The pharmacokinetics of diminazene aceturate after intramuscular administration in healthy dogs

D M Miller, G E Swan, R G Lobetti and L S Jacobson

ABSTRACT
The pharmacokinetics of diminazene aceturate following intramuscular (i.m.) administration at 4.2 mg/kg was evaluated in 8 healthy German Shepherd dogs. Blood samples were collected at 19 intervals over a period of 21 days. Diminazene plasma concentrations were measured using a validated HPLC method with UV detection and a sensitivity of 25 ng/ml. The in vitro and in vivo binding of diminazene to blood elements was additionally determined. Diminazene pharmacokinetics showed a large inter-individual variation after i.m. administration. It had a short absorption half-life (K01-HL of 0.11 ± 0.18 h), resulting in a Cmax of 1849 ± 268.7 ng/ml at Tmax of 0.37 h and a mean overall elimination half-life (T½α) of 5.31 ± 3.89 h. A terminal half-life of 27.5 ± 25.0 h was measured. At 1 h after i.m. injection, 75 % of the diminazene in whole blood was in the plasma fraction. The results of this study indicate that diminazene is rapidly distributed and sequestered into the liver, followed by a slower terminal phase during which diminazene is both redistributed to the peripheral tissues and/or renally excreted. It is recommended that diminazene administered i.m. at 4.2 mg/kg should not be repeated within a 21-day period.

Key words: Babesia canis, babesiosis, canine, diminazene, liver sequestration, pharmacokinetics, pharmacology.


INTRODUCTION
Diminazene is the antibabesial drug of choice for the treatment of canine babesiosis in South Africa. Differences in the dosage described for diminazene and the occurrence of mortality at doses equal to or close to the recommended therapeutic dose for the treatment of canine babesiosis have been described. The diminazene products used to treat babesiosis in South Africa contain a combination of diminazene aceturate and antipyrine, mostly in a concentration of 45 % m/m and 55 % m/m, respectively.

Babesia canis is a tick-transmitted intracellular protozoan parasite responsible for high morbidity and mortality among dogs in South Africa. The incidence of canine babesiosis at the outpatients clinic of the Onderstepoort Veterinary Academic Hospital over a 6-year period was 11.7 % (1253 of 10710 sick dogs presented per year). In 1955, Fussgänger wrote of his work on the antibabesial drug diminazene: "Remarkable therapeutic success has been obtained during the last years in the treatment of protozoal diseases in domestic animals using a novel drug developed in the research laboratories of Fabwerke Hoechst A.G." How, little pharmacokinetic work with diminazene aceturate has since been done in dogs.

Bauer reported peak serum concentrations of diminazene (3 µg/ml) occurred at 3 hours (h) after i.m. administration in the dog and that all traces of diminazene were absent by 24 h. He concluded that diminazene was excreted via the kidneys within 24 h. Onyeilili and Anika looked at the influence of Trypanosoma congolense on the disposition of diminazene in the dog and reported that drug elimination followed a biphasic process. This was seen irrespective of infection, but infection significantly shortened the half-life of absorption T½α of diminazene from 0.17 h to 0.12 h, although the urinary excretion of the drug remained constant. That study used 3.5 mg/kg diminazene intramuscularly (i.m.) in both healthy dogs and in dogs with trypanosomiasis.

Mean plasma concentrations were 0.2 ± 0.008 µg/ml, but no peak concentrations were reported. No diminazene was found in the plasma after 48 h. Higher plasma concentrations were found in dogs infected with trypanosomes, and higher tissue concentrations were present in healthy dogs. In tissues sampled at 48 h, the highest concentrations of diminazene were found in the kidneys and liver in both groups and low diminazene concentrations were found in the brain. Diminazene persisted in the tissues for more than 10 days. The same authors subsequently reported a biological half-life (T½β) of 9.87 h in healthy dogs and 12.51 h in T. brucei-infected dogs. The T½β was significantly decreased after trypanosome infection (0.14 h vs 0.2 h).

