THE PHARMACOKINETICS OF DIMINAZENE IN DOMESTIC RUMINANTS AND THE PHENOTYPIC BASIS OF RESISTANCE TO DIMINAZENE IN *TRYPANOSOMA CONGOLENSE* INFECTIONS IN GOATS

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DECLARATION

I certify that all the experimental studies comprising the ten main chapters of this dissertation were carried out at the International Laboratory for Research on Animal Diseases (ILRAD), Nairobi, Kenya under the supervision of Dr. A.S. Peregrine of ILRAD and Prof. Y.O. Aliu of the Ahmadu Bello University, Zaria, Nigeria. This dissertation has not been previously submitted to any institution for the award of a certificate, diploma or degree of any sort.

CERTIFICATION

This dissertation entitled "**The pharmacokinetics of diminazene in domestic ruminants, and the phenotypic basis of resistance to diminazene in *Trypanosoma congolense* infections in goats**", by Mohammed Mamman meets the regulations governing the award of degree of **Doctor of Philosophy** of Ahmadu Bello University (ABU) and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

While an effective antitrypanosomal vaccine awaits development, tsetse control and efficient use of the rather limited repertoire of antitrypanosomal drugs have remained the only practical methods available for limiting the severe losses due to animal trypanosomiasis in sub-Saharan Africa. In order to maximise the therapeutic efficacy of the trypanocidal drug diminazene aceturate (Berenil®) against the disease, a series of experiments were carried out to properly characterise the pharmacokinetics of the drug in noninfected and trypanosome-infected cattle, goats and mice. Studies were also carried out to investigate the mechanisms of resistance of *Trypanosoma congolense* to diminazene *in vivo*.

In noninfected Boran *(Bos indicus)* cattle administered diminazene aceturate as a single intramuscular dose, 3.5 mg/kg body weight, the pharmacokinetics of the drug were characterised by rapid absorption *(K_a* = 2.56-11.60 h-^{1}), triphasic disposition, extensive systemic distribution *($V_{d(ss)}$* - 1.20-2.02 L/kg), long elimination half-life *($t_{1/2}X_z$* = 86.50-145.48 h) and slow total body clearance *(CI* - 1.24-1.70 ml/min/kg). Approximately 8.3% of the total administered dose was eliminated via the urine over the first 24 h of the treatment.

Absorption and disposition of diminazene in noninfected cattle were similar to those determined in cattle that were treated during the chronic stage of infection with a diminazene-sensitive *T. congolense* population. However, when the drug was administered to the cattle during the acute phase of an infection with a diminazene-sensitive *T. congolense* population, significant differences in the disposition were observed. In particular, absorption of the drug was more rapid, and a higher peak plasma concentration was attained but with lower systemic distribution. At 48 h of treatment, the mean concentrations of diminazene maintained in the plasma of the cattle were essentially the same being 0.43 ± 0.07 µg/ml in the noninfected state, 0.43 ±0.11 µg/ml during the acute phase of infection, and 0.44 ± 0.09 µg/ml in chronic

infection. Thus, the plasma concentration-versus-time profiles of diminazene in noninfected cattle were not significantly influenced by concurrent acute or chronic trypanosome infections. The data would appear to suggest that adjusting the dose of diminazene aceturate when treating acute or chronic trypanosome infections with diminazene-sensitive populations may not be necessary. However, a modified dosage regimen is required in the treatment of infections with trypanosome populations that are resistant to the recommended single doses of diminazene aceturate.

Based on the pharmacokinetic data obtained from the above studies, therefore, a two-dose regimen of diminazene aceturate with repeat treatments at 8 h or 24 h was designed and its efficacy tested against experimental diminazene-resistant trypanosome infections in goats. With the double treatment, the drug showed a higher plasma concentration-time profile and prolonged elimination half-life. The two-dose regimen also proved more effective than the single-dose treatment. However, while the response of drug-resistant infections to diminazene *in vivo* is dependent on the concentrations of diminazene, it is also dependent on a number of other factors. It was found that the time, after infection, at which the animals were administered diminazene aceturate significantly influenced the apparent respsonse of the parasites to the drug. The treatment was curative in all goats treated on day 1 of challenge but failed to eliminate infection in any goat when instituted after microscopic demonstration of parasites in peripheral blood on day 19 of infection.

Further experiments indicated that the ability of the parasites to reappear after treatment was not due to invasion of the central nervous system (CNS). In addition, compared to plasma, concentrations of diminazene in the cerebrospinal fluid were significantly lower and would appear unlikely to eliminate the parasites even if they were to invade the CNS.

Comparative studies on the pharmacokinetics of diminazene in the lymph and plasma were also undertaken in goats. The data suggested that trypanosomes at the level of the skin would be exposed to a greater concentration of the drug than

parasites located in the blood. However, whether or not this would explain the diminazene-sensitivity to the parasites present on day 1 of infection was not established. It was also not clear whether the efficacy of the treatment on day 1 of challenge was due to the relatively smaller number of parasites present at the level of the skin compared to that in the blood on day 19 of infection. Thus, additional studies were carried out in mice to determine the response of different parasite numbers to diminazene aceturate as well as the frequency of diminazene-resistant trypanosomes occurring *in vivo*. The results indicated that as the parasite number increased, the diminazene-sensitivity decreased. Diminazene-resistant trypanosomes occurred at an apparently low frequency in trypanosome populations which reappeared following treatment with the drug. Quite surprisingly, however, the frequency varied inversely with the parasite numbers.

In summary, the pharmacokinetic data described in the dissertation have enhanced our understanding of the disposition of diminazene in noninfected and trypanosome-infected animals. It also became apparent that, with the aid of an appropriate multiple dosage regimen, infections with diminazene-resistant trypanosome populations may be cured. The time at which treatment is commenced after initiation of a trypanosome infection as well as the number of parasites present at the time of the treatment significantly influence the apparent response of the parasites to the drug. The frequency of drug-resistant trypanosomes occurring *in vivo* is low.

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%	Percent
/	Per
<	Less than
>	Greater than
0-48 h	Value determined from zero to 48 hours
0-∞	Value determined from zero hour to infinity
™	Registered trade mark
®	Registered trade mark
A-T	Adenine-thymine
Ab-d10, Ab-d29	Relative amount of fluorescence bound to a population of parasites that were present in animals on days 10 and 29 of infection, respectively
AIC	Akaike information criterion
ANOVA	Analysis of variance
AUC	Area under the concentration-versus-time curve
AUFS	Absorbance units full scale
AUMC	Area under the concentration-time versus time curve
b.w.	Body weight
BLL	Bovine lymphocyte lysate
C_1	Zero time intercept of the rapid distribution phase
C_2	Zero time intercept of the slow distribution phase
cAMP	Cyclic adenosine monophosphate
CD_{50}	Dose of drug required to cure 50% of infected animals
Cl, CL	Apparent total body clearance
CL_r	Renal clearance
C_{max}	Maximum concentration
C_{mean}	Mean concentration

C_{min}	Minimum concentration
C_{mean}	Mean concentration
CNS	Central nervous system
Conc.	Concentration
C_p	Concentration in plasma
C_{rbc}	Concentration in red blood cell
CSF	Cerebrospinal fluid
C_{wb}	Concentration in whole blood
C_z	Zero time intercept of the terminal elimination phase
Da	Dalton units
DDT	Diethyl-dichloro-tetrachloroethane
DEAE	Diethylamino ethyl
DFMO	DL-α-difluoromethylornithine
DIC	Disseminated intravascular coagulopathy
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
F	Bioavailability
FACS	Fluorescent activated cell sorting
FAO	Food and Agriculture Organisation of the United Nations
Fig.	Figure
g.	Grams
g	Gravity (centrifugation)
G. m.	*Glossina morsitans*
G_0	Nonproliferating stage of the cell-division cycle
h	Hour
h^{-1}	Per hour

HCT	Haematocrit centrifuge technique
HPLC	High-performance liquid chromatography
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
IFAT	Indirect fluorescent antibody technique
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ILCA	International Livestock Centre for Africa
ILRAD	International Laboratory for Research on Animal Diseases.
IS	Inoculum size
iu	International units
K_{12}	Transfer rate constant from the central compartment to the shallow peripheral compartment
K_{13}	Transfer rate constant from central compartment to the deep peripheral compartment
K_{21}	Transfer rate constant from shallow peripheral compartment to the central compartment
K_{31}	Transfer rate constant from deep peripheral compartment to the central compartment
K_a	Absorption rate constant
kDNA	Kinetoplast DNA
K_{el}	Elimination rate constant from the central compartment
kg	Kilogram
kg^{-1}	Per kilogram
km^2	Square kilometer
l, L	Liter
λ_1	Exponent of the rapid distribution phase
λ_2	Exponent of the slow distribution phase
Log	Logarithm (base 10)

λ_z	The hybrid rate constant for the disappearance phase of the curve
M	Molar
µg	Micrograms
mg	Milligrams
min	Minute
µl	Microliter
ml	Milliliter
ml^{-1}	Per millilitre
mm	Millimeter
mol. wt.	Molecular weight
MRT	Mean residence time
mVAT	Metacyclic variable antigen type
n	Number
NA	Not available (or applicable)
ND	Not determined
nm	Nanometer
P	A probability value
p, pp	Page, pages
p	Proportion
PBS	Phosphate buffered saline
PCV	Packed red blood cell volume (per cent)
PSG	Phosphate saline glucose
θ	Theta
r^2	Correlation coefficient
rads	Radiation dose (roentgen units)
rbc	Red blood cell
RNA	Ribonucleic acid
rpm	Revolutions per minute

s.c.	Subcutaneous
SD	Standard deviation
SIC	Schwartz information criterion
sp., spp.	Species
T.	*Trypanosoma*
$t_{1/2}$	Half-life
$t_{1/2}K_a$	Absorption half-life
$t_{1/2}K_{el}$	Half-life for elimination from the central compartment
$t_{1/2}\lambda_1$	Half-life of the rapid distribution phase
$t_{1/2}\lambda_2$	Half-life of the slow distribution phase
$t_{1/2}\lambda_z$	Half-life of the terminal elimination phase
t_{max}	Time taken to attain maximum concentration
v/v	Volume per volume
VAT	Variable antigen type
V_c	Volume of the central compartment.
V_d	Apparent volume of distribution
VSG	Variable surface glycoprotein
V_{ss}, $V_{d(ss)}$	Apparent volume of distribution at steady state
w/v	Weight per volume
w/w	Weight per weight
WAH	World Animal Health
WHO	World Health Organization of the United Nations
WIIAD	Winrock International Institute for Agricultural Development
wt/vol	Weight per volume
°C	Degrees celsius

Chapter 1

INTRODUCTION

Over the last quarter of a century, the African continent has suffered enormous economic losses due to drought, pest infestation, livestock disease epidemics, socio-economic and political instability, and gross imbalance in world trade (FAO, 1987a). Consequently, the *per caput* food production in the continent fell by almost 20%; animal production was severely hampered, and the calories/head/day of 2299, declined to a level that was comparatively lower than in Asia, 2485; North and Central America, 3370; and Europe, 3397 (FAO, 1986; FAO, 1987b). At present, the annual animal meat intake *per caput* in Africa is 10 kg, compared to 80 kg in Europe (ILCA, 1990). Considering the present situation, therefore, the need to enhance food production in the continent cannot be over-emphasized. Several strategies have been outlined to meet this objective (FAO, 1987a; WIIAD, 1992).

In view of the economic significance of domestic animal species to the continent, one of the strategies to combat food shortage is directed at improving livestock production, particularly through the control of animal diseases which, annually, cause death of several millions of African cattle (WAH, 1991). At present, African trypanosomiasis, more than any other disease, causes the greatest hinderance to livestock development in sub-Saharan Africa (Murray and Gray, 1984; Morrison *et al.*, 1985).

In 1963 alone, it was estimated that trypanosomiasis caused an annual loss of US $5 billion in terms of animal meat production (Peregrine, 1989). In humans, at least 20,000 new cases of "sleeping sickness" are reported annually (WHO, 1986). Because of these socio-economic impacts, control of African trypanosomiasis has, therefore, been placed a matter of high priority in the mandate of the FAO (Finelle, 1980b).

1.1 Objectives of the study

The studies described in this dissertation were therefore carried out to:

1. Investigate the pharmacokinetics of diminazene in non-infected and *T. congolense*-infected animals in order to determine whether the current chemotherapeutic regimens for controlling animal trypanosomiasis can be improved.

2. Determine the mechanisms of resistance to diminazene in *T. congolense* operating *in vivo*. To this end, studies were carried out in goats to determine:

   (a) the therapeutic efficacy of two doses of diminazene aceturate against infections with *T. congolense* that are resistant to single doses of the drug;

   (b) the response of infections with diminazene-resistant *T. congolense* to diminazene aceturate at different stages of infection;

   (c) whether or not invasion of the CNS contributes to the resistance phenotype of diminazene-resistant *T. congolense*, and

   (d) if differences in the pharmacokinetics of diminazene in blood plasma, lymph and cerebrospinal fluid account for variation in the responses of diminazene-resistant *T. congolense* to diminazene aceturate at different stages of infection following tsetse challenge.

3. Estimate the *in vivo* frequency of diminazene-resistant *T. congolense* in animals.

It is hoped that the data from these studies will supplement other studies aimed at identifying the component(s) of drug resistance operating *in vivo*, thereby enabling the design of more effective control strategies for animal trypanosomiasis.

Chapter 2

REVIEW OF LITERATURE

2.1 Trypanosomes

2.1.1 Morphology

Trypanosomes are protozoan parasites which belong to the order *Kinetoplastida*, the family *Trypanosomatidae* and the genus *Trypanosoma*. Species of trypanosomes are broadly categorised into two sections, *Salivaria* and *Stercoraria*. Categorised under *Stercoraria* are genera of trypanosomes whose infective stages are reached in the posterior region of the insect vector and are transmitted to the definitive host through faecal contamination. *Salivarian* trypanosomes complete their developmental cycle in the anterior region of the vector and their infective stages are transmitted via the salivary medium by inoculation during bloodmeals. Pathogenic African trypanosomes of man and animals are *salivarian* and belong to three distinct groups, which include *Trypanosoma* (subgenus *Trypanozoon) brucei, T. (Nannomonas) congolense* and *T. (Duttonella) vivax*. Other *Salivarian* trypanosomes of veterinary importance include *T. (T.) equiperdum*, *T. (T.) evansi* and *T. (N.) simiae*. *T. (Pycnomonas) suis* is a specific pathogen of *suidae*.

