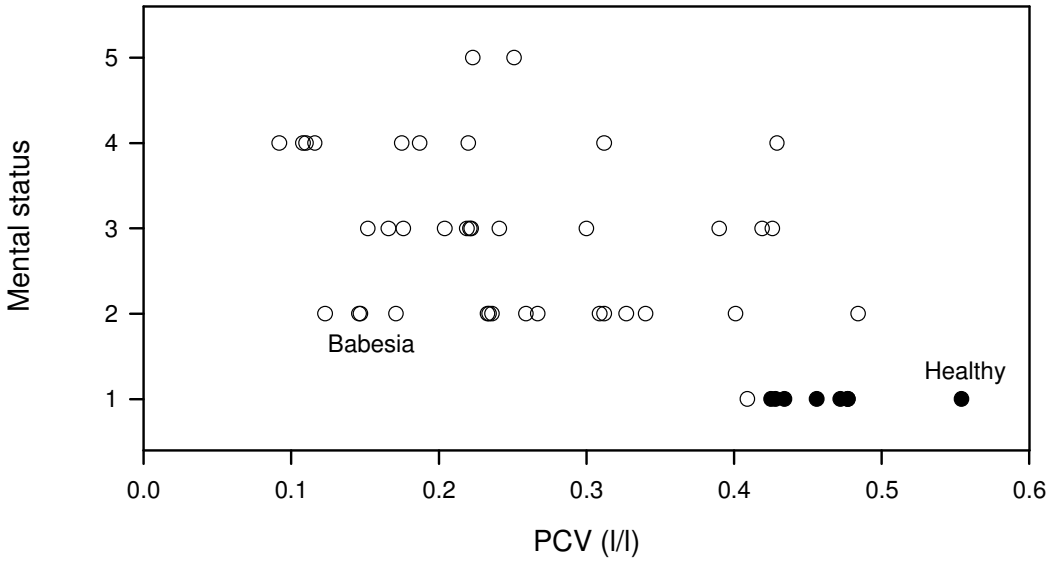


Figure 4.11: Scatter plot of the PCV against the mental status. Data are shown for both the healthy experimental and sick animals.

4.2.4 PCV and splenomegaly

There was insufficient power (power = 0.14) to detect a statistical difference in the PCV between those babesia dogs with and without splenomegaly (t-test, p = 0.18). Figure 4.12.

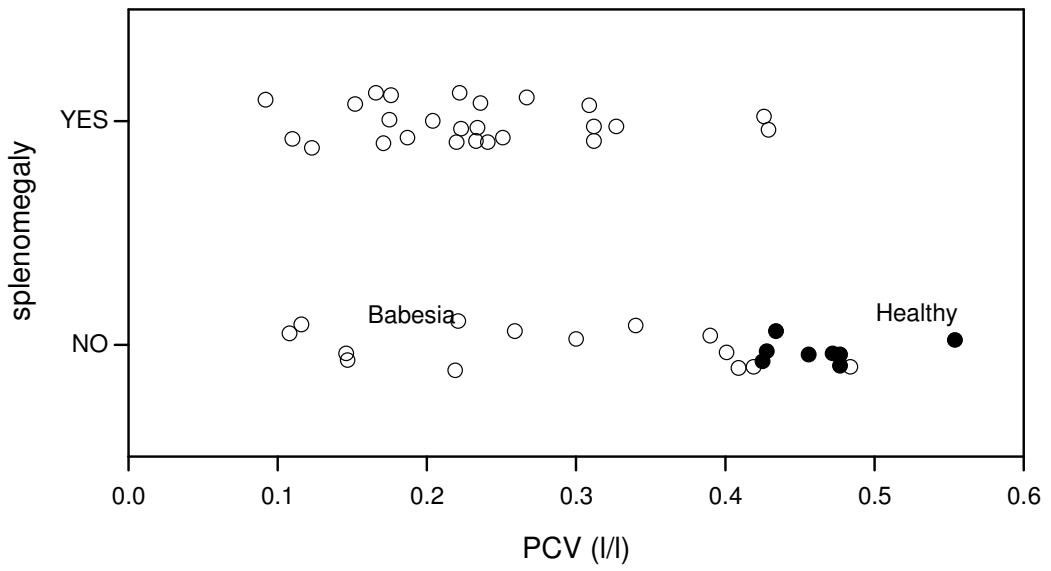


Figure 4.12: Scatter plot of the PCV against splenomegaly. Data are shown for both the healthy experimental and sick animals.

4.2.5 Albumin and mental status

There was insufficient power (power = 0.05) to show a significant difference in the albumin concentrations between babesia dogs with varying mental status (one-way ANOVA, p = 0.93). Figure 4.13.

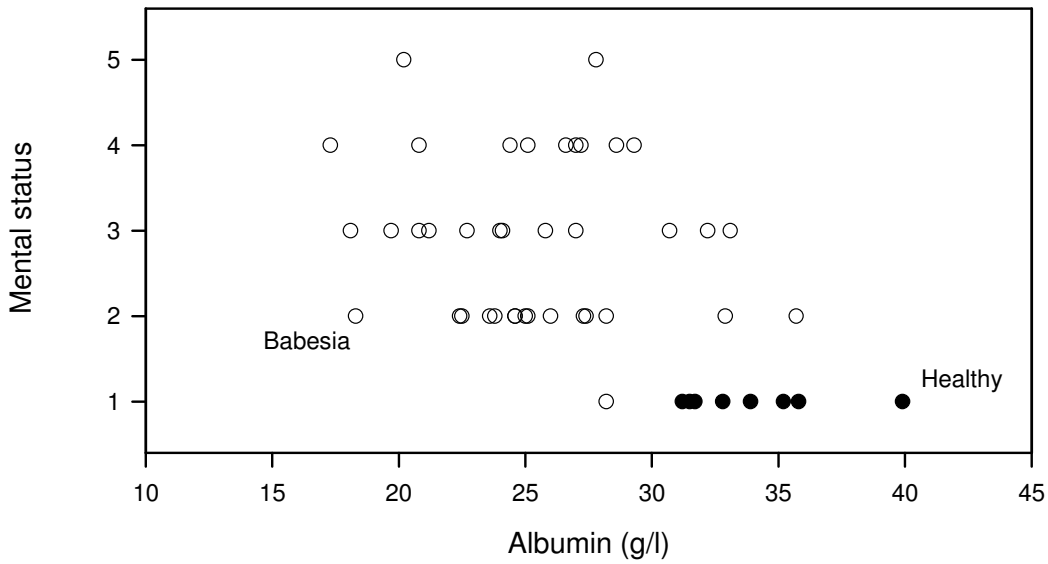


Figure 4.13: Scatter plot of the albumin concentration against the mental status. Data are shown for both the healthy experimental and sick animals.

4.2.6 Albumin and splenomegaly

There was a trend towards a statistical difference in the albumin concentrations between those babesia dogs with and without splenomegaly, with the latter having higher albumin concentrations (t-test, p = 0.064). There was, however, insufficient power (power = 0.34). Figure 4.14.

4.2.7 Albumin to PCV

There was a poor, but significant direct correlation, (r = 0.53, p = <0.001, Pearsons correlation) between the albumin and PCV (Figure 4.15) in the babesia infected animals.

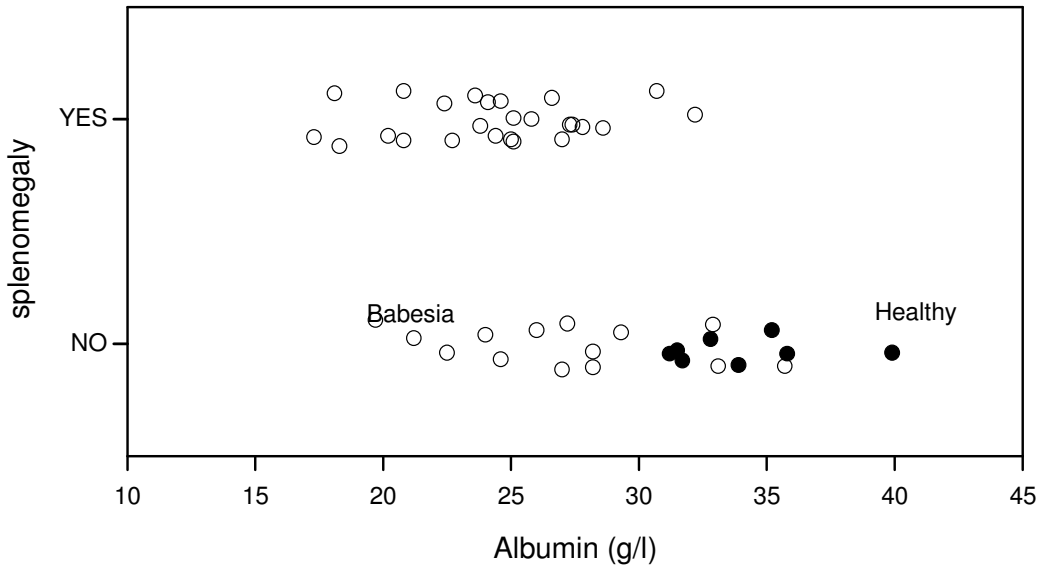


Figure 4.14: Scatter plot of the albumin concentration against splenomegaly. Data are shown for both the healthy experimental and sick animals.

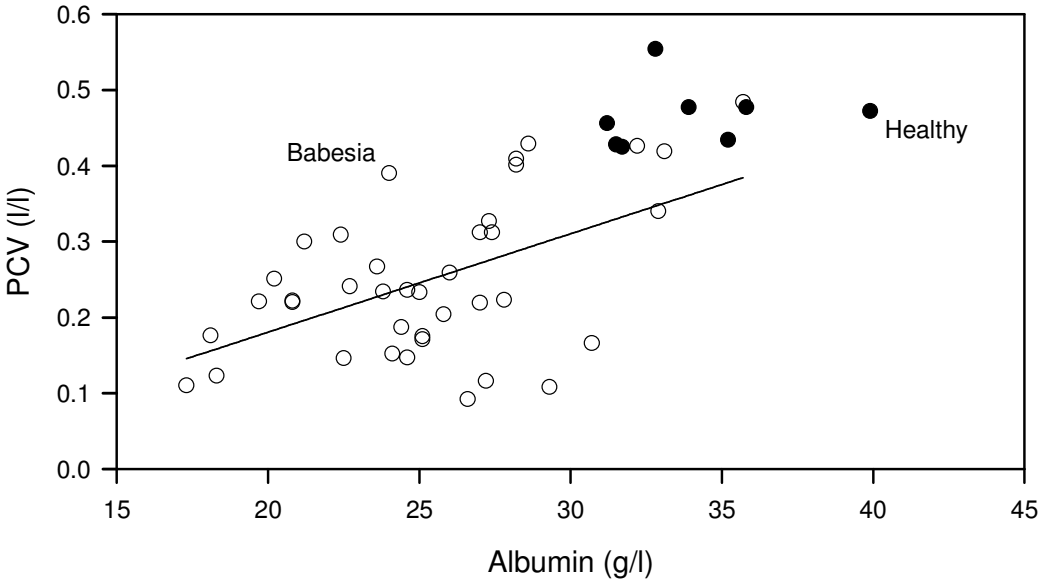


Figure 4.15: Scatter plot of the PCV against the albumin concentration. The correlation line is shown for the data of the babesia infected dogs only.

4.3 DIMINAZENE ACETURATE ANALYSIS AND PLASMA CONCENTRATIONS

4.3.1 HPLC (high performance liquid chromatography)

The limit of diminazene aceturate quantification by the HPLC was 200 ng/ml, while the limit of detection was 100 ng/ml. Accuracy was between 80 to 109%, while the precision (co-efficient of variation) was <25% at concentrations greater than 200 ng/ml.

4.3.2 Prior administrations of phenamidines

No dog had received diminazene aceturate (or phenamidine or other aromatic diamidine) during the 2-month period prior to blood collection.

4.3.3 Diminazene reconstitution and administration

All doses of diminazene aceturate were given by intra-muscular injection. The volume of water used for diminazene aceturate reconstitution ranged from 2.36 – 2.54 ml with a median of the 2.437 ml. The final concentration of the reconstituted diminazene aceturate was between 41,34 and 44,49 mg/ml. The mean dose of diminazene aceturate injected was 4.31 mg/kg and 109.23 mg/m^2 with a range of 4.06 - 4.45 mg/kg and 63.71 - 169.27 mg/m^2.

4.3.4 Diminazene aceturate results

Diminazene aceturate concentrations were measured in 151 blood samples from the 39 dogs (Figures 4.16 and 4.17). All samples collected were analysed as a single batch. Diminazene aceturate concentration could not be quantified in 9 samples. These were all the 4th samples. Five blood samples were not collected in three dogs: one dog (patient 1) was removed due to aggressive behaviour and inability to collect the 3rd and 4th sample, one dog (patient 4) died after the 3rd sample was collected, and one dog (patient 27) was removed after collecting the 2nd blood sample due to worsening anaemia. No problems with excessive blood loss due to blood collection were experienced. The one dog (dog 32) that was removed from the trial due to anaemia fulfilled the PCV criteria for

inclusion based on the Outpatients PCV, but was removed after the full blood count results revealed a PCV that fell to within the exclusion criteria.

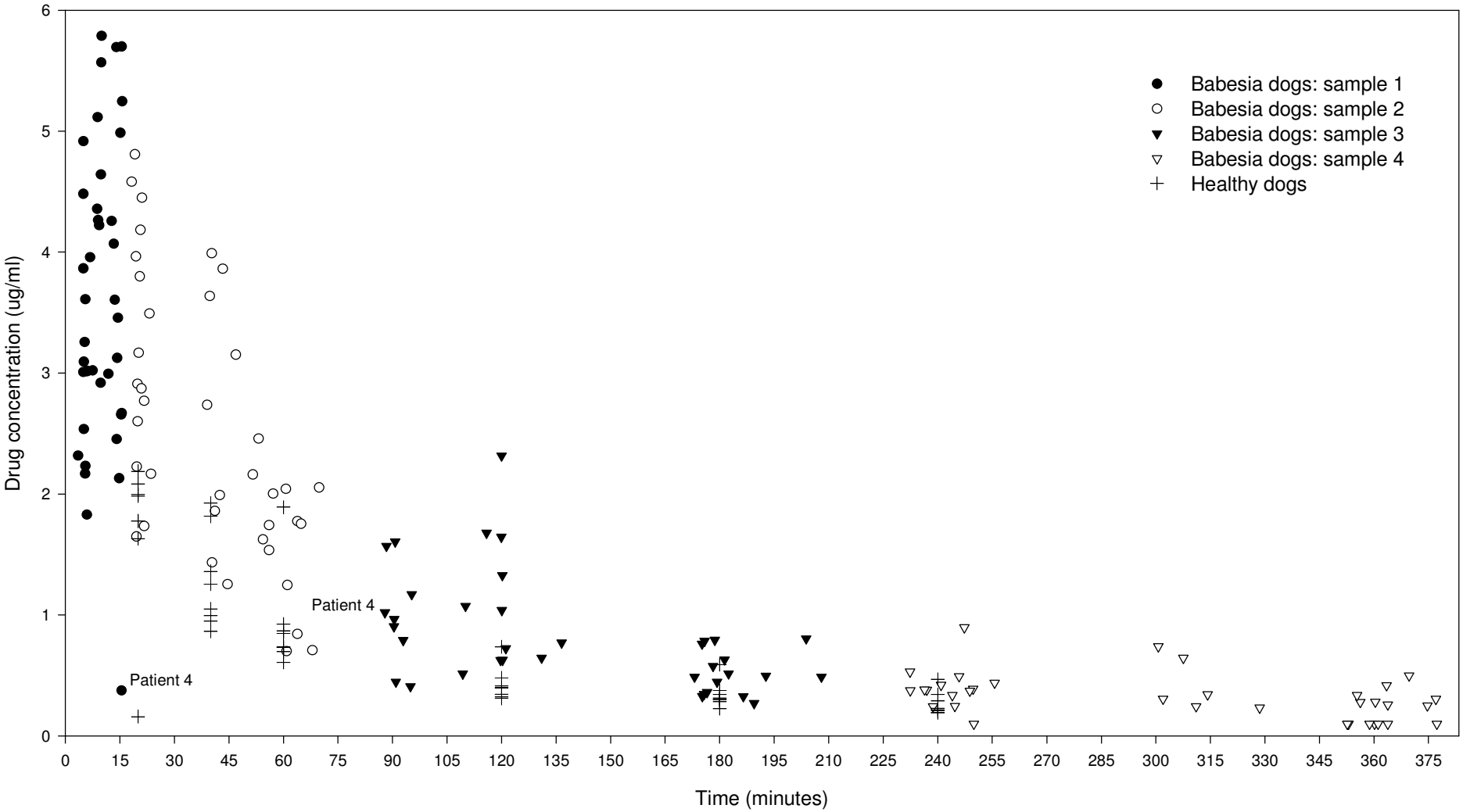


Figure 4.16: Diminazene blood concentrations (linear scale) showing the data for both babesia infected dogs and healthy dogs for comparison.

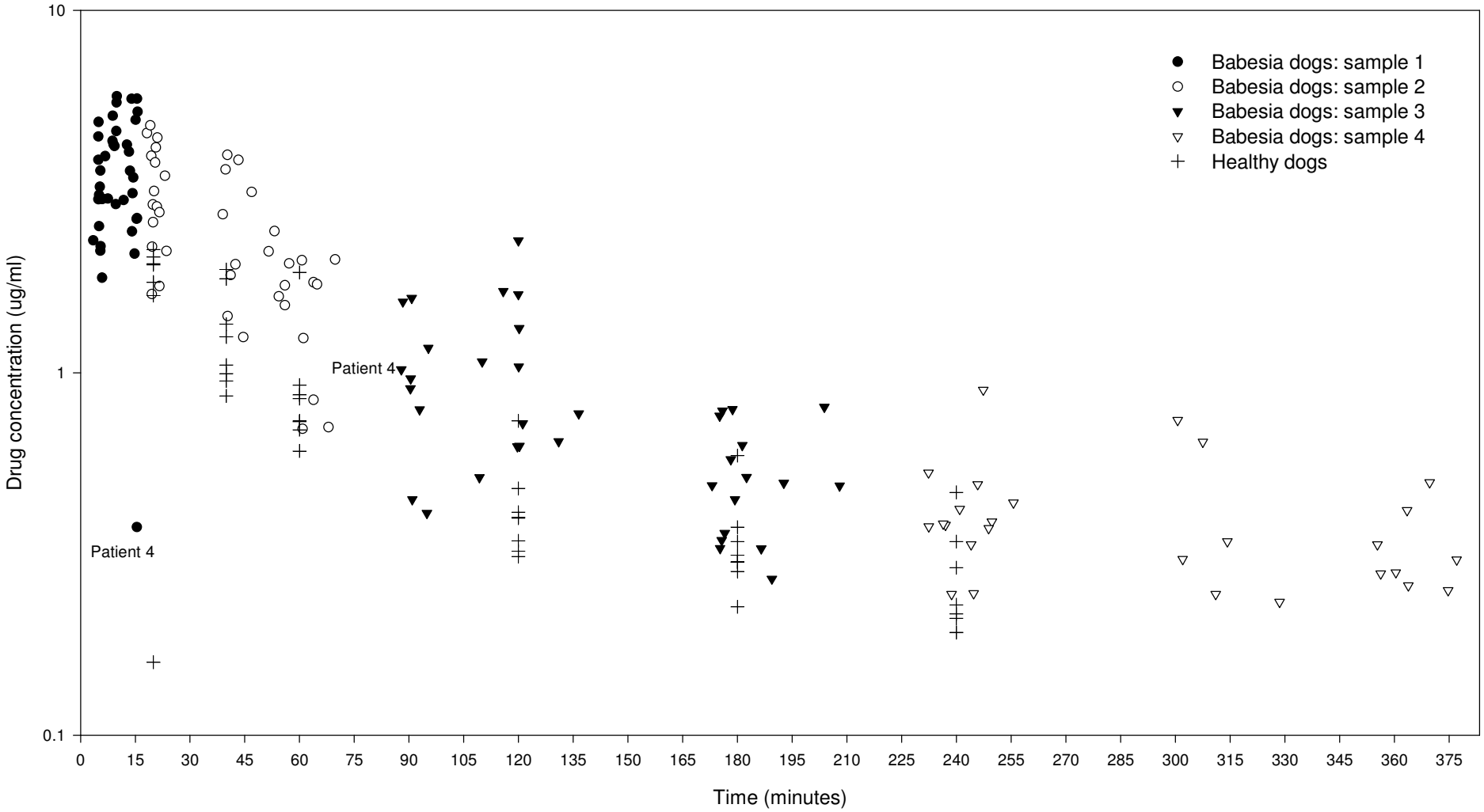


Figure 4.17: Diminazene blood concentrations (semi log scale) showing the data for both babesia infected dogs and healthy dogs for comparison.

4.4 POPULATION PHARMACOKINETIC MODELLING

A one-compartment model (WinNonMix Model 3) with no lag period and first order elimination was used. A two-compartment model could not be fitted to the data. Sigma^2 was estimated by WinNonMix using the first order method with the variance functional model at 1. Optimization was performed with the Quasi-Newton Algorithm. Standard errors of fixed parameters were calculated using the Sandwich Method. Improvements in models were based on a decrease in the minimum objective function (MOF) value and visual assessment of the predicted individual concentration versus time graph as discussed in the materials and methods.

The general format of the mixed effect specification modelling is given as:

parameter = parameter1 + parameter2 * covariate ... + random effect1.

For example:

*VF = VF_0 + VF_1*Haemoglobin + VF_ETA0*

Where VF refers to volume of distribution and ETA refers to the random effect. The numerical suffix "_0, _1…" refers to the specific mixed effect covariate (i.e. in this case, VF_1 is the volume of distribution to be multiplied by the haemoglobin).

