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A bioequivalence and pharmacokinetic evaluation of two commercial diminazene aceturate formulations administered intramuscularly to cattle

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ABSTRACT

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The bioequivalence of the diminazene formulation Veriben (Centaur) was determined in cattle (n = 10) by means of a single-dose, randomized cross-over experiment. The results of nine statistical procedures commonly used for bioequivalence evaluation are discussed. Veriben was found to be equivalent to Berenil (Hoechst) with respect to the area under the plasma concentration versus time curve, but not in terms of the maximum plasma drug concentration and the time to maximum plasma drug concentration. Pharmacokinetic parameters were calculated in which bioequivalence data (n = 10) together with data from an additional four cattle were used. A two-compartment model best described the pharmacokinetic behaviour of diminazene in cattle. Peak concentrations of diminazene (3,24 ± 0,16 µg/mℓ) were reached 49,8 (± 7,6) min after intramuscular injection of 3,5 mg/kg drug, with absorption proceeding rapidly ($t½_{\alpha}$ = 1,93 ± 0,95 h). Diminazene was slowly eliminated ($t½_{\beta}$ = 222 h), resulting in a mean residence time of 13,27 d. The safe interval necessary between successive treatments of diminazene or before live babesia vaccines should be administered, and a recommended pre-slaughter withdrawal period are also discussed.

INTRODUCTION

"Remarkable therapeutical 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 Farbwerke Hoechst A.G." wrote R. Fussgänger in 1955 about his work on diminazene aceturate (Berenil), an aromatic diamidine compound, discovered 16 March 1954. Today, nearly 40 years later, the exact mechanism of action and *in vivo* behaviour of the drug is still unknown. In the South African context, diminazene aceturate has long been an important treatment of babesiosis, a major tick-borne protozoal disease in various animal species. The need to understand the pharmacokinetics of this drug in cattle is therefore of importance, especially when treatment may interfere with the efficacy of live protozoal vaccines that are often administered soon after treatment.

Renewed interest in the drug has been stimulated with the expiry of the original patent on Berenil resulting in the manufacture of generic products. The registration of generic products in South Africa requires that pharmaceutical companies are able to demonstrate that their new generic product substitutes the original product in all respects. For this

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purpose, bioequivalence can be shown either by means of extensive comparative clinical endpoint studies or by means of comparative bioavailability. The main objective of this work was to evaluate the bioequivalence of a newly introduced diminazene aceturate formulation Veriben (Centaur) to Berenil (Hoechst) by the use of comparative bioavailability studies to provide the necessary data to meet the requirements for the registration of Veriben as a stock remedy in South Africa in terms of Act 36 of 1947. A secondary objective was to find out more about the pharmacokinetics of diminazene in cattle and thus provide users of the drug with more accurate information on its behaviour in cattle. Such information would enable better prophylactic use of the drug in cattle and better use of live babesia vaccines which are often used concurrently with diminazene to control epidemics.

MATERIALS AND METHODS

Experiment 1: bioequivalence study

Animals

Ten healthy adult Brahman bulls from the Onderstepoort Veterinary Institute (OVI), of approximately the same live mass and age, were used for the bioequivalence trial. A full clinical examination, including the clinical pathology of each animal, was performed 7 d before, and at 1 and 5 d after each treatment. One animal was injured during the second treatment phase and died as a result of suffocation after falling and twisting its neck in the crush.

The animals were housed together in a single camp and were fed a standard ration supplied by the OVI. Each animal was ear-tagged and numbered. On the day before each treatment, after a 24-h fasting period, the mass of the animals was determined and the animals treated accordingly.

Trial design and allocation

A single-dose, randomized, cross-over design was used. At the start of the trial the animals were ranked according to live mass, and replicates (a replicate in this instance being one animal on each treatment) were formed starting from the heaviest and proceeding to the lightest animal. Within each replicate, animals were allocated randomly to treatment groups by means of a table of random numbers. Two treatment phases were separated by a drug-free interval (washout period) of four months, at which time the treatments administered to each group were crossed over.

Treatment

At each treatment phase each animal received one of the following treatments:

- Reference product (R)—Berenil
- Test product (T)—Veriben

The products were in the form of soluble granules, containing 44,5 % m/m diminazene aceturate and 55,5 % m/m dimethylphenyl pyrazolone (antipyrine). The doses (3,5 mg active ingredient kg^{-1} for each animal, at each treatment phase) were measured into separate bottles the day before treatment. On the day of treatment the dose was diluted with 20 mℓ sterile water and injected intramuscularly into the gluteal muscle.

Blood samples

Blood was collected from the jugular vein in 10-mℓ heparinized vacuum tubes according to the following time schedule: before drug administration (0) and at 0,25, 0,5, 0,75, 1, 2, 3, 6, 12, 24, 36, 48, 72, 120, 168 and 336 h after drug administration. The blood samples were centrifuged as soon as possible after completion of collection at each treatment interval. From each sample two aliquots of plasma were transferred to labelled polyethylene tubes and stored at –20 °C until assayed.