Healthy dogs given i.m. diminazene showed severe clinical signs associated with damage to the central nervous system (CNS) and then died. Interestingly, 1 dog was resistant to the toxic effects of diminazene despite repeated daily i.m. treatments at 3.5 mg/kg for 15 and 30 doses, while other dogs showed typical neurological clinical signs after only 2 doses. At necropsy the brains were oedematous and showed bilaterally symmetrical haemorrhages together with malacic lesions of the cerebellum, midbrain and thalamus. Healthy dogs treated with 15 mg/kg of diminazene i.m. had brain lesions mimicking those of cerebral babesiosis. The incidence of CNS toxicity is not known but it has been reported to occur both with overdose and at the recommended dose.

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

The unpredictability of diminazene toxicity, with mortalities reported at doses equal to or close to the recommended therapeutic dose for the treatment of canine babesiosis, dictated the need...
for further study. A better understanding of the pharmacokinetics and macromolecular binding features of diminazene in dogs is needed to explain the cause for the variation in the clinical responses seen. The current study was undertaken to examine the pharmacokinetics of diminazene acetate after intramuscular injection in healthy adult dogs.

MATERIALS AND METHODS

Animals

The Animal Use and Care Committee of the Faculty of Veterinary Science approved this study (Study No. 36.5.274).

Eight male German Shepherd dogs, of approximately the same age (18 months) and weight range (30 ± 2.5 kg) were used. The dogs were obtained from the Roodeplaat dog breeding station, of the South African Police Service. Once the study was completed, the dogs were returned to the breeding station, to continue their normal duties.

Dogs were included in this prospective study if they were fully vaccinated, were found to be clinically healthy and were easy to handle. Dogs that had been treated for canine babesiosis in the preceding 3 months, received any medical therapy for any disorder within a 3-week period, or had been dipped for ectoparasite control within a 2-week period prior to the start of the study, were excluded.

Full clinical examination, urine analysis, complete haematology and serum biochemistry (total serum protein, albumin, globulin, alkaline phosphatase, alanine aminotransferase, urea and creatinine) were performed to rule out any underlying disease that might affect diminazene pharmacokinetics. All the test results were within normal limits.

Treatment

Multiple bottles of diminazene (Berenil, Hoechst Roussel Vet, Halfway House, South Africa), from the same batch, were repackaged by Kyron laboratories (Kyron Laboratories, Benrose, South Africa) specifically for the study. The bottles were weighed to ensure that they contained the correct amount of content. A sample of the contents of each bottle was kept to analyse the drug concentration after dilution. The mixture was prepared by reconstituting the 1.05 g diminazene with 25 ml sterile water to give a resultant volume of 25 ml and a concentration of 42 mg/ml.

All the dogs were weighed 2 days prior to the start of the study, following a 12-h period of food withdrawal and after the dogs had been taken out to urinate and defaecate. The dose of diminazene for each dog was calculated and recorded according to its fasted body weight.

The dogs were injected with freshly diluted diminazene at the standard dose of 4.2 mg/kg. Feed was withdrawn from all the dogs 12 h before to 4 h after treatment. Intramuscular injections were performed in the M. biceps femoris, midway between the hip and the stifle joints.

Blood collections

Five ml blood samples were collected 1–2 min pre-treatment (Time 0), and at 0.33, 0.66, 1, 2, 3, 4, 8, 12, 18, 24, 36, 48, 72, 120, 168, 240, 336 and 504 h post-treatment. The sample timing was based on available diminazene pharmacokinetic data, with special attention given to the peak concentration (C max) and overall elimination half-life (T½β), reported in the literature. Blood was drawn into evacuated, uniquely identified heparinized tubes (Vacutainer, BD Vacutainer Systems, Preanalytical Solutions, Belliver, Plymouth, UK) from either the cephalic or jugular veins. The heparinized blood was stored on ice until centrifuged. Samples were centrifuged within 30 min of collection at 3000 r.p.m for 15 min, the plasma transferred to polycarbonate tubes and stored at −20°C until analysed.

