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Studies in rabbits on the disposition and trypanocidal activity of the anti-trypanosomal drug, diminazene aceturate (Berenil)

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1 After intramuscular injection of 3.5 mg kg^{-1} to rabbits, diminazene aceturate shows biphasic pharmacokinetics with maximum blood and interstitial fluid concentrations occurring after 15 min and 3 h respectively.

2 Seven days after treatment, 40–50% of the dose had been excreted in the urine and 8–20% in faeces.

3 Highest diminazene residues were determined in liver: 7 days after dosage, residues of 40.53 ± 4.00 $\mu g\, g^{-1}$ were present, corresponding to 35–50% of the dose.

4 The recommended dose of 3.5 mg kg^{-1} was not curative for *Trypanosoma congolense* infections of rabbit but did cause the parasitaemia to become subpatent. A limited prophylactic effect was observed.

Introduction

African trypanosomiasis, as sleeping sickness in man and ngana in cattle, is a parasitic disease of considerable economic and epidemiological importance (WHO, 1979). One of the few drugs available for chemotherapy of animal infections of the haemoflagellate parasitic protozoa *Trypanosoma brucei brucei, Trypanosoma congolense* and *Trypanosoma vivax* is diminazene aceturate (Berenil: N-1,3 diamidino-phenyl triazene diaceturate, tetrahydrate) whose trypanocidal activity was first reported by Bauer (1955a,b) and Fussganger (1955).

The trypanocidal activity of diminazene aceturate was discussed in detail by Fussganger & Bauer (1958) and the report of these workers that a single intramuscular dose of 3.5 mg kg^{-1} to cattle cured *T. congolense* and *T. vivax* infections was followed by an increase in the use of the drug for treatment of bovine trypanosomiasis (Fairclough, 1963; Williamson, 1970). The latter author summarized three reasons for the success of diminazene: (1) it has a much higher therapeutic index in cattle than any other active curative or prophylactic trypanocide; (2) it is often active against cattle infections resistant to other drugs and (3) even subcurative doses have been reported not to give rise to drug resistance (Fussganger & Bauer, 1960). Whiteside (1962) took advantage of these last two properties of diminazene to use the drug as one of a 'sanative pair' in a control regimen devised to minimize the development of drug-resistant trypanosome strains. After the success of this approach it was suggested by Fairclough (1963) that a reason for the low incidence of drug resistance following diminazene use was the rapidity with which it was metabolized and excreted. Fairclough reported no residual protection against reinfection 24 h after treatment of infected cattle with 3.5 mg kg^{-1} diminazene. This finding was in accord with those of Bauer (1958) who found that maximum diminazene concentrations in the serum of dogs treated with a 7.0 mg kg^{-1} i.m. dose occurred 1–3 h after treatment and then fell to less than 1 $\mu g\, ml^{-1}$ 16 h later. A detailed kinetic study using a chemical assay, by Raether, Hajdu, Seidenath & Damm (1972) revealed a rapid fall in blood drug levels from 11.4 $\mu g\, ml^{-1}$, 15 min after a subcutaneous 25 mg kg^{-1} dose to rats, to 0.2 $\mu g\, ml^{-1}$ 18 h later. Analysis revealed the drug to be handled according to the two compartment model with half lives of 1.4 and 7 h respectively. Rapid excretion after intramuscular injection to small animals was also reported by Fussganger & Bauer (1958). However, in contrast to these findings biological assays of trypanocidal activity in cattle blood (Van Hoeve, Cunningham & Grainge, 1964) and rabbit blood (Goodwin & Tier-

ney, 1977) suggest that some trypanocidal activity is retained for 2–3 weeks following intramuscular dosage of 7 mg kg^{-1} (cattle) or 15 mg kg^{-1} (rabbits). Furthermore, some prophylactic activity of diminazene was reported by Williamson (1957), Cunningham, Harley, Van Hoeve & Okori (1964), Lumsden, Herbert and Hardy (1965), Raether *et al.* (1972) and more recently, by Wellde & Chumo (1983).

There is little evidence available of the fate of therapeutic doses of diminazene and this paper reports pharmacokinetic data on the distribution and excretion of the drug in uninfected and *T. congolense* infected rabbits, and presents the results of investigations of the curative and prophylactic efficacy of diminazene against *T. congolense* infections in rabbits. Preliminary results of these studies have been published (Gilbert & Newton, 1982a).