Experiment 2

Experimental animals

To compensate for the death of the bull in experiment 1, a second experiment was carried out using four Simmentaler bulls with masses between 294 and 340 kg. This trial was conducted completely separately from the original trial, with these cattle being handled, housed and fed separately. Although described as a second experiment, this experiment was carried out to supplement experiment 1 and was not designed as a second bioequivalence trial, hence the small sample size.

Allocation and treatment

Allocation procedures and treatments were performed similarly to those of experiment 1. A washout period of 70 d between treatments was allowed for this trial.

Blood sample collection

The blood sample collection procedure was the same as for experiment 1.

Diminazene plasma assay

The plasma samples were analyzed for diminazene content by HPLC technique 19–23 months after collection. The long storage period for plasma samples was as a result of the time taken to modify Aliu & Odegaard's (1985) method. A modified paired-ion extraction technique, using a Supelco C18 solid-phase extraction column, described by Gummow (1993) was

used. The technique was validated for recovery, accuracy/precision and reproducibility (Gummow 1993). A detection limit of 0,202 μg diminazene $m\ell^{-1}$ plasma was achieved.

Pharmacokinetic analysis

Bioequivalence parameters

The rate and extent of diminazene absorption was compared by means of the maximum plasma drug concentration (C_{max}), the time to maximum plasma drug concentration (T_{max}) and the area under the concentration (AUC) versus time profile, 0–336 h (AUC_{0-336}). C_{max} and T_{max} were read directly from the observed concentrations, while AUC_{0-336} was calculated by the linear trapezoidal rule for the collection period.

Statistical analysis of bioequivalence parameters

A study of the literature showed that many statistical tests have been developed to test for bioequivalence (Anderson & Hauck 1983; Hauck & Anderson 1984; Liu & Wang 1991; Schuirmann 1987; Steinijans & Dilletti 1983; Westlake 1972; Westlake 1979). Each of these has its own set of assumptions, strengths and weaknesses, and much debate centres on what the ideal test should be (Dettelbach 1986; Lamy 1986; Metzler 1989; Pabst & Jaeger 1990; Roche 1984; Westlake 1979). Because of this debate, it is becoming standard practice to apply several statistical procedures to a set of results. The following set of statistical procedures were therefore applied to the bioequivalence parameters.

- Analysis of variance (ANOVA) with treatment, subject and period as the main effects.
- A point estimate for the ratio of the test product relative to the reference product, with period as the main effect, calculated as the geometric mean of the individual ratios (Steinijans & Dilletti 1983).
- The 90 % conventional t-confidence intervals for the true differences between the product means
- The 95 % symmetrical confidence intervals of Westlake (Westlake 1976)
- The two one-sided test procedure (Schuirmann 1987)
- The power approach (Schuirmann 1987)
- The interval hypothesis of Anderson & Hauck (1983)

In addition to these parametric tests, the non-parametric Wilcoxon rank test and Mann-Whitney tests (Steinijans & Dilletti 1983) were also applied to the bioequivalence parameters obtained for experiment 1. These were used primarily as a means of comparing the more descriptive parameter of T_{max}.

Determination of additional pharmacokinetic parameters

The curve-fitting program PCNONLIN (version 3, SCI Software) was used to approximate non-linear mathematic functions. A two-compartment model (Model 13) with first-order input, first-order output, no lag time and macro-constants as primary parameters was applied to the average concentration-time data for each part of each experiment. In addition to this, all the concentration-time results for experiment 1 and experiment 2 were combined and averaged according to time, and the results used as the basis for a two-compartment model for diminazene after intramuscular injection in cattle.

RESULTS AND DISCUSSION

Bioequivalence analysis

The C_{max}, T_{max} and AUC_{0-336} for individual animals are summarized in Table 1, together with the sequence of formulation administration. These values were used to test for bioequivalence. Mean plasma diminazene concentrations of Berenil and Veriben for experiment 1 over time are shown in Fig. 1. The inset is a magnification of the first 24-h period on a more appropriate time scale.

Analysis of variance (ANOVA)

The primary purpose of ANOVA is to identify and isolate those sources of variability that are not of interest, thereby allowing more precise estimation of the factors of primary interest (Metzler 1989). The ANOVA approach requires a complete data set if period effects are to be evaluated (Pabst & Jaeger 1990). Since it is recommended that period effects be evaluated (Pabst & Jaeger 1990), animal 845 was excluded from the data set to enable the carrying out of a balanced design analysis. It is further recommended that logarithmic transformation of bioequivalence data be carried out, as such manipulation gives a better representation of the true nature of events in cross-over studies (Pabst & Jaeger 1990). For this reason only log-normal (ln) transformed data were used for the ANOVA and succeeding bioequivalence tests. The end results of the ANOVA carried out on C_{max}, AUC and T_{max} are shown in Table 2. The results for formula, period and subjects are expressed as the probability that the F-test value will be greater than the value determined by ANOVA. They show that any variation that may have occurred between groups as a result of formulation, period or subject, could not be proved at the 0,05 % significance level.