Diminazene binding to blood elements

The binding of diminazene to plasma and red blood cell contents was examined both in vitro and on ex vivo samples. For the in vitro study 425 mℓ of blood, collected in acid-citrate dextrose was drawn from a dog chosen following the same selection criteria as used for the dogs in the pharmacokinetic study. Three 50 ml bags of the blood were fortified to 3 different concentrations of diminazene, 0.5, 1.5 and 3 µg/ml. The blood bags were placed in a refrigerator at 4°C for 24 h to allow drug to red blood cell binding to occur. They were turned every 4 h to ensure mixing of the diminazene and blood. After 24 h, two 10 ml samples of blood was withdrawn from each bag, centrifuged and divided into plasma and packed RBC. The packed RBC from each individual bag was washed 3 times using physiological saline and then pooled. Half of the plasma was microcentrifuged through a 10 000 micropore filter at 10 000 rpm for 30 min (Beckman CS-15R centrifuge with Beckman F1010 head, radius 8 cm, Beckman Coulter, Midrand, South Africa). This procedure left 3 samples of (1) washed packed RBC; (2) plasma and (3) filtered plasma (water fraction). All samples for the in vitro study were processed on the same day and 6 replicates were collected for each fraction. These samples also acted as the in vitro quality control to test for the repeatability of the analyses.

Additional 5 ml heparinised sample were collected from all trial animals at 1 h after treatment for ex vivo analysis. These were separated into fractions as described above and pooled. The pooled fractions were then split into as many 1 ml aliquots as the available volume allowed. Five replicates of each pooled fraction were analysed and the average concentration for each fraction determined.

Diminazene assay

Diminazene concentrations were determined by a validated high performance liquid chromatographic (HPLC) method with UV detection following paired ion extraction3. The method had a limit of quantification of 25 ng/ml.

Pharmacokinetic analysis

Pharmacokinetic analysis was performed by non-linear compartmental analysis and non-compartmental analysis for extravascular administration of the diminazene plasma concentration versus time data. The analysis was performed by means of PC Nonlin Version 4.2 (Statistical Consultants, Inc., New York, USA) using the Nelder-Mead algorithm. Initial pharmacokinetic parameter estimates, used for the non-linear analysis, were derived automatically by initial linear analysis performed by the program. Akaike’s information criterion7, based on the mean values of the final estimates of the associated pharmacokinetic parameters and lack of systematic deviations around the fitted disposition curve, was used to determine the number of exponential terms that best described the data.

Primary pharmacokinetic parameters were derived by 2-compartment analysis with 1st-order input, 1st-order output and lag time (Model 13) of the plasma concentration-time data for each individual animal, yielding the microconstants K01 and K10. Secondary disposition parameters, including area under the curve (AUC), K01 half-life (K01-HL), K10 half-life (K10-HL), overall absorption and distribution half-life (T½α) and overall elimination half-life (T½β), were derived from the primary parameters utilising standard procedures5.

The area under the plasma concentration versus time curve (AUC, zero-moment) and the 1st non-normalised moment (AUMC) were calculated according to the trapezoidal method from time zero to the last sample time as derived by non-compartmental analysis. Extrapolation of AUC to infinity (AUC∞) was performed using the slope of the terminal phase (β). Since AUMC to infinity could not be
RESULTS

None of the dogs showed any pain reaction at the time of injection and no muscle swelling was seen at the injection site. Three dogs developed diarrhoea within 20 min of diminazene administration. One of these dogs had diarrhoea again after 45 min while 2 other dogs developed diarrhoea after 1 h and after 1 h 15 min. All the dogs in the trial ate (when they were offered food) after the 4 h sample had been drawn and none of the dogs exhibited diarrhoea again for the duration of the study.