The diminazene plasma concentration was calculated according to the formula:

$$C(T) = D*K_{01}/V/(K_{01}-K_{10})*(EXP(-K_{10}*T)-EXP(-K_{01}*T))$$

Where C = plasma concentration at time T with dose D, using volume of distribution (V), K_{01} and K_{10}.

4.5 WINNONMIX – WITH NO MIXED EFFECT COVARIATE DATA ADDED

Experimental intramuscular diminazene pharmacokinetic data ("healthy" group) from Miller[39] was added to this study's data. These were healthy adult German Shepherd dogs of both genders. In total there were 47 subjects (39 from this study, the "babesia" group, and 8 from the experimental set, the "healthy" group). In total there were 198 observations (142 from this study and 56 from the experimental set). The 9 plasma diminazene concentrations below the level of detection in the babesia data set were excluded during the modelling. The initial parameters and modelling limits for VF (fractional volume of distribution), K_{01} (first order absorption rate constant) and K_{10} (first order elimination rate constant) are given in Table 4.3. These were based on work by Miller[39], Onyeyilli and Anika[46] and Anika and Onyeyilli[7]. Miller and co-workers estimated the mean K_{01} to be 43, which proved to result in a poorer fit (MOF 5.8; Figure 4.18) than when an initial estimation of 20 was used (Figure 4.19). The model was initially run using only babesia infected animals (Table 4.4). Adding healthy individuals to the data set improved the model fit (MOF decreased from –13.5 to –70.2). The results for the model using only the experimental dogs (MOF – 108.4) are shown in Table 4.4. The

final pharmacokinetic parameter estimates and individual observed versus predicted plasma concentrations for the combined data sets are given in Table 4.4 and Figure 4.19, respectively.

Table 4.3: The initial parameters, and their limits, used for the non-covariate population model.

Parameter	Initial value	Low limit	High limit
VF_0	0.5	0	15
K_{01}_0	20	0	400
K_{10}_0	0.9	0	10

Table 4.4: Final mixed effects modelling parameter estimates, with no covariate data added, using the combined babesia and healthy dog data.

Parameter	Final estimate	Standard error	CV%
VF_0 (Babesia infected dogs)	1.00	0.06	5.56
VF_0 (Healthy dogs)	2.32	0.22	9.49
VF_0 (All dogs)	1.10	0.07	5.96
K_{01}_0 (Babesia infected dogs)	21.88	4.20	19.19
K_{01}_0 (Healthy dogs)	9.44	10.23	108.40
K_{01}_0 (All dogs)	22.32	4.10	18.36
K_{10}_0 (Babesia infected dogs)	0.84	0.07	7.86
K_{10}_0 (Healthy dogs)	0.60	0.05	8.83
K_{10}_0 (All dogs)	0.85	0.06	7.40
$Sigma^2$ (Babesia infected dogs)	0.09	0.01	15.26
$Sigma^2$ (Healthy dogs)	0.03	Not estimated	Not estimated
$Sigma^2$ (All dogs)	0.07	0.01	14.41
Where	VF = VF_0 + VF_ETA0		
	K_{01} = K_{01}_0 + K_{01}_ETA0		
	K_{10} = K_{10}_0 + K_{10}_ETA0		

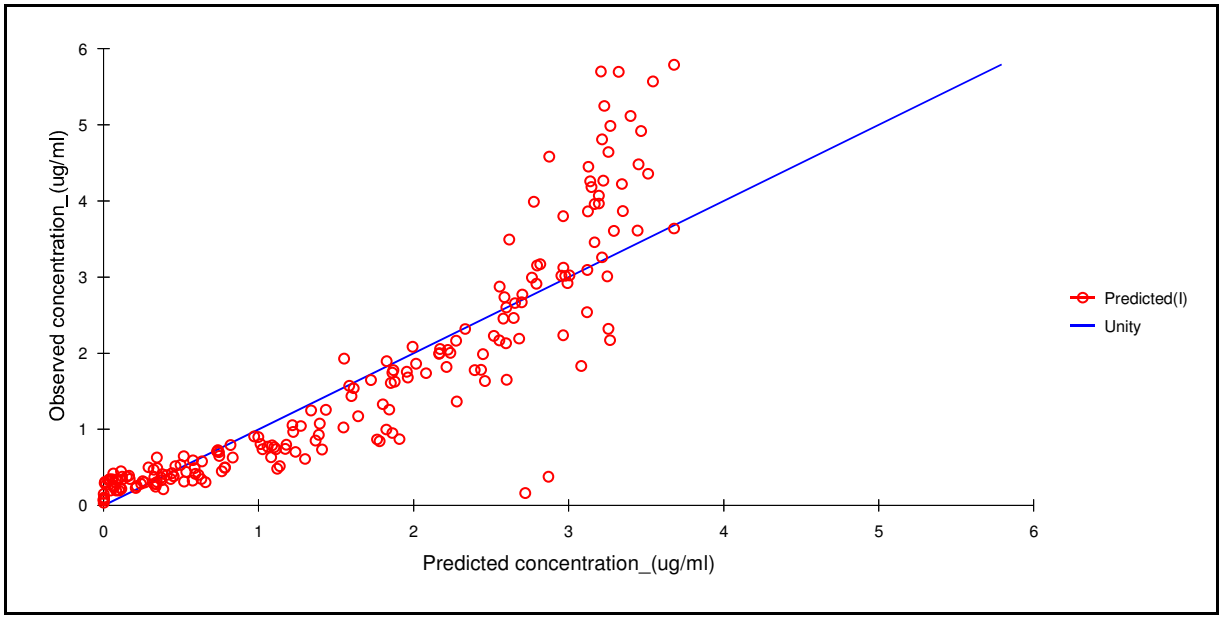


Figure 4.18: The individual predicted versus observed plasma diminazene concentration when an initial K_{01} of 43 was used in the combined data sets of babesia and healthy dogs.

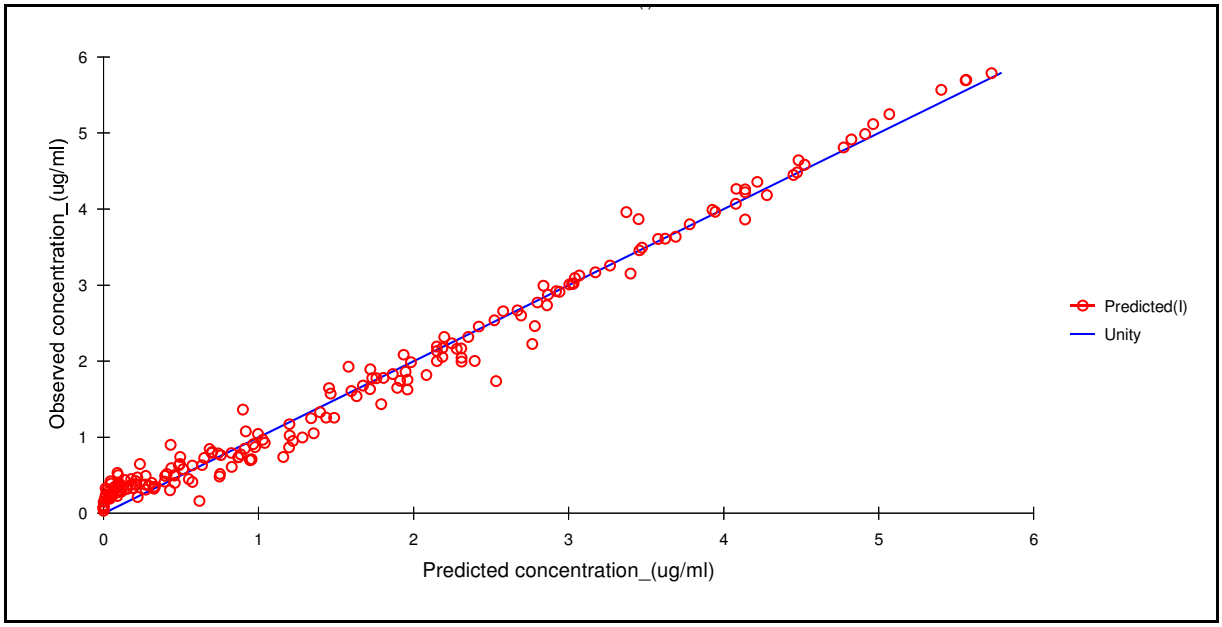


Figure 4.19: The individual predicted versus observed plasma diminazene concentration when an initial K_{01} of 20 was used in the combined data sets of babesia and healthy dogs.

4.5.1 Variations in random specification

Different inter-individual random specifications were tested using the 'Mixed Effects Specification' dialog box in WinNonMix. The base model's additive inter-individual random specification ($\theta_j=\theta+\eta_j$) was changed to $\theta_j=\theta\bullet(1+\eta_j)$ or $\theta_j=\theta\bullet\eta_j$ or $\theta_j=\theta\bullet(1+\exp(\eta_j))$ or $\theta_j=\theta+\exp(\eta_j)$. These changes had no effect on delta MOF or worsened the base model. The additive model was therefore retained and the parameter estimates showed reasonable approximation to normality (see later, Figure 4.40)

The 'Between Subject Variance' in the 'Error Structure Specification' dialog box in WinNonMix was left blank. This is the default setting and allows WinNonMix to generate estimates.

Different forms of intra-individual residual error variance in the 'Error Structure Specification' dialog box were attempted. Although there were significant improvements in the MOF (Figure 4.20), the additive model was retained as the most appropriate as the individual observed versus predicative diminazene plasma concentration scatter plots showed loss of unity at higher concentrations (plasma concentrations were under predicated) as compared to the base model with the additive error structure (Figure 4.19), indicating inappropriate model specification despite the statistical improvement.

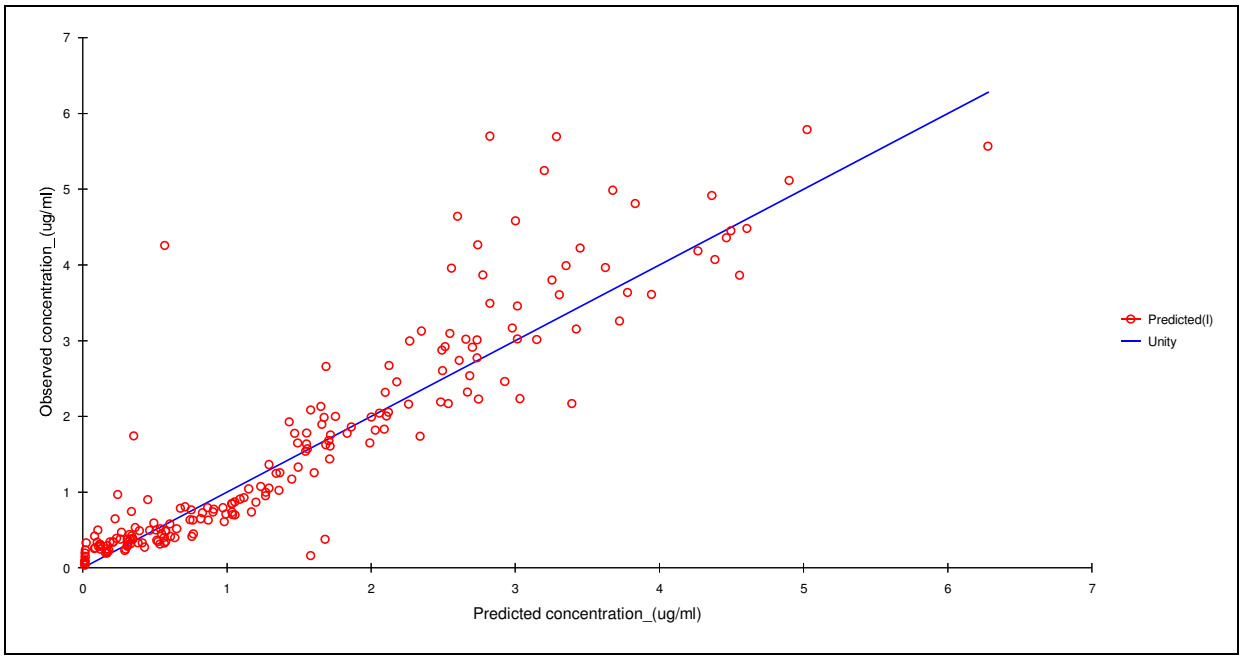


Figure 4.20: The constant power residual error model had the best MOF at 123. The observed versus predicted plasma concentration plots show significant divergence from the unity line indicating inappropriate model specification despite the statistical improvement.

4.5.2 Outliers

The dog that died showed a flip-flop effect on the plasma diminazene versus time plot (Figure 4.17). When the base model was run, with that dog's data excluded, there was a significant improvement in the model (MOF = -74.8). This dog's data was included in all other analyses as it represents a real clinical event.

4.6 WINNONMIX – MIXED EFFECT MODELLING WITH SINGLE COVARIATES ADDED

The full data set (39 subjects from this study and 8 from the experimental work) was used for all analyses, unless otherwise stated.

Mixed effect modelling was not performed on those parameters with too few samples: interbreed variation; serum haemolysis (5 dogs); icteric serum (6 dogs); other illnesses (1 dog); in-saline agglutination (1 dog); vomiting (5 dogs); diarrhoea (4 dogs). Mucus membrane was not assessed due to its subjective nature and its poor prediction of PCV, seen by the large overlap of PCVs at different mucus membrane colours (Figure 4.4). The effect of an immature age on the pharmacokinetics could not be investigated as there were no dogs less than 3 months of age. The leukocyte and platelet parameters were also not added to the mixed effects model. Thrombocytopenia is commonly associated with babesiosis and therefore is a reflection of the health status in this study.[28]

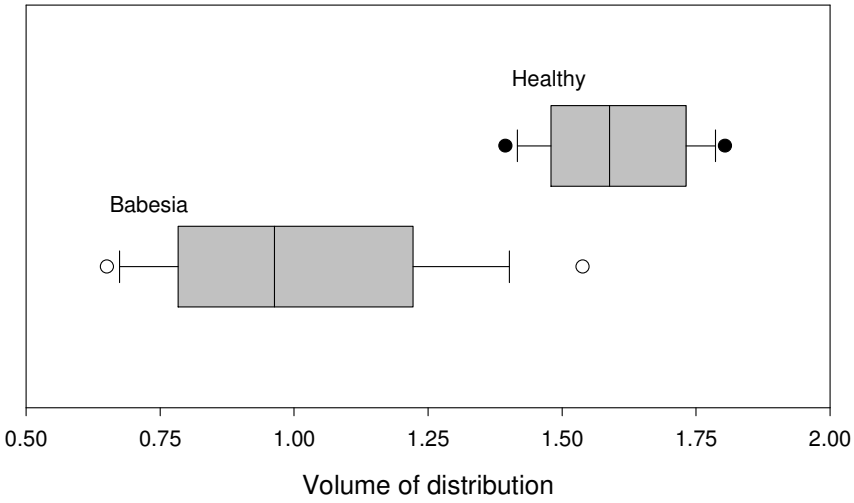
4.6.1 Health status

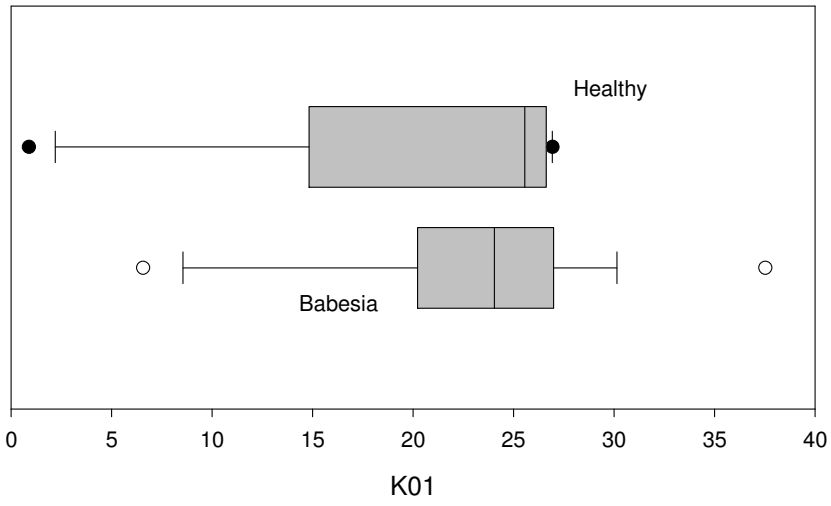
There was a significant difference (Mann-Whitney Rank Sum, p = <0.001) for VF between the healthy and babesia infected dogs (medians of: 1.589 and 0.963, respectively). There was no statistical difference in K_{01} between the two groups (Mann-Whitney Rank Sum, medians of: 25.563 and 24.044, respectively). A t-test for K_{10} showed insufficient power (0.232) to detect a significant difference (p = 0.107) between healthy and babesia infected animals (means of: 0.981 and 0.866, respectively). Figure 4.21.

The WinNonMix *AsClass* function was used to investigate whether the disease status influenced the pharmacokinetic parameters. Only VF was significantly influenced (Table 4.5). The MOF decreased (from –70.2) to –92.7, –73.5 and –72.7 for VF, K_{01} and K_{10}, respectively when disease status was added as a covariate. When VF and K_{01} were modelled concurrently, there was further significant improvement in the model (MOF –101.1), despite the fact that disease status on K_{01} alone did not significantly improve the model.

Table 4.5: Parameter estimates for the mixed effect modelling with health status used as covariate on VF.

Parameter	**Final estimate**	**Standard error**	**CV%**
VF_0	1.96	0.15	7.65
VF_1(health status=babesia)	-0.95	0.15	15.86
K_{01}_0	22.12	4.59	20.75
K_{10}_0	0.83	0.06	7.11
$Sigma^2$	0.08	0.01	18.21
Where	VF = VF_0 + AsClass(health status)*VF_1 + VF_ETA0		
	$K_{01} = K_{01}$_0 + K_{01}_ETA0		
	$K_{10} = K_{10}$_0 + K_{10}_ETA0		





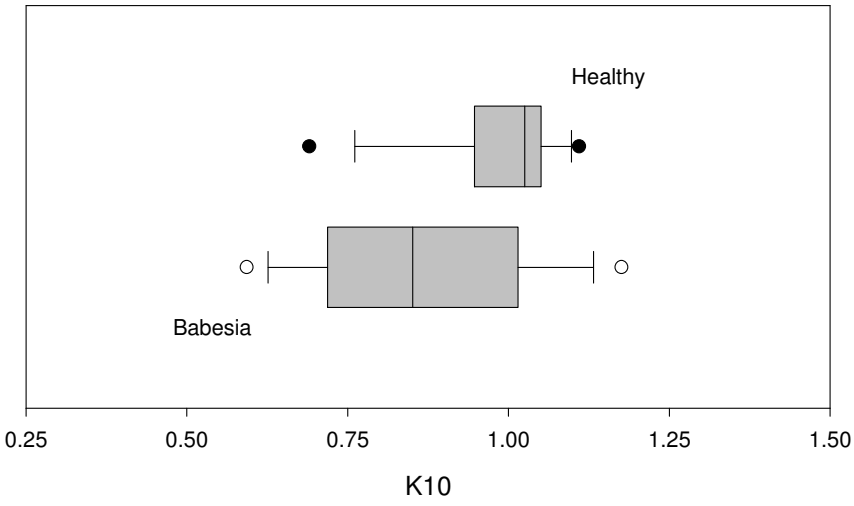


Figure 4.21: Box chart showing the 5th, 10th, 25th, median, 75th, 90th and 95th percentiles of the pharmacokinetic parameters for volume of distribution, K_{01} and K_{10}, as determined by the base model, for the babesia infected and healthy dogs.

4.6.2 Haemoglobin and red blood cells

Only the regression of VF to the erythrocyte parameters (PCV, haemoglobin concentration and red cell count) showed sufficient power. For both K_{01} and K_{10} the power was <0.38 and the r^2 <0.06. The r^2 for VF to PCV, red blood cell count and haemoglobin was 0.35, 0.38 and 0.38, respectively (Figure 4.22, Figure 4.23, and Figure 4.24).

Red blood cells, haemoglobin and PCV all decreased the MOF to a similar degree (MOF: –79.3 to –81.4) when modelled for the VF. Since PCV was considered the most practical measure (as micro centrifuges are commonly available in general veterinary practice), only this parameter was further evaluated.

Only VF showed a significant improvement when PCV was added to the mixed effects modelling (Table 4.6). The MOF for the PCV was –79.3, –73.2 and –72.0 for VF, K_{01} and K_{10}, respectively.

Healthy animals were removed from the model to investigate the direct effect of PCV. The PCV is correlated to the health status as babesiosis causes anaemia. Therefore, PCV may not be an independent variable. There was no significant improvement to the model. The MOF for VF, $K_{01}$and K_{10} was –15.2, –16.7 and –14.1, respectively.