The ANOVA also provides a means of calculating confidence intervals for formulation means. These are

TABLE 1 Pharmacokinetic results of experiment 1 used for the testing of bioequivalence

No.	Seq.	Veriben (T)			Berenil (R)		
		C_{max} (μg/mℓ)	T_{max} (h)	AUC_{0-336} (μg·h/mℓ)	C_{max} (μg/mℓ)	T_{max} (h)	AUC_{0-336} (μg·h/mℓ)
850	RT	6,90	1,00	468,44	4,26	0,75	485,44
854	RT	2,29	0,75	480,84	5,52	0,50	308,46
857	RT	3,61	1,00	289,63	6,77	0,50	321,22
844	RT	2,97	1,00	420,13	2,44	24,00	314,62
880	RT	6,35	1,00	396,23	6,07	0,50	420,34
855	TR	2,13	48,00	425,62	2,75	0,75	370,87
851	TR	6,56	0,50	387,47	1,60	24,00	398,77
849	TR	3,26	0,50	429,11	3,23	1,50	427,70
856	TR	3,12	0,75	354,33	3,21	24,00	425,25
845	–	–	–	–	3,05	6,00	446,00
Mean		4,13	6,05	405,76	3,89	8,25	391,87
SD		1,91	15,70	55,04	1,70	10,99	57,71

TABLE 2 Estimated sources of variation for log-normal transformed data, after ANOVA (α = 0,05), expressed as a probability

Parameter	Formula		Periods		Subjects		MSE
	F	Probability	F	Probabilty	F	Probability	
C_{max}	0,082	0,782	0,991	0,353	0,963	0,527	0,218
AUC_{0-336}	0,428	0,543	0,829	0,393	1,409	0,332	0,021
T_{max}	0,497	0,504	0,216	0,656	0,508	0,819	0,714

F = F-test value

MSE = Mean square error

based on the mean square of error (MSE) (Steinijans & Dilletti 1983) (Table 2), i.e. the ratio of the residual (error) sum of squares (SSE) divided by the error degrees of freedom (DFE). However, if period effects are evaluated, as was the case here, then both the numerator (SSE) and denominator (DFE) of the quotient are reduced, and no accurate prediction of confidence intervals is possible, because one cannot ascertain whether the MSE was increased or decreased (Pabst & Jaeger 1990).

Point estimates

Point estimates were calculated for AUC, C_{max} and T_{max} using the ratio of the geometric means of the test product, Veriben, over the reference product, Berenil. The results were:

C_{max} : 104 %

AUC : 105 %

T_{max} : 55 %

The point estimates reflect the direction in which the sample difference or ratio has been found, and in this case show Veriben to have a marginally higher C_{max} and AUC than Berenil, but a greatly decreased T_{max}.

The 90 % conventional t-confidence intervals

The conventional 90 % confidence interval for the expected difference $(\mu_2 - \mu_1)$, is symmetrical about the estimated mean difference $(\bar{x}_2 - \bar{x}_1)$ and not symmetrical about zero (Schuirmann 1987). Similarly, the conventional 90 % confidence interval for the bioequivalence ratio is symmetrical about the point estimator and not unity (Schuirmann 1987).

The 90 % conventional t-confidence intervals for the ratio of the means at the 10 % level of significance were estimated to be:

C_{max} : $70\% \leq \mu_t/\mu_r \leq 162\%$

AUC : $92\% \leq \mu_t/\mu_r \leq 119\%$

T_{max} : $9\% \leq \mu_t/\mu_r \leq 297\%$

From these results it can be seen that the point estimates lie within the estimated confidence intervals.