Morphologically, trypanosomes are unicellular and, as bloodstream forms, consist of an elongated body with a centrally placed oval nucleus. An undulating membrane is present but its features vary with the genus: it is inconspicuous in *Nannomonas*, only slightly developed in *Duttonella*, but well developed and quite prominent in *Trypanozoon*. The organisms also possess a single locomotory flagellum which is seen distinctly at the anterior region in *Trypanozoon* and *Duttonella*, but not in *Nannomonas*. The shape, size, as well as characteristics of motility of trypanosomes, are all used for species characterisation (Vickerman, 1969).

Infectivity and growth characteristics in laboratory animals provide additional distinguishing features (Stephen, 1986). Finally, with the availability of highly sensitive and specific techniques such as isoenzyme analysis and DNA-probes, it has

become possible to further classify trypanosomes into subspecies, zymodemes and clones (WHO, 1978):

— a zymodeme is a clonally-derived group of trypanosomes with distinct enzyme characterisitcs.

— a clone is a trypanosome population which arises from a single trypanosome.

2.1.2 Distribution

The regions in which African trypanosomes occur closely reflect the distribution of tsetse fly within the continent. Tsetse flies (*Glossina* species) infest approximately 10 million km^2 of the African continent in a region which spans 37 countries and covers 40% of the sub-Sahara north of the Kalahari desert. In this region, *Glossina* species transmit human and animal trypanosomiases leading to subclinical as well as clinical diseases and, not uncommonly, death (WHO, 1986; WAH, 1991).

2.1.3 Infection

Natural infection of a tsetse fly occurs during a bloodmeal from a trypanosome-infected host. Bloodstream trypanosomes thereafter lose their surface coat in the fly and undergo successive development through the procyclic and epimastigote stages into infective, metacyclic, form. A susceptible host becomes infected when fed on by an infective tsetse fly. Tsetse flies can be infected with, and transmit, more than one trypanosome species (Clarke, 1969; Stephen, 1986).

2.1.4 Transmission

Salivarian trypanosomes are generally transmitted cyclically by blood-sucking tsetse flies. On the basis of habitat preferences, tsetse flies are broadly classified into three species; namely, *G. morsitans* **(savanna),** *G. fusca* **(forest)** and *G. palpalis* **(riverine)**. Each species comprises several subspecies (Potts, 1954; Katondo, 1984; Moloo, 1985). Significant differences exist within and between these groups with respect to their habitat and host preferences, trypanosome infection rates (Roberts and

Gray, 1972b; Baldry and Molyneux, 1980), vectorial capacity (Wilson *et al.*, 1972), and virulence of transmitted infections (Hoare, 1970).

Mechanical (non-cyclical) transmission by biting flies is a normal means by which *T. evansi* is spread between infected and susceptible hosts (Stephen, 1986). Although there is evidence that transmission of *T. vivax* occurs by non-tsetse biting flies (Hoare, 1972; cited by Stephen, 1986), the significance of this mode of transmission on the epidemiology and spread of the disease within, and beyond, tsetse-infested areas has been questioned (Wells, 1972; Leeflang, 1975). However, several workers have presented unequivocal evidence for the existence of *T. vivax* in South America and other regions outside the tsetse-infested regions of sub-Saharan Africa (Wells *et al.*, 1982a,b; Agudelo *et al.*, 1984; Wells, 1984).

2.1.5 Infectivity

Trypanosome species affect a wide spectrum of domestic and wild animals but show considerable variation in their infectivity and pathogenicity for the different host species (Hoare, 1972). The variation is due to a combination of factors which relate to the vector, parasite, host and the environment (Leach and Roberts, 1965; cited by Stephen, 1986).

Different species and subspecies of tsetse flies show considerable variation in their vectorial capacity. In cattle for instance, *G. morsitans* was shown to be more competent than *G. tachinoides* in transmitting *T. congolense* and *T. simiae*, but both vector species were found to be equally efficient in their capacity to transmit *T. vivax* (Roberts and Gray, 1972b). In addition, tsetse flies show preference for certain hosts as sources of bloodmeals, thereby influencing the prevalence of tsetse-transmitted infection in different animal species existing in the same environment (Jordan *et al.*, 1962; Leach 1973). However, the major factor determining the prevalence of trypanosome infection in animals is the level of infective tsetse-fly challenge to which animals are exposed (Mulatu *et al.*, 1991; Leak *et al.*, 1993). Therefore, changes in climatic factors, such as rainfall, which influence fly populations, may cause significant

variation in disease prevalence (Gill, 1976; Leak *et al.*, 1993). Furthermore, factors such as the mean ambient temperature (Ford and Leggate, 1961) and availability of bloodmeal sources (Leach, 1973) may also influence the trypanosome infection rate in tsetse flies (Leach and Roberts, 1981).

2.1.6 Virulence

The virulence of the diseases caused by trypanosomes may vary within and between the same parasite species. In cattle, it was shown that the same strain of *T. vivax* could lead to acute or chronic infection (Krampitz, 1970). In another investigation conducted in cattle, it was observed that morphologically longer strains of *T. congolense* produced relatively more virulent infections than shorter strains (Godfrey, 1961). It would appear therefore that differences in virulence may exist between parasites of the same species which occur in dissimilar geographical areas. This may be exemplified by *T. vivax*.

The diseases caused by *T. vivax* species in West African cattle are generally acute (Unsworth, 1953). In contrast, in East Africa, cattle infections with *T. vivax* are generally relatively mild (Hudson, 1944). The severity of disease caused by a given trypanosome species may also vary significantly with the host. For instance, *T. simiae* produces a fulminating disease in the pig; only mild clinical signs in sheep, and is non-infective to cattle (Stephen, 1970).

In addition to factors associated with the vector and parasite, susceptibility to challenge also depends on the host's immune status. For instance, animals that have developed immunity to *T. brucei* or *T. congolense* are immune to homologous challenge for at least 3 months (Cunningham, 1968; Wilson, 1971; Akol and Murray, 1983, 1985; Nantulya *et al.*, 1984). Innate resistance to the disease is also shown by some indigenous African breeds of ruminants and wild animals (ILCA, 1979; Murray *et al.*, 1982).

Clinical manifestations of trypanosomiasis are also more severe in animals on low plane of nutrition and/or with intercurrent haemoprotozoal or helminth infections

(Griffin *et al.*, 1981; Specht, 1982; Akol and Murray, 1983, 1985; Kaufmann *et al.*, 1992).

2.2 Clinical Manifestation of Trypanosomiasis

2.2.1 Clinical Signs

The major clinical signs of trypanosomiasis in domestic livestock include lethargy, fever, inappetance, anaemia, lachrymation, weight loss, decreased productivity, emaciation and death (Fiennes *et al.*, 1946). Pyrexia is a well recognized feature of trypanosomiasis and is usually of low grade and intermittent in chronic infections (Fiennes, 1970; Valli *et al.*, 1978b). However, severe fever in excess of 41.7°C is known to occur with some *T. vivax* infections in cattle (Hudson, 1944; Stephen, 1986).

Trypanosoma brucei brucei (Bruce, 1895; cited by Hoare, 1970) affects all domestic animals and causes mild to virulent infections in equidae, sheep, goats and dogs but, unlike *T. b. gambiense* and *T. b. rhodesiense*, is not infective for man (Hoare, 1970). *Trypanosoma b. brucei* causes mild disease in ruminants but severe manifestations may occur in pigs (Hoare, 1972; Stephen, 1986; Agu and Bajeh, 1987), cats (Hill, 1955; Losos and Ikede, 1972), horses and dogs (Richardson and Kendall, 1957; cited by Hoare, 1970). Corneal opacity, conjunctivitis and keratitis may be observed in dogs (Koch, 1904; Parkin, 1935), cattle (Ikede and Losos, 1972; Ilemobade and Schillhorn Van Veen, 1974) and horses (Yorke, 1911).

Trypanosoma equiperdum, the causative agent of *dourine*, a venereal disease, is a specific parasite of the *equidae* and is naturally transmitted through coitus (Hoare, 1972; Stephen, 1986). *Trypanosoma evansi* is infective to all domestic animals and causes a disease termed *surra*. The severity of the disease may, however, vary greatly for a given host depending on the virulence of the parasite strain and host-susceptibility in different geographical regions (Hoare, 1972). For instance, in Indonesia, *T. evansi* infections in horses, unlike in cattle, are acute and result in 100%

mortality (Dieleman, 1986). In India, cattle seldom show clinical signs due to *T. evansi* (Verma *et al.*, 1973).

Both *T. congolense* and *T. vivax* are primarily animal pathogens and cause moderate to severe clinical disease in a wide variety of domestic animals. Occasionally, *T. vivax* infections in cattle run a severe course which is characterised by a haemorrhagic syndrome (Hudson, 1944; Wellde *et al.*, 1983; Gardiner, 1989; Gardiner *et al.*, 1989). Natural *T. vivax* infections in the pig and dog are rare. Consequently, these animals are considered refractory to this trypanosome species (Stephen, 1986).

Trypanosoma suis is infective to the pig and, probably wild *suidae* (Stephen, 1986). *T. simiae* causes fulminating disease in pigs with high mortality rates (Hoare, 1936). While wild *suidae* such as the bush-pig and warthog are the reservoir hosts of *T. simiae*, infections with the parasite can be induced, experimentally, in sheep, goats and rabbits (Stephen, 1986).

Trypanosomiasis also affects the reproductive capacity of male and female animals (Ikede *et al.*, 1988; Edeghere *et al.*, 1992). Anoestrus was observed in *T. vivax*-infected zebu heifers (Ogwu *et al.*, 1984). When the infection was initiated during the first trimester, abortion occurred in one out of four heifers. However, when the infection was induced in the second trimester, all four heifers delivered normally. Infection during the third trimester resulted in stillbirths and prematurely-delivered calves which died soon after birth (Ogwu *et al.*, 1985; Ogwu and Njoku, 1987). Some of the observed effects would appear to be direct since transplacental transmission of *T. vivax* is known to occur in goats (Pigneur, 1932) and cattle (Ogwu *et al.*, 1985). Infection with *T. vivax* or *T. congolense* in bulls causes a severe decrease in semen volume and concentration (Sekoni *et al.*, 1988).

2.3 Pathogenesis

2.3.1 The Chancre

The pathognomonic sign of primary syphilis in man is a localized skin reaction termed the "chancre" (Stargardt, 1914). Tsetse bites have been known to cause similar lesions in cattle (Livingstone, 1857) and man (Manson, 1903, 1908). After Kleine (1909) had established that the tsetse-fly was the vector of trypanosomiasis, the chancre became clinically important as the earliest recognizable sign of the disease in man (Burtt and Fairbairn, 1945; De Raadt *et al.*, 1966).

Successful deposition of trypanosomes in mammalian skin may initially be indicated by the appearance of the chancre and, in the later stages, microscopic detection of trypanosomes in peripheral blood (Akol, 1985). The chancre could be induced in several animal species by the bite of trypanosome-infected tsetse flies (Dwinger *et al.*, 1988), or intradermal inoculation of *in vitro*-derived metacyclic trypanosomes (Gray *et al.*, 1985; Akol *et al.*, 1986).

The chancre has also been observed in tsetse-transmitted infections with *T. congolense* in cattle, sheep and goats (Roberts *et al.*, 1969; Gray and Luckins, 1980; Emery and Moloo, 1981); *T. brucei* in goats (Emery and Moloo, 1980); *T. vivax* in goats (Emery *et al.*, 1980); *T. b. rhodesiense* (Corson, 1932; Fairbairn and Godfrey, 1957) and *T. gambiense* in man (Godfrey and Killick-Kendrick, 1967).

In animals, however, manifestation of the chancre is not an essential pre-requisite for development of trypanosome infections following tsetse-transmission. For instance, cattle which were challenged with some East African stocks of *T. vivax* through the bites of infected tsetse flies developed high parasitaemia without showing local skin reactions (Dwinger *et al.*, 1986). Similarly, the chancre is absent in tsetse-transmitted trypanosome infections in the guinea-pig (Willet and Gordon, 1957), rat (Gordon and Willet, 1958) and mice (Emery and Moloo, 1980). Apparently, the thin skin in these rodent species allows direct penetration of the proboscis to the

subcutaneous tissue where the metacyclic trypanosomes are deposited (Morrison *et al.*, 1985).

Intracutaneous Stage: The series of events that occur from the time infective tsetse flies deposit metacyclic trypanosomes in the skin to the time when the parasites become detectable in the blood have been extensively investigated (Emery and Moloo, 1980, 1981; Gray and Luckins, 1980; Akol and Murray, 1982; Barry and Emery, 1984; Dwinger *et al.*, 1988, 1990). Ultrastructural studies indicate the occurrence of a distinct stage in the skin which is intermediate between metacyclic and bloodstream form trypanosomes (Gray *et al.*, 1985; Dwinger *et al.*, 1988). This distinct "extravascular" or "intracutaneous" stage has been recognised in the skin of goats infected with *T. congolense* and *T. brucei*, but not with *T. vivax* (Dwinger *et al.*, 1988).

Parasite Migration: Following a tsetse bite, trypanosomes multiply in the skin resulting in a variable number of parasites in the chancre which may be few (*T. vivax*) or large (*T. brucei*) (Otieno and Darji, 1979). The parasites then migrate away from the skin to invade the general circulation via the lymphatics draining the areas of the tsetse bites. Dwinger *et al.* (1990) observed *T. congolense* in the lymph of goats on day 4 following a tsetse bite, preceding by 2 days the appearance of a detectable skin reaction or parasitaemia. In the same animal species, the period between fly challenge and first detection of parasites in the lymph was 5-6 days for *T. vivax* (Emery *et al.*, 1980) and 2 days for *T. brucei* (Barry and Emery, 1984). In goats infected through tsetse-bite with *T. vivax* or *T. brucei*, parasitaemia was detected before the chancre reached its peak size (Emery and Moloo, 1980, 1981; Barry and Emery, 1984).

2.3.2 Anaemia

The mechanisms which surround the development of anaemia in trypanosomiasis, and the extent to which the syndrome plays a role in the pathophysiology of the disease are still poorly understood (Murray, 1979). Although

anaemia is commonly detected after microscopic demonstration of parasitaemia, it is still unclear whether the initial changes in the packed red blood cell volume percent (PCV) occur with, or after, the first peak of parasitaemia (Murray and Dexter, 1988). However, a direct relationship has been established between anaemia and severity of parasitaemia (Jennings *et al.*, 1974; Wellde *et al.*, 1974; Holmes and Mamo, 1975; Dargie *et al.*, 1979b; Murray, 1979; Saror, 1980).