A semilogarithmic plot of mean diminazene concentrations versus time was constructed (Fig. 1). The derived compartmental and non-compartmental data are summarised in Tables 1 and 2. Compartmental pharmacokinetic analysis revealed a very rapid rate of absorption as measured by the half-life of absorption (K01-HL) 0.11 ± 0.18 h and rapid overall absorption and distribution half-life (T½α) 0.36 ± 0.19 h (Table 1). The distribution into the peripheral compartment was more rapid than the distribution back into the central compartment. A mean overall elimination half-life (T½β) of 5.31 ± 3.89 h was derived. There was large inter-subject variation in the pharmacokinetic results (% CV range of 37–163 for the various pharmacokinetic parameters).

Peak plasma concentrations (Cmax) of 1849.9 ± 268.7 ng/mL occurred at 0.37 ± 0.12 h (Tmax) after intramuscular administration. A terminal half-life of 27.5 ± 24.96 h and MRT of 10.32 ± 5.44 h were observed following non-compartmental analysis.

The binding of diminazene to blood elements (Table 3) revealed that the drug was predominantly bound to plasma. Seventy-five per cent of diminazene in whole blood was present in the plasma 1 h after i.m. injection while 24 h after the in vitro blood/diminazene admixture, 85–94.5 % of the diminazene was extracted from the plasma fraction. The mean percentage of diminazene in the filtrate (water fraction) was 17 % of the total plasma diminazene, for the 3 concentrations of diminazene in the in vitro study and 24 % for ex vivo 1 h samples. The plasma concentration of diminazene from the in vitro study, was corrected for a haematocrit of 50 % so that the equations reflected the actual volume of plasma per mL of blood rather than the diminazene concentrations per mL of plasma. Seventy-six per cent of the diminazene was extracted from the plasma fraction and the percentage of diminazene bound to the red blood cells was 18.5 % of the total diminazene in the blood.

DISCUSSION

This is the first comprehensive study of the pharmacokinetics of diminazene following i.m. administration in dogs.

Table 1: Pharmacokinetic results following intramuscular administration of diminazene aceturate at 4.2 mg/kg in dogs (n = 8) derived by 2-compartmental analysis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Individual dog results</th>
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<tbody>
<tr>
<td></td>
<td>Dog 1</td>
</tr>
<tr>
<td>A (ng/mL)</td>
<td>25063</td>
</tr>
<tr>
<td>B (ng/mL)</td>
<td>393</td>
</tr>
<tr>
<td>K01</td>
<td>3.50</td>
</tr>
<tr>
<td>α (h⁻¹)</td>
<td>2.87</td>
</tr>
<tr>
<td>β (h⁻¹)</td>
<td>0.0840</td>
</tr>
<tr>
<td>K10</td>
<td>0.80</td>
</tr>
<tr>
<td>K12</td>
<td>1.85</td>
</tr>
<tr>
<td>K21</td>
<td>0.3015</td>
</tr>
<tr>
<td>K10-HL (h)</td>
<td>0.87</td>
</tr>
<tr>
<td>K11-HL (h)</td>
<td>0.20</td>
</tr>
<tr>
<td>T½α (h)</td>
<td>0.24</td>
</tr>
<tr>
<td>T½β (h)</td>
<td>8.25</td>
</tr>
</tbody>
</table>

A, distribution phase intercept (initial serum drug concentration); B, elimination phase intercept; K01, rate at which drug enters the central compartment; α, overall absorption and distribution constant; β, overall elimination constant; K10, rate at which drug leaves the central compartment; K12, rate constant for drug removal/distribution from central compartment; K21, rate constant for distribution from peripheral compartment; K10-HL, half-life of K10; K11-HL, half-life of K11; T½α, overall absorption and distribution half-life; T½β, overall elimination half-life.
The pharmacokinetics of diminazene were characterised by a very rapid rate of absorption and short overall elimination half-life (T½β, 5.31 ± 3.89 h). The maximum plasma concentrations (Cmax; 1850 ± 269 ng/mL) measured at 22.2 ± 6 min (Tmax) were most likely an underestimation since the peak plasma concentrations were already measured in the 1st blood samples collected, at 20 min after treatment, in 7 of the 8 dogs.