Methods

Analysis of radioactivity in blood and urine

Samples (0.5 ml) of urine and plasma were chromatographed on a 30 cm × 1.5 cm column of Sephadex G-25 (fine grade – Pharmacia GB Ltd) with 0.2 M phosphate buffer (Gomori, 1955). Untreated samples of urine and plasma and samples of radioactive material eluted from the gel chromatography column were chromatographed on thin layer cellulose glass backed plates using a mixture of ethyl-acetate : glacial acetic acid : water (10 : 4 : 4 v/v).

Sample collection

Four rabbits were housed individually in metabolism cages (Aluminium Alloy, North Kent Plastic Cages Ltd) permitting the separation of urine and faeces. Diminazene aceturate (dissolved in 1 ml sterile water) was given intramuscularly at the recommended therapeutic dose of 3.5 mg kg^{-1}. Blood was obtained by puncture of an ear vein and tissues were removed immediately after the animals had been killed. Tissue fluid was obtained by the method of Calnan, Holt, Ford & Pflug (1972) in which soft plastic perforated hair curlers (length 6 cm, diameter 1 cm) were implanted subcutaneously on the flanks of New Zealand White adult male rabbits (Morton Commercial Rabbits) of weight approx. 4 kg. The operation was performed under sodium pentobarbitone anaethesia ('Sagatal', May and Baker Ltd) and at least one month was allowed for the wound to heal before rabbits were used in pharmacokinetic experiments. Blood free interstitial fluid was removed from the implanted tissue fluid cages with a 21 gauge needle attached to a 5 ml syringe. Blood and tissue fluid samples (0.3 ml each) were obtained in triplicate 0.25, 0.5, 1, 2, 3, 5, 7, 12, 15, 18, 24, 72, 120 and 168 h after injection of diminazene and all urine and faeces excreted were collected daily throughout the experiment.

Treatment of samples

Blood samples (0.3 ml) were mixed in glass vials with 1.5 ml of Soluene 350 tissue solubilizer (Packard Instruments Ltd), diluted 1/1 (v/v) with propan-2-ol and incubated for 30 min at 50°C. After cooling to room temperature, 0.5 ml hydrogen peroxide (35 vol %) was added, followed by 15 ml of acid scintillant comprising a 9 : 1 (v/v) mixture of Instagel (Packard Instruments Ltd) and 1 M HCl. Urine and tissue fluid samples (0.3 ml) were added directly to 15 ml of Instagel in glass vials.

Faeces were broken down to a fine powder with a pestle and mortar, mixed and dried to constant weight at 100°C; 10–20 mg samples of faeces were then digested in 2 ml Soluene 350 at 55°C for 2 h, after cooling, 1 ml propan-2-ol and 0.5 ml hydrogen peroxide (35 vol %) were added, and mixed thoroughly, before incubating for a further 2 h at 55°C. After cooling, 20 ml of acid scintillant was added.

Tissue samples, up to 100 mg, were digested in 2 ml Soluene 350 at 55°C for 2 h. After cooling, 1 ml propan-2-ol and 0.5 ml hydrogen peroxide (35 vol %) were added, and the mixture incubated for a further 2 h before the addition of 20 ml acid scintillant.

All samples were prepared in triplicate and radioactivity was measured in a Packard Tri-Carb 460C Liquid Scintillation Counter using external standard quench correction. Samples were counted for 100 min or until 10^4 d.p.m. had been registered.

Infection of rabbits

Rabbits were infected subcutaneously with 10^6 *Trypanosoma congolense* diluted from heavily infected mouse blood in Trypanosome Dilution Buffer (T.D.B. – aqueous solution of 0.005 M KCl, 0.08 M NaCl, 0.001 M $MgSO_4.7H_20$, 0.02 M Na_2HPO_4, 0.002 M NaH_2PO_4 and 0.02 M glucose : pH 7.7). The stock used was *T. congolense* TREU 1290, originally isolated from an ox at Zaria, Nigeria in 1967; cryopreserved stabilates were provided by Dr A.R. Gray (The Centre for Tropical Veterinary Medicine, University of Edinburgh). The parasitaemia in samples of rabbit ear blood was estimated by counting the number of trypanosomes per high power field (× 400) in a thick blood film.