During the course of a trypanosome infection, the kinetics of the anaemia may be divided into two phases (Murray and Dexter, 1988). The first of these two phases is characterised by haemolytic anaemia and varies with the degree and duration of the parasitaemia (Jennings *et al.*, 1974; Holmes and Jennings, 1976; Dargie *et al.*, 1979b). In this stage, chemotherapy is usually followed by elimination of the parasites; recovery by the animal; and re-establishment of normal haematological values. There appears to be several factors which may lead to haemolysis in trypanosomiasis. It may be due to the production of haemolytic factors such as free fatty acids and phospholipases (Tizard and Holmes, 1976). In other studies, a pronase-sensitive factor produced *in vitro* by *T. brucei* was also shown to be haemolytic (Chi *et al.*, 1975, 1976). It is also probable that trypanosomes produce neuraminidase (Esievo *et al.*, 1982) which may cleave sialic acid from the surface of erythrocytes, rendering the surface susceptible to osmotic damage followed by erythrophagocytosis. Other factors that may promote haemolysis and/or erythrophagocytosis include pyrexia (Murray, 1979), adsorption of trypanosome antigen to red blood cells (Woo and Kobayashi, 1975), and production of trypanosome-specific immunoglobulins which adhere to erythrocyte surfaces (Kobayashi *et al.*, 1976) as a prelude to removal by the reticuloendothelial system.

In cattle, the role of haemodilution in anaemia appears to be significant (Fiennes, 1954; Clarkson, 1968; Naylor, 1971; Holmes and Mamo, 1975; Anosa and Isoun, 1976; Valli *et al.*, 1978a). In contrast, results from studies conducted in cattle that were infected with *T. brucei* and *T. congolense* (Dargie *et al.*, 1979a,b) indicated

that anaemia occurred not due to haemodilution, but as a result of decreased red blood cell volume, blockage of reticuloendothelial iron release and bone marrow dysfunction.

In the second phase of anaemia, significant erythrophagocytosis proceeds even in the absence of the parasites (Murray and Dexter, 1988). The continued presence of the anaemia is, possibly, due to dyshaemopoeisis (Dargie *et al.*, 1979a; Anosa *et al.*, 1992) and persistent activity of the mononuclear phagocytic system (Jandl *et al.*, 1965). In this chronic stage of infection, animals show poor growth, body wasting, decreased productivity and death. Unlike in the first phase, the response to treatment with trypanocidal drugs at this stage of infection is usually unsatisfactory (Murray and Dexter, 1988).

2.3.3 Thrombocytopoenia

In addition to anaemia, thrombocytopoenia is a frequent finding in trypanosome-infected animals (Anosa, 1988a,b). Thrombocytopoenia has been observed in experimental infections with *T. vivax* in goats (Veenendaal and Van Miert, 1976) and *T. brucei* in cattle, goats and buffalo (Davis, 1982). Severe thrombocytopoenia also features in the haemorrhagic syndrome due to acute *T. vivax* infection in cattle (Wellde *et al.*, 1983; ILRAD, 1984; Olubayo and Mugera, 1985; Gardiner, 1989; Assoku and Gardiner, 1989). It has been shown experimentally that, regardless of the trypanosome species or the mode of infection (fly challenge or syringe inoculation), the degree of thrombocytopoenia varies directly with the level of parasitaemia (Davis, 1982) and can be reversed following treatment with diminazene aceturate (Wellde *et al.*, 1978).

The factors which lead to the development of thrombocytopenia in African trypanosomiasis are unclear (Assoku and Gardiner, 1989). However, the possible role of disseminated intravascular coagulopathy (DIC) has been suggested (Barret-Connor *et al.*, 1973; Robins-Browne *et al.*, 1975; Van Den Ingh *et al.*, 1976b; Forsberg *et al.*, 1979; Davis, 1982). DIC or DIC-like lesions have been described in

infections with *T. simiae* in pigs (Isoun, 1968; Van Dijk *et al.*, 1973) and *T. vivax* in goats (Van den Ingh *et al.*, 1976a).

Another factor that may play a role in the development of thrombocytopoenia in trypanosomiasis is the sequestration of platelets in the spleen of infected animals. The sequestration, as shown in *T. brucei*-infected rabbits, occurred only in the intact, but not splenectomised, animals (Jenkins *et al.*, 1974). However, it remains to be established whether, in addition to the rabbit, sequestration of platelets in the spleen plays a role in the thrombocytopoenia observed in trypanosome infections in other species.

2.3.4 Immune Response

The host immune response during trypanosome infection is primarily humoral and is mediated by IgM and IgG (Morrison *et al.*, 1981; Musoke *et al.*, 1986) which are produced in response to trypanosome variant surface glycoprotein (VSG) and non-VSG antigens (Flynn *et al.*, 1992; Williams *et al.*, 1992). IgM may be the most important antibody type for control of parasitaemia and protection in laboratory animals (Campbell *et al.*, 1978) and cattle (Luckins, 1976). In zebu cattle exposed to infection by natural challenge, serum levels of IgM and IgG increased 9-fold and 2-fold, respectively (Luckins, 1972). Similar increases in serum IgM concentrations were observed in *T. vivax*-infected cattle (Clarkson *et al.*, 1975).

Based on studies carried out by De Gee (1980) and Vos *et al.* (1988a,b), development of immunity in animals challenged with *T. vivax* appears to be different from that described for *T. congolense* and *T. brucei* (Cunningham, 1968; Wilson, 1971; Akol and Murray, 1983, 1985; Nantulya *et al.*, 1984). In cattle infected through tsetse challenge with *T. brucei* or *T. congolense*, treatment with diminazene aceturate administered within 3-4 weeks of infection is followed by recovery and the animals are subsequently immune to homologous challenge. But in goats infected by tsetse challenge with *T. vivax*, treatment with diminazene aceturate 3-4 weeks after the challenge did not confer immunity to homologous or heterologous challenge

regardless of whether the challenge and treatment protocol was repeated as many as three times. The different picture presented with *T. vivax* infections may be because only few metacyclic trypanosomes are extruded by *T. vivax*-infected flies, hindering inoculation of all mVATS in the serodeme. It would also appear that unlike *T. brucei* and *T. congolense*, tsetse-transmitted *T. vivax* may not revert to a constant mVAT repertoire (De Gee, 1980). Finally, the extruded metacyclic population may undergo rapid antigenic variation that may hinder expression of all the mVATs, and in sufficient numbers, to allow adequate stimulation of protective immune response.

2.3.5 Antigenic Variation

The surface coat of metacyclic and bloodstream trypanosomes comprises a glycoprotein (Vickerman, 1969) and, during infection, forms an important protective barrier for the parasite against the host's immune response. As early as 1905, it was observed that, *in vitro*, serum collected during the early phase of infection with *T. equiperdum* in a horse did not neutralise parasites isolated from the same host later in infection. With further observations indicating the ability of trypanosomes to survive and establish chronic infections in the presence of host antibodies, it became clear that trypanosomes are able to change their surface coat. These observations have since been validated in *in vivo* studies with *T. brucei* (Gray, 1970, 1972), *T. congolense* (Wilson, 1967; Uilenberg and Giret, 1972) and *T. vivax* (Clarkson and Awan, 1969; Jones and Clarkson, 1971). The phenomenon has also been shown to occur *in vitro* (Doyle *et al.*, 1980).

The ability of a subpopulation of trypanosomes to express an antigenically different surface glycoprotein is referred to as "antigenic variation" (Englund *et al.*, 1982; Turner, 1984). The subpopulation which expresses an antigenically different VSG survives the host's immune response, re-establishes parasitaemia from which a subgroup with a further different VSG arises so that there is always a residual antigenically variant trypanosome subpopulation which the host's immune response fails to eliminate, except when a variable antigenic type is re-expressed during the

course of infection (Nantulya *et al.*, 1979). Serologically, 101 variant-specific surface antigens were shown to be expressed by a clone of *T. equiperdum* (Capbern *et al.*, 1977). Furthermore, based on gene cloning and DNA hybridisation studies conducted with *T. brucei* (Van der Ploeg *et al.*, 1982), it was estimated that the number of VSG genes contained in a single trypanosome was 1000-2000.

Since genes encoding VSGs may be very similar, and the genetic repertoire is rapidly expansible, the capacity of antigenic variation would appear to be infinite (Lamont *et al.*, 1986). Antigenic variation is one of the reasons that has frustrated efforts directed at developing VSG-based anti-trypanosomal vaccines (Morrison *et al.*, 1985).

2.4 Control of Trypanosomiasis

Since a field vaccine against trypanosomiasis is not available (Morrison *et al.*, 1981), control of the disease has mainly involved the use of trypanocidal drugs, control/eradication of tsetse flies, and the use of trypanotolerant cattle.

2.4.1 Anti-trypanosomal Drugs

Anti-trypanosomal drugs used in domestic livestock include diminazene aceturate (Berenil®; Hoechst AG, Frankfurt, Germany), homidium bromide (Ethidium®; FBC Ltd., Cambridge, England), homidium chloride (Novidium®; May and Baker, Dagenham Ltd., England), isometamidium chloride (Samorin®; May & Baker, Dagenham Ltd., England), quinapyramine sulphate (Trypacide®; May & Baker, Dagenham Ltd., England), suramin (Naganol®; Bayer Co., Leverkusen, Germany) and Melarsomine (Cymelarsan®; Rhone Merieux, Lyon, France).

Curative Treatment: In cattle, sheep and goats, Berenil® is the recommended drug of choice for routine treatment of isolated trypanosome infections in animal herds. Owing to reasons of toxicity, however, the drug is contraindicated in camels (Leach, 1961). Berenil® is often used alone or in alternation with homidium or isometamidium for strategic herd treatment (Whiteside, 1958; Trail *et al.*, 1985). This

approach has been found to be quite satisfactory (Leach and Roberts, 1981) except in cases of multi-drug resistance (Gitatha, 1979; Rottcher and Schillinger, 1985; Schonefeld *et al.*, 1987; Mohamed-Ahmed *et al.*, 1992).

Chemoprophylaxis: Samorin® is the primary chemoprophylactic trypanocide currently used in bovine trypanosomiasis. Chemoprophylaxis with this drug is the recommended approach especially in endemic areas. Administered as a single intramuscular (i.m.) dose, 0.5-1.0 mg kg^{-1} body weight (b.w.), isometamidium has been reported to confer prophylaxis varying in duration between less than 1 month (Peregrine *et al.*, 1987) to 7.5 months (Wiesenhutter *et al.*, 1968) in cattle. The wide variation in the duration would appear to result from several factors including the drug dose as well as the strain and drug-sensitivity of the parasite (Kinabo and Bogan, 1988).

In trypanosomiasis of camels and horses, Trypacide® sulphate is used as a curative while Trypacide prosalt® (a mixture of quinapyramine sulphate and quinapyramine chloride) is used for chemoprophylaxis. *T. evansi* infections in camels may be also treated with a recently introduced arsenical, Cymelarsan® (Raynaud *et al.*, 1989).

2.4.2 Vector Control

Since tsetse flies are essential for the transmission and maintenance of African trypanosomiasis (Allsopp, 1984), control programmes directed against these vectors have constituted an important strategy in the eradication of the disease. In areas cleared of tsetse flies, trypanosomiasis no longer constitutes a problem (Putt *et al.*, 1980). Strategic tsetse fly control/eradication programmes as means of controlling African trypanosomiasis have employed a variety of techniques.

Hand Catching: Hand-catching, the oldest of the methods (Glasgow and Potts, 1970), has been quite successful in the control of *G. palpalis* (Austen and Hegh, 1922; cited by Glasgow and Potts, 1970) and *G. fuscipes fuscipes* (Glasgow and

Duffy, 1947). However, failure to effectively isolate areas cleared of tsetse from re-infestation have, in some cases, rendered hand-catching exercises ineffective (Symes and Vane, 1937). Although the specific insect is destroyed, this technique is relatively expensive and has largely been replaced by methods which use chemical insecticides (Glasgow and Potts, 1970).

Chemical Insecticides: The major organochlorine insecticides that have been extensively used in tsetse control programmes are DDT (diethyl-dichloro-tetrachloroethane), dieldrin and endosulfan. In practice, these compounds are sprayed on target areas either from the ground or aerially (from aircrafts). Wettable powders and solutions of these compounds are available for short-term effects and the choice of insecticide is varied depending on the climate. Because of long-term persistence (Baldry, 1963) in sprayed environments, DDT has also been used as an emulsion spray to achieve residual effects on tsetse habitat (Burnett, 1970). Of the three compounds, DDT is the most suitable in localities with long dry season, but for less extreme conditions dieldrin is the choice (Burnett, 1970).

There are several reports on the successful control of tsetse flies through the use of chemical insecticides (Allsopp, 1984), notably in Nigeria (Davies and Blasdale, 1960; Davies, 1964, 1971; Spielberger *et al.*, 1977), Zimbabwe (Robertson *et al.*, 1972; Chapman, 1976) and Botswana (Davies and Bowles, 1979). Success in areas involved in these countries has been credited to tsetse fly eradication as well as the implementation of several measures which effectively prevented tsetse fly re-infestation. In Nigeria, for instance, reclamation and human settlement in tsetse decolonized areas have so far successfully prevented fly re-infestation (Putt *et al.*, 1980; Jordan, 1987).

There are, however, problems associated with the use of chemical insecticides in the control of tsetse flies. These include tsetse fly re-invasion into areas from where they have been cleared (Hursey, 1972; Finelle, 1980a; Itard, 1980), and environmental pollution (Koeman *et al.*, 1971). To minimise the problem of

environmental pollution, use of ultra-low volume (ULV) preparations have been introduced (Spielberger *et al.*, 1977).

Traps and Screens: Use of traps (Harris, 1930) and screens (Shircore, 1916) were among the earliest methods employed in the control of tsetse flies (Burnett, 1970). Over the years, large scale control of tsetse flies through the use of traps and screens impregnated with insecticides has been successfully achieved in several areas (Ryan *et al.*, 1981; WHO, 1986; Kupper, 1987). This appears to be an important change in control strategy (Kuzoe, 1991) because, in addition to their proven efficacy, traps and screens are relatively cheap, non-polluting to the environment and simple to use by inhabitants of rural communities. However, successful field use of traps and screens requires thorough supervision to prevent theft or their destruction in case of heavy rains and/or river flooding.

Predator Parasites: Other methods of controlling tsetse flies include the use of predator parasites of *Glossina* species, release of sterilized male tsetse flies, clearance of vegetation comprising tsetse fly habitats, fire exclusion and elimination of wild reservoir hosts (Potts, 1970).