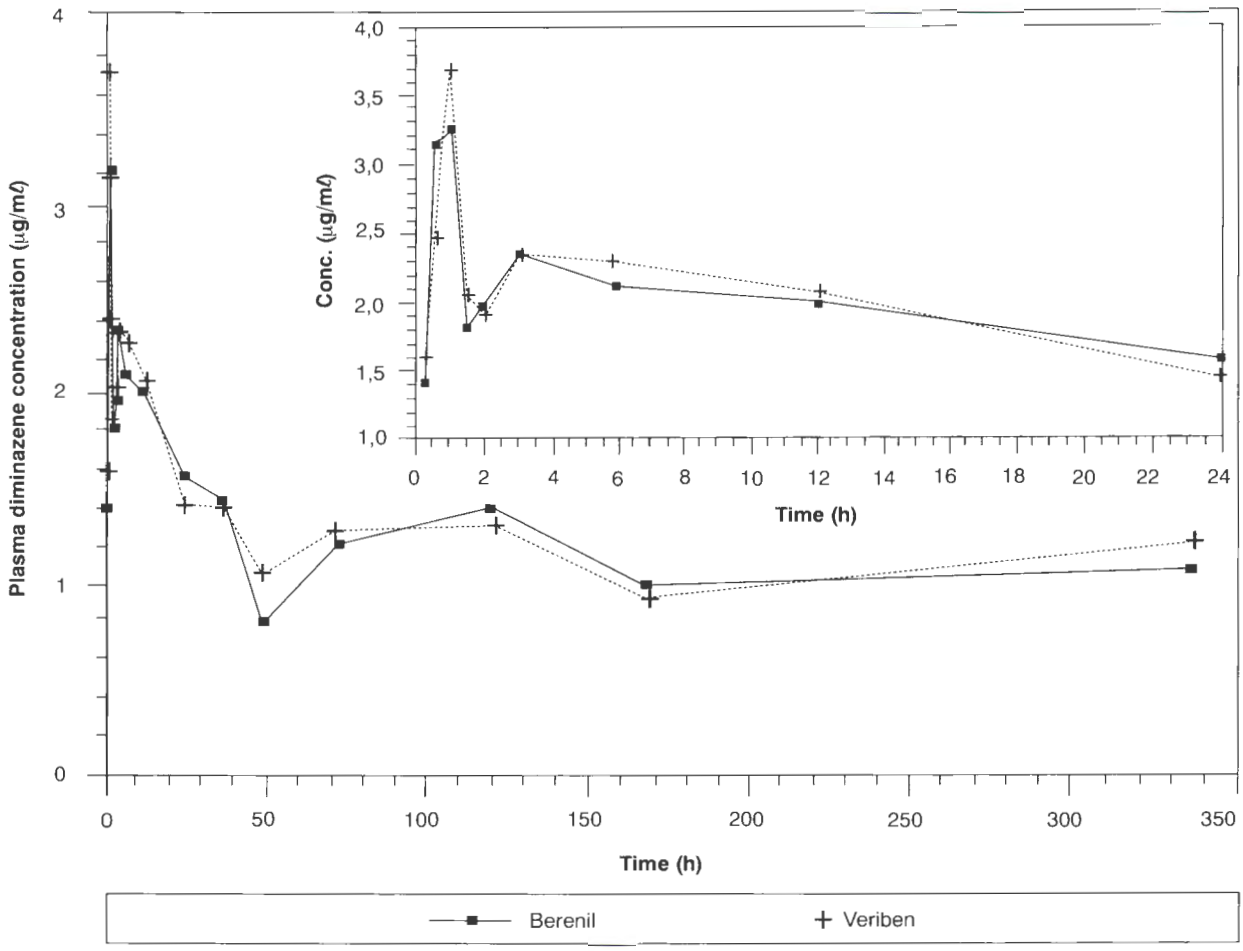

FIG. 1 Mean plasma diminazene concentrations of Berenil and Veriben for experiment 1 over time

Based on a permissible difference of ± 20 % between the true product means, a satisfactory confidence interval for testing bioequivalence in the case of the t-test, would be ± 20 % of the point estimate, i.e.:

C_{max} : 84–124 %
AUC : 85–125 %
T_{max} : 35–75 %

Therefore, the criteria for bioequivalence can be met only for AUC, and the products are not bioequivalent with respect to C_{max} and T_{max}. The implications of this will be discussed later. The fact that the conventional confidence intervals are not symmetrical about zero or unity is one of the major criticisms of the use of this method for bioequivalence assessment (Pabst & Jaeger 1990). However, conventional confidence intervals, like point estimators, help to reflect the direction in which the sample difference or ratio has been found (Pabst & Jaeger 1990).

The 95 % symmetrical confidence intervals of Westlake

With particular reference to comparative bioavailability trials, Westlake (1972, 1976, 1979), shifted the emphasis from estimation to decision making. Usually, if the 95 % confidence limits fall within acceptable limits, as recommended by a regulatory agency, the test formulation will be accepted as being bioequivalent and, if not, it will be rejected. As acceptable limits are given in a symmetrical form, say 0,8–1,2, the use of a confidence interval symmetrical about zero for differences, or unity for ratios, was proposed by Westlake (1976) and the conventional 95 % confidence interval was modified accordingly. It has subsequently been pointed out, however, that if data have been analyzed geometrically, i.e. after logarithmic transformation, then limits of acceptance (such as 80 %, 120 %) would lead to an asymmetrical decision scheme if Westlake's method is used (Metzler 1989). To compensate for the multiplication character

of the log-normal distribution, it is recommended that, in evaluating confidence intervals derived after logarithmic transformation, seemingly asymmetrical limits of acceptance should be applied (i.e. 80 %, 125 %) to Westlake's method (Pabst & Jaeger 1990; Steinijans & Dilletti 1983).