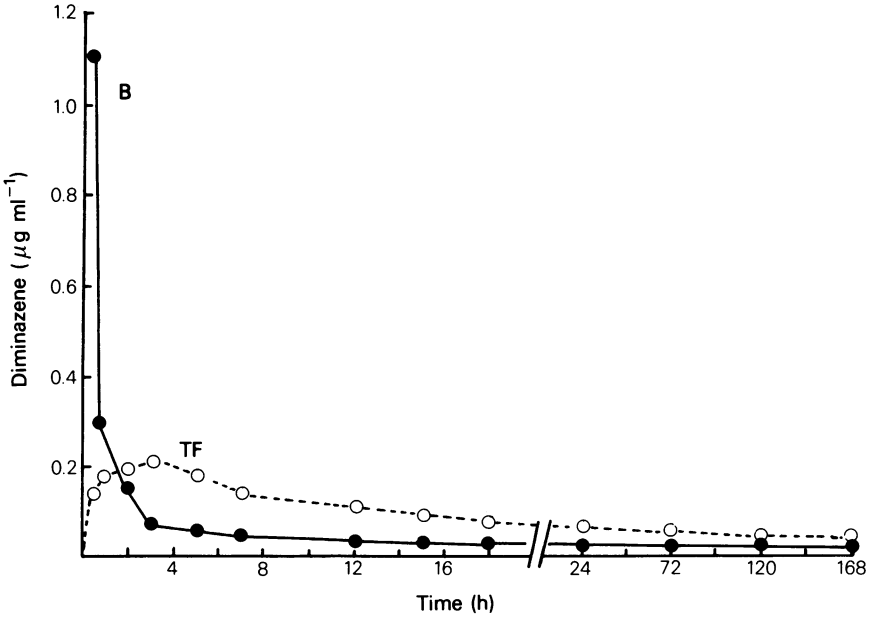

Figure 1 Levels of radioactivity in blood (B, ●) and tissue fluid (TF, ○) of rabbit after intramuscular injection of 3.5 mg kg^{-1} [^{14}C]-diminazene aceturate (specific activity 18.4 mCi g^{-1}). Radioactivity in blood was measured after addition of 0.3 ml blood to 1.5 ml Soluene 350, dilution 1:1 (v/v) with propan-2-ol and incubation for 30 min at 50°C. After cooling 0.5 ml hydrogen peroxide (35 vol %) was added, followed by 15 ml of acid scintillant. Samples of tissue fluid were added directly to scintillant. All radioactivity is assumed to be in the form of [^{14}C]-diminazene and each point represents the mean of 3 determinations. Standard deviations were in the range 4–9% of the values shown.

Calculation of pharmacokinetic parameters

Kinetic constants were calculated from the mean plasma diminazene concentrations of all four rabbits used. The two compartment pharmacokinetic model was demonstrated using a log-linear plot of drug concentration against time and least squares analysis linear regression. The biological half life was calculated from the gradient of the regressed line, elimination constants by using the formula $K_{el} = 0.693/t_{\frac{1}{2}}$ and zero time intercepts by extrapolation.

Drugs

Radioactively labelled diminazene aceturate (*bis* phenyl [U-^{14}C]-diminazene aceturate: specific activity 18.4 mCi g^{-1}) and unlabelled diminazene aceturate were generous gifts of Dr F. Bauer, Hoechst Aktiengesellschaft, West Germany.

Results

Chromatography of urine and plasma from [^{14}C]-diminazene-treated, uninfected and *T. congolense*-infected rabbits shows all the radioactivity to chromatograph in the same position as [^{14}C]-diminazene aceturate in both the gel and thin layer systems used. Acidification causes the chemical breakdown of diminazene (Williamson, 1970), but in both chromatographic systems, [^{14}C]-diminazene added to urine or plasma from untreated rabbits could be separated from the radioactive components of [^{14}C]-diminazene which had been acidified to pH 4 with HCl, suggesting that these breakdown products were not present in the rabbit samples examined.