Sterile Male Technique: Male tsetse flies can be sterilised by chemicals (Chadwick, 1964) or irradiation (Potts, 1958; Tenabe *et al.*, 1986). When released into the field, such sterile males compete with normal tsetse flies for mating with female tsetse flies. With time this results in a large ratio of sterile wild to normal male tsetse flies (Dame, 1970). Within nine months of release of sterile male tsetse flies, a 98% control of *G. m. morsitans* was achieved in Zimbabwe (Dame and Schmidt, 1970). However, this method is considered very expensive (Roelants and Williams, 1982).

Integrated Control: In areas of high trypanosomiasis risk, an integrated chemoprophylactic and tsetse control strategy has proved to be the most suitable approach as was shown by Mulatu *et al.* (1991) and Leak *et al.* (1993). The

integrated approach is especially indicated for areas in which, owing to drug-resistance or very high tsetse fly challenge, use of drugs alone does not suffice but requires, additionally, reduction of the fly population to a level that decreases the disease prevalence to a minimum in the exposed animals (Mulatu *et al.*, 1991; Leak *et al.*, 1993).

2.4.3 Trypanotolerant Breeds of Ruminant Animals

The term *trypanotolerance* is ascribed to some animal breeds which are able to survive and maintain satisfactory productivity in tsetse-infested areas of moderate to high trypanosomiasis risk in which other breeds readily succumb to the disease (Foy, 1911; MacFie, 1913). In addition to wild game, the trypanotolerant trait has been observed in some indigenous African breeds of cattle (N'dama, Muturu), sheep (Djallonke) and goats (West African Dwarf) which are found mainly in West and Central parts of Africa (ILCA, 1979). In natural and experimental challenge with *T. vivax* and *T. congolense*, N'dama cattle have consistently been shown to be superior to zebu cattle in their ability to control parasitaemia and anaemia (Stephen, 1966; Roberts and Gray, 1972a; Murray *et al.*, 1979a,b). This comparative advantage is the basis for preferential use of trypanotolerant breeds in tsetse-infested areas (Dwinger *et al.*, 1992). Investigation into the genetic and immunologic basis for trypanotolerance has been an area of extensive research (Stewart, 1951; Desowitz, 1959; Roberts and Gray, 1973; Letenneur, 1978; Toure *et al.*, 1978; Murray *et al.*, 1981; Teale, 1991; Dwinger *et al.*, 1992). Such studies are aimed at the identification and characterisation of the specific factors that are responsible for the superior trait of trypanotolerant animals, and possibly, the use of such information to improve the resistance of susceptible cattle to trypanosomiasis (Murray *et al.*, 1982; Trail *et al.*, 1987; Teale, 1991).

However, trypanotolerance does not confer total protection. For instance, N'Dama cattle are known to succumb to trypanosomiasis under very severe challenge (Stewart, 1937; Williams *et al.*, 1992). Also, due to their relatively small body size,

trypanotolerant cattle are thought to be less productive than zebu breeds (Morrison *et al.*, 1981). However, the productivity of trypanotolerant (N'dama) cattle was found not to differ significantly from that of trypanosusceptible (zebu) breeds both under laboratory (Roberts and Gray, 1973) and field (ILCA, 1979; Murray *et al.*, 1981) conditions.

2.5 Chemotherapy of Animal Trypanosomiasis

During the past four decades, chemotherapy of animal trypanosomiasis has depended on drugs belonging to four principal classes: the *phenanthridine*, homidium; the *phenanthridine aromatic amidine*, isometamidium; the *aromatic diamidine*, diminazene; and the *quinoline pyrimidine*, quinapyramine. Information on the indications and dosage regimens of drugs used in the chemotherapy and chemoprophylaxis of animal trypanosomiasis is summarised in Tables 1 and 2.

2.5.1 Suramin

Suramin is the only sulphonated naphthylamine derivative which successfully emerged as an effective trypanocide from field trials (Williamson, 1970). Introduced by Mayer and Zeiss in 1920, suramin is also the oldest of all the current trypanocidal drugs and has been widely used both as a chemotherapeutic and as a chemoprophylactic in animal trypanosomiasis.

Chemistry: Physically, suramin is a pinkish white powder with a high degree of solubility in water. Suramin is extremely hygroscopic. For this reason it is recommended that the drug be kept in a dessicator for at least 24 hours prior to use so as to prevent quantitative errors during weighing (Stephen, 1986). It is also recommended that the drug be administered slowly by the i.v. route. Suramin is highly anionic, and due to the presence of its naphthylamine trisulphonic groups

Table 1

Anti-trypanosomal Drugs Used as Chemotherapeutics in Animal Trypanosomiasis.

Anti-trypanosomal drug	Trade Name	Conditions of use		Indications	Animal	Treatment of relapses
		Dose (mg/kg)	Route			
Homidium bromide	Ethidium®	1.0	i.m.	*T. vivax*, *T. congolense*	Ruminants *equidae*, pigs	Diminazene
Homidium chloride	Novidium®	1.0	i.m.	*T. vivax*, *T. congolense*	Ruminants, *equidae*, pigs	Diminazene
Isometamidium chloride	Samorin® Trypamidium®	0.25-0.5	i.m.	*T. vivax*, *T. congolense*, (*T. brucei*)	Cattle	Diminazene
Diminazene aceturate	Berenil® Veriben®	3.5-7.0	i.m.	*T. vivax*, *T. congolense*, (*T. brucei*, *T. evansi*)	Ruminants, dogs	Isometamidium
Quinapyramine dimethy-lsulphate	Trypacide® (Sulphate)	3.0-5.0	s.c.	*T. evansi*, *T. equinum*, *T. equiperdum*, *T. brucei*, (*T. congolense*, *T. vivax*)	Ruminants, *equidae*, camels, pigs, dogs	Isometamidium
Suramin	Naganol®	7.0-10.0	i.v.	*T. evansi*, *T. brucei*, *T. equiperdum*	Camels, *equidae*	Quinapyramine
Melarsomine	Cymelarsan®	0.25	s.c., i.m.	*T. evansi*,	Camels,	Not reported

i.m. (intramuscular); s.c. (subcutaneous); i.v. (intravenous); ( ) less susuceptible.

Table 2

Anti-trypanosomal drugs used as chemoprophylactics in animal trypanosomiasis.

Anti-trypanosomal drug	Trade Name	Conditions of use		Period of prophylaxis	Indications	Animal	Treatment of relapses
		Dose (mg/kg)	Route	(months)			
Isometamidium chloride	Samorin® Trypamidium®	0.5-1.0	i.m.	1-6	*T. vivax*, *T. congolense*, *T. brucei*	Cattle	Diminazene
Quinapyramine dimethyls-ulphate:chloride (3:2 w/w)	Trypacide® (Prosalt)	3.0-5.0	s.c.	2	*T. evansi*, *T. brucei*	Cattle, *equidae*	Suramin
Suramin-quinapyramine complex		40.0 (quinap-yramine)	s.c.	3-6	*T. simiae*, *T. evansi*	Pigs, *equidae*, camels	Isometamidium Diminazene

i.m. (intramuscular); s.c. (subcutaneous).

(Spinks, 1948) binds, very strongly to plasma proteins *in vivo*, thereby remaining in circulation for as long as 3 months and, thus, its prophylactic activity (Dewey and Wormall, 1946). Suramin's extensive binding to plasma proteins may also account for its poor penetration across the blood-brain barrier and its lack of efficacy against trypanosome infections that involve the central nervous system.

Spectrum of Activity: In cattle, suramin has limited efficacy against infections with *T. vivax* and *T. congolense* but is effective against infections with *T. brucei* in ponies and *T. evansi* in camels (Hawking, 1963a).

Mechanism of Action: Despite its early introduction, very little is known about the mode of action of suramin. However, it is established that the drug enters trypanosomes via endocytosis (Fairlamb and Bowman, 1977; 1980), inhibits a variety of trypanosomal enzymes (Hawking, 1963a; Fortes *et al.*, 1973; Fairlamb and Bowman, 1977), and impairs the functions of ribosomes (Williamson and MacAdam, 1965) and lysosomal enzymes (Hart and Young, 1975; Jacques *et al.*, 1975).

Toxicity: Although adverse reactions such as circulatory collapse and skin rash may be observed immediately after administration of suramin, toxicity due to the drug is usually cumulative and results from accumulation of the drug in the blood when the drug is used chronically. Lesions occur in several organs but are most severe in the kidneys and in animals with nephritis, in which, therefore, treatment with suramin is contraindicated (Hawking, 1963a).

2.5.2 Suramin Complexes

Suramin possesses 6 anionic groups which enable it to readily combine with basic diamidines, phenanthridines and quinoline derivatives forming complexes that are termed "suraminates". Drugs that have been used in the formation of these complexes included pentamidine (Guimaraes and Lourie, 1951; Beaudiment *et al.*,

1954; Cosar *et al.*, 1954), quinapyramine (Watson and Williamson, 1958), antimalarials (Thompson *et al.*, 1963) and quinuronium sulphate (Ryley, 1964).

Advantages: Suramin complexes offer several advantages over the individual drugs. For instance, pentamidine-suraminate is less toxic than either drug used alone (Beaudiment *et al.*, 1954; Cosar, *et al.*, 1954, Williamson and Desowitz, 1956; Desowitz, 1957) and, unlike suramin which is given by slow i.v. injection, the combination can be administered by the i.m. route. Clinically, quinapyramine-suramin complex is effective against *T. simiae* infections in pigs (Watson and Williamson, 1958), and against infections with *T. evansi* in ponies (Gill and Malhotra, 1971) and camels (Rutter, 1967; Mahmoud and Gray, 1980). Furthermore, the duration of prophylaxis can be enhanced as was shown with quinapyramine-suraminate (Stephen and Gray, 1960; Gray, 1961) and homidium-suraminate (Desowitz, 1957) which were reported to confer protection against *T. vivax* and *T. simiae* for at least 6 months. In practice, however, these complexes cause tissue sloughing (Stephen, 1958; Stephen and Williamson, 1958; Williamson, 1958). This, along with the very high cost of production (Williamson, 1970), has resulted in their discontinuation.

2.5.3 Quinapyramine

Chemistry: Jensch (1937) demonstrated that the activity of Surfen C against *T. congolense* was due to the presence of the 4,6-diamino quinaldine nucleus. Subsequently, Barret *et al.* (1953) synthesised a related pyridine derivative with weak trypanocidal activity. The activity, which depended on the quaternization of the pyrimidine nitrogen, was greatly enhanced in quinapyramine in which a bis-quaternary group was included (Curd, 1950; Davey, 1950; Ainley *et al.*, 1953). As the dimethylsulphate (*Trypacide® sulphate*), quinapyramine is soluble in water and has been widely used as a curative at a dose of 5 mg/kg administered subcutaneously (Davey, 1957). As quinapyramine prosalt (*Trypacide® Prosalt*), a mixture of quinapyramine dimethylsulphate and its chloride analogue in a 3:2 w/w ratio, the

formulation confers prophylaxis for up to 6 months, depending on the degree of challenge (Marshall, 1958; Robson, 1962; Whiteside, 1962a).

Spectrum of Activity: The spectrum of activity of quinapyramine encompasses *T. congolense* (Curd and Davey, 1949) and *T. vivax* (Davey, 1950), as well as members of the subgenus *Trypanozoon* including *T. brucei, T. rhodesiense, T. gambiense* and *T. evansi* (Curd and Davey, 1949; Leach, 1961; Hawking, 1963a). At doses of 3-5 mg/kg, Wilson (1949a,b) reported cures of *T. simiae* infections in pigs infected by syringe inoculation. In contrast, the drug was found to be ineffective against tsetse-transmitted *T. simiae* infections in the same animal species (Unsworth, 1952; Williamson, 1958).

Use of quinapyramine for treatment of infections with *T. congolense* and *T. vivax* has been drastically reduced because it has been observed that quinapyramine-resistant strains cross-resist diminazene, isometamidium and homidium (Whiteside, 1962b; Williamson, 1970; Leach and Roberts, 1981). Field application of quinapyramine is therefore now mainly confined to treatment of *T. brucei* and *T. evansi* infections in horses and camels.

Mechanism of Action: Quinapyramine is a cationic compound without trypanocidal activity and, *in vitro*, appears to act by reducing the growth rate of flagellates including *Crithidia* and trypanosomes (Sen *et al.*, 1955; Newton, 1966). In this way it suppresses the parasites to a level sufficient enough for the defence mechanisms of the body to take over and control the infection (Newton, 1980). *In vitro*, incorporation of radiolabelled adenine into RNA of *Crithidia* was inhibited by quinapyramine (Newton, 1966). The drug also interferes with ribosomal functions, protein synthesis and polyamine function (Kahan *et al.*, 1968; Bacchi, 1981).

Toxicity: Toxic signs associated with quinapyramine are curare-like and include excessive salivation, sweating and uncontrolled muscle tremors (Ormerod and Paton, 1950; Garner, 1950). Quinapyramine poisoning in cattle is characterised by kidney

damage, haemorrhagic gastroenteritis, pulmonary oedema and hydropericardium (Davey, 1957).

2.5.4 Phenanthridines

Chemistry: Compounds in this group took their origin from early experiments with numerous compounds by Ehrlich. The studies revealed that the activity of the triphenylmethane dye, Trypanosan, was due to the presence of an acridine compound present in the material as an impurity (Williamson, 1970). Subsequent studies with a variety of acridine and phenanthridine derivatives (Morgan and Walls, 1931) resulted in the synthesis of **phenidium chloride** and establishment of its therapeutic activity against *T. congolense* (Browning *et al.*, 1938).

A structural analogue, **dimidium** (Walls, 1947), was found to be more active and water soluble. Extensive use of the two compounds in East and West African zebu cattle resulted in drug-resistance and, in addition, dimidium caused delayed photosensitisation (Hornby *et al.*, 1943; Burdin and Plowright, 1952; Davey, 1957; Marshall, 1958). Replacement of a methyl group on the quaternary nitrogen in dimidium by an ethyl group yielded **homidium bromide** (Ethidium®) (Watkins and Woolfe, 1952; Short *et al.*, 1953). In addition to enhanced activity, ethidium was devoid of the delayed toxic effects of dimidium. Ethidium® is soluble in hot or boiling, but not cold water. This practical disadvantage was overcome by the introduction of **homidium chloride** (Novidium®) which is soluble in cold water (Wragg, 1955).

Two other phenanthridine compounds, **prothidium** (Short and Watkins, 1957; Watkins 1958a,b) and **metamidium** (Wragg *et al.*, 1958) with both curative and prophylactic activities were later introduced. Metamidium is a hybrid compound formed through the linkage of a portion of the aromatic diamidine diminazene to homidium. The compound is made up of two isomers; the red isomer, which is more water soluble and efficacious than the purple isomer, and is known as

isometamidium and, as the chloride salt, is marketed as Samorin® (May & Baker, Dagenham Ltd., England).