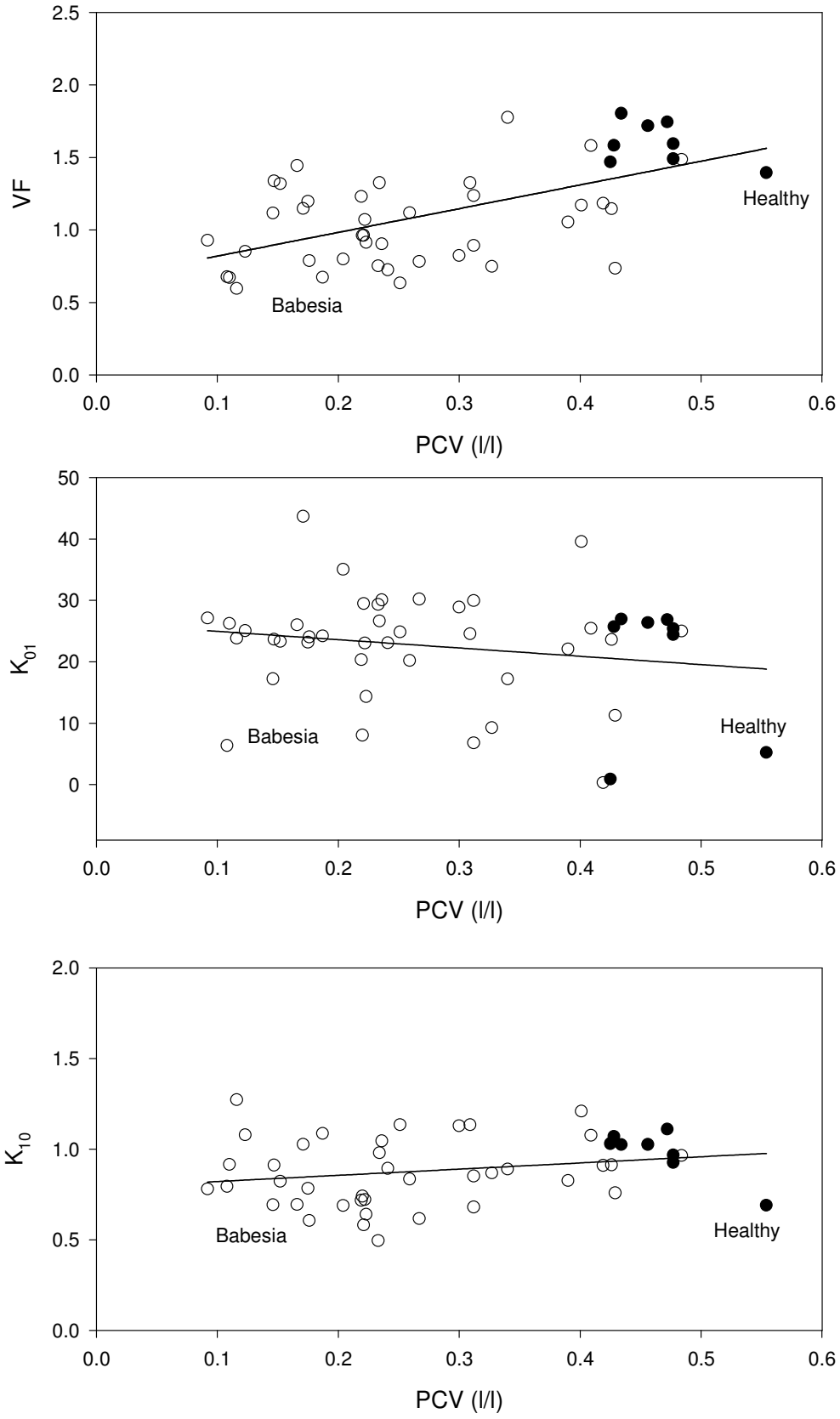


Figure 4.22: The VF, K_{01} and K_{10} plotted against the PCV with the regression line.

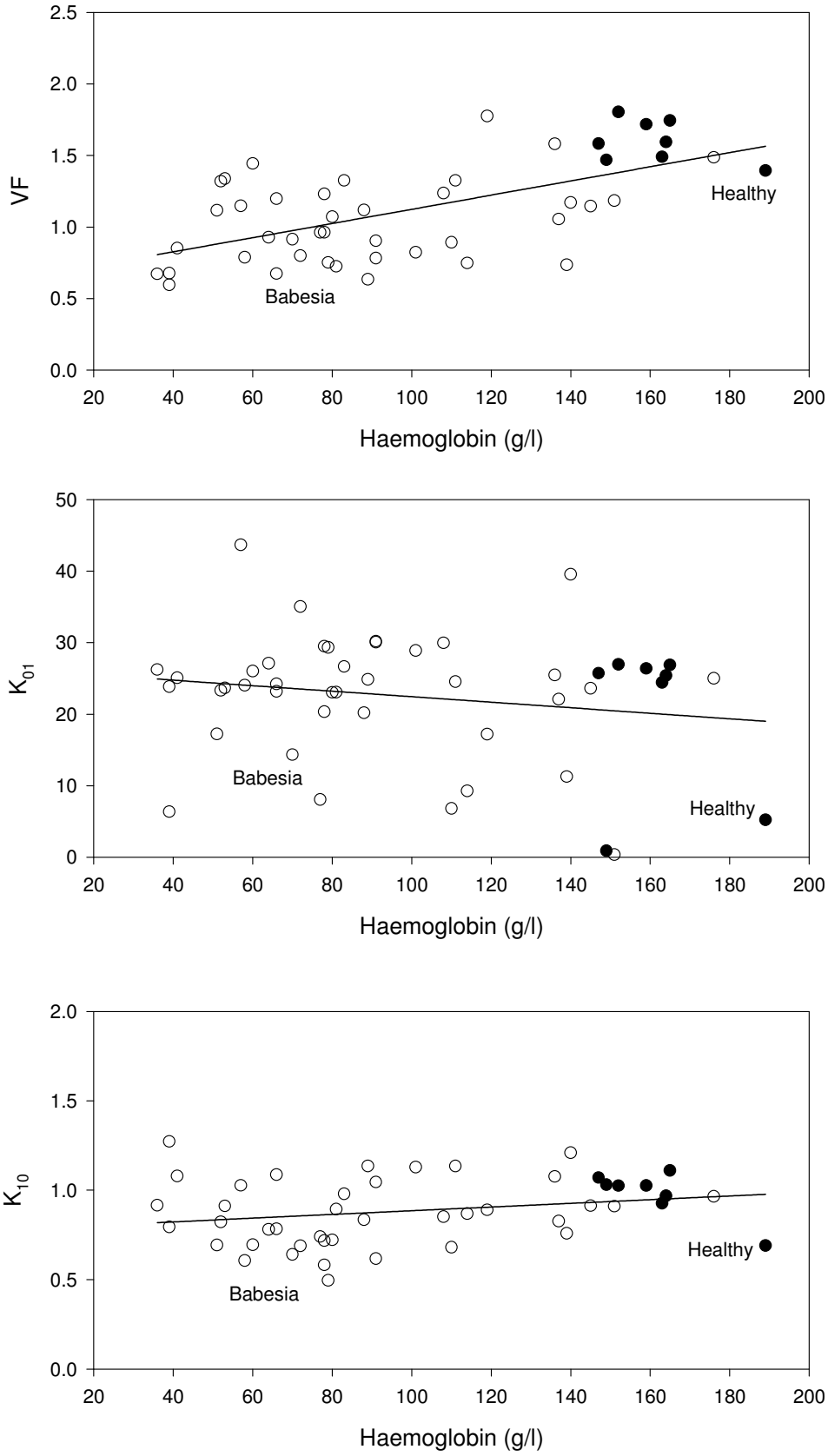


Figure 4.23: The VF, K_{01} and K_{10} plotted against the haemoglobin concentration with the regression line.

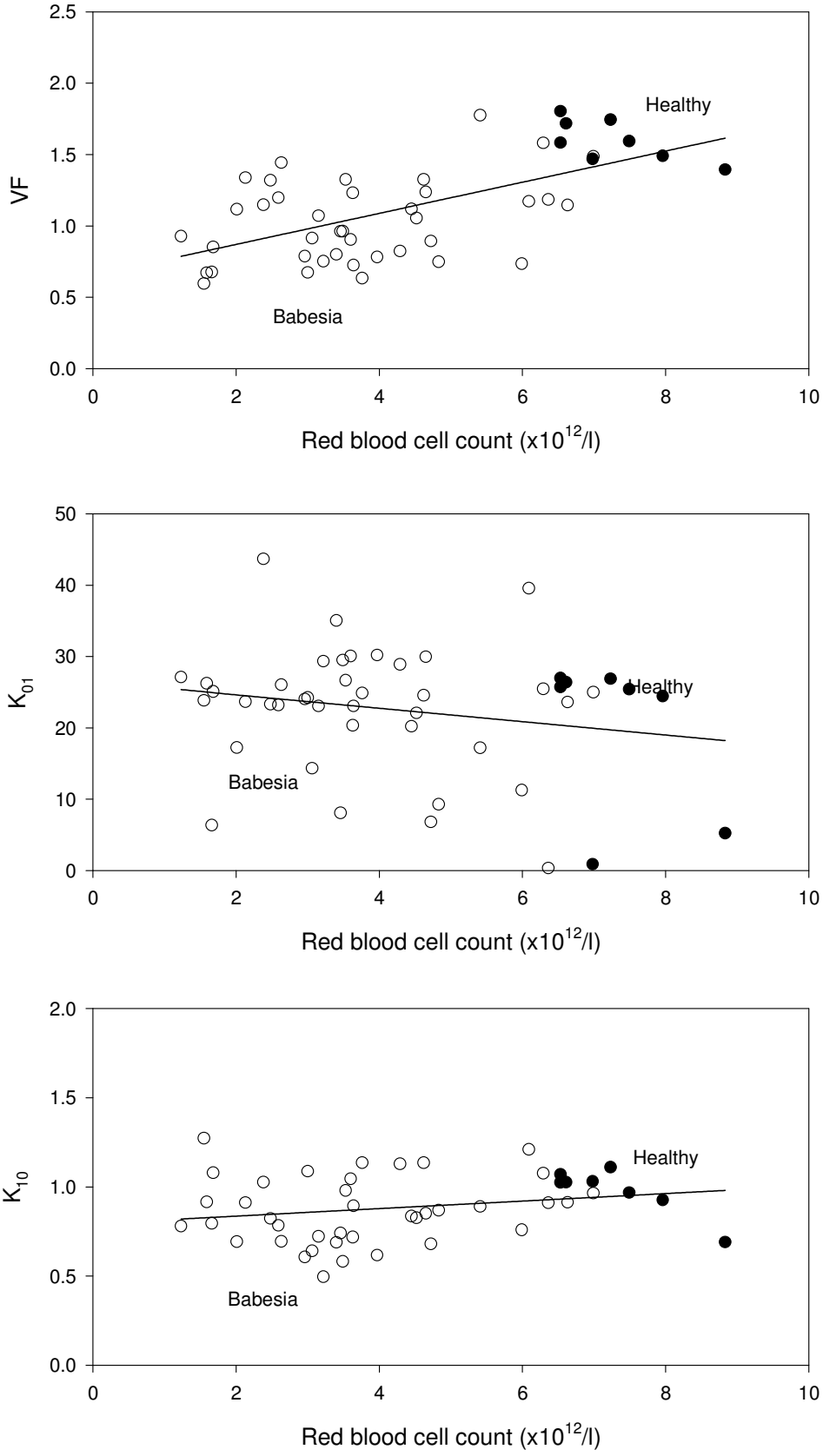


Figure 4.24: The VF, K_{01} and K_{10} plotted against the red blood cell count with the regression line.

Table 4.6: Final parameter estimates for the mixed effect modelling with packed cell volume as covariate.

Parameter	Final estimate	Standard error	CV%
VF_0	0.80	0.12	15.20
VF_1(PCV)	1.28	0.47	36.46
K_{01}_0	21.93	4.64	21.16
K_{10}_0	0.84	0.06	7.06
$Sigma^2$	0.07	0.01	15.67
Where	VF = VF_0 + PCV*VF_1 + VF_ETA0		
	K_{01} = K_{01}_0 + K_{01}_ETA0		
	K_{10} = K_{10}_0 + K_{10}_ETA0		

4.6.3 Albumin

The VF was positively correlated to albumin (r^2 = 0.45, Figure 4.25). The regression analysis for both K_{01} and K_{10} showed insufficient power (r^2 = 0.04 and power = 0.26 for both).

Including albumin into the population model resulted in the MOF decreasing to –81.6, –70.7, and –71.7 for VF, K_{01} and K_{10}, respectively. Table 4.7 shows the parameter estimates for VF.
The model was run with healthy animals removed from the data set. Albumin is a negative acute phase protein and decreases with inflammation. Babesiosis is an inflammatory condition and therefore hypoalbuminaemia may show colinearity to health status and may not be an independent covariate. There was a statistical difference between the albumin concentrations of healthy dogs and babesia dogs. When only ill dogs were analysed, the addition of albumin concentrations did not significantly improve the model for VF, K_{01} or K_{10} (MOF –15.8, –14.0 and –13.5, respectively).

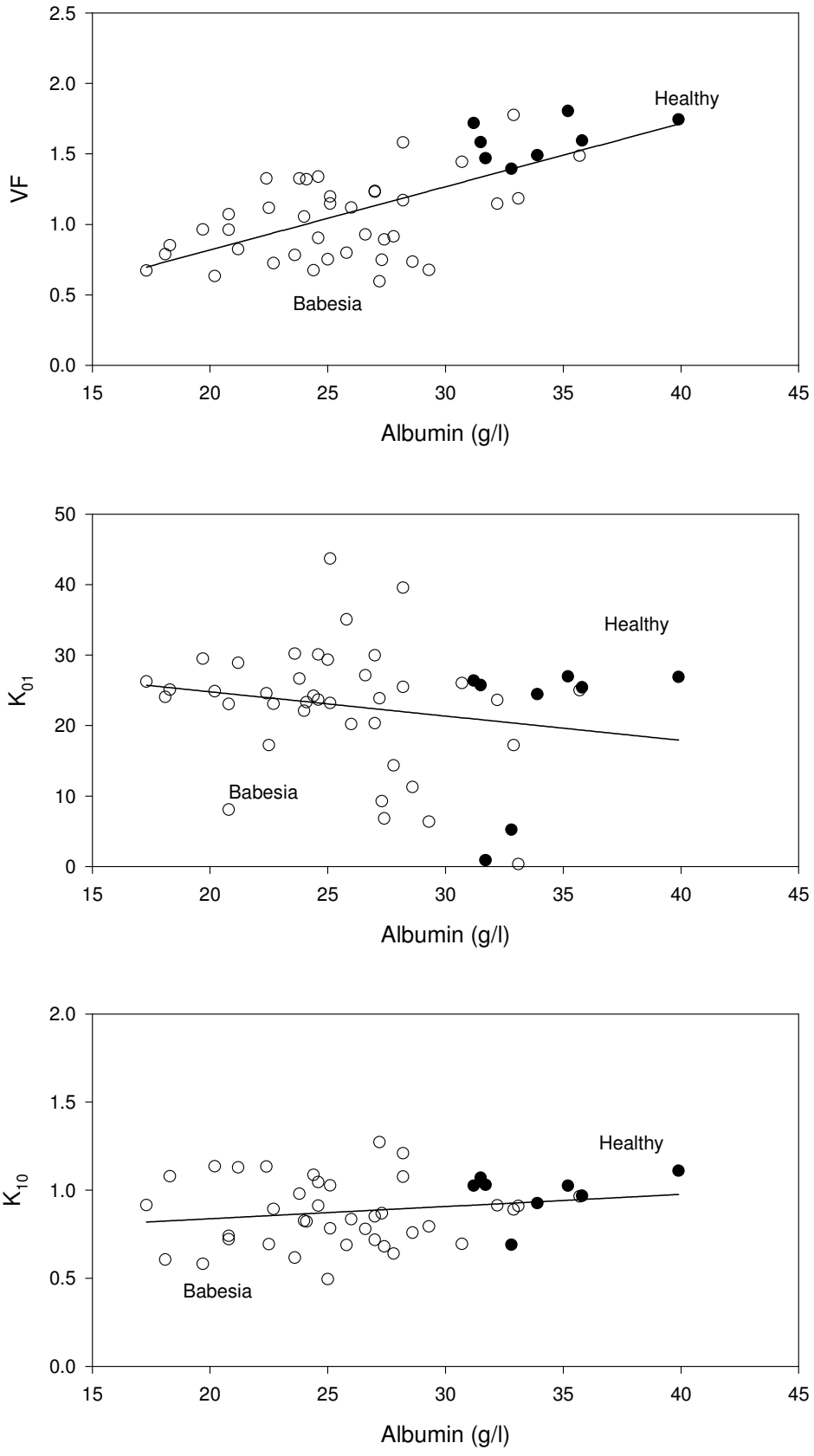


Figure 4.25: The VF, K_{01} and K_{10} plotted against albumin with the regression line.

Table 4.7: The parameter estimates for the mixed effect modelling with serum albumin concentration as covariate.

Parameter	Final estimate	Standard error	CV%
VF_0	0.19	0.27	138.54
VF_1(albumin)	0.04	0.01	30.40
K_{01}_0	22.89	4.56	19.94
K_{10}_0	0.84	0.06	7.26
$Sigma^2$	0.08	0.01	14.91
Where	VF = VF_0 + albumin*VF_1 + VF_ETA0		
	K_{01} = K_{01}_0 + K_{01}_ETA0		
	K_{10} = K_{10}_0 + K_{10}_ETA0		

4.6.4 Gender

There was no significant difference between male and female animals for VF (t-test, p = 0.714), K_{01} (Mann-Whitney Rank Sum Test, p = 0.452) and K_{10} (t-test, p = 0.635), although there was insufficient power for both t-test results.

There was no improvement in the mixed effects model when the gender was added (MOF for VF = –70.7; K_{01} = –71.1; K_{10} = –72.0.).

4.6.5 Mental status

There was a tendency for the VF to decrease with a worsening of the mental status amongst the babesia animals (One Way ANOVA, p = 0.076). There was, however, insufficient power (power = 0.352) to draw any conclusions. There was no significant difference in the K_{01} on a Kruskal-Wallis ANOVA on Ranks (p = 0.802). There was insufficient power (power = 0.049) in the One Way ANOVA for K_{10} (p = 0.522**).** Figure 4.26.

The MOF decreased to –100.8 and –76.4 for VF and K_{10}, respectively when the mental status was added to the mixed effect specification. A reasonable fit could not be found for K_{01}. The MOF for VF was significant at 4 degrees of freedom (delta MOF > 9.49).Table 4.8.

Healthy dogs were defined as having a mental status of 1. Since there was only 1 babesia dog with a mental status of 1, mental status may have acted as a surrogate marker for health status. The model

was therefore run on babesia infected dogs only. Although there was no significant improvement in the MOF (MOF: -21.8), when mental status was added to the model for VF there was a small, but progressive, decrease as the dogs were rated sicker (i.e. an increasing MS score). Table 4.9.

Table 4.8: The parameter estimates for the mixed effect modelling with mental status as covariate to VF. (MS: mental status)

Parameter	Final estimate	Standard error	CV%
VF_0	1.95	0.13	6.84
VF_1(MS=2)	0.87	0.15	17.86
VF_1(MS=3)	0.90	0.14	15.27
VF_1(MS=4)	1.04	0.13	12.92
VF_1(MS=5)	1.08	0.15	14.06
K_{01}_0	22.67	3.51	15.49
K_{10}_0	0.83	0.06	6.88
$Sigma^2$	0.08	0.01	18.13
Where	VF = VF_0 – AsClass(MS)*VF_1 + VF_ETA0		
	$K_{01} = K_{01}$_0 + K_{01}_ETA0		
	$K_{10} = K_{10}$_0 + K_{10}_ETA0		

Table 4.9: The parameter estimates for the mixed effect modelling with mental status as covariate for VF in the babesia infected dogs only. (MS: mental status)

Parameter	Final estimate
VF_0	1.87
VF_1(MS=2)	0.80
VF_1(MS=3)	0.83
VF_1(MS=4)	0.97
VF_1(MS=5)	1.01
Where	VF = VF_0 – AsClass(MS)*VF_1 + VF_ETA0

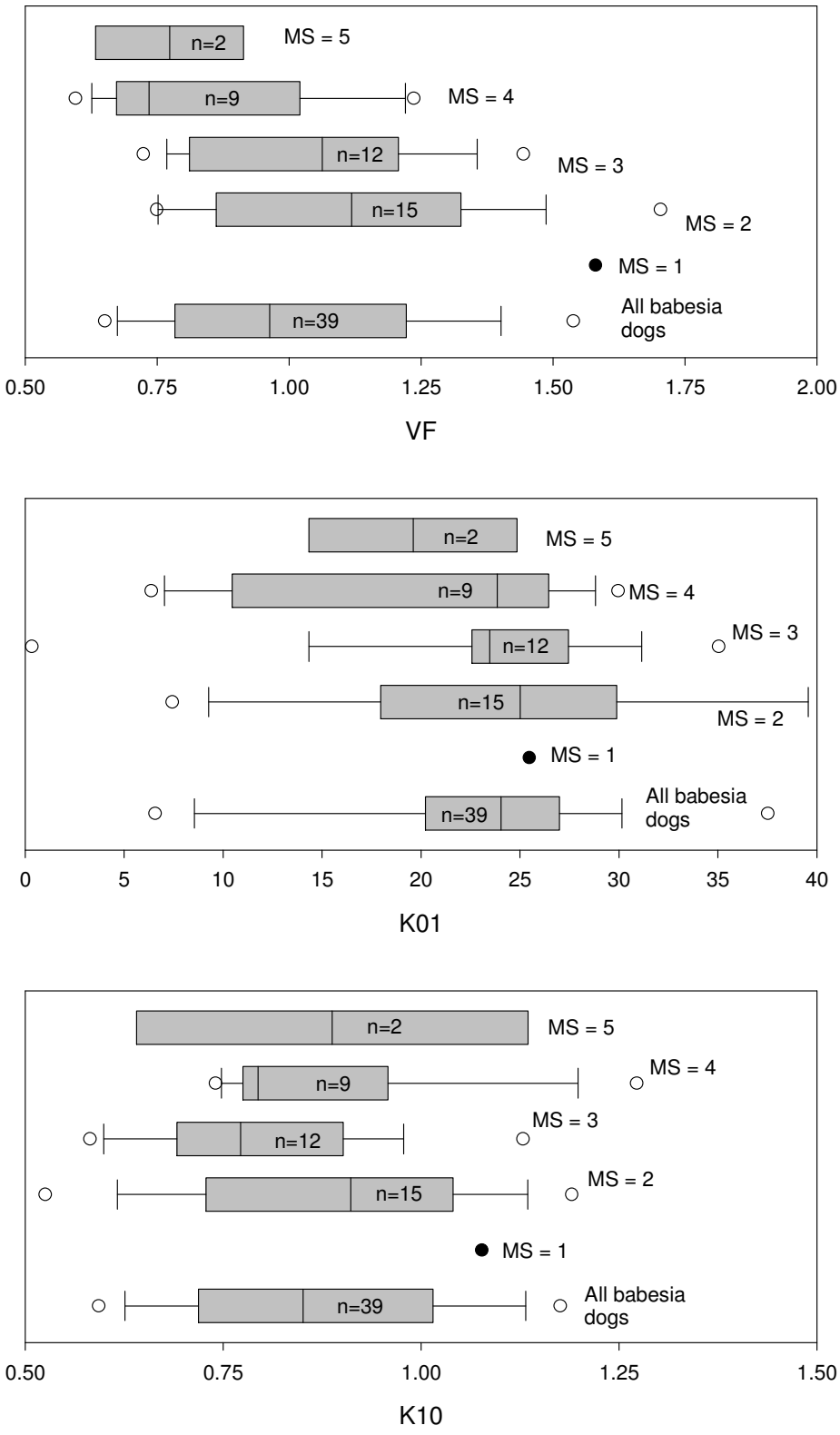


Figure 4.26: Box chart showing the pharmacokinetic parameters (as determined by the non-covariate model) for the all the babesia infected dogs concurrently and then separated into the five categories of mental status (MS). There was only one dog with a MS of 1 (black dot). Key as for Figure 4.21.