The confidence intervals (expressed as a %) obtained when the data was evaluated according to Westlake's method were:

C_{max} : (38 %, 162 %)
AUC : (81 %, 119 %)
T_{max} : (0 %, 200 %)

Hence, only AUC fell within the accepted limit of (80 %, 125 %) for bioequivalence, while the products could not be considered equivalent in terms of C_{max} and T_{max}.

The two one-sided procedure

The two one-sided tests procedure (Schuirmann 1987), as the name implies, consists of decomposing the interval hypotheses H_0 and H_1 into two sets of one-sided hypotheses as follows:

H_{01} : $\mu_t/\mu_r \leq 0{,}8$ $\quad$ H_{02} : $\mu_t/\mu_r \geq 1{,}25$
H_{11} : $\mu_t/\mu_r > 0{,}8$ $\quad$ H_{12} : $\mu_t/\mu_r < 1{,}25$

The two one-sided tests procedure consists of rejecting the interval hypothesis H_{01} and thus concluding equivalence of μ_t and μ_r, if both H_{01} and H_{02} are rejected at a nominal level of significance α which, in this case, was chosen to be 0,05. The logic underlying the two one-sided tests procedure is that if one may conclude that $0{,}8 < \mu_t/\mu_r$, and may also conclude that $\mu_t/\mu_r < 1{,}25$, then it has in effect been concluded that $0{,}8 < \mu_t/\mu_r < 1{,}25$.

This procedure, therefore, turns out to be operationally identical to the procedure of declaring bioequivalence if the 90 % conventional confidence interval for μ_t/μ_r x 100 % is completely contained in the bioequivalence range 80–120 %. This decision rule ensures that the probability of erroneously accepting bioequivalence is, at most, 5 %.

The results of the two one-sided tests procedure were as follows:

C_{max} : Prob. $\mu_t/\mu_r < 80\%$ = 0,118
Prob. $\mu_t/\mu_r > 125\%$ = 0,247

AUC : Prob. $\mu_t/\mu_r < 80\%$ = 0,003
Prob. $\mu_t/\mu_r > 125\%$ = 0,017

T_{max} : Prob. $\mu_t/\mu_r < 80\%$ = 0,671
Prob. $\mu_t/\mu_r > 125\%$ = 0,187

When the hypotheses H_{01} and H_{02} are tested at a significance level of 0,05, it is apparent that only AUC complies with the decision rule for bioequivalence and the products cannot be considered equivalent for C_{max} and T_{max}. These findings therefore support the findings of the t-test and symmetrical confidence intervals of Westlake.

The power approach

The power of the test, i.e. the probability of detecting a deviation of 20 % in the mean of the reference product, should this difference really exist, was calculated and compared with a desired power of 0,80. Although there are certain pitfalls in this method if it is used as a tool for testing bioequivalence (Schuirmann 1987), it provides a rough estimate of the sensitivity of the inference statistics used in the analysis of the data.

The results of the power test were as follows:

C_{max} = 0,14
AUC = 0,80
T_{max} = 0,48

Hence only AUC meets the necessary requirements for a desired power of 0,80.

The Hauck and Anderson test

Anderson & Hauck (1983) and Hauck & Anderson (1984), calculated P-values that could be interpreted as the largest confidence level for which the confidence interval could still be contained within the region of acceptance (80–125 %). This translates into, the probability of falsely rejecting the null hypothesis that the products are not bioequivalent based on a (symmetrical) bioequivalence range of 80–125 %.

The Hauck and Anderson P-values for experiment 1 data were as follows:

C_{max} = 0,18
AUC = 0,01
T_{max} = 0,48

The condition used for rejecting the H_0 in favour of equivalence was $P \leq \alpha$ (0,05). Hence only AUC meets the conditions for rejecting H_0 in favour of equivalence.

Non-parametric procedures

For data which do not follow normal distribution even after some transformation, a non-parametric evaluation may be indicated. Parameters like T_{max}, that, by their very nature, cannot be normally or log-normally distributed, also have to be evaluated by non-parametric methods. The poor sensitivity of the non-parametric tests procedures must, however, be noted, especially when applied to small numbers of cases, and for this reason non-parametric tests were applied only to experiment 1 data.

With non-parametric procedures, if the design is unbalanced, there is no way to correct this imbalance (Pabst & Jaeger 1990), and for this reason, animal 845 was not included in the non-parametric tests.

Because some of the assumptions of the ANOVA model, such as additivity of period, subject and formulation effects, or homogeneity of variances for subjects and residuals, respectively, are neither obvious nor easily verifiable, a non-parametric method to obtain a confidence interval under less restrictive assumptions, was chosen. Such a method is the Wilcoxon signed rank test (Tukey) which is the non-parametric analogue of the paired t-test.