Samorin® has been used extensively both as a curative and as a prophylactic against *T. congolense* and *T. vivax* infections in domestic livestock (Hawking, 1963a; Williamson, 1970; Leach and Roberts, 1981). Experimentally, it has been shown that the duration of chemoprophylaxis with isometamidium varies with the degree of resistance of the parasite (Peregrine *et al.*, 1987, 1991b) and dose of the drug, but is unaffected by the parasite load or the presence of a concurrent trypanosome infection (Peregrine *et al.*, 1988).

Isometamidium is used at the recommended doses of 0.25-0.5 mg/kg and 0.5-1.0 mg/kg, administered once i.m. for therapeutic and prophylactic purposes, respectively (Kinabo and Bogan, 1988c). Homidium is used as a curative at a recommended single i.m. dose of 1.0 mg/kg. At this dose, the drug has been reported to confer prophylaxis in cattle for up to 4 months (Whiteside, 1962a). However, this duration has been shown to vary inversely with the trypanosome prevalence (Dolan *et al.*, 1990, 1992).

Mechanism of Action: The mode of action of phenanthridines appears to involve inhibition of DNA synthesis (Newton, 1957; Wagner, 1971; Kinabo and Bogan, 1987). Through intercalation with adjacent base pairs, homidium causes a distortion of the DNA double helix structure causing a loss of the template function which effectively prevents RNA synthesis and DNA replication (Newton, 1980). Isometamidium inhibits DNA polymerase (Marcus *et al.*, 1982), RNA polymerase (Richardson, 1973) and incorporation of nucleic acid precursors into DNA and RNA (Lantz and Van Dyke, 1972).

The mechanism of action of isometamidium may also be explained by its inhibitory effects on replication of kinetoplast DNA (Opperdoes, 1985), glycoprotein biosynthesis (Casero *et al.*, 1982), lipid metabolism (Dixon *et al.*, 1971; MacAdam

and Williamson, 1974; Kinabo and Bogan, 1987) and membrane transport systems (Girgis-Takla and James, 1974).

Toxicity: Field observations on toxicity with phenanthridine compounds have been reported only with dimidium (Williamson, 1962). Principally due to liver damage, dimidium causes delayed toxicity which begins to manifest some 6 weeks after treatment. The observed signs include photosensitization, lachrymation, nasal discharge, dermatitis, jaundice and emaciation (Findlay, 1950; Davey, 1957). At necropsy, periportal fatty infiltration was the major lesion observed (Burdin and Plowright, 1952; Thorold *et al.*, 1952). Unless when administered in toxic doses, homidium and isometamidium do not cause liver damage (Ford *et al.*, 1954; Unsworth, 1954; Robson, 1958).

Phenanthridine compounds cause local tissue damage when given by the subcutaneous route (Kinabo and Bogan, 1988b), especially in toxic doses (Williamson, 1962; Philips *et al.*, 1967; Braide and Isoun, 1980; Ward and Wallace, 1983; Bell, 1987; Kinabo and Bogan, 1987). In cattle, transient salivation, diarrhoea, urination, lachrymation, tachycardia and increased heart rate may be observed following i.v. administration of isometamidium at doses of 0.5-1 mg/kg.

Owing to its interaction with the DNA (Le Pecq and Poletti, 1967; Prutz, 1984), ethidium is mutagenic to *Saccharomyces cerevisiae* and *Salmonella typhimurium* (Slonimski *et al.*, 1968; MacGregor and Johnson, 1977; Le Cointe *et al.*, 1981; Fukunaga *et al.*, 1984; Fayeulle, 1985).

Mutagenic effects of isometamidium on *S. typhimurium* has also been documented (Crithon *et al.*, 1977). When given intravenously to pregnant rats between days 5 to 17 after mating, isometamidium caused toxic signs in the dams and was found to be teratogenic to the foeti (Copping and East, 1986). The lesions in the foeti included micro-ophthalmia, hydrocephalus and vertebral abnormalities.

2.5.5 Melarsomine

Recently, a new trivalent arsenical, melarsomine (Cymelarsan®, RM110, Mel Cy), was introduced as a therapeutic for *T. evansi* infections in domestic livestock. The therapeutic efficacy of the drug has been demonstrated against experimental *T. evansi* infections in camels when administered at single s.c. or i.m. doses of 0.3-0.6 mg/kg (Zelleke *et al.*, 1989). Another report from South China indicated that at a single i.m. dose of 0.5-3.0 mg/kg, Cymelarsan® was curative against natural *T. evansi* infections in buffaloes (Lun *et al.*, 1991). In both studies, however, treated animals showed swellings localized at the sites of drug administration, but which spontaneously disappeared after a few days (Zelleke *et al.*, 1989) or months (Lun *et al.*, 1991).

2.5.6 Aromatic Diamidines

Yorke *et al.* (1929) showed that glucose was essential for *in vitro* maintenance of trypanosomes. This work was extended by Poindexter (1935) who demonstrated that survival and multiplication of trypanosomes *in vivo* were diminished by a synthetic analogue of insulin, Synthalin (Jansco and Jansco, 1935; Schern and Artagaveytia-Allende, 1935). *In vitro* demonstration of a direct effect of Synthalin on trypanosomes (Lourie and Yorke, 1937) pioneered the synthesis of numerous Synthalin-related compounds. Maximal trypanocidal activity in these compounds was found in aliphatic derivatives possessing a diamidino group (King *et al.*, 1937). A series of trials with several of these compounds led, eventually, to the introduction of the aromatic diamidine, **diminazene aceturate** as a chemotherapeutic for animal trypanosomiasis (Jensch, 1954, 1955).

2.6 Diminazene

Chemistry: Diminazene is 1,3-bis(4-amidinophenyl)triazene and, as the diaceturate tetrahydrate ($C_{22}H_{29}N_9O_6.4H_2O$, mol. wt. = 587.6; mol. wt. base = 281.3), is

marketed as Berenil®. The compound consists of two amidinophenyl moieties linked by a triazene bridge (Fig. 1) and is produced as a yellow odourless powder which in

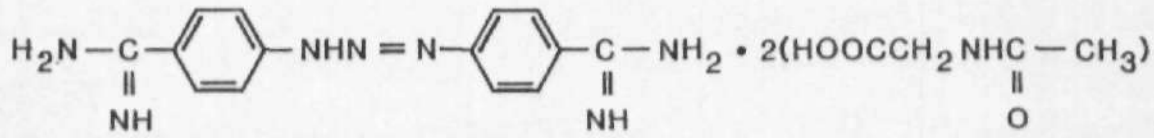


Fig. 1. Structure of Diminazene aceturate

aqueous solution is stable for 2–3 days (Fairclough, 1962). Because of this short duration of stability, the commercial preparation Berenil® consists of diminazene aceturate combined with the stabiliser phenyldimethyl pyrazolone (antipyrine). Solutions of the resultant formulation can be used for up to 10–15 days when stored at room temperature without loss of activity (Fairclough, 1962).

2.6.1 Spectrum of Activity

Following initial experimental and field trials (Bauer, 1955a,b; Fussganger, 1955), diminazene administered at a single i.m. dose of 3.5 mg/kg was found to be highly effective against *T. congolense* and *T. vivax* infections in cattle (Fussganger and Bauer, 1958). In infections with *T. brucei* a higher dose, 5 mg/kg, may be required. However, when examined *in vitro*, *T. congolense, T. vivax* and *T. brucei* showed equal sensitivity to the drug (Van Hoeve and Grainge, 1966).

Doses of 3-5 mg/kg were ineffective against *T. simiae* infections in pigs (Bauer, 1955b). However, at a dose of 7 mg/kg, diminazene aceturate was curative against *T. simiae* infections in sheep (Joshua and Kayit, 1984). Diminazene aceturate is also effective against *T. evansi* infections in buffaloes when administered i.m. in doses varying from 3.5 mg/kg b.w. (Lun *et al.*, 1991) to 16 mg/kg b.w. (Bhathacharjee and Sinha, 1971).

Although diminazene is not recommended for use in camels because of problems with toxicity (Leach, 1961), doses of 1.25 mg/kg b.w. and 5 mg/kg b.w. have been shown to eliminate *T. evansi* infections in this animal species in India (Raisinghani and Lodha, 1980) and the U.S.S.R. (Khamiev and Tulegenova, 1981), respectively. However, in similar studies carried out in Sudan, diminazene aceturate at a dose of 3.5 mg/kg b.w. failed to eliminate such infections in camels (Leach, 1961). Differences in efficacy amongst these studies may reflect variation in diminazene sensitivity of the different trypanosome populations. Alternatively, it may reflect differences in the pharmacokinetics of the drug between Bactrian and dromedary camels.

Trypanosoma equinum, a dyskinetoplastic variant of *T. evansi* which infects equidae in South America, has been shown to be susceptible to treatment in mules with diminazene aceturate at an i.m. dose of 3.5 mg/kg b.w. However, the same dose was ineffective in horses (Mancebo and Monz-n, 1986) and may reflect differences in disease pathogenesis.

The causative agent of *dourine*, *T. equiperdum*, is transmitted venereally, at coitus, between horses. Because of this mode of transmission, therefore, treatment of dourine is contraindicated, and a policy of "test and slaughter" aimed at eradicating the disease is the preferred mode of control adopted in affected countries (Stephen, 1970; Leach and Roberts, 1981). However, single i.m. administration of diminazene aceturate at a dose of 5 mg/kg b.w. appeared to cure 30 natural clinical cases of

dourine in Kazakhstan (Sabanshiev, 1984); trypanosomes were not detected in any of the treated animals for up to 364 days following treatment.

Trypanosoma theileri is a ubiquitous trypanosome that is generally apathogenic, except in young cattle. Reiter *et al.* (1987) have demonstrated activity of diminazene against pathogenic infections at a dose of 7.0 mg/kg b.w., but have recommended that such treatment be limited to animals with high levels of parasitaemia that are exhibiting clinical signs.

Diminazene aceturate has been used extensively across the African continent with very satisfactory results (Fairclough, 1962; MacLennan, 1968; Leach and Roberts, 1981). Its popularity in cattle has been largely due to its therapeutic efficacy with an index that was found to be higher than with other anti-trypanosomal drugs used in animals (Williamson, 1970). In addition, diminazene is often effective against infections that have proved refractory to treatment with other trypanocidal drugs (Fairclough, 1962; Whiteside, 1962b; Williamson, 1970). And for about two decades after extensive field usage, the reported incidence of resistance to diminazene has remained low (Williamson, 1976).

In addition to its trypanocidal activity, diminazene is highly effective against *Babesia* species (Taylor *et al.*, 1956). The tick-borne disease, "babesiosis", caused by these protozoan parasites is of considerable importance in several regions of the world including Africa. In cattle, i.m. treatment with diminazene aceturate at doses of 3-8 mg/kg b.w., has been shown to control infections with *B. bigemina*, *B. bovis* and *B. divergens* (Kuttler, 1981). Furthermore, cross-bred calves treated with diminazene aceturate at a dose of 3 mg/kg b.w. have been immunised against *B. bigemina* when the treatment was administered on either day 3, or days 3 and 6, following infection (Sharma and Bansal, 1984).

In horses, *B. caballi* is sensitive to treatment with diminazene aceturate when administered at a dose of 5 mg/kg b.w. on 2 occasions within a 24-hour interval

(Kirkham, 1969). In contrast, doses of 3.5-10 mg/kg b.w. appear to be ineffective for *B. equi* infections in donkeys (Singh *et al.*, 1984; Kaggwa and Lawal, 1989).

Single i.m. treatment with diminazene aceturate at doses of 0.2-3.5 mg/kg b.w. has also been used to control *B. canis* infections in adult dogs (Thibault and Jaussaud, 1987). Doses as high as 10 mg/kg b.w. appear to be required for *B. gibsoni*. However, problems with toxicity may be encountered at this higher level. In contrast, two doses of 3 mg/kg b.w., given 24 hours apart, were shown to be effective for both *B. canis* and *B. gibsoni* in adult dogs (Farwell *et al.*, 1982) and problems with toxicity were not reported. However, when the dosage regimen was administered to 3-week old puppies, acute toxicity was observed in some animals (Harvey *et al.*, 1988). These results suggest that diminazene's maximum tolerated dose is reduced in juvenile animals.

Bactericidal (Bauer, 1956) and Leishmanicidal (Meith, 1966; Rees *et al.*, 1985; Lynen and Van Damme, 1992) properties have also been demonstrated with diminazene aceturate. It appears to exhibit activity against francaiellosis in cattle (Tursunov *et al.*, 1981) and *Pneumocystis carinii* infection in rats (Walzer *et al.*, 1988).

Although diminazene aceturate has not been registered for use in man, the compound has been used instead of suramin (Williamson, 1970) for the treatment of early stage infections with both *T. b. gambiense* (Hutchinson and Watson, 1962) and *T. b. rhodesiense* (Temu, 1975; Abaru and Matovu, 1981). Treatment in humans involves administration of the compound by deep i.m. injection using a 2% (w/v) solution in sterile 5% (w/v) glucose. The dose regimen used is either 7 daily injections of the drug at a dose of 2 mg/kg b.w., or 3 doses of 5 mg/kg b.w. given at one or more days' interval (Temu, 1975; Abaru and Matovu, 1981).

The above studies have demonstrated diminazene's efficacy against early-stage sleeping sickness. However, the drug appears to have no advantage over suramin for treating *T. b. rhodesiense* and *T. b. gambiense* infections, or over pentamidine

(Williamson, 1970) for treating *T. b. gambiense* infections, except for diminazene's relatively short treatment regimen (Apted, 1980). Attempts to simplify the route of administration have demonstrated that diminazene exhibits trypanocidal activity when given *per os* (Bailey, 1968). Oral administration also appears to reduce the incidence of reactive encephalopathy when patients with late stage *T. b. rhodesiense* infections are subsequently treated with the organic arsenical, melarsoprol (Bailey, 1968).

2.6.2 Combination Regimens

Many of the trypanocidal compounds used for human trypanosomiasis have a low therapeutic index. Drug toxicity is, therefore, often observed. In an attempt to reduce drug doses, and therefore the incidence of toxicity, diminazene has been combined with a variety of compounds to determine if this has an additive or synergistic effect. Combination with various nitroimidazoles was shown to have at least an additive effect against *T. b. brucei* infections in mice (Jennings *et al.*, 1980) and against *T. simiae* infections in pigs (Zweygarth and Rottcher, 1987) that appear to have accessed the central nervous system (CNS). Similar effects have also been demonstrated when diminazene has been combined with either α-difluoromethylornithine (Jennings, 1992) or suramin (Williamson *et al.*, 1982) for the treatment of *T. brucei* sp. CNS infections.