4.6.6 Body weight and body surface area

Linear regression analysis showed poor correlation between the pharmacokinetic parameters and body weight (r^2 of 0.103, 0.031 and 0.011 for VF, K_{01} and K_{10}, respectively, Figure 4.27) and body surface area (r^2 of 0.122, 0.030 and 0.015 for VF, K_{01} and K_{10}, respectively, Figure 4.28). Power was, however, insufficient in all cases (power < 0.68)

Including the body weight and body surface into the mixed effects specification did not significantly improve the model. Population modelling using mg/m^2 rather than mg/kg as dose in the base model worsened the model and increased the MOF to 424.7.

4.6.7 Spleno- and hepatomegaly

There was a statistical difference in the VF and K_{10} between all dogs with splenomegaly and those without (T-test; p = 0.001 and p = 0.016, respectively). Dogs without splenomegaly had a larger VF and K_{10} than dogs with splenomegaly (mean ± standard deviation: 1.343 ± 0.385 versus 0.959 ± 0.243 and 0.976 ± 0.167 versus 0.838 ± 0.180, respectively). Figure 4.29. When healthy animals were removed from analysis to prevent bias (healthy dogs should not have splenomegaly), this statistical difference was lost for both VF and K_{10} (p = 0.174, power = 0.145 and p = 0.072, power = 0.315, respectively), although the there was insufficient power for K_{10}. There was no statistical difference for K_{01}.

There were only 2 dogs that had hepatomegaly, which prevented meaningful comparative pharmacokinetic analysis between non-hepatomegaly and hepatomegaly dogs.

For the mixed effect modelling, animals missing data where assumed not to have splenomegaly. The MOF decreased significantly when splenomegaly was added to the population model to K_{01} (Table 4.10). The MOF for VF, K_{01} and K_{10} was -73.9, –79.2 and –73.2, respectively.

The effect of splenomegaly on babesia infected dogs only was determined to remove the bias of health. There was no significant improvement in the MOF (VF = 14.2; K_{01} = 13.5 K_{10} = 14.8).

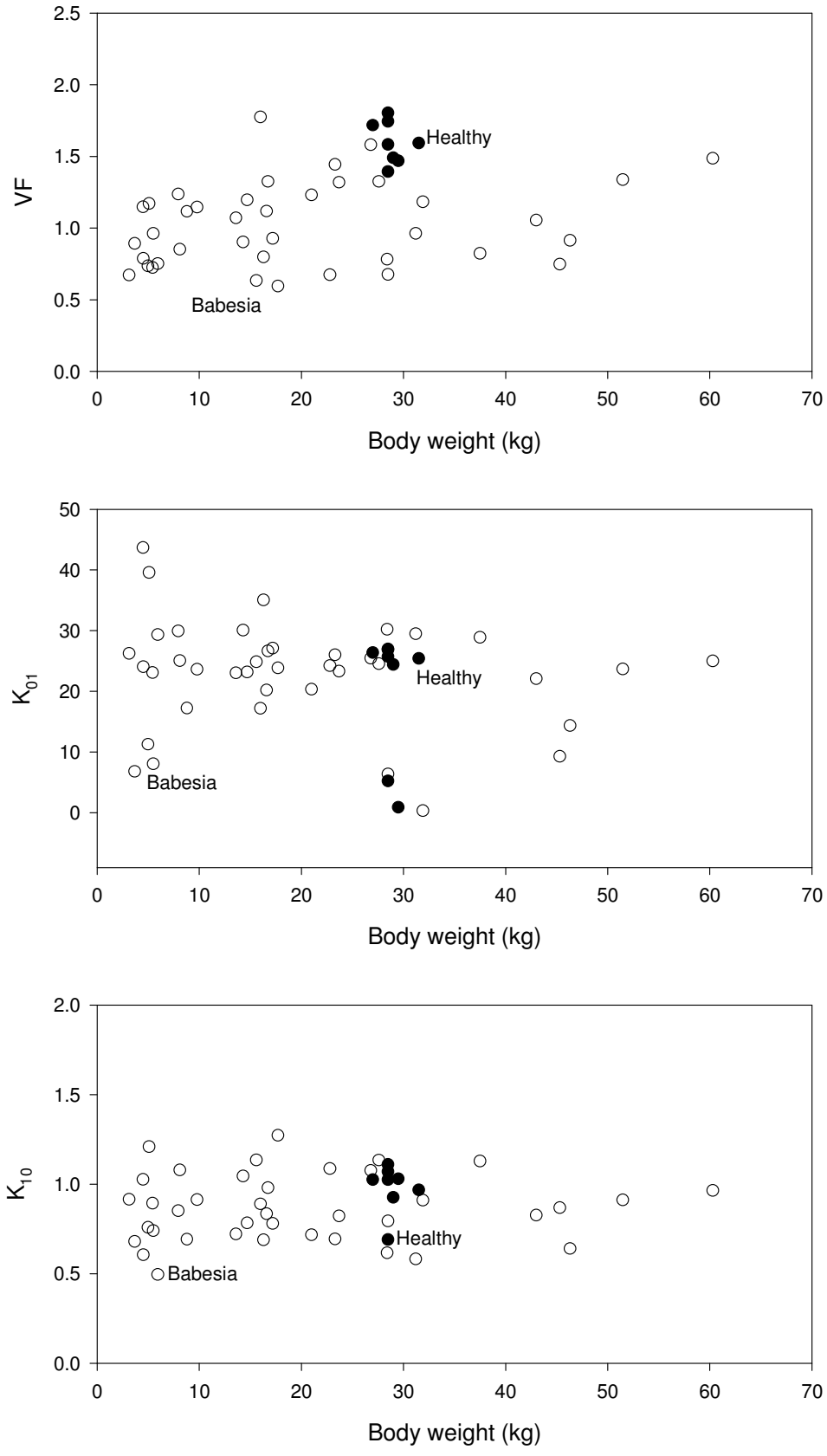


Figure 4.27: The VF, K_{01} and K_{10} plotted against body weight.

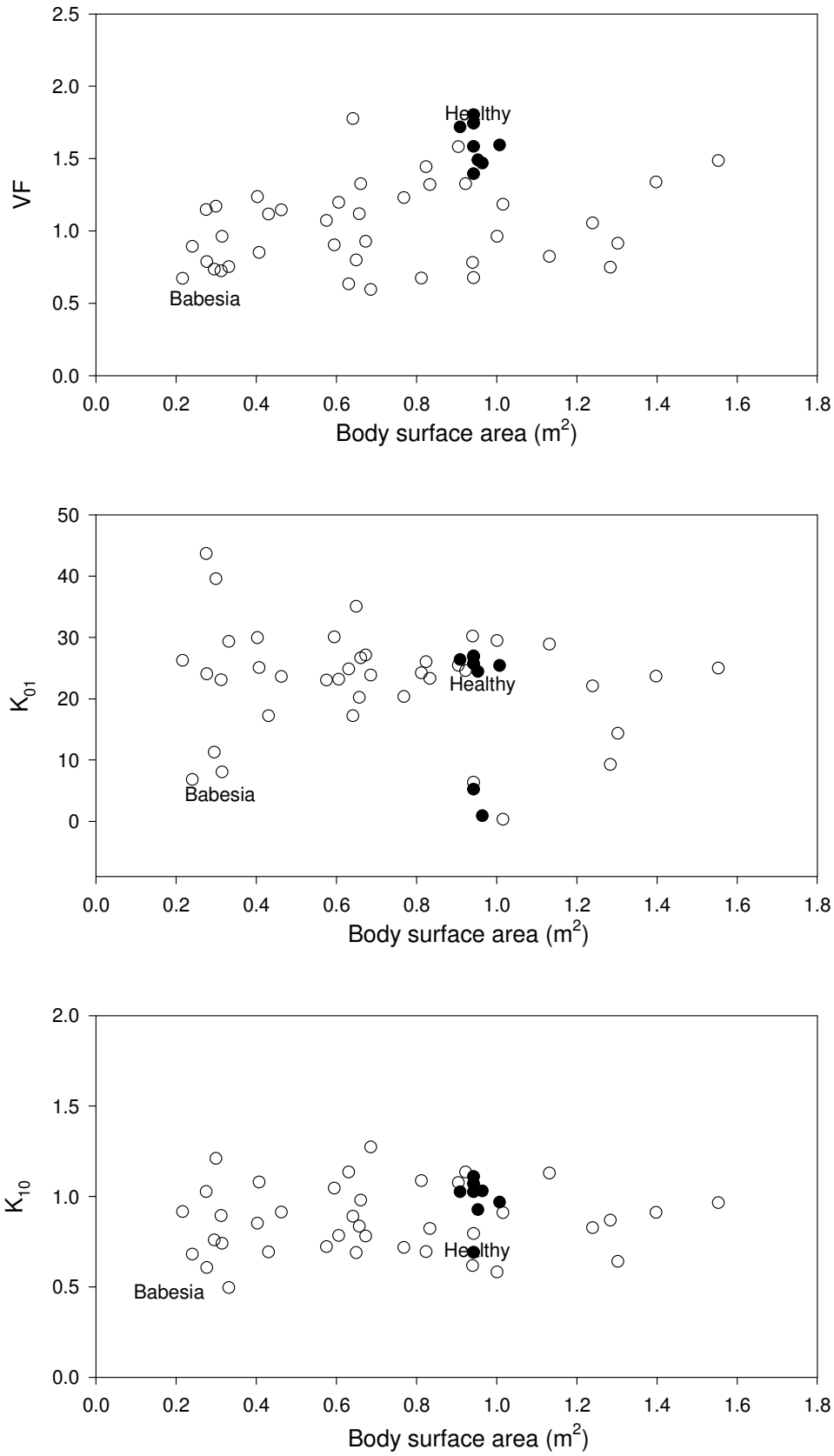


Figure 4.28: The VF, K_{01} and K_{10} plotted against body surface area.

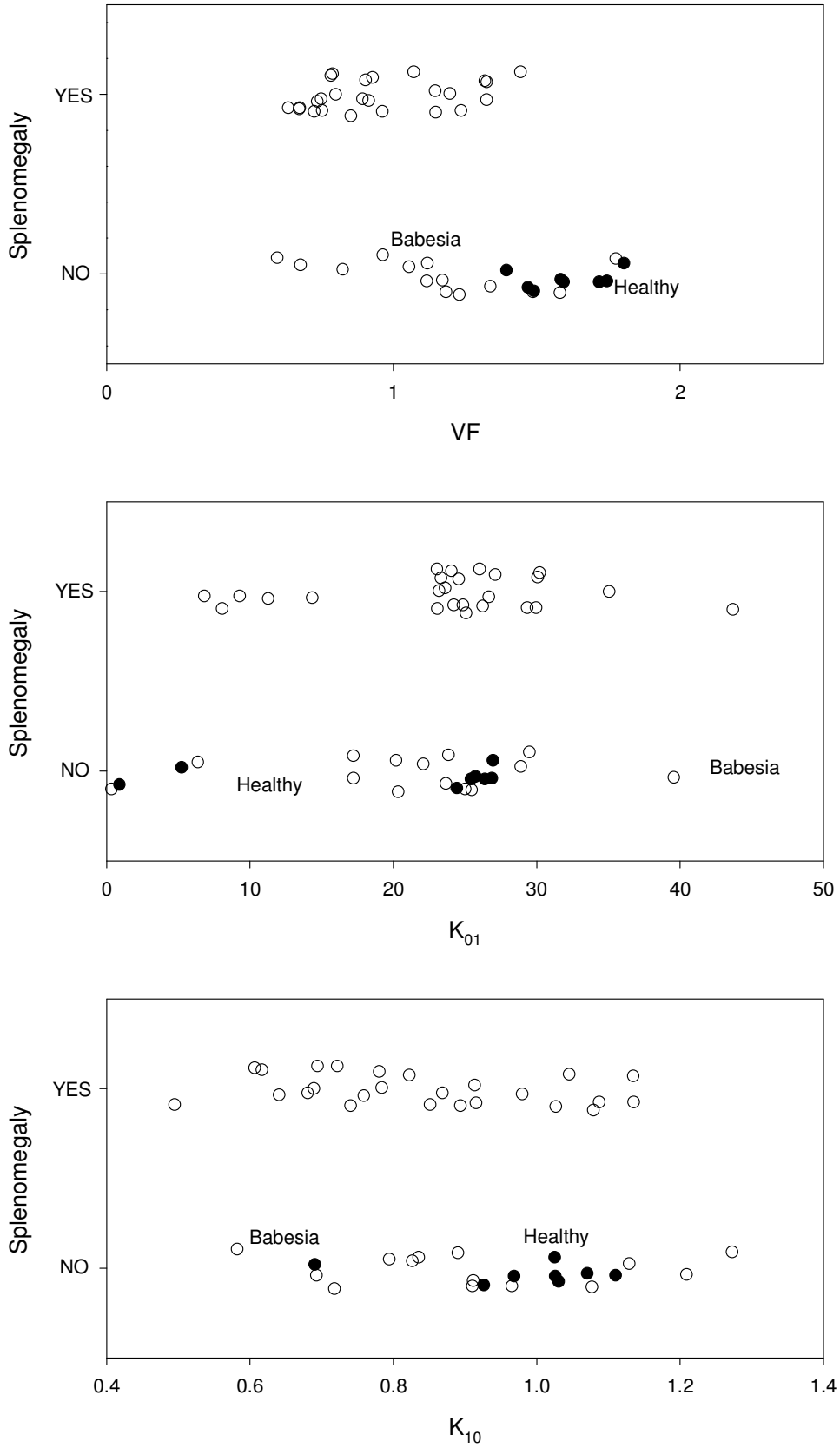


Figure 4.29: Scatter plot of the VF, K_{01} and K_{10} plotted against the presence of splenomegaly.

Table 4.10: Final parameter estimates for the mixed effect modelling with splenomegaly as covariate.

Parameter	Final estimate	Standard error	CV%
V_F_0	1.14	0.08	7.19
K_{01}_0	19.87	4.27	21.48
K_{01}_1(splenomegaly=no)	-8.43	3.69	43.73
K_{10}_0	0.79	0.07	8.89
$Sigma^2$	0.06	0.01	25.52
	VF = VF_0 + VF_ETA0		
Where	K_{01} = K_{01}_0 + AsClass(splenomegaly)*K_{01}_1 + K_{01}_ETA0		
	K_{10} = K_{10}_0 + K_{10}_ETA0		

4.6.8 Age

The exact ages for the experimental health dogs were not available. An age of 18 months was used. There was poor correlation between age and the pharmacokinetic parameters (r^2 = 0.03, 0.11 and 0.0 for VF, K_{01} and K_{10}, respectively) - Figure 4.30. The power was, however, less than the desired level of 0.8 (power < 0.62). There were no animals under 3 months of age.

Adding age to the mixed effects model did not improve it.

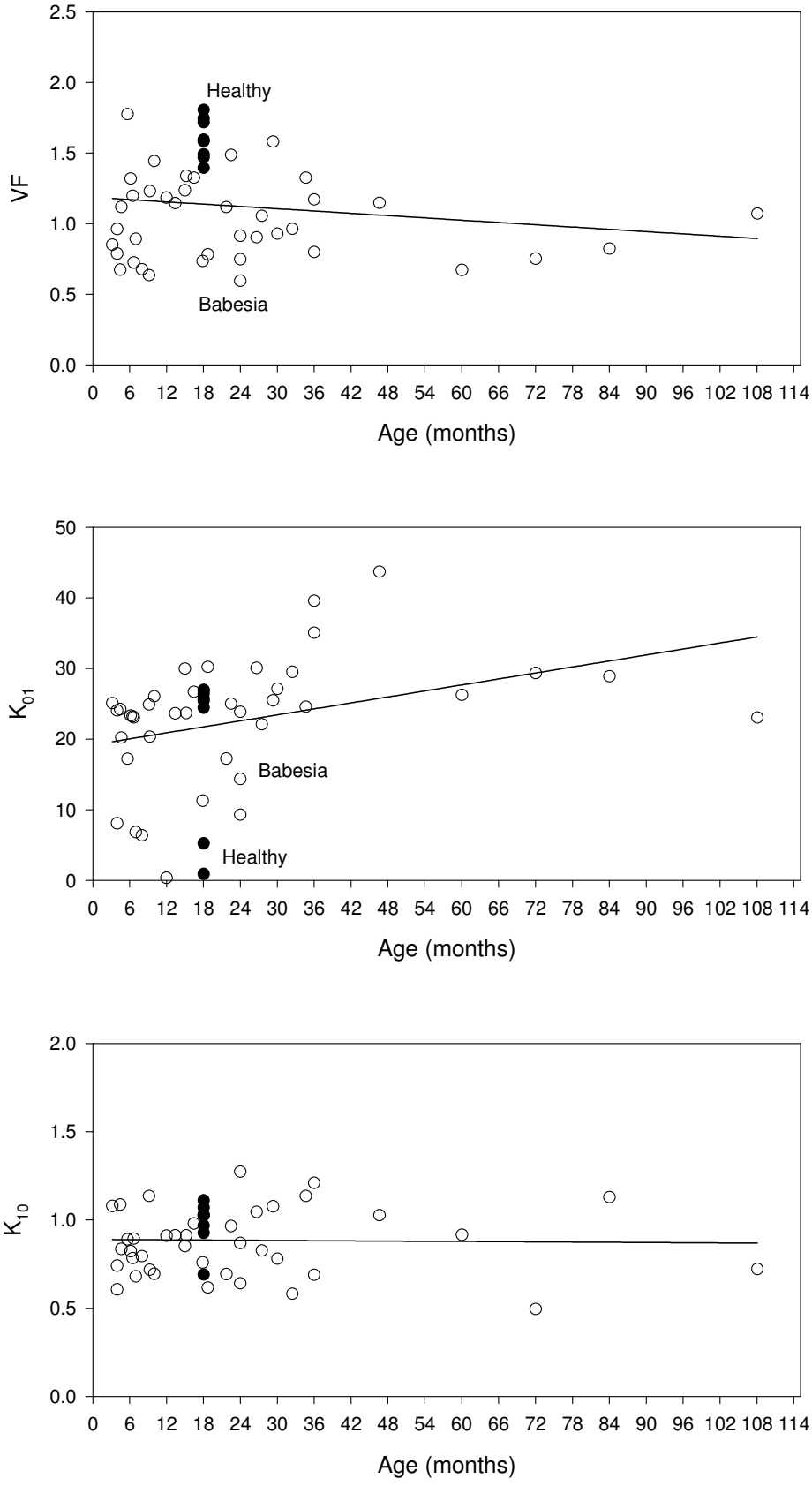


Figure 4.30: The VF, K_{01} and K_{10} plotted against age with the regression line.

4.6.9 Creatinine

There was poor correlation between the pharmacokinetic parameters and creatinine (Figure 4.31). The r^2 was <0.08 for all parameters, however the power was less than 0.5 in all cases.

There was no significant improvement in the model when creatinine was added to the mixed effects. The MOF for VF, K_{01} and K_{10} was –70.8; –71.7 and –73.6, respectively.

4.6.10 Other medication

There was insufficient power to determine a statistical difference in the VF, K_{01} and K_{10} between the babesia infected dogs that had not received other medication prior to diminazene administration and those that had (t-test, p = 0.74, power = 0.05; Mann-Whitney, p = 0.104 and t-test, p = 0.09, power = 0.26, respectively).

Mixed effect modelling on only the babesia infected dogs resulted in a minimal effect on MOF for the VF (–13.9), K_{01} (–13.9) and K_{10} (–14.4).

4.6.11 Non-linear specification

Non-linear specifications for significant non-categorical covariate – parameter interactions (PCV and albumin on VF) were investigated. These showed no advantage over and above the additive specifications.