The results of the Wilcoxon method (α = 0,10) with the use of logarithmically transformed ratios were as follows:

C_{max} : 71–148 %
AUC : 73–121 %
T_{max} : 12–200 %

These confidence intervals therefore do not fall within the 80–120 % range necessary for bioequivalence, implying that on the basis of the Wilcoxon non-parametric analysis, the products are not bioequivalent. To verify this finding, a second non-parametric test was carried out, the Mann-Whitney procedure, which is a similar non-parametric test designed specifically to compare the means of two independent random samples, and takes a possible period effect into consideration. The assumption is that the distribution of the two populations are identical except for a possible shift between them. The results of the Mann-Whitney procedure at the 10 % significance level were:

C_{max} : 64–207 %
AUC : 90–124 %
T_{max} : 12–980 %

These results therefore, supported the parametric analyses procedures which showed the products to be equivalent in terms of AUC, but not with respect to C_{max} or T_{max} for experiment 1.

Discussion (bioequivalence analyses)

The results of the parametric and non-parametric tests for bioequivalence, in general, show Veriben to be "bioequivalent" to Berenil with respect to AUC, but not in terms of C_{max} or T_{max}. The failure to show equivalence with T_{max} can be explained by the marked variation between animals with respect to T_{max} for both Veriben and Berenil (Table 1). This variation obviously skews the distribution making the mean an unreliable measure of central tendency and hence an inaccurate way of comparing the two sample groups. In this case the median is probably the best measure of the central value and is calculated to occur at 1 h for Veriben and at 0,63 h for Berenil, leaving a 22 min difference between the products, which is much closer than the 2,2 h difference shown between the means of the two products (Table 1). It is unlikely that a 22 min difference in T_{max} would have any bearing on the efficacy of the drug. In view of the long elimination half-life of diminazene, it was felt that the difference in $T_{max's}$ could, with good reason, be considered close enough for Veriben to still be regarded as bioequivalent to Berenil, provided AUC and C_{max} met the bioequivalence criteria. Since C_{max} failed to meet the bioequivalence criteria the products are, strictly speaking not bioequivalent, but this does not necessarily mean that Veriben is an inferior product to Berenil. The significance of C_{max} is that the magnitude of the drug levels is determined, thus, side effects due to excessively high levels (rapid influx) or therapy failure due to subminimal activity levels may be recognized even when no differences can be detected in the AUC values. The data shows Veriben to have a higher C_{max} than Berenil and therefore therapy failure should not occur with Veriben. The relative difference in magnitude between the two C_{max} is expressed in the point estimate which is 104 %. This value is not large, as reflected by a point estimate of 105 % for AUC, and therefore it is unlikely that Veriben will result in overdosage of diminazene, particularly since the drug has a fairly wide margin of safety (Bauer 1967). Hence it can be concluded, on the basis of experiment 1 data, that although Veriben is not in all respects bioequivalent to Berenil, it is unlikely that Veriben will be less effective than Berenil from a therapeutic point of view.

Pharmacokinetics

To obtain an estimation of the *in vivo* behaviour of diminazene, the concentration-time results of the two experiments were averaged (Table 3) and fitted to a two-compartment model (Fig. 2). The fitted model showed a good correlation between observed and estimated values (r^2 = 96,2 %). This was confirmed by the use of Akaike criteria (= 8,6) which aid in picking the model with the fewest number of parameters that best fits the data (Evans, Schentag & Jusko 1987).

The results of the two-compartment analysis, together with their standard errors, are shown in Table 4. It is estimated from the study that a peak concentration of 3,24 (± 0,16) µg diminazene per mℓ plasma is reached approximately 49,8 (± 7,6) min after intramuscular injection. This corresponds to the findings of Klatt & Hadju (1976) who reported an estimated C_{max} of 3,23 µg/mℓ and T_{max} of 30 min for cattle. The results also indicate that peak diminazene concentrations in cattle are approximately half of those reported for sheep (C_{max} = 6,71 µg/mℓ) (Aliu & Odegaard 1985). No lag time was apparent and absorption appeared to proceed as a first-order process

TABLE 3 Plasma concentrations of diminazene with time after intramuscular injection in cattle

Berenil (µg/mℓ)																
Time (h)	0,25	0,50	0,75	**1,00**	1,50	2	3	6	12	24	36	48	72	120	168	336
Experiment 1	1,40	3,16	3,19	3,25	1,83	1,98	2,35	2,12	2,01	1,58	1,44	0,80	1,22	1,41	1,00	1,08
Experiment 2	2,84	3,70	3,87	3,84	3,50	2,57	2,67	2,08	1,71	1,99	1,42	1,19	1,34	1,25	0,92	0,44
Mean ($\bar{x}_B$)	2,12	3,43	3,45	3,55	2,67	2,28	2,51	2,10	1,86	1,79	1,43	1,00	1,28	1,33	0,96	0,76
Veriben (µg/mℓ)																
Experiment 1	1,60	2,45	3,19	3,68	2,05	1,92	2,34	2,30	2,07	1,44	1,40	1,05	1,29	1,33	0,94	1,21
Experiment 2	2,50	3,18	3,23	3,87	3,20	3,12	2,91	2,12	1,71	1,48	1,32	1,16	0,90	0,75	0,63	0,42
Mean ($\bar{x}_V$)	2,05	2,82	3,21	3,78	2,63	2,52	2,63	2,21	1,89	1,46	1,36	1,11	1,10	1,04	0,79	0,82
Mean of $\bar{x}_B$ and $\bar{x}_V$	2,09	3,13	3,33	3,67	2,65	2,40	2,57	2,16	1,88	1,63	1,40	1,06	1,19	1,19	0,88	0,79
Estimated two-compartment	2,28	3,04	3,23	3,22	3,02	2,71	2,45	1,90	1,63	1,54	1,48	1,43	1,32	1,14	0,97	0,58