Figure 1 shows the estimated levels of diminazene aceturate present in blood and tissue fluid from a drug-treated, uninfected rabbit. Maximum blood levels were attained 15 min after dosage and were followed by a rapid fall to less than 0.1 μg diminazene ml^{-1} after 7 h. A log-linear plot indicated biphasic kinetics consistent with drug distribution according to the two compartment model (Teorell, 1937; Kruger-Thiemer, 1977). Interstitial fluid drug levels reached a maximum after 3 h and thereafter decreased steadily. Table 1 shows the values of the kinetic parameters calculated from this data.

Measurement of radioactivity in urine (Figure 2) indicated an almost uniform rate of renal excretion over 7 days following diminazene administration, ac-

Table 1 [^{14}C]-diminazene plasma levels, half life, rate constants and theoretical zero time plasma concentrations following 3.5 mg kg^{-1} (i.m.) dosage of rabbits

Time after dosage (h)	*Plasma diminazene concentration* (ng ml^{-1})	*Elimination constant* (k_2 h^{-1})	*Biological half life* ($t_{\frac{1}{2}}$; h)	*Theoretical zero time plasma concentration* (ng ml^{-1})
.25	1,299 ± 131			
.5	761 ± 92			
1	339 ± 44	0.55	1.3	1,194
3	116 ± 2	±0.10	±0.1	
5	83 ± 1			
12	53 ± 9			
24	44 ± 7			
48	40 ± 2	0.0067	103	532
72	30 ± 3	±0.0008	±4	
96	28 ± 2			

Plasma concentrations expressed as mean ± s.e.mean ($n = 4$); kinetic parameters calculated from mean data from all animals.

counting for 40–50% of the administered dose. Faecal excretion occurred more slowly: only 8–20% of the dose was excreted by this route.

Determination of ^{14}C present in rabbit tissues 7 days after diminazene treatment showed that 35–50% of the dose was present in the liver. (Table 2).

Although radioactivity significantly above background was detected in all tissues examined, this corresponded to diminazene residues of not greater than 1 μg g^{-1} tissue and probably represents less than 1% of the dose.

The effect of diminazene treatment on the parasitaemia of a *T. congelense* infected rabbit is shown in Figure 3. The 3.5 mg kg^{-1} dose, administered intramuscularly, caused the parasitaemia to become subpatent after 24 h but subinoculation of rabbit blood to mice subsequently gave rise to patent infections, which were always cured by a 3.5 mg kg^{-1} i.m. diminazene dose. The prophylactic efficacy of diminazene against inocula of approx. 10^6 *T. congolense* to rabbits is summarized in Table 3: absolute protection against this large challenge was only demonstrated up to 24 h after diminazene treatment.

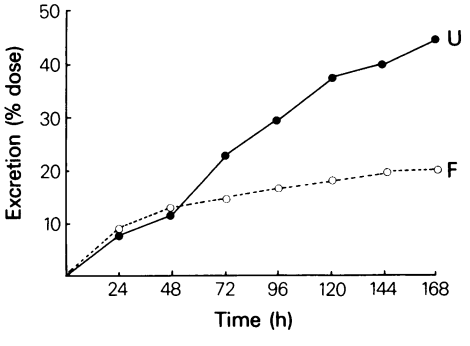

Figure 2 Excretion of radioactivity by rabbits after intramuscular injection of 3.5 mg kg^{-1} [^{14}C]-diminazene aceturate (specific activity 18.4 mCi g^{-1}). Levels of radioactivity were measured after addition of 0.3 ml urine to 15 ml Instagel scintillant or addition of 10–20 mg of faeces to 2 ml Soluene 350 and digestion at 55°C for 2 h, followed by cooling, addition of propan-2-ol (1 ml) and hydrogen peroxide (0.5 ml: 35 vol%) and further incubation for 2 h at 55°C, before addition of 20 ml of acid scintillant. Urinary (U, ●) and faecal excretion (F, ○) are shown, each point represents the mean of 3 determinations and standard deviations were in the range of 3–10% of the values shown.