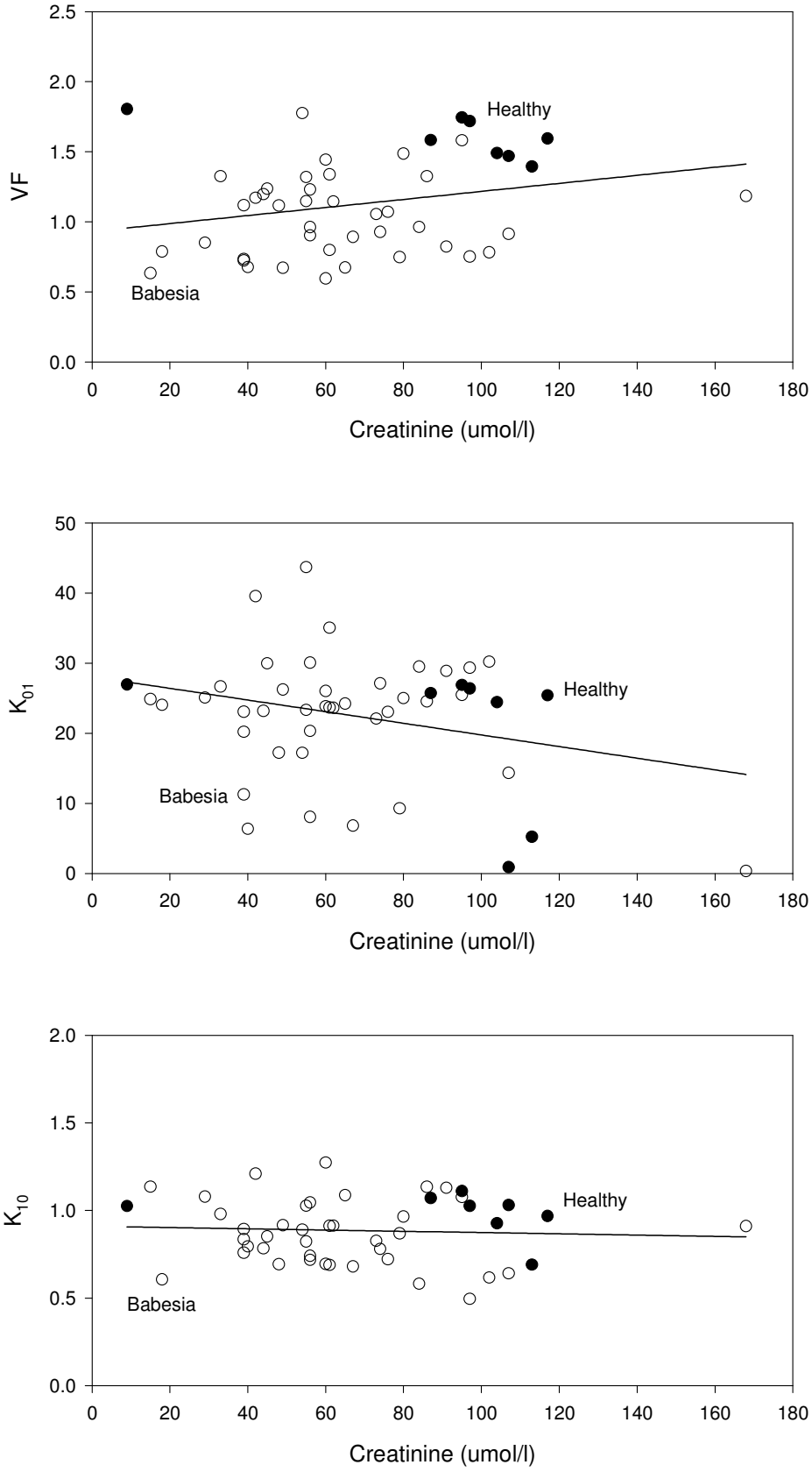


Figure 4.31: The VF, K01 and K10 plotted against creatinine with the regression line.

4.7 WINNONMIX – MIXED EFFECT MODELLING – COMBINATIONS OF COVARIATES

Health status (for VF and K_{01}), albumin (VF) and PCV (VF) in combination were investigated. No attempt was made to add splenomegaly or mental status to the final model due to the issues of colinearity. Furthermore, these are subjective assessments and therefore prone to errors. Health status for K_{01} was retained. This was done, since although health status on K_{01} was not significant on its own, the modelling of health status to K_{01} in addition to VF, significantly improved the model (MOF -101.1). Using either PCV or albumin in place of health status for K_{01} resulted in a poorer fit. Albumin and PCV were not modelled concurrently due to colinearity. The additive mixed effect and inter- and intra- individual specification was retained. The final model is shown in Table 4.11 and Figure 4.32, Figure 4.33 and Figure 4.35. The MOF decreased to –126.5. Removing individual covariates from the final model resulted in > 10.83 increase in MOF (i.e. $p < 0.001$). Figure 4.34 and Figure 4.36 from the original model (i.e. section 4.5, all dogs, Figure 4.19) are also shown as a comparison. The initial parameters and limits used in the model are shown in Table 4.12. The primary and secondary pharmacokinetic parameters estimates for the healthy and babesia animals are shown in Table 4.13.

The parameter estimates for K_{01} were normally distributed, but both VF and K_{10} were not (Figure 4.37). Of the inter-individual error parameters, only VFeta was normally distributed, centred on zero. K_{01}eta was normal but not centred around zero, while K_{10}eta was centred on zero, but was not normally distributed (

Figure 4.39). The base model histogram for the error specification and parameter estimates as shown in Figure 4.38 and Figure 4.40 as a comparison.

Table 4.11: Final parameter estimates for the mixed effect modelling of the final model.

Parameter	**Final estimate**	**Standard error**	**CV%**
V_F_0	1.75	0.41	23.47
V_F_1(health=babesia)	-0.92	0.21	22.71
V_F_3(albumin)	0.02	0.01	86.64
K_{01}_0	8.44	1.17	13.84
K_{01}_1(health=babesia)	2.41	1.80	74.60
K_{10}_0	0.63	0.04	6.21
$Sigma^2$	0.03	0.01	21.27
Where	V_F = V_F_0 + V_F_1*Asclass(health) + V_F_3*alb + V_F_ETA0		
	K_{01} = K_{01}_0 + K_{01}_1*AsClass(health) + K_{01}_ETA0		
	K_{10} = K_{10}_0 + K_{10}_ETA0		

Table 4.12: The initial parameter estimates, with limits used, for modelling the final combined model.

Parameter	Initial value	Low limit	High limit
V_F_0	2	0	15
V_F_1(health=babesia)	0	–2	15
V_F_3(albumin)	0.0449	0	15
K_{01}_0	8	0	400
K_{01}_1(health=babesia)	0	–10	20
K_{10}_0	0.9	0	10

Table 4.13: The primary and secondary pharmacokinetic parameter estimates for healthy and babesia animals. Values are given as mean ± standard deviation for normally distributed results and median (25%-75%) for non-normally distributed data.

Parameter	Healthy	Babesia
VF (l/kg)	2.16 ± 0.32†	1.10 ± 0.35†
K_{01} (h^{-1})	16.07 (10.65 - 16.71)‡	23.34 (20.02 – 27.52)‡
K_{10} (h^{-1})	0.67 (0.66 – 0.69)	0.68 (0.64 – 0.75)
AUC (µg.h/ml)	2.76 (2.59 – 3.21)‡	5.66 (4.66 – 7.59)‡
Fractional Clearance (l/kg/h)	1.44 ± 0.27†	0.78 ± 0.25†
C_{max} (ug/ml)	1.66 (1.49 – 1.86)‡	3.54 (3.00 – 4.73)‡
T_{max} (h)	0.21 (0.20 – 0.30)‡	0.16 (0.14 – 0.17)‡

VF: Fraction volume of distribution; K_{01}: absorption constant; K_{10}: elimination constant; AUC: area under the curve; C_{max}: peak plasma concentration; T_{max}: time to peak plasma concentration; †: significant difference (t-test, $p < 0.01$); ‡: significant difference (Mann-Whitney Rank Sum Test, $p < 0.01$)

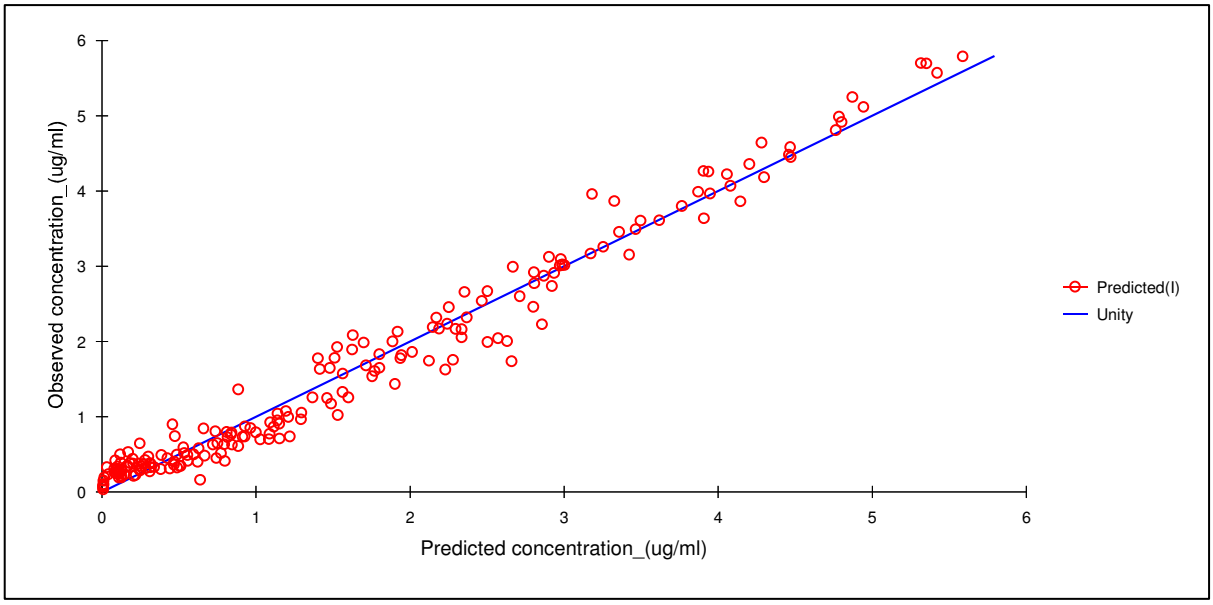


Figure 4.32: The individual predicted versus observed plasma diminazene plasma concentrations of the final model using babesia and healthy dog data.

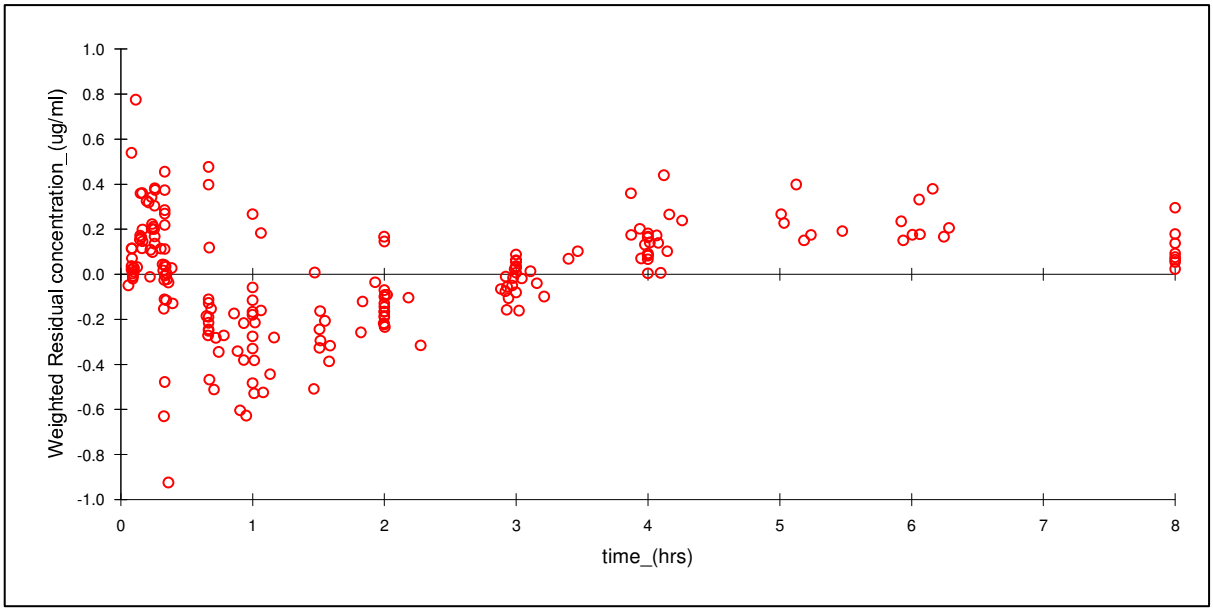


Figure 4.33: The weighted residual of the predicted, individual, diminazene plasma concentration over time of the final model, using the combined data sets of babesia and healthy dogs.

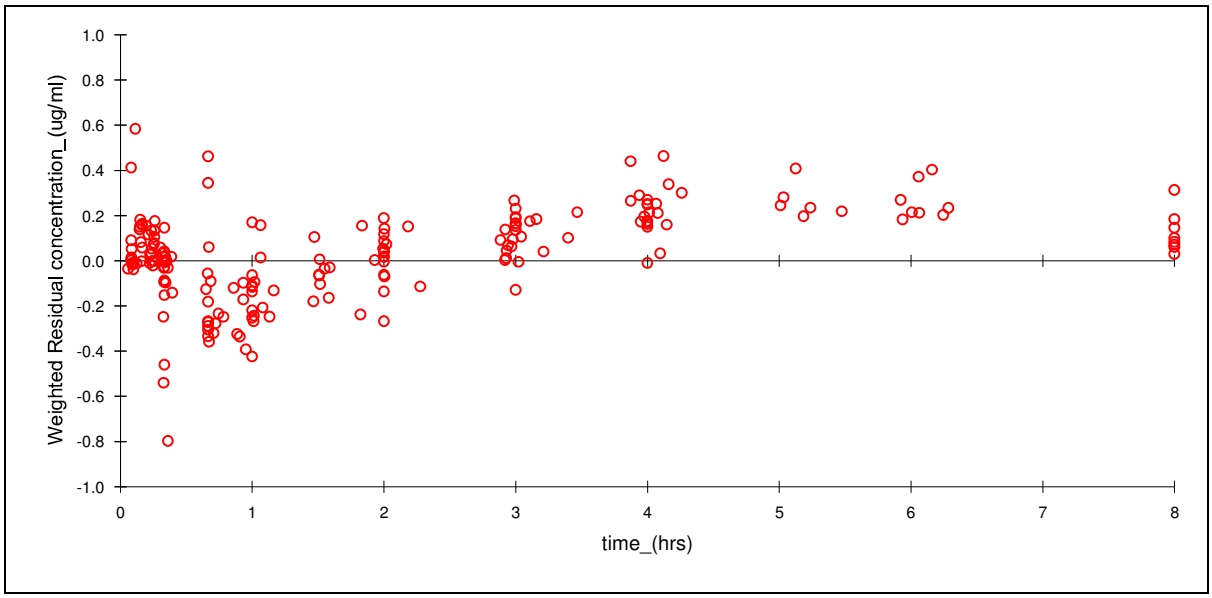


Figure 4.34: The weighted residual of the predicted, individual, diminazene plasma concentration of the initial non-covariate model over time, using the combined data sets of babesia and healthy dogs.

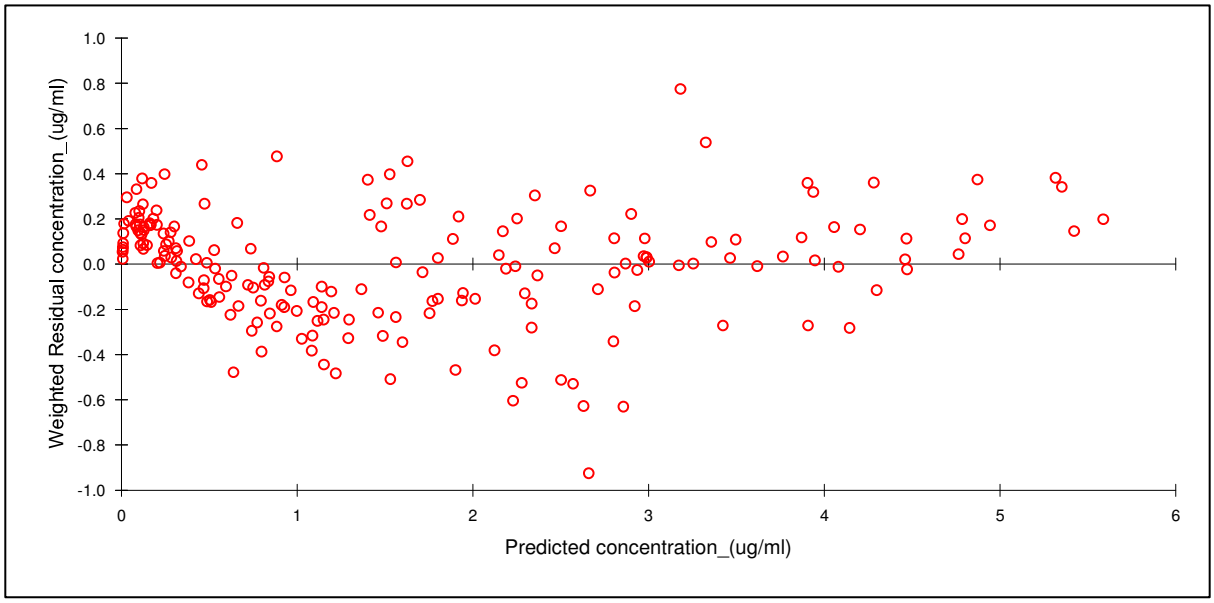


Figure 4.35: The weighted residual of the predicted, individual, diminazene plasma concentration plotted against the predicted concentration of the final combination model, using the combined data sets of babesia and healthy dogs.

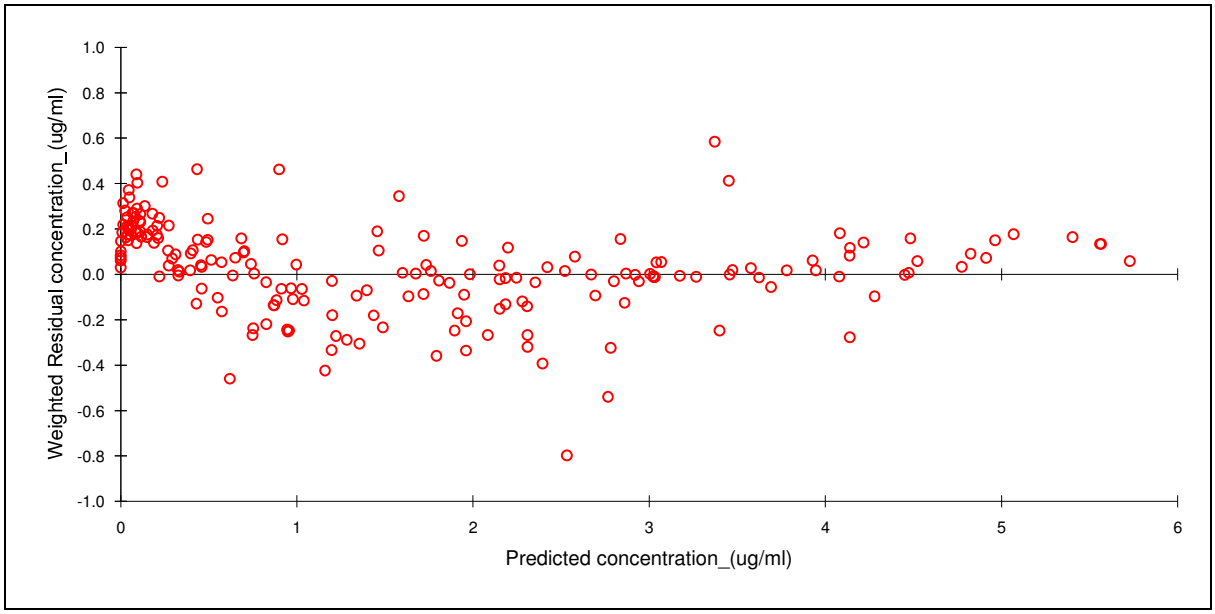


Figure 4.36: The weighted residual of the predicted, individual, plasma diminazene concentration plotted again the predicted concentration of the initial non-covariate model, using the combined data sets of babesia and healthy dogs.

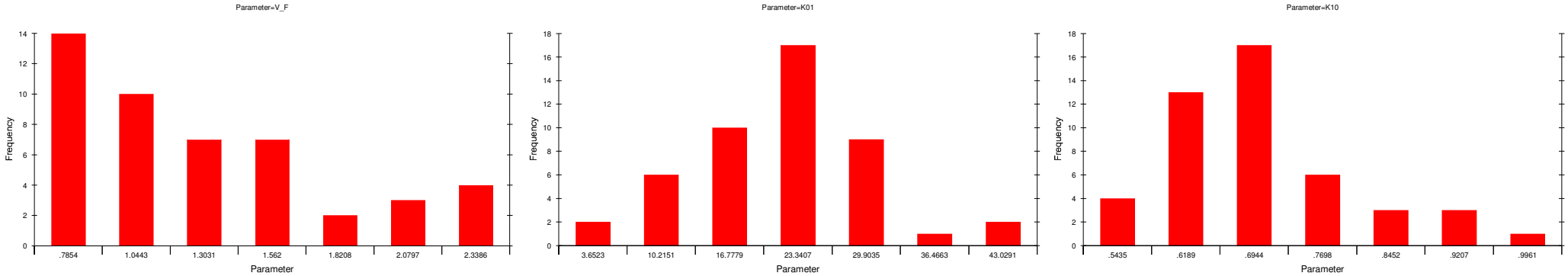


Figure 4.37: Histogram of the parameter estimates in the final model, where $\theta_j = \theta+\eta_j$.