The precipitous drop in plasma concentrations was ascribed to rapid distribution of diminazene into the peripheral compartment. It seems likely that the liver serves as an initial sump for diminazene. Onyeyili and Anika21 found that 7 kg dogs, given 3.5 mg/kg of diminazene, had 81 µg/g of diminazene in their livers 48 h after diminazene administration. This accounts for 78.8% of the total drug if one takes the liver weight at 3.4% of body weight5. Gummow found that injection site reactions occurred in cattle1 and reasoned that this could result in secondary peaks. It is possible that the same reaction is responsible for the biphasic absorption seen in this current study, but as the distribution phase is faster than renal excretion (normal creatinine clearance rates 2.8 ± 0.96 ml/min/kg to 4.09 ± 0.52 ml/min/kg22) sequesteration within the liver is the more likely hypothesis. This would also explain the differences in the rate of distribution to and from the peripheral compartment (K12 versus K21).

Higher plasma concentrations of diminazene were found in dogs infected with trypanosomales while higher tissue concentrations were present in healthy dogs5. The total body clearance was significantly lower in healthy dogs and the distribution half-life was significantly decreased in infected dogs5. Onyeyili et al.21 found significantly higher diminazene residues in the tissues of healthy dogs than in dogs infected with T. congolense in all tissues tested except in the brain. The distribution half-life was significantly reduced after infection and infection increased the rate at which diminazene was distributed to the body5,21. Mamman et al.22, in a study of healthy cattle and those with acute and chronic T. congolense infection, found that Cmax was significantly greater and Tmax significantly shorter in the acute infection group. Similar studies have, however, not been reported in Babesia-infected dogs or cattle.

In dogs with babesiosis, factors such as hypotension, anaemia, acidosis, changes in albumin concentrations and altered endothelial integrity23, as well as possible altered drug absorption from the injection site may alter the pharmacokinetics of diminazene. Alterations in hepatic and renal perfusion may have a large influence on diminazene distribution and thus may influence plasma concentrations of diminazene and play a role in either potentiating or decreasing the chances of toxicity. Furthermore a definite diagnosis of diminazene toxicity is complicated by the difficulty in distinguishing this from cerebral babesiosis, as well as from the clinical signs of hypoglycaemia in severe babesiosis5.

The CNS toxicity of diminazene is dose-related, and repeated doses of diminazene can also cause CNS toxicity due to cumulative effects. The current study demonstrates that diminazene is extensively distributed, but it is not known how this translates into tissue concentrations within the brain or what effect it may have on transport mechanisms in the blood–brain barrier.20,24 From the study results, a washout period of at least 21 days is recommended. This conservative approach is suggested by the finding that most of the drug is distributed to the peripheral compartment and that the limit of detection was not low enough to determine the terminal phase and the AUC correctly. In one canine study, diminazene persisted in the tissues for over 10 days25. In addition, the babesiosis disease process may alter diminazene pharmacokinetics, as alluded to above, and due to the fact that bile acid levels were raised in a third of severely ill animals with babesiosis26. Hypotension is a common phenomenon in clinical babesiosis27 and is usually associated with splanchnic [and thus hepatic] vasoconstriction to ensure cerebral blood flow. Apparent differences in the susceptibility of dogs to diminazene toxicity have been implied in South Africa and this is sup-

Table 2: Pharmacokinetic results following intramuscular administration of diminazene acetateur at 4.2 mg/kg in dogs (n = 8) derived by non-compartmental analysis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Individual dog results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog 1</td>
<td>Dog 2</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>0.33</td>
</tr>
<tr>
<td>Cmax (ng/mL)</td>
<td>1998 ± 6283</td>
</tr>
<tr>
<td>AUC (ng/h/mL)</td>
<td>330 ± 2769</td>
</tr>
<tr>
<td>Vc/f (L/kg)</td>
<td>48 ± 310</td>
</tr>
<tr>
<td>Cl/f (mL/kg/h)</td>
<td>0.6 ± 0.6</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>10.01 ± 11.52</td>
</tr>
</tbody>
</table>