with a half-life ($t½_\alpha$) of 1,93 ± 0,95 h. This was almost the same as the absorption half-life previously described for cattle by Kellner, Echert & Volz (1985), but shorter than that given by Fouda (1978) who had values of 2 h and 5 h, respectively. The discrepancy in Fouda's (1978) results probably occurred because of the small sample size (n = 3) that was used in that study and of what appears to be an absence of sampling points in the first hour after treatment. The absorption half-life in cattle is therefore longer than that reported for sheep (Aliu & Odegaard 1985) and goats (Aliu, Odegaard & Signen 1984) which had values of 5,03 and 15,9 min, respectively. This longer absorption half-life probably explains the lower C_{max} seen in cattle.

On the basis of the two-compartment model (Fig. 2) it would appear that diminazene has a rapid absorption phase (K_{01} = 3,84) with an estimated $t½_{01}$ = 10,86 (± 3,3) min and a slow terminal elimination phase (K_{10} = 0,007) having an estimated $t½_{10}$ = 97,76 (± 36,7) h. Removal of drug from the central compartment is only slightly more rapid (K_{12} = 0,197) than removal of drug from the peripheral compartment (K_{21} = 0,158), which suggests that no significant accumulation of drug in a deep peripheral compartment is occurring. Closer examination of the results, however, indicates that this may not, in fact, be entirely true. If experiment 1 is considered separately from experiment 2 then it becomes apparent that during experiment 1, the rate of removal from the central compartment was approximately 2–3 times more rapid than removal of drug from the peripheral compartment, which implies that drug was in fact accumulating or being sequestered in a peripheral compartment(s) in experiment 1 animals. It

TABLE 4 Estimated pharmacokinetic parameters for diminazene

Parameter	Diminazene (SE)
Rate constant β (h^{-1})	0,00311
Intercept B (µg/mℓ)	1,65
$AUC_{0-\infty}$ (µg/mℓ/h)	535,8 (± 181,47)
Rate constant α (h^{-1})	0,357
Intercept A (µg/mℓ)	2,35
$t½_\beta$ (h)	222,14 (± 91,61)
$t½_\alpha$ (h)	1,93 (± 0,945)
T_{max} (h)	0,83 (± 0,13)
C_{max} (µg/mℓ)	3,24 (± 0,16)
Volume (ℓ/kg)	0,92 (± 0,115)
K01	3,84
K10	0,0071 (± 0,0027)
K12	0,197 (± 0,108)
K21	0,1586 (± 0,093)
$t½_{K01}$ (h)	0,181 (± 0,055)
$t½_{K10}$ (h)	97,76 (± 36,7)

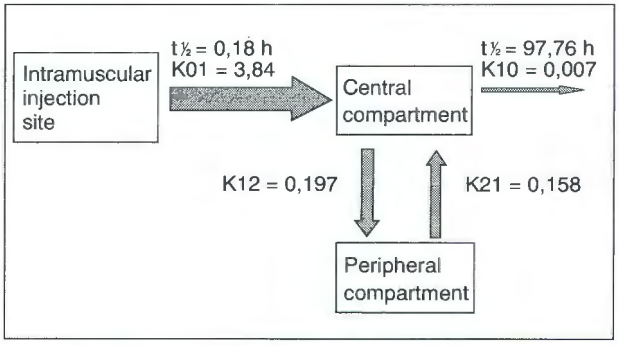

FIG. 2 Two-compartment model for i.m. injection of diminazene in cattle

could be that age or breed is influencing the behaviour of drug in the peripheral compartment, but the answer remains uncertain. If accumulation was occurring in a peripheral compartment, then this is probably related to diminazene's known affinity for proteins (Alvi, Haqqi & Hadi 1985), since the drug is reportedly 65–85 % protein bound in the case of sheep (Aliu & Odegaard 1985).