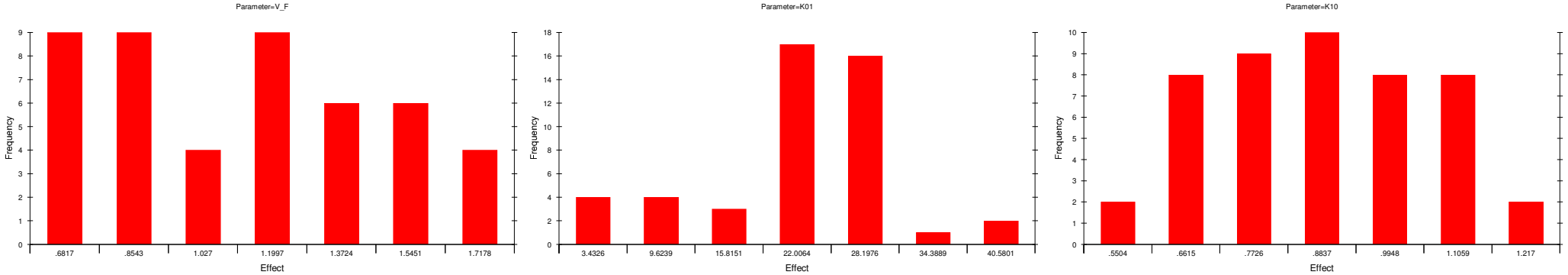


Figure 4.38: Histogram of the parameter estimates in the base model, where $\theta_j = \theta+\eta_j$.

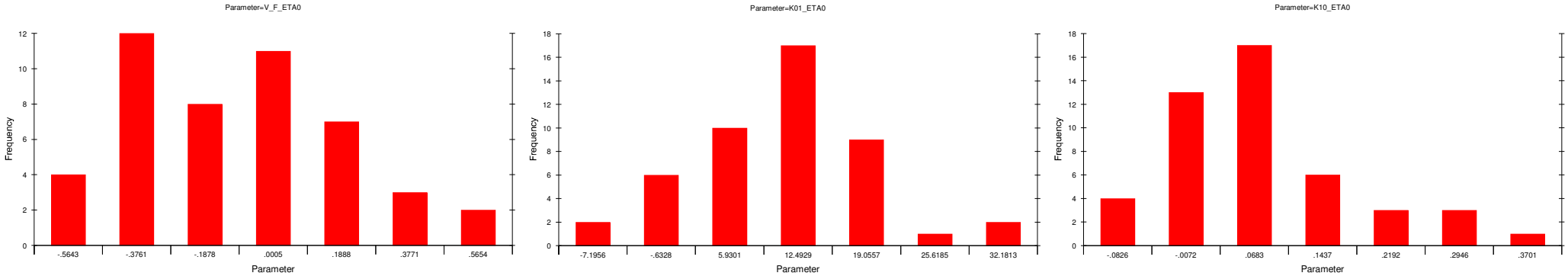


Figure 4.39: Histogram of the error estimates in the final model.

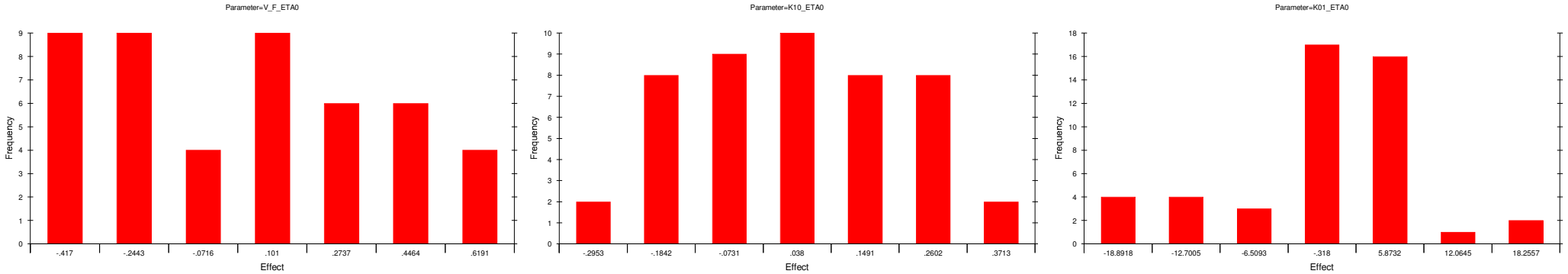


Figure 4.40: Histogram of the error estimates in the base model.

CHAPTER 5: DISCUSSION

The aim of this study was to provide a descriptive model of the population pharmacokinetics of diminazene aceturate in dogs naturally infected with *Babesia canis*. No attempt was made to establish a predictive model. Therefore formal model verification was not undertaken. It is unlikely that the data from this study could indeed be used for predictive purposes. Ribbing and Jonsson suggested that small to moderate sized data sets (< 50-100 subjects) should not be used for predictive models. Intra-individual pharmacokinetic changes over time were not an issue in this study as each patient was treated only once for *Babesia canis* infections.[49] Furthermore, should any dog require future treatment with diminazene aceturate such data should be seen as an independent event with no co-dependency on the first treatment.

5.1 BASE MODEL

The residual plots and observed versus predicted plots seem to indicate model misspecification and that the data may better fit a two-compartment model. In previous non-population pharmacokinetic work on diminazene in experimental dogs, 2-compartmental models were fitted to the pharmacokinetic data.[7,39,46] In this study a 2-compartment model could not be fitted to the data, despite adding Miller's[39] data to this study's data. Inadequate sample numbers is the most probable reason that a 2-compartmental model could not be fitted. It is unlikely that the initial parameter values were inappropriate as numerous values were tried and tested prior to accepting the values used.

5.2 MIXED EFFECT MODELLING WITH SINGLE COVARIATES

Of the three pharmacokinetic parameters in the combined data set, VF was influenced the most by covariates (health status, PCV, albumin and mental status). K_{01} was influenced by a single covariate, namely splenomegaly. When only the babesia infected animals were modelled, none of the above significantly improved the model. The small sample size is likely responsible for this. Unfortunately, it is not possible to prospectively estimate sample numbers required to attain sufficient power in population studies.

Previous studies have examined the effect of illness on the pharmacokinetics of diminazene.[7,46] They showed a significant differences in the VF[7,46] and clearance of the central compartment[7], with sick animals having a lower values. There are no published studies in the mainstream English literature that have looked at the pharmacokinetics (using population methods or otherwise) of diminazene in experimental or non-experimental, *Babesia* infected dogs. The similar findings between the

experimental studies[7,46] and this work suggest that the influence of illness is a generalised, rather than a disease specific effect (i.e. babesiosis versus trypanosomiasis).

5.3 COLINEARITY BETWEEN ILLNESS AND COVARIATES

Colinearity between illness and covariates was not addressed in the trypanosomiasis work.[7,46] Factors such as blood protein concentrations and haemoglobin concentrations may influence the pharmacokinetics of a drug. Both trypanosomiasis and babesiosis may induce anaemia and lower plasma albumin concentrations by virtue of their pathophysiology and inflammatory nature. It was not clear whether the change in diminazene pharmacokinetics between healthy dogs and dogs with trypanosomiasis was as a result of the disease or specific covariates (e.g. PCV) affecting the pharmacokinetic parameters. Diseased animals may have different pharmacokinetics from healthy individuals by virtue of covariates that are independent, but also correlated, to the disease status, rather than due to illness *per se*. This is borne out by the loss in the significant effects of the covariates on the models when babesia animals were modelled alone (as compared to the models that had data from both the diseased and experimental dogs). There were not enough healthy animals, and with enough variation in the PCV and albumin, to investigate the influence of these covariates on the pharmacokinetic parameters, thereby eliminating the colinearity of disease.

The influence of disease needs to be broken down into 2 components: either the absence or presence of infection and the degree or severity of the illness (i.e. mild, moderate or severe). Within the babesia population, both splenomegaly and mental status could be markers of disease severity, if one makes the assumption that more severely ill animals have a greater proportion of splenomegaly or a lower mental status. Albumin, as a negative acute phase protein, may also serve as an indicator of disease severity.

5.3.1 Colinearity and splenomegaly

When the healthy animals were removed from the data set, the addition of splenomegaly to the population model was no longer significant, suggesting that the effect of splenomegaly on the full data set may be due to the colinearity between disease and splenomegaly. Unfortunately, the initial cursory statistical analysis of the pharmacokinetic parameters to splenomegaly status in the babesia infected dogs only demonstrated a lack of power. A larger sample size may therefore give a different result on the inclusion of splenomegaly in the babesia only population model.

5.3.2 Colinearity and mental status

Mental status significantly improved the population model for VF, but not when healthy dogs were removed. A lack of power in the investigatory analysis of mental status in the babesia animals alone again suggests that a larger sample size may result in a different result. In the (significant) full model, it is noteworthy that the VF estimates became progressively smaller as the mental status worsened. There is, therefore, good circumstantial evidence that the *degree* of illness influences the parameter estimates. Furthermore, only those animals requiring outpatient treatment were admitted into the study and therefore the lack of significance may be a consequence of the selection criteria. The effect of severity of illness (as assed by mental status) warrants further investigation.

5.3.3 Colinearity and red blood cell parameters

Packed cell volume had a significant effect on the VF in the population model. The VF decreased with a decrease in the PCV. There was however a significant difference in PCV of healthy dogs to babesia infected dogs, such that colinearity to illness was likely. The effect of PCV on VF became non-significant when only babesia animals were modelled. There was no statistical difference in the PCV between those babesia infected dogs with and without splenomegaly or with differing mental status, although power was insufficient in both cases. If the latter are real findings (i.e. not a type II error), it would lend argument that the red blood cell status has no direct effect on parameters, and its influence on the population model is due to its colinearity with health status. The final combination model provides good support that PCV's influence on the model was due to colinearity. This is further supported by previous work in cattle infected with *T. congolense*.[33]

5.3.4 Colinearity and albumin

Albumin had a significant effect on the population model. Only VF was significantly affected, increasing as the albumin concentrations increased. This effect is counterintuitive. Increasing albumin concentrations should result in more protein drug binding, higher plasma concentrations and hence a lower VF. The opposite effect occurred, and would suggest colinearity to illness (by virtue of albumin being a negative acute phase protein). Diminazene has been shown to bind strongly to albumin. Miller[39] showed that more than 75% of diminazene is plasma bound and of that fraction, < 24% is found free in the water component.[5] There was a statistically significant difference in albumin concentration between healthy dogs and babesia dog. It is likely that the significant effect of albumin on the population model was associated with its correlation to health *status*. There is some evidence that influence of albumin on the model is not limited to its colinearity to health status, despite that

when healthy animals were removed from the population model, the significant effect of albumin on VF was lost. There was a trend towards a statistical difference in the albumin concentration between those babesia infected dogs with and without splenomegaly and colinearity with disease *severity* should remain as a possible consideration.

5.4 NON SIGNIFICANT COVARIATES

The addition of gender into the model had no effect. This could potentially have been a type II error due to the small sample size and the additional influence of neutering on gender status.

All dogs, save one (patient 4 – the dog that died), had normal creatine concentrations. This prevented comparing dogs with normal renal function to dogs with abnormal renal function. It was therefore not surprising that the addition of creatinine had no influence on the pharmacokinetics.

Age did not influence the model. There were no animals in the 0-3 month age category, the period in which the greatest age related metabolic changes occur.[10] There were too few dogs (6) in the 3-6 month age category to allow meaningful categorical (young versus adult) analysis. Similarly, there were too few old dogs (1 dog > 8 years).

The administration of other medications by owners had no effect on the population pharmacokinetics. This was not unexpected due to the small number animals that received medication and the heterogeneous nature of the products used. Furthermore, non-linear mixed effect modelling has been suggested to be a poor method of evaluating the influence of other drugs in population pharmacokinetic studies.

5.5 OUTLIERS

The one patient that died, Dog 4, was more ill than initially appreciated. It was also the only dog with a raised creatinine. The flip-flop effect seen in this dog's plasma diminazene concentration is best explained by a decrease in the absorption due to poor circulation. This dog's data was, however, retained in the final model as such events are inevitable in clinical work.

5.6 FINAL MODEL

The two previous works on the effect of illness on the pharmacokinetics of diminazene aceturate were both experimental, non-population pharmacokinetic, intravenous studies with small animal numbers (n = 5), using trypanosomiasis as the infectious model.[7,46] The results of this study similarly showed that

disease affected the pharmacokinetics of diminazene, with a significant decrease in the VF and fractional clearance. Other significant differences shown in this study included a shorter time to maximum plasma concentration, higher maximum plasma concentrations, larger area under the curves and a larger K_{01} for the babesia animals. The K_{el} constant was not markedly different in any of the three works.

In this study, both the plasma albumin concentration and the PCV had significant effects on the VF. There was a poor but significant correlation between these two covariates. Covariate colinearity in population pharmacokinetics has the effect of increasing the unreliability of the final parameter estimates when they are modelled concurrently, with an r >0.5 being suggested as the threshold value.[9] When covariates show no colinearity, the addition of a second covariate should have minimal influence on the first covariate's estimates.[9] The influence of colinearity between albumin and PCV was seen when these two parameters were modelled concurrently. The inclusion of PCV into the albumin model, resulted in an increase in the PCV coefficient of variation of the final estimate without significantly improving the model. This was not the case when albumin and health status were added to the model, despite the evidence for colinearity between albumin and health status. Correlation between covariates increase the probability of selecting the incorrect covariate for inclusion into the model.[49] The inclusion of only one of two significantly correlated, but independent, covariates in a population pharmacokinetic model may not in itself be a problem, proved that the model performs well. Albumin was retained due to its underlying physiological effect on VF, which would most likely still have been presence, but hidden, by the correlation between albumin and health status. In other words, albumin carried unique information on the pharmacokinetics – that of protein binding, regardless of its correlation to health. Indeed, the significant improvement in the model with the addition of albumin to health status supports this.

5.7 MODEL CRITIQUE

A simple additive inter-individual and residual error model fitted the data best. However, the weighted residual versus time and concentrations scatter plots showed that they were not evenly distributed, despite using various error specifications and was probably a result of needing to use a 1-compartmental model. It is likely that the non identity residual error models had significantly improved MOF, despite poorer predicated versus observed plasma concentration plots due to the relative weighting of the predications at the lower concentrations. Since there are more of the lower concentration plots, an improvement here will be reflected in the MOF. Yet, it is the high plasma concentrations are of clinical importance. The spreading out of these plots will count less towards the MOF as they are fewer in number. Additionally, a large improvement in the lower concentrations will not really reflect graphically in the graph, as a large change of a small number is still a small number. Lastly, it is inappropriate to compare models with different error structures by the value of the MOF. The MOF should only be used to compare nested models.

The inability to find a greater relationship between covariates and parameters may stem from the study design. Inter-occasional variability could not be assessed in this study. This results in increasing the random inter-individual variability, rather than the intra-individual variability as seen when inter-occasional samples are analysed. In effect, this leads to larger parameter variability, potentially creating expectations that covariate – parameter relationships exist, when they don't.[56]

There is evidence that the final model was suboptimal as only K_{01} showed a normal distribution, and VFeta was the only random effect that was normally distributed, centred on zero. The lack of normality is likely a reflection of the small sample size and model misspecification, as normality was shown for some of estimated parameters. The use of a parametric modelling technique is, therefore, probably still appropriate.

A statistical improvement between two models is based on the difference in the MOF between the two, which approximates the X^2 distribution.[62,63] A difference of > 3.84 in the MOF (with 1 degree of freedom) would be significant at the p = 0.05 levels. Wahlby *et al* tested this assumption and reported that when using the first order method of estimation, the actual MOF required to show significance was higher. Differences in MOF of 8 to 17 may be needed to achieve significance for the inclusion of covariates with different parameters.[63] Almost all the covariates that were defined as significant, had delta MOF of > 8. This would support the robustness for their selection, and minimise a type I error. Albumin and health status for VF showed delta MOF of 11.4 and 22.5, respectively. The addition of health status to K_{01}, as discussed above, resulted in delta MOF of 8.4. Categorical data seem to have significance levels similar to that estimated by the X^2 distribution.[64] Therefore, splenomegaly with a delta MOF of 3.7 for VF approached, and may even have been significant.

Numerous effects of the small sample size of this study on the model results have been discussed. Some of these have only been quantified in the literature since inception of the study.[49,62-64] Despite this shortcoming, and as discussed above, the large delta MOF observed for selected covariates, suggest the findings of this study to be more likely to suffer from type II errors, and not type I errors.

Systematic errors may have biased the results of this study. The diminazene work in the healthy animals was done at another time, and blood concentrations were measured by another laboratory, although using the same method. Both the healthy and sick blood samples were, however, analysed as single batches, eliminating systematic error within each group. Animals in the healthy group were of a uniform breed, and there may be unknown breed associated differences in the pharmacokinetics of diminazene. However, the findings of this study showed similar changes to what both Onyeyilli and Anika showed in sick animals: decrease in clearance, increase in C_{max}, decrease in VF, increase in AUC and no significance change in K_{el}[7,46]. This would suggest that the results in this study are not significantly affected by a systematic error during determination of the diminazene plasma concentrations.

5.8 PHARMACOKINETIC PARAMETERS

5.8.1 Pharmacokinetic parameter estimations as compared to previous works

The direct comparison of pharmacokinetic parameters determined by this study to other studies is not possible due to the difference in the compartmental model (i.e. 1 versus 2). The estimates in this study for the healthy animals (using Miller's data set) are different to those determined by Miller.[39] In the non-compartmental work, Miller showed a lower (fractional) clearance of 0.83 l/kg/h (versus 1.44 l/kg/h in this study).[39] In this study only the first 6 hours of plasma samples were collected / or analysed and about half of these were taken during the rapid distribution phase (approximately 120 min, Figure 4.17), artificially increasing the clearance, which should rather be seen as an apparent clearance. Therefore, the clearances determined for ill animals by the model in this study are likely to be overestimations.

The C_{max} of healthy dogs as determined by Miller[39] was higher than our estimation for the healthy dogs (1.85±0.27 versus 1.64 ± 0.37 respectively) and is probably accounted for by our overestimation of clearance.[5] The C_{max} estimation for Miller's animals may have been inappropriate (low) due to collecting the first sample later (i.e. after the C_{max} and T_{max}) than in this study.[39] However, a similar result has been seen in cattle that were injected with diminazene intramuscularly.[33] C_{max} and T_{max} were higher and shorter, respectively, in those cattle acutely infected with *Trypanosoma congolense*. This would support the findings that the relative shifts of C_{max} and T_{max} between the healthy and diseased dogs (not necessarily the absolute values) of this study are real.

5.8.2 The influence of disease on pharmacokinetic parameters

The shorter T_{max} in sick animals is explained by an increase in the absorption rate. Possible mechanisms include an increase in lymphatic flow or venous return or a decrease in the tissue binding allowing for an increased absorption rate and higher plasma concentrations. The work on tissue diminazene concentrations in dogs infected with *Trypanosoma* supports this interpretation. Onyeyilli and Anika showed that healthy dogs had higher tissue concentrations of diminazene in the liver, kidney, heart and skeletal muscle than infected dogs.[7] The cause of the change in tissue binding is unknown, but a change tissue pH associated with disease may be one potential mechanism. This similarly explains the higher C_{max} seen in diseased animals.

Clearance decreased with illness. This is similar to what was shown in the trypanosomiasis work.[7,46] The decrease in clearance was attributed to an increase in the AUC, ascribed to a decrease in tissue

uptake, which has been shown to occur in dogs infected with *Trypanosoma*[47], especially the liver, which Miller[39] estimated to hold 79% of the administered drug in healthy animals. Renal function (as estimated by plasma creatine concentration) was normal in all babesia infected (save one) and healthy animals in this study. Furthermore, in the work on trypanosomiasis, urinary recovery of diminazene at 72 hours was not significantly different between the healthy and infected dogs, indicating that the decrease clearance cannot be attributed to a decrease in renal function.[7,46] Decreased tissue perfusion, and hence a prolonged distribution phase, cannot account for the decrease[47] in tissue uptake as the shorter T_{max} and higher C_{max} implies the contrary.

The K_{10} was unaffected by covariates. Nor was there a significant difference in the K_{10} of healthy and sick animals, which is similar to the findings in work done by Onyeyilli and Anika.[7,46] The lower VF seen in the babesia infected dogs is attributed to higher plasma concentrations caused by the decrease in tissue binding in diseased animals.[47,59]

5.9 CLINICAL RELEVANCE

Recommendations for dose adjustments based on the results of this work are not possible. A larger sample size would be required and model validation would be essential. Secondly, it is not known whether diminazene has its anti-protozoal effect based on peak concentrations or time exposure, and what the minimum values are. Based on the rapid drop in diminazene plasma concentrations post injection, C_{max} is most likely responsible for the antiprotozoal effect. The results of this study would suggest that the risk of toxic side effects when diminazene is administered to healthy animals is less than that of sick animals due to the lower C_{max}.

CHAPTER 6: CONCLUSIONS

The illness status (i.e. healthy versus infected dogs), PCV and albumin concentration all had a significant effect of the population pharmacokinetics of diminazene in dogs when modelled individually. Colinearity between all three covariates was possible. Pharmacokinetically, the protein binding effect of hypoalbuminaemia on VF was opposite to the expected results, suggesting the influence of albumin was related to its colinearity with illness. Illness status had the highest single influence on the modelling and was therefore retained in the final model. The red blood cell parameters had a significant influence on the population model by virtue of their colinearity with health. The effect of albumin on the population model was attributed to the colinearity to health status and less clearly to disease severity. It was, however, retained in the final model due to further significantly improving the MOF. The results of this study are in concordance with previous work on the influence of disease on the pharmacokinetics of diminazene.

Theoretically, knowing all the covariates that influence the pharmacokinetic parameters should enable one to model accurately the population pharmacokinetics without needing to resort to including the health status of the animal. However, it may be difficult to identify all the covariant parameters that alter the pharmacokinetics in an individual. Alternatively, modelling for countless specific effects (e.g. the cytokine involved in inflammation) may be practically prohibitive. Mixed effect specifications using the health status may circumvent these hindrances by including in the model a variable that is correlated to the mechanistic causes (e.g. plasma concentrations of inflammatory mediators) and prevents the negative influence that colinearity has on models, even if the covariates are independent.