Table 2 Radioactivity residual in rabbit tissues 7 days after intramuscular dosage of 3.5 mg kg^{-1} [^{14}C]-diminazene aceturate

Tissue	*Residue* (μg g^{-1} wet weight)
Liver	40.53 ± 4.00
Kidney	3.27 ± .31
Lung	1.95 ± .24
Spleen	1.79 ± 0.35
Heart	0.60 ± 0.13
Brain	2.45 ± 0.07
Fat	0.76 ± 0.05
Testis	1.37 ± 0.34
Cornea	1.27 ± 0.16
Ileum	0.73 ± 0.09
Skeletal muscle	2.06 ± .26
Skin	0.37 ± 0.07

Results expressed as μg diminazene g^{-1} wet weight of tissue, ± s.e.mean ($n = 4$), assuming all radioactivity to be present as unchanged drug.

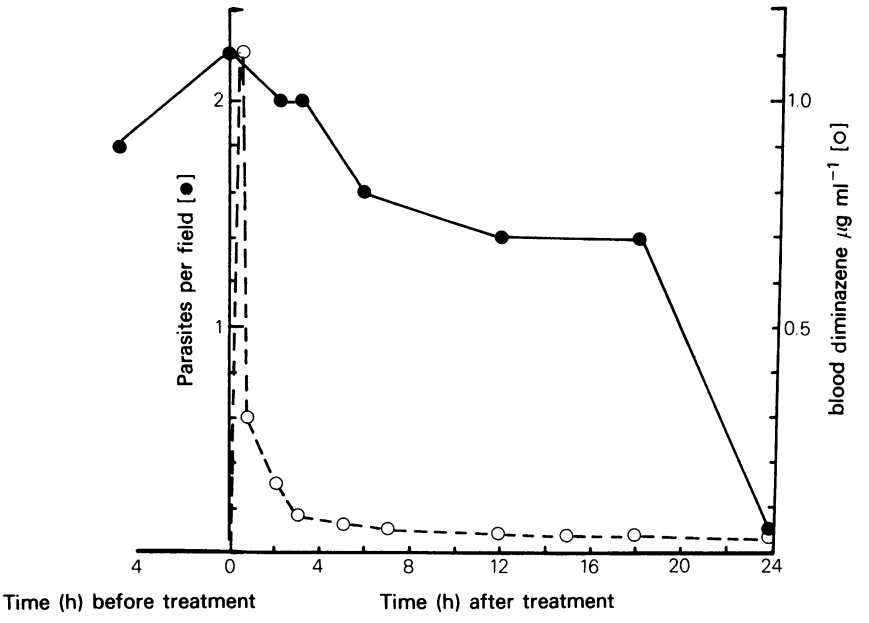

Figure 3 The effect of an intramuscular dose of 3.5 mg kg^{-1} [^{14}C]-diminazene aceturate on the parasitaemia of a *Trypanosoma congolense* infected rabbit. Blood levels of diminazene (○) were determined as for Figure 1 and the parasitaemia (●) determined by microscopic examination after subcutaneous inoculation of 10^6 parasites, and expressed as the number of trypanosomes per high power microscope field (× 400) in a thick blood film. Rabbits were treated with diminazene 6–10 days after infection and untreated, control, *T. congolense* infections remained patent throughout the experiment. Presented are typical data from a set of 4 experiments.

Thereafter the establishment of a patent parasitaemia was delayed by diminazene but not abolished.

Discussion

The biphasic kinetics of diminazene in the blood of treated, uninfected and *T. congolense* infected rabbits is in agreement with the findings of Raether *et al.* (1972) whose investigations were made using *T. rhodesiense* infected rats. Maximum diminazene levels detected in blood after a 3.5 mg kg^{-1} i.m. dose were 0.65–1.48 μg ml^{-1} which is lower than those detected by Raether and co-workers who used much higher, non-therapeutic, doses of 25–100 mg kg^{-1} administered subcutaneously.