The molecular basis for these observations have not been elucidated but may be due to inhibition of different biochemical pathways. Lastly, combination with either mepyramine maleate or piroxicam has been shown to enhance the therapeutic activity of diminazene against *T. vivax* infections in sheep (Joshua and Babalola, 1983) and *T. b. brucei* infections in mice (Abatan, 1991), respectively. Since both mepyramine and piroxicam are anti-inflammatory compounds, their co-administration with diminazene may increase the bioavailability of the trypanocide.

2.6.3 Mechanisms of Action of Diminazene

Diminazene binds to trypanosomal kDNA (Newton, 1972; MacAdam and Williamson, 1972). This binding does not occur by intercalation (Newton, 1972; Benard and Riou, 1980) but via specific interaction with sites rich in adenine-thymine (A-T) base pairs (Newton, 1972; Brack and Delain, 1975). Nonintercalative binding of diminazene to DNA with a strong affinity for A-T base pair regions has similarly been demonstrated *in vitro*, using DNA obtained from various other sources (Waring, 1965; Lane *et al.*, 1991). Such studies have shown that the molecule binds with higher affinity to 5′–AATT–3′ than to 5′–TTAA–3′ regions of DNA (Hu *et al.*, 1992). Furthermore, that binding to double helix DNA occurs via the minor groove (Gresh and Pullman, 1984; Laughton *et al.*, 1990; Hu *et al.*, 1992), and that attachment to specific sites occurs via electrostatic and hydrogen-bond forces (Gresh and Pullman, 1984; Brown *et al.*, 1990; Yoshida *et al.*, 1990; Lane *et al.*, 1991). Through this specific interaction in trypanosomes, diminazene inhibits synthesis of RNA primers, resulting in accumulation of replicating intermediates, thereby inhibiting kDNA replication (Newton, 1972; Brack and Delain, 1975). In other work, Shapiro and Englund (1990) have shown that diminazene specifically inhibits mitochondrial type II topoisomerase in viable trypanosomes. Thus, inhibition of DNA replication may also occur via this interaction.

Finally, although diminazene selectively fragments kinetoplast, but not nuclear, DNA (MacAdam and Williamson, 1972), it is still not established whether kDNA is the primary target for diminazene *in vivo* (Newton, 1980). Indeed, activity of diminazene and other diamidines at sites other than kDNA may explain the diverse changes observed in the structure and function of trypanosomatids exposed to these drugs. Such alterations include aggregation and loss of ribosomes (Wallis, 1966); modification of lysosomes and the cytoplasmic membrane (MacAdam and Williamson, 1972); inhibition of basic amino acid transport (Gutteridge, 1966); inhibition of DNA and RNA polymerases (Waring, 1965; Marcus *et al.*, 1982), and interference with

polyamine uptake, synthesis and function (Wallis, 1966; Bachrach *et al.*, 1979; Bacchi, 1981). In addition, *in vivo* and *in vitro* studies indicate that diminazene interacts with cholinergic and histaminergic receptors by which the drug appears to induce cardiovascular changes when administered in high doses (Arowolo, 1981; Arowolo and Eyre, 1984).

2.6.4 Analytical Techniques of Diminazene

Of the several analytical techniques (Table 3) that have been reported for drugs used against animal trypanosomiasis, six have been used to determine the concentration-time profiles for diminazene *in vivo* (Table 4). A colorimetric approach with a relatively low level of sensitivity of 0.5 µg/ml (Raether *et al.*, 1972) was used to investigate the pharmacokinetics of diminazene in rats (Raether *et al.*, 1972), monkeys (Raether *et al.*, 1974), cattle (Klatt and Hajdu, 1976) and dogs (Anika and Onyeyili, 1989; Onyeyili and Anika, 1989, 1991). Using a bioassay, disposition of diminazene was examined in cattle (Cunningham *et al.*, 1964) and in rabbits (Goodwin and Tierney, 1977). However, data from the latter technique are difficult to interpret since anti-trypanosomal activity can be exhibited by serum from untreated animals. In attempts to increase the sensitivity of diminazene assays, radiometric methods have been described by Gilbert (1983) and Kellner *et al.* (1985). Although these assays can detect diminazene in concentrations as low as 0.028 µg/ml of plasma, or 0.18 µg/g of tissues, they are deficient in specificity since they measure total radioactivity and do not distinguish intact from modified drug (Aliu and Ødegaard, 1983). Finally, gas chromatography mass spectrometry (Fouda, 1978), high-performance liquid-chromatography (Fouda, 1977; Aliu and Ødegaard, 1983) and thin-layer chromatography (Gluth *et al.*, 1986)

have proven to be the most sensitive and specific methods for extraction and detection of diminazene.

Table 3
Analytical techniques for drugs used against animal trypanosomiasis.

	Suramin	Homidium	Isometamidium	Quinapyramine	Diminazene	Pentamidine
Bioassay	Goodwin and Tierney (1977)	NA	Goodwin and Tierney (1977)	NA	Goodwin and Tierney (1977)	Bernard *et al.* (1985), Collins *et al.* (1986), Donnelly *et al.* (1988)
High-performance liquid chromatomatography	NA	Perschke and Vollner (1985)	Perschke and Vollner (1985) Kinabo and Bogan (1988d)	Perschke and Vollner (1985)	Fouda (1977) Aliu and *degaard (1983) Murilla and Kratzer (1989)	Dickinson *et al.* (1985), Conte *et al.* (1986), Lin *et al.* (1986), Berger *et al.* (1989, 1990), Ericsson and Rais (1990), Bronner *et al.* (1991)
Colorimetry	NA	NA	NA	NA	Raether *et al.* (1972) Klatt and Hajdu (1976) Onyeyili and Anika (1989) Anika and Onyeyili (1989)	NA
Radiometry	NA	Gilbert and Newton (1982a)	NA	NA	Gilbert (1983) Gilbert and Newton (1982b) Kellner *et al.* (1985)	NA
Spectrophotometry	NA	NA	Philips *et al.* 1967	NA	NA	NA
Radioimmunoassay	NA	NA	Kinabo and Bogan (1988a)	NA	NA	NA
Thin-layer chromatography	NA	NA	NA	NA	Kellner *et al.* (1985) Moffat (1986)	NA
Gas-chromatography mass spectrophotometry	NA	NA	NA	NA	Fouda (1978)	Berger *et al.* (1990)
ELISA	NA	NA	Whitelaw *et al.* (1991)	NA	NA	NA

NA = Not available.

2.6.5 Pharmacokinetics of Diminazene

The availability of highly sensitive analytical techniques for diminazene (Table 4) has facilitated the generation of a substantial body of data on the pharmacokinetics of diminazene in different animal species (Table 5).

Absorption of diminazene occurs following oral administration to rats (Raether *et al.*, 1972; Kellner *et al.*, 1985), monkeys (Raether *et al.*, 1974) and humans (Bailey, 1968). In all species studied, i.m. administration is followed by rapid absorption with mean C_{max} and t_{max} values of 1.30 ± 0.13 μg/ml and 15.00 - 30.00 min, respectively, in rabbits (Gilbert, 1983), 5.11 ± 1.63 μg/ml and 30.00 ± 15.00 min, respectively, in cattle (Klatt and Hajdu, 1976; Kellner *et al.*, 1985), 4.2 ± 0.42 μg/ml and 48.30 ± 40.10 min, respectively, in goats (Aliu *et al.*, 1984), and 6.70 ± 0.58 μg/ml and 31.25 ± 10.31 min, respectively, in sheep (Aliu and Ødegaard, 1985). Values of hybrid pharmacokinetic parameters determined from these studies vary widely, principally due to differences in experimental methods and approaches to data analyses.

Distribution of diminazene occurs rapidly and appears to be either biphasic (Klatt and Hajdu, 1976; Fouda, 1977; Gilbert, 1983; Kellner *et al.*, 1985) or triphasic (Aliu *et al.*, 1984; Aliu and Ødegaard, 1985) in different animal species (Table 5). Following i.m. administration, distribution half-life values were shortest in the sheep, 0.41 h (Aliu and Ødegaard, 1985), and longest in the goat, 1.88 h (Aliu *et al.*, 1984). However, the apparent volumes of distribution (V_d) in the two species were similar (Table 5).

Elimination half-life values of 10-30 h in sheep and goats (Aliu *et al.*, 1984; Aliu and Ødegaard, 1985) are shorter than in rabbits, 103 h (Gilbert, 1983), and in cattle, 88-145 h (Klatt and Hajdu, 1976; Kellner *et al.*, 1985). Consistent with these long half-life values, low concentrations of diminazene persist in the plasma of goats and cattle for several weeks following treatment with a single dose of diminazene (Fouda, 1977, 1978; Aliu *et al.*, 1984; Kellner *et al.*, 1985). Experiments to

Table 4

Analytical techniques for diminazene.

Technique	Assay procedure	Limit of detection		References
		Plasma/Serum (μg/ml)	Tissues (μg/g)	
Colorimetry	Colorimetric analysis of diazotized diminazene.	0.50	ND	Raether *et al.* (1972)
		0.50	ND	Klatt and Hajdu (1976)
		0.20	0.09	Onyeyili and Anika (1991)
Bioassay	Evaluation of trypanocidal activity of plasma or tissue fluid.	—	—	Goodwin and Tierney (1977)
Radiometry	Measurement of ^{14}C-diminazene in biological samples.	0.028	0.37	Gilbert (1983)
		0.18	0.18	Kellner *et al.* (1985)
Gas chromatography mass spectrometry	Reduced diminazene (4-aminobenzamidine) is derivatized and analysed using a mass spectrometer coupled to a gas chromatograph.	0.10	ND	Fouda (1978)
High-performance liquid chromatography	Spectrophotometric analysis of extracted 4-aminobenzamidine or intact diminazene.	0.10	ND	Fouda (1977)
		0.05	ND	Aliu and Ødegaard (1983)
		0.10	0.72	Murilla and Kratzer (1989)
Thin-layer chromatography	Fluorodensitometric analysis of diamidines in fluid and tissue sample extracts.	0.01	0.05	Gluth *et al.* (1986)

— = specific values not given; ND = not determined.

Table 5

Pharmacokinetic data for diminazene.

Animal species	Drug administration Dose (mg/kg)	Route	Plasma fall-off	$t_{1/2}k_a$ (min)*	$t_{1/2}\lambda_1$ (h)*	$t_{1/2}\lambda_z$ (h)*	Vd (L/kg)	References
Goat	3.5 diminazene base	i.m.	Triphasic	9.56	1.88	16.10	0.09 ± 0.27	Aliu *et al.* (1984)
	2.0 " "	i.v.	Triphasic	NA	6.09	10.26	0.39 ± 0.07	"
Sheep	3.5 " "	i.m.	Triphasic	4.65	0.42	11.39	0.76 ± 0.18	Aliu and Ødegaard (1985)
	2.0 " "	i.v.	Triphasic	NA	0.41	9.58	0.56 ± 0.04	"
Cattle	3.5 diminazene aceturate	i.m.	Biphasic	ND	1.22	74.29	ND	Klatt and Hajdu (1976)
	3.5 " "	i.m.	Biphasic	ND	2.38	200.96	ND	Kellner *et al.* (1985)
Rabbit	3.5 diminazene aceturate	i.m.	Biphasic	ND	1.26	104.20	ND	Gilbert (1983)
Dog	3.5 " "	i.v.	Biphasic	ND	0.18	10.64	2.17 ± 0.31	Anika and Onyeyili (1989) Onyeyili and Anika (1989)

min = minutes; * = values are given as geometric means; h = hour; L/kg = litres/kilogram; i.m. = intramuscular; i.v. = intravenous; NA = not applicable; ND = not determined.

determine the route(s) by which diminazene is eliminated have shown that the molecule is excreted in the urine (Gilbert, 1983; Aliu *et al.*, 1984; Kellner *et al.*, 1985; Anika and Onyeyili, 1989; Onyeyili and Anika, 1989), faeces (Gilbert, 1983; Kellner *et al.*, 1985) and milk (Aliu *et al.*, 1984). However, except for the study conducted in goats (Aliu *et al.*, 1984), in which only intact diminazene was assayed, it is not clear from the other reports whether diminazene is eliminated intact or in a modified form.

To date, an unequivocal report on the recovery of metabolites of diminazene from animals has not been published. With regard to pentamidine, however, *in vitro* studies with rat tissue homogenates indicate that the drug is readily metabolised by liver microsomes to seven primary and two secondary metabolites (Berger *et al.*, 1992). In contrast, although diminazene binds to microsomal enzymes it does so at sites that are not involved with drug metabolism. It would therefore appear that it neither induces nor inhibits microsomal mixed function oxidases (Dalvi, 1988).

2.6.6 Toxicity Due to Diminazene

Treatment of domestic livestock with standard therapeutic doses of diminazene aceturate (3.5-7.0 mg/kg b.w.) rarely results in signs of toxicity. Furthermore, since diminazene's therapeutic index in most animal species is relatively great, cattle, for instance, can tolerate i.m. doses as high as 21.0 mg/kg b.w. without exhibiting signs of systemic toxicity (Fairclough, 1963).

In camels, however, a single dose of 7.0 mg/kg b.w. can be highly toxic (Leach, 1961). Diminazene is also relatively toxic in dogs. Experimentally, i.m. administration of doses in excess of 10.0 mg/kg b.w., once or repeatedly, results in severe signs of disturbance in the gastrointestinal tract, respiratory, musculoskeletal and nervous systems in both camels (Homeida *et al.*, 1981) and dogs (Losos and Crockett, 1969). In laboratory rodents, hypotension is also commonly observed (Steinmann *et al.*, 1986). The hypotensive response appears to result from direct (Steinmann *et al.*, 1987) and indirect (Ellis *et al.*, 1970; Arowolo; 1981; Arowolo and Eyre, 1984)) effects on the cardiovascular system. At necropsy, gross and

histopathological lesions have been described in the liver, kidneys, urinary bladder, lungs, heart and brain of dogs (Losos and Crockett, 1969), donkeys (Boyt *et al.*, 1971), camels (Homeida *et al.*, 1981) and rats (Akanji and Ngaha, 1989).