APPENDIX 1: OWNER INFORMATION SHEET

Population pharmacokinetic study of Diminazene in dogs suffering from Babesiosis: Information Sheet

Dear sir/ madam

Your dog has been diagnosed with babesiosis (billary, bosluiskoors). This is caused by a small parasite (called *Babesia canis*) that invades the red blood cell. This disease is very similar to human malaria; however, babesiosis is transmitted by ticks and not by mosquitoes, as in the case of malaria.

As part of the fight against *Babesia canis*, the Section of Small Animal Medicine is doing ongoing research on babesiosis. This will help dogs survive this terrible disease. In this research project, we are investigating the blood level of the drug (diminazene) that is used to treat babesia in pets. We want to determine if the blood levels differ between dogs, depending on how ill they are. How will this knowledge help you? If we show that some dogs need less or more of the drug because of how ill they are, we would be able to adjust the dose that your pet would receive. This will decrease the chances of your dog having side effects from the treatment.

We would like your help by being allowed to include your pet into this project. Your dog will be treated the same as any other patient would be. We will take five blood samples for analysis of the drug concentration in the blood, as well as a urine sample. As these samples need to be taken over 6 hours we will need to keep your pet at the Outpatients Clinic. There will be no extra cost to you over and above the normal cost of treating your dog for babesiosis.

If you agree, you will be requested to fill in a questionnaire and give written consent to allow us to take blood and urine from your dog. These procedures are safe and routine.

You may remove you pet from the trial at any time. The Ethics Committee of the Faculty of Veterinary Science has passed this study.

Thank you in advance.

Dr. Frank Kettner
Department of Companion Animal Clinical Studies
Faculty of Veterinary Science
Onderstepoort
0110
Tel: (012) 529 8483
E-mail: fkettner@op.up.ac

APPENDIX 2: OWNER CONSENT FORM

Population pharmacokinetic study of Diminazene in dogs suffering from Babesiosis: Consent form

I,_____, the undersigned owner / authorised representative (please delete), hereby give permission for the pet dog under my care:

Name: _____

Breed: _____

Age: _____

Sex: _____

Colour: _____

to participate in the study of monitoring diminazene blood levels.

I understand that blood and urine will collected from the above animal. I further understand that this is a routine and safe procedure. I am aware that the above pet will be admitted to the Outpatients Clinic of the Onderstepoort Veterinary Academic Hospital, for at least 6 hours to have blood samples taken from him/her. I also understand that the cost pertaining to this study is not my responsibility and that I am only liable for cost relating to the diagnosis, treatments and any complications or other cost that relate directly to the above pet suffering from babesiosis.

This study has been explained to me and I have been given the Information Sheet. I am further aware that I may remove my pet from this study at any time at my request and this will in no way jeopardise the proper care of my dog.

In the unfortunate case of my pet dying, I give permission for a full post-mortem to be done.

Signed at Onderstepoort on this day_____ of the month of _____ in the year _

_____ _____

Name of owner or authorised representative Signed

_____ _____

Name of witness Signed

APPENDIX 3: RANDOMISATION TABLE

Patient no.	Sampling group	Random no.			
1	c	0.050413226			c
2	b	0.066801756		b	
3	a	0.066842254	a		
4	c	0.068661086			c
5	a	0.070423951	a		
6	a	0.070599256	a		
7	c	0.103538894			c
8	a	0.112962695	a		
9	b	0.118223711		b	
10	a	0.133605269	a		
11	c	0.154333675			c
12	b	0.165178220		b	
13	a	0.170178074	a		
14	a	0.175639838	a		
15	a	0.189836401	a		
16	b	0.201918825		b	
17	a	0.203057766	a		
18	a	0.247694496	a		
19	c	0.252783871			c
20	c	0.256103747			c
21	b	0.269183185		b	
22	a	0.271376523	a		
23	b	0.280372544		b	
24	c	0.281665911			c
25	c	0.285891207			c
26	b	0.286394661		b	
27	b	0.293217202		b	
28	c	0.319615417			c
29	b	0.348678234		b	
30	b	0.352260641		b	
31	a	0.400158217	a		
32	c	0.406228367			c
33	a	0.416134739	a		
34	c	0.422086463			c
35	c	0.423420763			c
36	a	0.426628885	a		
37	a	0.431381241	a		
38	a	0.437212005	a		
39	a	0.451502373	a		
40	c	0.452240761			c
41	b	0.465976211		b	
42	a	0.467536153	a		
43	c	0.490249049			c
44	c	0.494149420			c
45	c	0.495985168			c
46	c	0.522480873			c
47	a	0.530189566	a		
48	c	0.531847971			c
49	c	0.562962767			c
50	a	0.563693755	a		
51	b	0.566616368		b	
52	a	0.580261550	a		
53	a	0.594618443	a		
54	b	0.617957877		b	
55	c	0.626637894			c
56	b	0.647655637		b	
57	c	0.667843622			c
58	a	0.669098875	a		
59	b	0.741850619		b	
60	c	0.749385893			c
61	b	0.750081044		b	
62	b	0.757263200		b	
63	b	0.763081752		b	
64	c	0.780117917			c
65	c	0.801728059			c
66	a	0.806497047	a		
67	a	0.811184312	a		
68	a	0.816709259	a		
69	a	0.818824976	a		

Patient no.	Sampling group	Random no.			
91	c	0.001943916			c
92	c	0.006264506			c
93	b	0.021264582		b	
94	c	0.022619333			c
95	b	0.024376043		b	
96	c	0.029782408			c
97	b	0.050790454		b	
98	b	0.060924797		b	
99	b	0.094150602		b	
100	c	0.105051049			c
101	a	0.175991868	a		
102	a	0.176881109	a		
103	a	0.182057787	a		
104	a	0.200856368	a		
105	a	0.204360206	a		
106	a	0.205655634	a		
107	b	0.210038141		b	
108	a	0.211015861	a		
109	a	0.232912771	a		
110	a	0.259240372	a		
111	c	0.263498468			c
112	b	0.272408022		b	
113	a	0.286805127	a		
114	c	0.298771534			c
115	a	0.301595635	a		
116	c	0.302524556			c
117	c	0.304989749			c
118	b	0.334657979		b	
119	a	0.335366183	a		
120	c	0.338670955			c
121	b	0.349275032		b	
122	b	0.350371521		b	
123	c	0.355795444			c
124	a	0.360693914	a		
125	c	0.375846675			c
126	c	0.388173136			c
127	c	0.401521621			c
128	b	0.423301577		b	
129	b	0.424177741		b	
130	b	0.441413637		b	
131	c	0.446559061			c
132	b	0.456112200		b	
133	c	0.459697016			c
134	c	0.478379444			c
135	c	0.485299597			c
136	a	0.485353675	a		
137	c	0.489710977			c
138	a	0.496421134	a		
139	c	0.515647780			c
140	b	0.516126955		b	
141	a	0.528675699	a		
142	b	0.530509922		b	
143	b	0.553756590		b	
144	c	0.555164848			c
145	a	0.563282052	a		
146	a	0.586258922	a		
147	a	0.598102501	a		
148	a	0.608449347	a		
149	a	0.627458917	a		
150	b	0.654798713		b	
151	c	0.661750552			c
152	c	0.662830164			c
153	b	0.689243568		b	
154	b	0.702394140		b	
155	b	0.704357099		b	
156	c	0.735038315			c
157	c	0.735647586			c
158	b	0.751770598		b	
159	b	0.779992343		b	

70	b	0.818973723		b		160	c	0.790800369			c
71	b	0.837341465		b		161	a	0.792362023	a		
72	a	0.842709973	a			162	b	0.807304800		b	
73	b	0.844950230		b		163	a	0.821716269	a		
74	b	0.852456828		b		164	a	0.842611251	a		
75	b	0.859381997		b		165	b	0.852924210		b	
76	b	0.883882027		b		166	a	0.860202415	a		
77	a	0.891419548	a			167	b	0.864393847		b	
78	b	0.893296919		b		168	c	0.866365897			c
79	b	0.897470508		b		169	c	0.867862579			c
80	c	0.922345142			c	170	a	0.869768285	a		
81	b	0.926258279		b		171	b	0.887343038		b	
82	c	0.930178477			c	172	c	0.895722811			c
83	c	0.947233110			c	173	c	0.917678884			c
84	c	0.948868658			c	174	a	0.924479926	a		
85	b	0.950677106		b		175	a	0.955309059	a		
86	a	0.959052593	a			176	b	0.958521698		b	
87	b	0.960008164		b		177	a	0.976882757	a		
88	c	0.982595923			c	178	b	0.989486528		b	
89	c	0.441649425			c	179	b	0.995907742		b	
90	b	0.993881162		b		180	a	0.998754257	a		

APPENDIX 4: DATA COLLECTION FORM

Population pharmacokinetic study of diminazene in dogs suffering from babesiosis: Questionnaire

Ensure that no exclusion criteria exist before commencing.

Patient no

Admission details

Owner's name:	
Patient's name:	
Owner no:	
Patient no:	
Date:	
Breed:	
Age: (y / m / w):	
Body weight:	

Name of the Outpatients clinician:	
Name of the veterinary student:	

Patient Information

sex: (circle appropriate choice)	male / female			
Neutered (circle appropriate choice)	yes / no			
Mucus membranes (circle most appropriate choice)	congested, normal, pale pink, very pale pink, white			
Capillary refill time in seconds (circle most appropriate choice)	(< 0.5sec) (0.5–1) (1-1.5) (1.5–2) (2-2.5) (2.5–3) (> 3)			
Heart rate				
Respiratory rate				
How would you describe the mental status: (circle most appropriate choice)	1: No change / normal, 2: Mildly depressed, still eating and drinking 3: Depressed, not eating < 24hrs 4: Depressed, not eating > 24hrs 5; Collapsed but can get up if encouraged 6: > 5			
Outpatients haematocrit	%			
Is serum icteric?	none	mild	moderate	severe
Is there haemolysis?	none	mild	moderate	severe
Outpatients TSP (measured on the refractometer)				
Vomiting over the last 48 hours?	yes / no – give details of type and quantity			
Diarrhoea over the last 48 hour?	yes / no – give details of type and quantity			
Body score based on the attached scoring system? (circle most appropriate choice)	1 / 2 / 3 / 4 / 5 / 6 / 7 / 8 / 9			
Did the confirming clinician palpate any of the following? (please circle appropriate one)	Splenomegaly: yes / no / not sure Hepatomegaly: yes / no / not sure			
Does the patient suffer from any other illness or disease?	yes / no – give details			
Entire female dogs	Pregnant No Not sure In heat			
Any other ongoing / chronic illness	yes / no – give details			

Is this patient on any other medication or has it received any medication over the last week? (give details)	
Ask specifically about: "disprin / aspirin / panado / paracetamol / or any head ache pill or any other tablet normally given to people" (give details)	

Diminazene administration

give diminazene into the biceps femoris muscles, half way between hip and knee – left or right hand side
use the smallest appropriate syringe for accurate dosing.

Diminazene bottle 1	Weight before water……weight after water……..
Diminazene bottle 2	Weight before water……weight after water……..
Time diminazene was given	Hour Minute
Volume of diminazene injected	
Body temperature at time of administering diminazene (retake the temperature).	
Was serum and EDTA blood collected prior to injecting Berenil?	yes / no
Was the diminazene given into the *biceps femoris* muscle?	Definitely i.m. Might have gone in sub cut

Collecting blood for Diminazene concentration measurements
Dogs 2,0 - 5kg - please use 0.5ml and 1ml paediatric EDTA and serum tubes.
Dogs 2 – 2,5 kg - omit taking last sample.

Stick sticker here that assigns patients

Table for timing of blood collection after diminazene injection

Group	A	B	C
1st sample	5 minutes	10 minutes	15 minutes
2nd sample	20 minutes	40 minutes	1hour
3rd sample	3 hours	2 hours	1.5 hours
4th sample	6 hours	5 hours	4 hours

All blood must be collected from the jugular vein in heparin (green topped) vacutainers .

Note the actual time of drawing blood for diminazene concentration determination accurately (use the designated stop watch please)	1st sample: hrs	min	sec
	2nd sample: hrs	min	sec
	3rd sample: hrs	min	sec
	4th sample: hrs	min	sec

Side effects of diminazene
any of the following signs within 6 hours after injecting diminazene? (circle appropriate choice)
provide details below incl. how long after administering diminazene.

Reaction to injection (ie pain etc)	yes / no	
Swelling at injection site	yes / no	
Muscle pain at injection site	yes / no	
Diarrhoea	yes / no	
Salivation	yes / no	
Vomiting	yes / no	
Muscle twitching at the injection site or else where (note where)	yes / no	
Collapse or weakness	yes / no	
Hyperaesthesia	yes / no	
Other	yes / no	
No abnormalities seen	yes / no	

Date Initial

Body condition scoring system†

1. **EMACIATED**
   Ribs, lumbar vertebrae, pelvic bones and all bony prominences evident from a distance. No discernable body fat. Obvious loss of muscle mass.

2. **VERY THIN**
   Ribs, lumbar vertebrae and pelvic bones easily visible. No palpable fat. Some evidence of other bony prominence. Minimal loss of muscle mass

3. **THIN**
   Ribs easily palpated and may be visible with no palpable fat. Tops of lumbar vertebrae visible. Pelvic bones becoming prominent. Obvious waist and abdominal tuck

4. **UNDERWEIGHT**
   Ribs easily palpable, with minimal fat covering. Waist easily noted, viewed from above. Abdominal tuck evident.

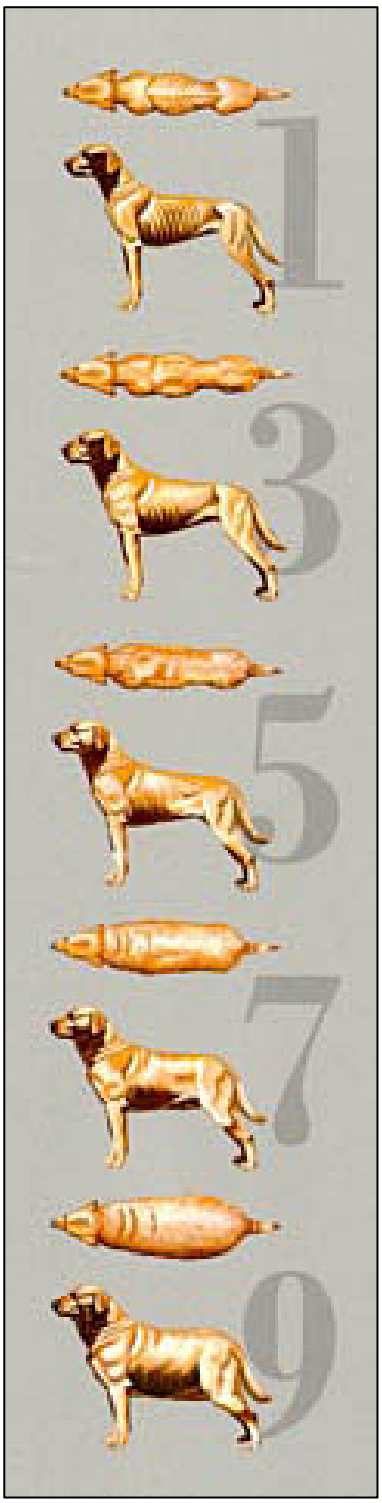
5. **IDEAL**
   Ribs palpable without excess fat covering. Waist observed behind ribs when viewed from above. Abdomen tucked when viewed from the side.

6. **OVERWEIGHT**
   Ribs palpable with slight excess fat covering. Waist is discernable viewed from above but is not prominent. Abdominal tuck apparent.

7. **HEAVY**
   Ribs palpable with difficulty, heavy fat cover. Noticeable fat deposits over lumbar area and base of tail. Waist absent or barely visible. Abdominal tuck may be absent.

8. **OBESE**
   Ribs not palpable under heavy fat cover, or palpable only with significant pressure. Heavy fat deposits over lumbar area and base of tail. Waist absent. No abdominal tuck. Obvious abdominal distension may be present.

9. **GROSSLY OBESE**



† www.purina.com

APPENDIX 5: POST MORTEM REPORT FOR DOG 4

Ref**: PM1057.02**

Outpatients
Dr. F. Kettner

Pathology
Fax (012) 529 8303
October 26th, 2002.

NECROPSY REPORT Test result: Pathology Laboratory

Your reference:
Our reference: PM1057.02
Date received: October 18th, 2002
Owner: [redacted]

Species: Canine; Breed: CB; Sex: : Age: ; Colour: Tan
ID / **Name:** Tiger
Specimen(s): Complete carcass

History: Diagnosed with babesiosis. Found dead in cage.

Microscopic examination of blood smear: Moderate to severe (3+) *Babesia canis* parasitaemia. No distinct anaemic changes of red blood cells visible.

Macroscopic pathological changes: Mild post mortem decomposition**.**

Mild to moderate general congestion of carcass.
Lungs: Moderate diffuse congestion and oedema.
Heart: Severe serosanguinous hydropericardium.
Liver and spleen: Moderate hepato- and splenomegaly.
Kidneys: Moderate diffuse bilateral haemoglobinuric nephrosis.
Urinary bladder: Severe haemoglobinuria.

Histopathological changes: Mild post mortem decomposition.

Lungs: Severe diffuse congestion and oedema.
Liver: Severe congestion and hepatosis with severe hydropic degeneration of hepatocytes and necrosis of single individual hepatocytes.
Kidneys: Severe diffuse nephrosis with severe tubular degeneration and necrosis as well as marked deposition of protein- and haemoglobin casts into renal tubules.

Only few *Babesia* sp. parasites could be seen in red blood cells of all organ sections examined.

No other significant changes present in other organs examined..

DIAGNOSIS: Canine babesiosis with fatal pulmonary oedema, hepatosis and haemoglobinuric nephrosis.

Thank you for referring the case to us.

Dr. J.A. Neser
Section Pathology
Department of Paraclinical Sciences

APPENDIX 6: DATA FOR THE 39 CLINICALLY DISEASED DOGS AND 8 HEALTHY EXPERIMENTALLY TREATED DOGS

Patient no	Date	Breed	Date of birth	Age (days)	Body weight (kg)	Gender (male=0 ; female=1)	Neutered (yes=0 ; no=1)	Mucus membranes (cong=0 ; norm=1 ; pale=2 ; very=3 ; white=4)	Capillary refill time (0-6)	Heart rate	Respiratory rate	Mental status (1-6)	Op ht	Op tsp	Isa (neg=0; pos=1)	Icterus (none=0 ; mild=1 ; mod=2 ; sev=3)
1	29-Sep-2002	Boerboel	14-Jun-2000	837	43.00	female	no	2	2	132	panting	3	31	58	0	1
2	29-Sep-2002	German shepherd	30-Sep-1995	2556	37.50	male	no	2	2	88	panting	3	25	50	0	0
3	10-Oct-2002	cross breed	1-Jan-2002	282	21.00	female	no	1	2	184	112	3	28	62	0	0
4	17-Oct-2002	cross breed	17-Oct-2001	365	31.90	male	no	2	2	90	48	3	41	58	0	1
5	28-Oct-2002	Bull terrier	28-Dec-2001	304	23.30	male	no	2	1	184	44	3	18	52	0	0
6	28-Oct-2002	Spaniel	5-Jan-2001	661	8.80	female	no	2	1	120	36	2	17	60	0	1
7	31-Oct-2002	Boerboel	26-Jul-2001	462	51.48	male	no	2	3	132	36	2	17	70	0	0
8	7-Nov-2002	Boxer	19-May-2002	172	16.00	female	no	2	3	72	40	2	34	72	0	0
9	16-Nov-2002	Border collie	17-May-2000	913	17.20	male	yes	2	1	104	54	4	19	56	1	0
10	16-Nov-2002	Shar Pei	17-Nov-1999	1095	16.30	male	yes	3	1	150	panting	3	24		0	0
11	21-Nov-2002	German shepherd	2-Jan-2000	1054	27.60	female	no	2	2	102	panting	2	33	60	0	0
12	26-Nov-2002	cross breed	14-Jul-2001	500	16.74	male	no	2	1	124	24	2	27	55	0	0
13	27-Nov-2002	Boerboel	12-Jan-2001	684	60.28	male	no	1	2	114	panting	2	47	70	0	0
14	30-Nov-2002	English bulldog	17-May-2002	197	14.70	male	no	1	1	126	48	4	20	52	0	0
15	2-Dec-2002	Miniature pinscher	3-Dec-1999	1095	5.10	male	no	1	1	146	24	2	47	55	0	0
16	5-Dec-2002	Labrador	6-Apr-2002	243	28.50	female	no	2	0	136	panting	4	13	68	0	0
17	5-Dec-2002	cross breed	6-Aug-2002	121	5.50	male	no	2	4	200	32	4	23	54	0	0
18	22-Dec-2002	Boerboel	3-Aug-2002	141	16.60	male	no	1	1	174	60	2	27	54	0	0
19	27-Dec-2002	Corgi cross	27-Dec-1993	3287	13.60	male	no	2	1	78	panting	3	23	52	0	1
20	27-Dec-2002	Husky	27-Dec-2000	730	17.70	female	yes	2	1	132	44	4	13	77	0	1
21	27-Dec-2002	Husky cross	6-Jun-2001	569	28.40	male	no	2	1	114	panting	2	29	72	0	0
22	1-Jan-2003	Boerboel	1-Jan-2001	730	45.30	male	no	2	1	120	panting	2	42	56	0	0
23	14-Jan-2003	Labrador	2-May-2000	987	31.20	male	no	2	2	160	panting	3	22	48	0	0
24	13-Jan-2003	Fox terrier cross	1-Dec-2001	408	9.80	male	no	2	2	180	100	3	40	61	0	0
25	14-Jan-2003	Basset	10-Apr-2002	279	15.60	male	yes	1	2	132	56	5	21	48	0	1