Peak diminazene levels detected in tissue fluid were much lower than those found in blood, and distribution equilibrium was not reached. This is consistent with the possibility that interstitial fluid

Table 3 Prophylactic action of diminazene aceturate against *T. congolense*

Time of diminazene treatment (days)	*Time of inoculation with 10^6 parasites*	*Time of detection of parasitaemia* (days)	
		Subpatent	Patent
—	0	3	9
0	6 h	No parasites	detected
0	24 h	No parasites	detected
0	2 days	3	12
0	3 days	3	12
0	5 days	3	9

Rabbits were inoculated i.p. with 10^6 *T. congolense* at the times indicated after diminazene treatment. The presence of a patent parasitaemia was detected by microscopical examination of 10 high power fields (× 400) of a thick blood film taken from an ear capillary. Subpatent parasitaemias were detected by subinoculation of rabbit blood (0.3 ml) to mice.

sampled forms part of the second, peripheral compartment, comprising less well perfused tissues, as suggested by Riegelman, Loo & Rowland (1968).

The half life for diminazene in the first compartment of the two compartment kinetic model (1.3 h, Table 1) is close to that reported in rats by Raether *et al.* (1972); that for the second compartment, is however much longer than in rats. Whether this long half life of 103 h maintains blood diminazene levels high enough for a prophylactic action against *T. congolense* challenge is unknown. The concentration of diminazene required for trypanocidal activity in body fluids is unknown but measurements by Goodwin & Tierney (1977) of the dilution of a known concentration of the drug required to kill *T. brucei* in culture medium at 35°C indicated that 2 ng diminazene ml^{-1} was trypanocidal in a 24 h incubation. This concentration was reduced to 0.25 pg ml^{-1} when trypanosomes were exposed for 48 h. Primary concentrations detected throughout the current work were considerably higher than these values in both blood and tissue fluid.

The rate of excretion of 40–50% of the intramuscular diminazene dose in urine and 8–20% in faeces appeared not to be a direct reflection of the blood diminazene concentration and appreciable quantities of the injected dose had been sequestered in liver 8 days after dosage. This liver residue may indicate potential for prophylactic action of diminazene such as that demonstrated recently by Wellde & Chumo (1983). These workers showed diminazene to have a prophylactic effect against 10^5 *T. congolense* inoculated up to 12 days after treatment of cattle with i.m. doses of 7.0 mg kg^{-1}.

In the current work, the recommended dose of 3.5 mg diminazene aceturate per kg body weight was not curative for the *T. congolense* stock used. The fact that a 3.5 mg kg^{-1} dose of diminazene was curative for mice, which had been inoculated with blood from diminazene-treated rabbits, indicates that this failure was not due to drug resistance. Amongst factors which could be responsible for this reported difference in diminazene efficacy in rabbits and in the field is the possibility of species differences in rate of elimination or degree of protein binding. Other, non-pharmacokinetic factors which could explain this apparent discrepancy are differences in trypanosome strain and inoculum size, and the fact that many field reports have relied on microscopical examination of blood films for the detection of parasites, a method less sensitive than subinoculation of test blood to mice.

It is possible that the trypanocidal concentration of diminazene for *T. congolense* in rabbits is higher than that achieved in the second less well perfused compartment of the two compartment kinetic model. The zero time intercept for the second pharmacokinetic phase gives an apparent initial concentration of 0.532 μg diminazene ml^{-1} (Table 1) assuming instantaneous distribution of the drug through both compartments. Although this is considerably higher than the concentration of diminazene shown by Goodwin & Tierney (1977) to be trypanocidal *in vitro*, this amount of drug would only be present *in vivo* for a very short time and the period of contact between drug and parasite is likely to be very important in the expression of trypanocidal efficacy.

Similar kinetic factors would influence the prophylactic efficacy of diminazene and the development of different dosage formulations might allow this activity to be extended. The inoculum used in this work is considerably larger than that to which any animal is likely to be exposed in the field and a prophylactic action of longer duration might be present against a smaller challenge. It is also likely that the prophylactic action of diminazene, in the field, will be enhanced by the host immune response. The trypanocidal activity in blood and tissue fluid from diminazene-treated rabbits was shown by Goodwin & Tierney (1977) to be greater and longer lasting in *T. brucei* infected rabbits than in uninfected rabbits.

These results suggest that the use of depot or slow release formulations of diminazene could be of advantage in the use of this trypanocide in the field. Similar experiments with the antitrypanosomal drug ethidium bromide (Gilbert & Newton, 1982b) revealed great similarities between the pharmacokinetics of this drug in rabbits and Friesian heifers and in the light of the current work it is important that the pharmacokinetics and efficacy of diminazene be established under field conditions.

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