Diminazene is extensively distributed in the body of treated animals and residues of the compound may persist for several weeks, principally in the liver and kidneys, and also, to a lesser extent, in the gastrointestinal tract, lungs, muscle, brain and fat (Gilbert, 1983; Kellner *et al.*, 1985; Onyeyili and Anika, 1991). Based on pharmacokinetic (Aliu *et al.*, 1984) and residue (Kellner *et al.*, 1985) studies, a pre-slaughter withdrawal period of 21-35 days has been recommended for diminazene when treated animals are intended for human consumption.

Although diminazene has not undergone formal evaluation for toxicity in man (Apted, 1980), it is not recommended for use in humans by the manufacturers on the basis of the toxicity of the drug observed in some animal species (Abaru *et al.*, 1984). However, in 17 cases reported by Hutchinson and Watson (1962), no local or systemic toxicity was observed. Furthermore, amongst 99 cases reported by Abaru *et al.* (1984), although various transient side effects were observed, it was concluded that they were no more serious than those produced by other trypanocidal drugs, such as suramin. It is, however, noteworthy that acute idiopathic polyneuritis (Laudry-Guillain-Barré syndrome) developed in a human patient infected with *B. microti* after treatment with diminazene aceturate (Ruebush *et al.*, 1979). It is worthy of note that stilbamidine was withdrawn because it caused delayed neuropathy in man (Sati, 1949).

Although diminazene and related aromatic diamidines interact with DNA (Newton, 1980; Williamson, 1980), several workers have reported that they are neither mutagenic (Stauffert *et al.*, 1990) nor teratogenic (Rakhmatullin, 1989; Yoshimura, 1990). But other workers have reported diminazene-induced mutagenic effects on yeast (Mahler and Perlman, 1973; Perlman and Mahler, 1973; Villa and Juliani, 1980).

2.6.7 Resistance to Diminazene

Drug-resistance is a well recognized feature in animal trypanosomiasis (Leach and Roberts, 1981; Campbell and Rew, 1986) and remains important because it is a major hinderance to the development of domestic livestock in the affected areas.

Under field conditions, factors responsible for the emergence of drug-resistance appear to be multiple. While the occurrence of naturally resistant trypanosomes cannot be discounted (Leach and Roberts, 1981), field observations on the response of infected animals to treatment indicate that application of subcurative doses of trypanocidal drugs and cross-resistance contribute greatly to the emergence of drug-resistance in the field. In addition, it appears that trypanocides differ considerably in their efficacy towards different trypanosome species. For example, the sensitivity of *T. vivax* to homidium is generally greater than that shown by *T. congolense* to the same drug. Consequently, in Nigeria, extensive use of homidium in cattle eliminated *T. vivax* (Killick-Kendrick and Godfrey, 1963) and lead to the emergence of homidium-resistant *T. congolense* (Jones-Davies and Folkers, 1966). However, the overall picture in the field is often more complex and difficult to interpret due to lack of accurate data on the prevalence of the disease across the African continent (Williamson, 1980).

Experimentally, significant differences in the responses of drug-sensitive and drug-resistant trypanosomes have frequently been demonstrated *in vivo* (Waitumbi *et al.*, 1988; Moloo and Kutuza, 1990; Peregrine *et al.*, 1991a; Chitambo *et al.*, 1992) and *in vitro* (Borowy *et al.*, 1985; Kaminsky *et al.*, 1989). Resistance to diminazene may vary enormously between trypanosome species (Hawking, 1963a), stocks (Kaminsky *et al.*, 1989) and clones (Peregrine *et al.*, 1991a). In comparison to drug-sensitive populations which are readily eliminated by therapeutic doses, drug-resistant strains resist treatment even with relatively high doses (2-4 times higher than therapeutic doses) of diminazene aceturate (Jones-Davies, 1967b; Clausen *et al.*, 1992; Mohamed-Ahmed *et al.*, 1992).

2.6.8 Mechanisms of Resistance to Diminazene

So far no data has been published on the molecular basis of resistance to diminazene in trypanosomes. In studies with the closely related aromatic diamidine, pentamidine, Damper and Patton (1976b) concluded that the molecule is transported into bloodstream forms of *T. b. brucei* via a carrier-mediated process that is substrate specific, concentrative and energy-coupled. They also concluded that alteration in pentamidine transport is the primary mechanism responsible for pentamidine resistance in trypanosomes (Damper and Patton, 1976a). However, drug efflux mechanisms and drug metabolism were shown not to play a role in drug resistance (Damper and Patton, 1976b).

Whilst *in vitro* cultivation studies have demonstrated heterogeneity in diminazene-resistance phenotype amongst trypanosome strains, and, therefore, suggest a biochemical basis for variation in diminazene resistance amongst trypanosome populations (Kaminsky *et al.*, 1989), it appears that mechanisms other than these may play a role in the apparent resistance that is observed *in vivo*. For example, experiments in mice have shown that *T. b. brucei* populations are eliminated if animals are treated on days 3 or 7 of infection with diminazene aceturate at a dose of 40 mg/kg b.w. However, if treatment is delayed until day 14, infections in all animals relapse. This phenomenon was shown not to be associated with an alteration in resistance (Jennings *et al.*, 1977) but due to sequestration of trypanosomes into sites, such as the brain (Jennings *et al.*, 1979) which are believed to be inaccessible to diminazene (Gutteridge and Coombs, 1977). A similar phenomenon has also been reported for *T. vivax* in goats (Whitelaw *et al.*, 1988) and *T. b. brucei* in dogs (Chukwu *et al.*, 1990).

2.6.9 Techniques to Determine Diminazene Resistance in Trypanosomes

Laboratory rodents, ruminants and *in vitro* cultivation systems have all been used to characterise the diminazene resistance of trypanosome populations. Whilst mice and rats are useful laboratory systems for quantifying drug resistance, they are

not always suitable for characterising the resistance phenotype of field isolates since they do not support the growth of all trypanosome species (Hawking, 1963a). They also do not provide data which can be directly extrapolated to the definitive host (Sones *et al.*, 1988; Zhang *et al.*, 1992). For certain purposes, therefore, use of large domestic animals, especially ruminants, has been preferred (Gitatha, 1979; Peregrine *et al.*, 1991a), since drug sensitivity data derived from such animals are of direct relevance to the field. However, such an experimental system is expensive and may take many months to provide definitive data.

The development of *in vitro* cultivation techniques for trypanosomes (Hill and Hirumi, 1983) has facilitated the development of *in vitro* systems for studying drug-parasite interactions in the absence of the host. Compared to drug-sensitivity studies carried out *in vivo*, such systems are generally cheap, rapid and reproducible. Furthermore, they also can be applied to all stages of the trypanosome life cycle. Table 6 summarises the different methods that have been described: these include growth inhibition assays; a long term viability test; inhibition of incorporation of radiolabelled hypoxanthine; a drug incubation infectivity test, a photometric test, and an akinetoplast induction test. In each system, the effect of diminazene on trypanosomal growth, viability or ability to utilise various substrates is used to determine the drug sensitivity of trypanosomes. However, while *in vitro* techniques constitute a valuable tool for investigating drug resistance in the laboratory, utilisation of diminazene-sensitivity data, derived *in vitro*, as a basis for predicting the diminazene-sensitivity of trypanosome populations in domestic livestock, is not yet possible (Kaminsky, 1990). This discrepancy may be due to the selection process which occurs when trypanosomes are adapted to *in vitro* cultivation.

Table 6

In vitro techniques for determining the diminazene resistance of trypanosome populations.

Technique	Depends on the amount of diminazene required to:	Trypanosome		Minimum concentration (µg/ml medium) required to produce specified effect		Duration of assay	References
		species	life-cycle stage	sensitive	resistant		
Growth inhibition assay	inhibit parasite growth	*T. b. brucei*	bloodstream forms		(1.00)	36 hours	Borowy *et al.* (1985)
		T. b. brucei	bloodstream forms	0.01	0.50	24 hours	Kaminsky *et al.* (1989)
		T. evansi	bloodtsream forms	0.11	14.24	24 hours	Zhang *et al.* (1992)
		T. equiperdum	bloodstream forms	0.06	1.78	24 hours	Zhang *et al.* (1992)
		T. congolense	metacyclic forms	5.00	50.00	7 days	Gray and Peregrine (1993)
Long term viability assay	kill all parasites in culture	*T. b. brucei*	bloodstream forms	0.01	0.10	6-10 days	Kaminsky *et al.* (1989)
Incorporation of ^{3}H-hypoxanthine	decrease uptake of ^{3}H-hypoxanthine in parasites by certain percentages	*T. b. brucei*	bloodstream forms		(2.50)	24 hours	Brun and Kunz (1989)
		T. congolense	procyclic forms	0.01	10.00	48 hours	Ross and Taylor (1990)
		T. congolense	bloodstream forms	>0.01	>1.00	24 hours	Brun and Rab (1991)
Drug incubation infectivity test	render parasites non-infective to mice	*T. b. brucei* *T. evansi* *T. vivax*	bloodstream forms	0.01	>1.00	3-30 days	Kaminsky *et al.* (1990)
Photometric test	inhibit parasite growth and produce change in colour of pH indicator in medium	*T. b. brucei*	bloodstream forms		(0.16)	72 hours	Zinsstag *et al.* (1991)
Akinetoplast test	cause parasites to lose kinetoplast	*T. congolense*	bloodstream forms	0.10	1.00	4-10 hours	Chitambo and Arakawa (1992)

( ) = value for the only strain tested; > = greater than.

2.6.10 Reports of Resistance to Diminazene

Using cattle to characterise the diminazene-resistance phenotype of trypanosome populations, resistance to diminazene has been reported for cyclically transmitted trypanosomes occurring in West, Central and East Africa (Table 7). Significant levels of resistance to diminazene have also been reported for populations of *T. evansi* originating from China (Zhang *et al.*, 1992), but not from within Africa. While reports of resistance to diminazene in Africa originate almost entirely from Nigeria and East Africa, it is not clear whether this reflects the true prevalence of drug resistance or the interests of scientists in these particular parts of that continent.

2.6.11 Innate Resistance

Subsequent to the first usage of diminazene aceturate in the field, populations of *T. congolense* and *T. vivax* were described in Nigeria that appeared to be naturally resistant to diminazene (Jones-Davies, 1967a, 1968). Furthermore, Williamson (1960) has suggested that, naturally, West African populations of *T. vivax* express a higher level of resistance to diminazene than *T. congolense*. However, whether such resistance is a result of cross-resistance induced by other drugs, such as quinapyramine, is not clear (Gray and Roberts, 1971b).

2.6.12 Acquired Resistance

Widespread, long-term, use of diminazene aceturate for bovine trypanosomiasis has not appeared to result in the development of resistance to diminazene in trypanosome populations in the field (Boyt, 1971; Wilson *et al.*, 1975; Rushigajiki *et al.*, 1986). In the laboratory, various attempts have been made to induce resistance to diminazene in trypanosomes. Fussganger and Bauer (1960) and Bauer (1962) treated infected mice and cattle, respectively, with subcurative doses of diminazene but were unable to induce resistance. However, using a similar protocol, Hawking (1963b) increased the resistance of a *T. congolense* population to diminazene by 5-fold over a 40-month period in guinea pigs. It would therefore

Table 7

Reports of resistance to diminazene, characterised in cattle.

Country	Trypanosome species	Resistance to diminazene aceturate at	Reference
Nigeria	*T. vivax*	7.0 mg/kg b.w.	Jones-Davies, 1967a
Nigeria	*T. congolense*	3.5 mg/kg b.w.	Na'Isa, 1967
Nigeria	*T. congolense*	7.0 mg/kg b.w.	MacLennan and Jones-Davies, 1967
Chad	*T. vivax*	7.0 mg/kg b.w.	Graber, 1968
Nigeria	*T. congolense*	7.0 mg/kg b.w.	Jones-Davies, 1968
Nigeria	*T. vivax*	3.5 mg/kg b.w.	MacLennan and Na'Isa, 1970
Nigeria	*T. vivax*	14 mg/kg b.w.	MacLennan, 1971
Uganda	*T. vivax*	7.0 mg/kg b.w.	Mwambu and Mayende, 1971
Kenya	*T. congolense*	21 mg/kg b.w.	Gitatha, 1979
Tanzania	*T. congolense*	7.0 mg/kg b.w.	Njau *et al.*, 1983
Kenya	*T. vivax*	3.5 mg/kg b.w.	Rottcher and Schillinger, 1985
Kenya	*T. vivax*	3.5 mg/kg b.w.	Schonefeld *et al.*, 1987
Tanzania	*T. congolense*	14 mg/kg b.w.	Mbwambo *et al.*, 1988
Somalia	*T. congolense*	10.5 mg/kg b.w.	Ainanshe *et al.*, 1992
Ethiopia	*T. congolense*	7.0 mg/kg b.w.	Codjia *et al.*, 1993

appear that *T. congolense* can acquire resistance to diminazene, but only as a result of lengthy subcurative dosage regimens (Whiteside, 1963). In contrast, using *T. evansi*, Osman *et al.* (1992) were able to produce an 80-fold increase in diminazene resistance of a cloned population over a 9-week period by subcuratively treating immunosuppressed mice. Since attempts to induce resistance to diminazene with the same experimental protocol in immunocompetent mice were unsuccessful, it would appear that immunological status rather than trypanosome species was the main factor determining the rate of development of resistance.

2.6.13 Cross-Resistance

Field and laboratory studies have shown that quinapyramine and melarsomine (Raynaud *et al.*, 1989) appear to be unique in inducing cross-resistance to diminazene (Whiteside, 1962a; Osman *et al.*, 1992; Ndoutamia *et al.*, 1993). Thus, as a result of the relative difficulty in inducing resistance to diminazene in the laboratory, Gray and Roberts (1971b) and Mwambu and Mayende (1971) suggested that diminazene-resistant populations observed in the field may generally be directly resistant to quinapyramine and cross-resistant to diminazene.

From an analysis of the cross-resistance characteristics for the different trypanocides, Whiteside (1960) proposed the concept of "sanative" pairs of drugs, i.e., pairs of drugs that do not induce resistance to the other. Two such combinations are homidium (Williamson, 1970) and diminazene, and isometamidium (Williamson, 1970) and diminazene. Thus, since 1967 the treatment regime recommended for trypanosomiasis in northern Nigeria is the use of homidium from May to October (during the rainy season), and diminazene from November to April (the dry season) (Aliu, 1981). Such regimens have been used to maintain the productivity of domestic livestock in the field without the development of resistance to either compound (Bourn and Scott, 1978; Trail *et al.*, 1985).