26	18-Jan-2003	Fox terrier cross	29-Oct-2000	811	14.30	male	yes	2	2	142	40	2	26	40	0	0
27	20-Jan-2003	Maltese	20-Jan-1998	1826	3.14	female	yes	3	2	156	48	4	15	60	0	0
28	24-Jan-2003	Fox terrier	5-Jul-2002	203	5.44	male	no	2	1	160	48	3	25	60	0	0
29	28-Jan-2003	Boerboel	24-Jul-2002	188	23.70	female	yes	1	1	132	72	3	15	50	0	0
30	29-Jan-2003	Jack Russell	29-Jan-1997	2191	5.96	female	yes	1	2	140	36	2	18	64	0	0
31	17-Feb-2003	Fox terrier cross	1-Apr-1999	1418	4.50	male	no	3	1	124	panting	2	17	50	0	0
32	Removed from trial															
33	10-Mar-2003	cross breed	9-Aug-2002	213	3.68	female	no	1	2	132	42	2	32	50	0	0
34	12-Mar-2003	Boerboel	27-Oct-2002	136	22.80	male	no	2	2	140	40	4	22	60	0	0
35	24-May-2003	German shepherd	16-Feb-2003	97	8.10	female	no	2	1	160	panting	2	15	42	0	0
36	24-May-2003	cross breed	23-Jan-2003	121	4.54	female	no	2	1	180	28	3	17	34	0	0
37	25-May-2003	Boerboel	25-May-2001	730	46.30	male	no	1	2	148	panting	2	24	60	0	0
38	25-May-2003	Dachshund	27-Nov-2001	544	5.00	male	no	1	2	168	32	4	37	48	0	0
39	10-Jun-2003	Jack Russell	11-Mar-2002	456	7.96	female	no	1		130	-	4	31	68	0	0
40	18-Aug-2003	cross breed	9-Mar-2001	892	26.80	female	yes	1	2	80	panting	1			0	0

Dr Miller's healthy dogs

1		gsd			29.00	male	no	1							0	0
2		gsd			28.50	male	no	1							0	0
3		gsd			28.50	male	no	1							0	0
4		gsd			31.50	male	no	1							0	0
5		gsd			28.50	male	no	1							0	0
6		gsd			27.00	female	no	1							0	0
7		gsd			29.50	male	no	1							0	0
8		gsd			28.50	male	no	1							0	0

cong: congested; Isa: in saline agglutination; mod: moderate; neg: negative; norm: normal; Op ht: Outpatient haematocrit; Op tsp: Outpatient total serum proteins; Patient no: patient number; pos: positive; sev: severe;

Appendix 6: continued

Patient no	Haemolysis (none=0; mild=1; mod=2; sev=3)	Vomition (yes=0 ; no=1)	Diarrhoea (yes=0; no=1)	Body condition score (1-9)	Splenomegaly (yes=0 ; no=1)	Hepatomegaly (yes=no; no=1)	Illness (yes=0; no=1)	Other medication (yes=0 ; no=1)	Body temp (℃)	Random group	Time of sample 1 (seconds)	Time of sample 2 (seconds)	Time of sample 3 (seconds)	Time of sample 4 (seconds)
1	0	1	1	5	no	no	1	liver tonic	39.1	c	868	3832		
2	1	1	1	4	no	no	1	1	41.1	b	587	3260	7184	14982
3	0	1	1	5	no	yes	1	Bob Martin	40.5	a	449	1299	10950	21830
4	0	1	1	5	-	-	1	1	40.0	c	926	3830	6560	
5	2	1	1	6	yes	no	1	1	40.9	a	304	1415	12481	21810
6	0	1	1	5	-	-	1	1	39.9	a	357	1210	10690	21320
7	0	1	1	5	-	-	1	1	39.1	c	929	3665	5576	14641
8	0	0	1	5	no	no	1	1	39.1	a	355	1175	10514	21158
9	0	1	1	5	yes	no	1	1	40.6	b	560	2340	7205	14454
10	0	1	1	5	yes	no	1	1	39.8	a	297	1240	10720	22624
11	0	1	1	5	yes	no	1	1	39.6	c	922	4079	5694	14995
12	0	1	1	5	yes	no	1	1	39.8	b	708	2420	7216	18119
13	0	1	1	8	-	-	1	1	41.1	a	847	2676	11191	21609
14	0	1	1	6	yes	no	1	1	40.2	a	295	1190	10385	21165
15	2	0	0	6	no	no	1	asprin	40.0	a	408	1300	11367	22640
16	1	1	1	6	no	no	1	1	39.9	b	798	2417	7213	18854
17	2	1	1	4	yes	yes	1	1	39.2	a	331	1387	11562	22484
18	0	1	1	5	no	no	1	1	40.1	a	360	1255	10540	21180
19	0	1	0	6	yes	no	1	1	40.2	c	816	3095	5300	14930
20	0	1	1	4	no	no	1	asprin	40.2	c	930	3890	5275	14320
21	0	1	1	4	yes	no	1	1	40.0	b	530	2815	6950	14840
22	0	1	1	6	yes	no	1	1	41.2	a	210	1093	8190	14220
23	0	1	1	5	-	-	1	1	39.5	b	527	3191	7197	18453
24	0	1	1	5	yes	no	1	Blue liquid, animalax	40.2	c	856	3360	5425	13945
25	0	0	0	4	yes	no	1	"pain tablet"	40.1	c	842	3641	5718	13940
26	0	1	1	4	yes	no	1	1	40.6	b	543	2548	7861	21529

27	0	1	1	5	yes	no	1	1	39.5	b	596	2384		
28	0	1	1	6	yes	no	1	1	40.8	c	908	4188	5445	15337
29	0	1	1	5	yes	no	1	1	40.4	b	580	2469	7270	18664
30	0	1	1	6	yes	no	1	asprin	40.1	b	593	2596	7200	18040
31	0	1	1	5	yes	no	1	paracetamol	39.0	a	295	1180	10760	21830
32														
33	0	0	1	5	yes	no	1	1	39.4	a	327	1228	10552	21374
34	0	1	1	5	yes	no	1	1	40.8	c	939	3430	6605	14182
35	0	1	1	5	yes	no	1	1	39.8	c	764	3361	5430	19712
36	0	1	1	4	yes	no	1	1	39.3	a	295	1265	12230	14750
37	0	1	1	5	yes	no	1	1	39.9	a	320	1165	10507	22179
38	0	1	1	4	yes	no	1	1	40.4	a	328	1147	10880	21625
39	0	0	1	4	yes	no	1	paracetamol	38.3	a	305	1192	10590	21667
40	0	1	0	4	no	no	Gastro-enteritis	Antibiotics, corticosteroids	38.9	c	890	3650	5455	14685

Dr Miller's healthy dogs							
1	0	1	1	1	1	1	1
2	0	1	1	1	1	1	1
3	0	1	1	1	1	1	1
4	0	1	1	1	1	1	1
5	0	1	1	1	1	1	1
6	0	1	1	1	1	1	1
7	0	1	1	1	1	1	1
8	0	1	1	1	1	1	1

mod: moderate; Patient no: patient number; sev: severe;

Appendix 6: continued

Patient no	Side effects (no=0 ; yes=1)	tsp	alb	glob	a/g	creat	hb	rcc	PCV	mcv	mchc	rdw	wcc	neuts s	neuts b	lymp	mono	eos	baso	thc	Body surface area (m^2)
1	0	56.8	24.0	32.8	0.73	73	137	4.52	0.390	71.4	35.1	13.8	6.9	3.24	0.14	2.48	0.83	0.21	0.00	1.3	1.2397
2	0	51.0	21.2	29.8	0.71	91	101	4.29	0.300	64.1	33.7	16.4	4.2	2.35	0.25	1.09	0.50	0.00	0.00	4.4	1.1316
3	1	52.5	27.0	25.5	1.06	56	78	3.63	0.219	60.3	35.6	16.5	3.2	1.79	0.00	1.28	0.06	0.06	0.00	2.0	0.7688
4	1	71.7	33.1	38.6	0.86	168	151	6.36	0.419	65.9	36.1	16.2	8.6	7.14	0.17	0.52	0.77	0.00	0.00	5.5	1.0159
5	0	63.8	30.7	33.1	0.93	60	60	2.63	0.166	63.2	36.2	16.2	4.6	2.44	0.51	1.47	0.18	0.00	0.00	2.5	0.8239
6	1	62.1	22.5	39.6	0.57	48	51	2.01	0.146	72.5	34.9	23.6	4.4	2.20	0.70	1.32	0.18	0.00	0.00	4.2	0.4305
7	0	64.9	24.6	40.3	0.61	61	53	2.13	0.147	69.2	35.9	15.3	5.9	4.74	0.00	0.73	0.42	0.01	0.00	4.4	1.3977
8	1	65.1	32.9	32.2	1.02	54	119	5.41	0.340	62.7	34.9	18.5	7.5	2.93	0.08	3.68	0.60	0.23	0.00	60.3	0.6413
9	0	50.6	26.6	24.0	1.11	74	64	1.23	0.092	75.0	68.8	13.7	3.6	2.50	0.00	0.52	0.54	0.01	0.03	3.0	0.6730
10	0	72.9	25.8	47.1	0.55	61	72	3.40	0.204	59.8	35.2	18.1	6.2	3.73	0.00	1.58	0.87	0.02	0.00	7.1	0.6493
11	0	66.7	22.4	44.3	0.51	86	111	4.62	0.309	66.9	35.9	17.7	9.1	3.75	0.18	4.41	0.46	0.29	0.00	149.0	0.9224
12	0	49.3	23.8	25.5	0.93	33	83	3.53	0.234	66.3	35.6	14.6	3.9	2.42	0.31	0.86	0.27	0.04	0.00	2.0	0.6609
13	0	68.8	35.7	33.1	1.08	80	176	6.99	0.484	69.2	36.3	16.1	5.3	4.68	0.00	0.20	0.37	0.01	0.04	67.8	1.5528
14	1	48.1	25.1	23.0	1.09	44	66	2.59	0.175	67.3	37.5	14.7	4.2	2.27	0.00	1.60	0.34	0.00	0.00	1.5	0.6061
15	0	51.9	28.2	23.7	1.19	42	140	6.09	0.401	65.7	35.0	15.2	4.6	2.71	0.05	1.38	0.41	0.05	0.00	3.0	0.2993
16	1	67.4	29.3	38.1	0.77	40	39	1.66	0.108	65.0	36.0	15.9	10.8	6.05	1.73	1.62	1.40	0.00	0.00	47.3	0.9424
17	0	46.3	20.8	25.5	0.82	56	77	3.46	0.220	63.6	35.2	15.5	10.7	1.09	2.79	0.93	0.00	0.00	0.00	5.0	0.3147
18	1	49.3	26.0	23.3	1.12	39	88	4.45	0.259	58.1	34.1	19.0	8.2	5.66	0.74	0.41	1.15	0.25	0.00	42.7	0.6572
19	1	51.3	20.8	30.5	0.68	76	80	3.15	0.222	70.7	36.2	13.4	7.9	6.09	0.32	0.55	0.88	0.06	0.00	14.3	0.5755
20	0	77.6	27.2	50.4	0.54	60	39	1.55	0.116	74.6	33.4	21.3	6.4	3.71	0.00	1.02	1.66	0.00	0.00	120.0	0.6860
21	0	76.5	23.6	52.9	0.45	102	91	3.97	0.267	67.3	34.2	16.0	9.5	6.81	0.19	1.41	0.79	0.30	0.00	8.9	0.9402
22	0	53.4	27.3	26.1	1.05	79	114	4.83	0.327	67.7	34.8	15.2	8.1	6.10	0.65	0.69	0.66	0.01	0.00	1.0	1.2835
23	1	38.5	19.7	18.8	1.05	84	78	3.49	0.221	63.3	35.0	15.0	4.2	2.81	0.46	0.67	0.25	0.00	0.00	3.2	1.0010
24	1	56.3	32.2	24.1	1.34	62	145	6.63	0.426	64.2	34.1	16.0	5.3	3.50	0.11	1.54	0.16	0.00	0.00	0.4	0.4625
25	1	43.2	20.2	23.0	0.88	15	89	3.76	0.251	66.8	35.6	15.3	13.8	8.42	1.79	0.28	3.31	0.12	0.00	1.4	0.6306
26	0	48.8	24.6	24.2	1.02	56	91	3.60	0.236	65.6	38.7	15.5	7.3	3.42	2.41	1.01	0.45	0.00	0.00	0.0	0.5950
27	0	82.7	17.3	65.4	0.26	49	36	1.59	0.110	69.4	33.1	27.9	7.6	5.09	0.76	0.91	0.84	0.00	0.00	44.0	0.2166

28	0	53.6	22.7	30.9	0.73	39	81	3.64	0.241	66.1	33.5	15.8	3.3	1.78	0.13	1.19	0.20	0.00	0.00	4.8	0.3124
29	0	46.2	24.1	22.1	1.09	55	52	2.48	0.152	61.5	34.0	16.6	5.7	3.36	0.51	1.25	0.57	0.00	0.00	2.1	0.8333
30	0	85.4	25.0	60.4	0.41	97	79	3.22	0.233	72.4	33.8	20.2	10.0	6.56	0.70	1.22	1.47	0.05	0.00	4.4	0.3320
31	1	60.8	25.1	35.7	0.70	55	57	2.38	0.171	71.8	33.2	17.9	6.2	4.59	0.19	0.87	0.31	0.25	0.00	0.2	0.2753
32																					
33	1	49.4	27.4	22.0	1.25	67	110	4.72	0.312	66.2	35.1	13.5	9.0	6.93	0.45	0.72	0.90	0.00	0.00	4.6	0.2407
34	1	49.3	24.4	24.9	0.98	65	66	3.00	0.187	62.5	35.0	18.1	7.9	5.61	0.08	1.66	0.55	0.00	0.00	7.5	0.8121
35	0	52.3	18.3	34.0	0.54	29	41	1.68	0.123	73.6	32.8	28.2	5.0	2.73	0.20	1.51	0.56	0.01	0.01	68.3	0.4074
36	1	42.4	18.1	24.3	0.74	18	58	2.96	0.176	59.5	32.8	18.1	6.0	4.20	0.24	0.96	0.60	0.00	0.00	1.7	0.2769
37	0	68.8	27.8	41.0	0.68	107	70	3.06	0.223	72.9	31.5	16.8	7.7	5.99	0.39	0.91	0.40	0.01	0.00	8.1	1.3023
38	1	55.4	28.6	26.8	1.07	39	139	5.99	0.429	71.7	32.3	14.4	10.6	7.74	0.64	1.17	0.85	0.21	0.00	12.4	0.2953
39	0	51.6	27.0	24.6	1.10	45	108	4.65	0.312	67.2	34.7		15.1	10.42	0.15	2.72	1.36	0.45	0.00	3.0	0.4027
40	1	76.6	28.2	48.4	0.58	95	136	6.29	0.409	65.1	33.3	18.2	5.9	2.60	0.06	2.01	0.77	0.47	0.00	141.0	0.9045
Dr Miller's healthy dogs																					
1		56.6	33.9	22.7	1.49	104	163	7.96	0.477	60.0	34.0	18.1	10.4	5.82	0.00	2.50	0.73	1.35	0.00	195.0	0.9534
2		79.1	32.8	36.3	0.90	113	189	8.83	0.554	62.8	34.1	16.3	17.0	9.52	0.17	4.93	1.02	1.36	0.00	217.0	0.9424
3		64.4	31.5	32.9	0.96	87	147	6.53	0.428	65.5	34.5	15.7	11.2	7.39	0.11	1.57	0.90	1.23	0.00	204.0	0.9424
4		57.2	35.8	21.4	1.67	117	164	7.49	0.477	63.7	34.4	17.1	14.3	9.58	0.00	2.57	0.86	1.29	0.00	244.0	1.0074
5		55.9	35.2	20.7	1.70	9	152	6.53	0.434	66.5	34.9	14.9	10.8	5.08	0.00	4.54	0.65	0.54	0.00	277.0	0.9424
6		63.6	31.2	32.4	0.96	97	159	6.61	0.456	68.9	34.8	15.5	15.3	10.10	0.00	1.99	1.07	1.99	0.15	273.0	0.9090
7		63.7	31.7	32.0	0.99	107	149	6.98	0.425	60.9	35.0	15.2	10.0	7.70	0.00	0.80	0.60	0.90	0.00	354.0	0.9643
8		65.8	39.9	25.9	1.54	95	165	7.23	0.472	65.3	35.0	15.2	14.9	9.54	0.15	2.68	0.60	1.94	0.00	353.0	0.9424

tsp: total serum proteins (g/l); alb: albumin (g/l); glob: globulin (g/l); a/g: albumin/globulin ratio; creat: creatinine (umol/l); hb: haemoglobin (g/); rcc: red cell count ($x10^{12}$/l); PCV: Packed cell volume (l/l); mcv: mean cell volume (fl); mchc; mean cell haemoglobin concentration (g/dl); rdw: red cell distribution width (%); wcc: white cell count ($x10^{9}$/l); neuts s: segmented neutrophil count ($x10^{9}$/l); neuts b: banded neutrophil count ($x10^{9}$/l); lymp: lymphocyte count ($x10^{9}$/l); mono: monocyte count ($x10^{9}$/l); eos: eosinophil count ($x10^{9}$/l); baso: basophil count ($x10^{9}$/l); thc: thrombocytes count ($x10^{9}$/l)

Appendix 6: continued

Patient no	Mg of diminazene injected	Calculated dose in mg/kg	Calculated dose in mg/m2	Diminazene plasma concentration in sample 1	Diminazene plasma concentration in sample 2	Diminazene plasma concentration in sample 3	Diminazene plasma concentration in sample 4
1	185.28	4.31	149.46	3.456	1.775		
2	161.58	4.31	142.79	4.642	1.624	0.626	0.388
3	90.48	4.31	117.70	3.021	2.770	0.515	0.259
4	137.88	4.32	135.72	0.375	0.842	0.514	
5	99.18	4.26	120.37	2.536	2.166	0.488	0.417
6	37.95	4.31	88.14	3.014	3.167	0.577	0.336
7	224.56	4.36	160.67	2.669	1.246	0.791	0.336
8	68.57	4.29	106.92	1.828	1.647	0.328	*
9	73.61	4.28	109.38	4.221	2.736	1.040	0.421
10	69.28	4.25	106.70	4.916	4.183	0.793	0.305
11	120.74	4.37	130.89	2.657	0.708	0.410	*
12	73.99	4.42	111.94	2.993	1.433	0.628	0.306
13	262.83	4.36	169.27	2.453	1.254	0.327	*
14	64.81	4.41	106.94	3.008	2.910	0.489	*
15	22.69	4.45	75.83	3.957	1.734	0.270	*
16	115.81	4.06	122.89	4.069	3.989	1.327	0.342
17	23.59	4.29	74.96	2.232	3.492	0.496	0.251
18	68.43	4.12	104.12	3.017	2.872	0.345	*
19	59.88	4.40	104.05	3.605	2.160	1.570	0.372
20	77.30	4.37	112.69	5.699	1.753	1.021	0.245
21	119.76	4.22	127.38	5.115	3.152	1.678	0.897
22	195.94	4.33	152.67	2.318	4.581	0.771	0.380
23	137.38	4.40	137.25	4.357	2.458	1.645	0.644
24	41.34	4.22	89.38	3.124	1.536	0.905	0.376
25	68.71	4.40	108.97	5.694	2.041	1.170	0.530
26	60.37	4.22	101.45	4.264	1.990	0.646	*
27	13.80	4.39	63.71	5.787	3.636		
28	23.38	4.30	74.84	4.985	2.053	1.606	0.438
29	103.28	4.36	123.93	2.919	1.859	0.724	0.245
30	26.03	4.37	78.41	5.568	3.862	2.316	0.740

31	19.52	4.34	70.92	3.865	2.226	0.447	*
32				No samples received			
33	16.09	4.37	66.82	2.168	3.799	0.785	0.279
34	98.98	4.34	121.88	5.246	2.002	1.073	0.383
35	34.57	4.27	84.87	4.257	1.741	0.964	0.233
36	19.21	4.23	69.36	4.481	4.449	0.804	0.492
37	198.52	4.29	152.44	3.257	3.964	0.761	0.498
38	21.58	4.32	73.07	3.610	4.808	0.631	0.281
39	34.15	4.29	84.80	3.093	2.601	0.361	*
40	114.18	4.26	126.23	2.129	0.700	0.447	0.246

Dr Miller's healthy dogs				**Time of sample collection in sec**	**1200**	**2400**	**3600**	**7200**	**10800**	**14400**	**28800**
121.80	4.20	127.76		diminazene plasma concentration	1.998	1.925	0.736	0.397	0.375	0.342	0.148
119.70	4.20	127.02		diminazene plasma concentration	2.188	1.816	1.891	0.736	0.590	0.468	0.327
119.70	4.20	127.02		diminazene plasma concentration	2.083	1.050	0.696	0.344	0.342	0.290	0.072
132.30	4.20	131.33		diminazene plasma concentration	1.983	1.255	0.924	0.412	0.314	0.229	0.102
119.70	4.20	127.02		diminazene plasma concentration	1.632	0.949	0.732	0.399	0.300	0.216	0.084
113.40	4.20	124.75		diminazene plasma concentration	1.779	0.994	0.848	0.322	0.283	0.192	0.031
123.90	4.20	128.49		diminazene plasma concentration	0.159	1.361	0.869	0.480	0.301	0.210	0.194
119.70	4.20	127.02		diminazene plasma concentration	1.775	0.864	0.608	0.311	0.226	0.192	0.061

*= below level of quantification

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