Calculation of the volume of distribution at steady state (Vd_{ss}) (Riviere, Craigmill & Sundlof 1991) allows the presentation of a proportionality constant relating serum drug concentrations to total amount of the body. Vd_{ss} is the only whole-body estimate of volume of distribution (Vd), the value of which is mathematically independent of the rate of drug elimination. With other Vd estimates (Riviere *et al.* 1991), a change in the rate of elimination, such as may occur with renal disease, will change the calculated value of Vd. Vd_{ss} is therefore probably the most reliable estimate of Vd and was calculated as:

$$Vd_{ss} = \text{Dose} \times \frac{(AUMC)}{(AUC)^2}$$
$$= 2{,}1\ \ell/kg$$

where AUMC is the area under the moment of first order curve.

Vd for cattle does not appear to have been previously published in such a way as to afford comparison. The Vd for cattle appears to be approximately 2,7 times that reported for sheep (Vd_{ss} = 0,76 ℓ/kg after i.m. injection) (Aliu & Odegaard 1985) and ten times that reported for goats (Vd_{ss} = 0,198 ℓ/kg after i.m. injection) (Aliu *et al.* 1984), which suggests that cattle may have a greater proportion of free diminazene in serum at any one time than sheep or goats. The greater proportion of free diminazene in cattle, indicates that less protein binding may be taking place in cattle which would explain the more rapid transfer of drug between peripheral and central compartments (Fig. 2). This is relevant to the efficacy of the drug since the drug is aimed at treating blood parasites.

The elimination half-life ($t\frac{1}{2}_{\beta}$) was estimated to be 222 h which was longer than the previously reported values of 68 h (Klatt & Hadju 1976) and 188 h (Kellner *et al.* 1985). This long elimination half-life is important as it provides evidence for the 2–3 week prophylactic activity reported by others (Cunningham, Harley, Van Hoeve & Okori 1964; Lumsden, Herbert & Hardy 1965) against trypanosomes. It is also important from a safety point of view, in that repetition of treatments could lead to toxicity problems, and it provides an estimated risk period during which live protozoal vaccines could be affected. If the elimination half-life is taken as 9 d, then a period of five half-lives could probably be regarded as a potential risk period for live vaccines or retreatments. Hence it could be recommended, on the basis of these findings, that animals not be vaccinated or retreated until 45 d after receiving the recommended dose of Veriben or Berenil, especially since therapeutic plasma concentrations of diminazene are uncertain.

Fractional clearance of diminazene was calculated as:

$$\frac{\text{Dose}}{AUC} = \frac{3500\ \mu g/kg}{535{,}8\ \mu g/m\ell/h}$$
$$= 6{,}53\ m\ell/h/kg$$
$$= 0{,}109\ m\ell/min/kg$$

Clearance of diminazene from cattle plasma was, therefore, found to be slower than from sheep and goats which had reported clearance values of 0,89 mℓ/min/kg and 0,624 mℓ/min/kg, respectively. This is consistent with the shorter elimination half-lives seen in sheep (9,3 h) (Aliu & Odegaard 1985) and goats (21,4 h) (Aliu *et al.* 1984), than in cattle.

The mean residence time (MRT) was calculated as:

$$\frac{AUMC}{AUC} = \frac{17\,0593{,}76}{535{,}8}$$
$$= 318{,}39\ \text{h or } 13{,}27\ \text{d}$$

This parameter has the advantage over the half-life parameters in that it takes into account all the processes which decide the fate of a drug in the body. Consequently, there are not several values for the MRT as there are for the half-life. However, the value of this parameter depends on the route of administration of drug and comparisons should be made accordingly. Comparative MRT were calculated by Aliu & Odegaard (1985) for sheep to be 14,16 ± 1,55 h, which is considerably shorter than the value calculated for cattle. This implies, therefore, that each drug molecule of diminazene remains in cattle nearly 23 times longer than in sheep.

A pre-slaughter withdrawal time was calculated by the use of the modified Nouws & Ziv (1978) formula:

$$t = \frac{(\ln R \cdot C_0 - \ln C_{lim}) \cdot t\frac{1}{2}}{\ln 2}$$

where C_0 (2,35 μg/mℓ) is the extrapolated zero-time plasma concentration; C_{lim} (0,202 μg/mℓ) is the detection limit of the assay method; and R is an accumulation factor calculated as:

$$\frac{(1{,}44 \times t\frac{1}{2}_{\beta})}{T_i}$$

where T_i is the interval between successive treatments.

If T_i is taken as 45 d, as discussed above, then

$$R = \frac{(1{,}44 \times 9{,}26\ d)}{45\ d}$$

$$= 0{,}296$$

and

$$t = \frac{(\ln 0{,}296 \cdot 2{,}35\ \mu g/m\ell - \ln 0{,}202\ \mu g/m\ell) \cdot 9{,}25\ d}{\ln 2}$$

$$= 16{,}5\ d$$

Nouws & Ziv (1978) have suggested that pre-slaughter withdrawal periods estimated from normal animals be multiplied by a safety factor of 4–5 (estimated from kidney concentrations of antibiotics in emergency-slaughtered cows). Hence a pre-slaughter withdrawal period based on their recommendation would be 66–83 d for diminazene diaceturate administered intramuscularly to cattle.

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