Tmax, time to peak plasma concentrations; Cmax, maximum plasma concentrations; K1, 1st-order rate constant associated with the terminal (log-linear) portion of the curve; AUClast, area under the plasma concentration curve to the last measurable plasma concentration; AUCinf, extrapolated to infinity; Vc,f, fractional volume of distribution of the central compartment; Cl/f, fractional total body clearance; MRT, mean residence time.

Table 3: Macromolecular binding of diminazene in bovine blood examined in vitro and in vivo.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Diminazene concentrations (ng/mL)</th>
<th>Binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>Filtrate</td>
</tr>
<tr>
<td>In vitro</td>
<td>500 ng/mL</td>
<td>330</td>
</tr>
<tr>
<td></td>
<td>1500 ng/mL</td>
<td>1152</td>
</tr>
<tr>
<td></td>
<td>3000 ng/mL</td>
<td>2138</td>
</tr>
<tr>
<td>In vivo</td>
<td>1 h sample*</td>
<td>913 ± 408**</td>
</tr>
</tbody>
</table>

*Mean of all dogs. **Mean of 2 animals.
ported by the fact that some dogs were resistant to 20 mg/kg diminazene i.m. repeatedly, while other dogs showed signs of diminazene toxicity at the recommended dosage\(^1\). The role of the blood–brain barrier extrusion pumps in the occurrence of ivermectin toxicity has been implicated in certain breeds\(^8\), raising the question of whether this may also be applicable to diminazene toxicity. These factors are of significance taking into consideration the distribution pharmacokinetics of diminazene, in particular the role of the liver and the increased distribution of diminazene to the brain in *Trypanosoma*-infected dogs\(^20\). A lower dose might be considered for dogs with compromised liver function, which could be at higher risk for toxicity. Collapse, salivation and diarrhoea have been described in clinically healthy dogs given large doses of diminazene i.v.\(^9\) but have not previously been reported following i.m. administration, and diarrhoea following diminazene administration in dogs with babesiosis might erroneously be ascribed to the disease. The fact that 5 of the 8 dogs in this study displayed bouts of diarrhoea after i.m. diminazene administration is worth taking note of.

Alvi et al.\(^1\) reported that diminazene bound to a number of blood proteins and could cross the red cell membrane to bind to haemoglobin. This was refuted by the current study. The binding of diminazene to red blood cells is markedly less than plasma, and is not expected to play an important role in the T\(_{1/2}\). Therefore no dose adjustments should be necessary for anaemic patients.

**CONCLUSION**

Diminazene is most likely first seques-
tered in the liver, then is slowly released back into the central compartment and redistributed into less well perfused peripheral tissues before finally being eliminated. The slower redistribution to and from these peripheral tissues is likely to be mainly responsible for the longer elimination half-life of diminazene as measured by non-compartmental analysis. An apparent low bioavailability could be explained by retention of a portion of the dose at the site of injection as was seen in cattle and to the fact that the C\(_{max}\) might have been missed in some of the dogs which could also have resulted in a smaller AUC measured after i.m. administration.

Since the terminal portion of the diminazene plasma concentration versus time curve of the i.m. data could not be clearly defined it is advised that a longer withdrawal time and that care should be taken in dogs with decreased liver mass. With the knowledge gained of the pharmacokinetics of diminazene in healthy dogs, a population pharmacokinetic study in dogs with babesiosis is recommended. This will allow us to more fully appreciate alterations in pharmacokinetics of diminazene and the potential covariants playing a role. A study of this nature would help to elucidate if there is any therapeutic implication due to large inter-individual variation. This may lead to the possible contributing causes of diminazene toxicity.

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