2.6.14 Stability of Resistance to Diminazene

The duration of time over which drug-resistant phenotype persists in trypanosomes varies. Fulton and Grant (1955) showed that resistance to atoxyl remained stable in a strain of *T. rhodesiense* that has not been exposed to the drug for 24 years. Stability in drug-resistance may vary with the trypanosome strain (Bishop, 1959; Peregrine *et al.*, 1991a) but is unaffected due to transfer between different host species (Yorke and Hawking, 1932; Yorke *et al.*, 1933; Murgatroyd and Yorke, 1937). In experiments carried out in cattle, a strain of *T. congolense* was shown to retain its resistance to diminazene aceturate at a dose of 3.5 mg/kg b.w. after a series of passages in 3 animals for 230 days. Similarly, a strain of *T. vivax* maintained its resistance to diminazene aceturate at 7.0 mg/kg b.w. for 164 days while passaged through 7 oxen (Gray and Roberts, 1971b). Similar observations concerning the stability of resistance to diminazene have been made with bloodstream forms of *T. b. brucei in vitro* (Kaminsky and Zweygarth, 1989).

Field observations indicate that drug-resistant trypanosomes disappear subsequent to withdrawal of cattle from, and suspension of the use of the resisted drug in, the affected areas (Whiteside, 1960). The disappearance does not appear to occur due to cyclical transmission since resistance of a *T. congolense* population to diminazene aceturate at a dose of 7.0 mg/kg b.w. in cattle was unaffected by 8 cyclical transmissions over 376 days (Gray and Roberts, 1971a). It would, therefore, appear that disappearance of diminazene resistance in the field does not result from loss of drug resistance as a result of long-term passage or tsetse transmission *per se* (Gray and Roberts, 1971b) but occurs due to some yet unidentified factors.

2.7 References

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MAIN CHAPTERS

CHAPTER 3

PHARMACOKINETICS OF DIMINAZENE IN FEMALE BORAN *(Bos indicus)* CATTLE

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Diminazene pharmacokinetics in Zebu cattle

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ABSTRACT

Aliu, Y.O., Mamman, M. & Peregrine, A.S. Pharmacokinetics of diminazene in female Boran *(Bos indicus)* cattle

The disposition kinetics and bioavailability of diminazene in five healthy heifers were determined after single intravenous (i.v.) and intramuscular (i.m.) administration of the drug in sequence with a wash-out period between administrations of six weeks. Intact diminazene in plasma, whole blood and urine samples was analysed using high-performance liquid chromatography. Nonlinear regression analysis of the i.v. and i.m. data indicated that, for either route, the plasma disappearance curves of diminazene were best described by triexponential equations. The i.v. bolus was followed by rapid and biphasic distribution with half-life values of 0.04 h and 0.58 h; $V_{d(ss)}$ was 1.91 ± 0.42 l/kg; elimination half-life was 31.71 h, while CL averaged 1.74 ± 0.40 ml/min/kg. Within 30 min of the i.v. dose, the erythrocyte/plasma partition ratio of diminazene was 0.30 ± 0.15. Diminazene was rapidly absorbed following i.m. administration: $t_{1/2}k_a$ was 0.60 h; C_{max}, 4.68 ± 1.12 µg/ml, was attained in 10–15 min and systemic availability was 102.42 ± 7.25%. The half-life of the terminal disappearance phase was 145.48 h. About 8.26% of the i.m. dose was excreted intact in the urine within the first 24 h of treatment. *In vitro*, diminazene was bound to bovine plasma albumin to the extent of 38.01–91.10%.

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INTRODUCTION

Since the introduction of diminazene diaceturate (Bauer, 1955a, 1955b; Fussganger, 1955), the drug has been widely accepted as an effective curative trypanocide (Leach & Roberts, 1981; Holmes & Scott 1982; Campbell & Rew, 1986). However, despite the long period that the drug has been in use, only a few pharmacokinetic studies have been conducted in cattle to establish the most efficacious dosage regimen for diminazene. Kinetic data so far reported for diminazene in cattle (Klatt & Hajdu, 1976; Kellner *et al.*, 1985) have shown that following i.m. administration at a dose of 2.0–3.5 mg/kg, absorption is rapid and peak plasma levels are attained within 15–45 min, the elimination half-life ranges from 40–205 h and urinary excretion is the principal process of elimination. Plasma levels of 1.15 µg/ml are maintained for a minimum of 24 h, and up to 0.36 µg/ml can be detected 20 days after administration of the drug. However, the methods used for quantification of diminazene in these studies – colorimetry (Klatt & Hajdu, 1976) and radiometry (Kellner *et al.*, 1985) – lack sufficient specificity because they do not distinguish between intact diminazene and its metabolites. Furthermore, pharmacokinetic-pharmacodynamic data, needed to establish effective diminazene dosage regimens that would be effective in eliminating both sensitive and resistant trypanosome infections of cattle, have not been documented. Since resistance to diminazene appears to have developed in a number of sites across sub-Saharan Africa (Jones-Davies, 1967, 1968; MacLennan & Jones-Davies, 1967; Gray & Roberts, 1971a, 1971b; Kupper & Wolters, 1983; Authie, 1984; Schönefeld *et al.*, 1987; Mbwambo *et al.*, 1988), and availability of other trypanocides is often restricted, it is important to determine whether the therapeutic activity of the drug might be enhanced through the use of more appropriate dosage regimens.

Aliu and Odegaard (1983) described a high-performance liquid chromatography (HPLC) technique with greater sensitivity than obtained with the

previously reported analytical techniques and used the assay to characterise the kinetics of diminazene in healthy goats and sheep (Aliu *et al.*, 1984; Aliu & Odegaard, 1985). The objectives of the present study were to determine the absorption and disposition kinetics of diminazene in cattle using the same technique and to evaluate the kinetic data obtained in order to optimize the dosage regimen for the drug.

MATERIALS AND METHODS

Animals

Five clinically healthy female Boran cattle, 11 – 14 months old and weighing 82–106 kg, were used in the study. The animals were screened for the presence of antibodies to *Trypanosoma, Babesia, Anaplasma* and *Theileria* species using an indirect immunofluorescent antibody test (Katende *et al.*, 1987). No antibodies were detected. As a precaution against helminthiasis all animals were treated with 7.0 mg/kg fenbendazole 6 weeks prior to the study. Animals were housed in individual pens and given hay and water *ad libitum* throughout the period of study. Concentrates (300–400 g/animal) (Young Stock, Belfast Millers, Nairobi, Kenya) were also fed to the animals on a daily basis.

Administration of drug

The study proceeded in sequence. In the first stage of the experiment, diminazene diaceturate, as the pure salt (Hoechst AG, Frankfurt, FRG), was injected as a 7.0% w/v solution in sterile normal saline into the left jugular vein of each animal at a dose equivalent to 3.5 mg diminazene base/kg body weight. A six-week wash-out period separated the i.v. dose and the second stage of the experiment in which the same dose of the same preparation was administered into the *rhomboideus* muscle on the left side of the neck. During, and after, each administration of drug, the cattle

were observed for signs of local and systemic toxicity. Transient adverse reactions – salivation, nasal discharge, lachrymation, urination, defaecation and muscle tremors – were observed for 15–30 min following i.v. injection of diminazene aceturate. In addition, there was evidence of pain at the site of i.m. injection which persisted for 0.5–1 h.

Collection of blood

Blood samples were drawn from the right jugular vein of each animal into ethylenediamine tetra-acetic acid (EDTA)-containing 10 ml evacuated tubes (Vacutainer, Becton Dickinson, USA) at 0, 5, 10, 15, 30, 45 min, and 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 21, 22, 24, 30, 36 and 48 h after administration of the drug by both the i.v. and i.m. routes. Following centrifugation of the samples at 1230 × *g* for 30 min, plasma was separated and stored at –70°C until analysed.

Collection of urine

One day before diminazene was administered to the cattle by the i.m. route, indwelling catheters (Inmed™, USA) were placed into the bladders of four of the animals. Complete urine collection was conducted over 2 h intervals, from 0 to 24 h, after administration of the drug. The volume of each 2 h fraction was measured and, after thorough mixing, a 10 ml aliquot was stored at –70°C for subsequent analysis.

Diminazene assay

Paired-ion extraction of intact diminazene from plasma, urine, whole blood, PBS/albumin mixture and ultrafiltrate of the PBS/albumin mixture was performed as a modified version of the method described by Aliu & Odegaard (1983).

Concentrations of intact diminazene in the extracts were determined by HPLC using filtered and degassed mobile phase (acetonitrile 20%: triethylamine 0.2%: water 79.8%) adjusted to pH 4.20 with orthophosphoric acid (all chemical reagents used were of HPLC grade). Extracts of standard solutions of diminazene and collected samples were chromatographed on an Ultrasphere-cyano column (Beckman, USA) of particle diameter 5 μm, internal diameter 4.6 mm and length 15 cm. The column was fitted with an Ultrasphere-cyano guard column of particle diameter 5 μm, internal diameter 4.6 mm and length 4.5 cm (Beckman, USA). The mobile phase flow rate was 1.0 ml/min. Diminazene was quantified at 254 nm and 0.05 absorbance units full scale (AUFS). The detection limit of the assay was 10 ng/ml. A chromatography software, System Gold™ V301 (Beckman, USA), was used to drive the HPLC system.

Pharmacokinetic calculations

A kinetic analysis computer programme, NONLIN (Version 3; Statistical Consultants Inc., KY, USA), was used to determine the least-squares best fit for the plasma diminazene concentration-time data from each animal. The data were not weighted. In addition to NONLIN, another kinetic programme, STATIS 3 (ClydeSoft, Glasgow, UK), was also used to analyse the i.m. data. Initial values used by NONLIN and STATIS 3 were obtained from an exponential curve stripping programme, JANA (Statistical Consultants Inc., KY, USA). The Akaike (Yamaoka *et al.*, 1978) and Schwartz (Schwartz, 1978) information criteria were used to select the model that best fit each data set. The coefficients and exponents of the equations best describing the absorption and disappearance curves were used to calculate the disposition half-lives and major pharmacokinetic parameters as commonly defined (Kinabo & McKellar, 1989). Half-life ($t_{1/2}\lambda_z$) of the terminal phase was estimated as $0.693/\lambda_z$ where λ_z is the hybrid rate constant for the disappearance phase of the curve. The area under the

plasma concentration-time curve ($AUC_{0\text{-}48\ h}$) and its first moment ($AUMC_{0\text{-}48\ h}$) were determined by the trapezoidal rule. Bioavailability (F), mean residence time (MRT), mean absorption time (MAT), total body clearance (CL), apparent volume of distribution at steady-state ($V_{d(ss)}$), half-life values for absorption ($t_{1/2}k_a$) and distribution ($t_{1/2}\lambda_1$ and $t_{1/2}\lambda_2$) were also calculated by standard equations (Gibaldi & Perrier, 1982; Riviere, 1988):

$$MRT = AUMC/AUC$$

$$MAT = MRT_{i.m.} - MRT_{i.v.}$$

$$F = AUC_{i.m.}/AUC_{i.v.}$$

$$CL = Dose/AUC$$

$$V_{d(ss)} = CL \times MRT$$

For heifers treated i.m., the peak plasma concentration (C_{max}) and time taken to attain peak concentrations (t_{max}) were obtained directly from the plasma concentration-time data profiles. Renal clearance (Cl_r) was estimated as the slope following regression of the urinary excretion rate on the plasma concentration (Gibaldi & Perrier, 1982; Saunders *et al.*, 1989). The symbols used in this paper are as defined by Allen *et al.* (1982). Half-life values are expressed as geometric means; values of other parameters are presented as arithmetic mean ± SD.

Partition of diminazene between red blood cells and plasma

At 0.5, 1 and 12 h following administration of diminazene by the i.v. route, blood samples were drawn into evacuated tubes containing EDTA to determine the concentration of diminazene in whole blood. The packed red blood cell volume percent (PCV) of each sample was determined. Subsequently, each whole blood sample was haemolysed with an equal volume of sterile distilled water and kept at -

70°C until analysis. The partition of diminazene between red blood cells and plasma was estimated according to equation 1:

$$C_{rbc} \times PCV = C_{wb} - C_p \times (1-PCV) \qquad (1)$$

where C_{rbc}, C_{wb} and C_p are the concentrations of diminazene in the red blood cell, whole blood and plasma fractions, respectively.

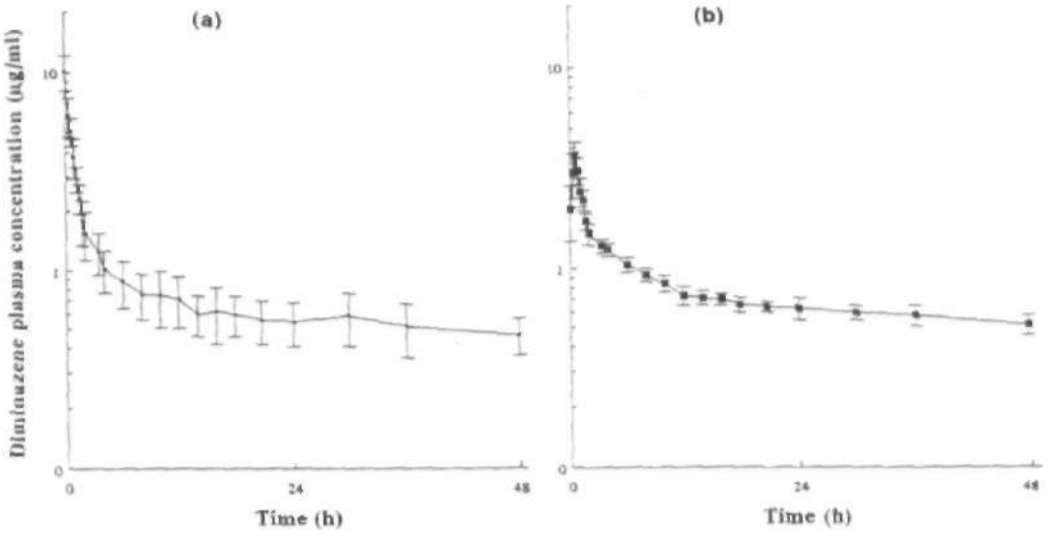
Binding of diminazene to albumin

Binding of diminazene to bovine plasma albumin (Fraction V, BDH Chemicals, Poole, England) was determined according to the method described by Riond & Riviere (1989). A stock solution of 50 μg diminazene/ml 0.2 M phosphate buffered saline (PBS; pH 7.4) was mixed 1:1 with 2×10^{-4} M albumin (in 0.2 M PBS) to obtain final concentrations of 0.5, 1, 2.5, 5 and 10 μg diminazene/ml. The final albumin concentration was thus 1×10^{-4} M. Separation of bound and free fractions was achieved by ultra-filtration of 4 ml of each dilution using an Amicon (Danvers, Massachusetts, USA) microseparation system with a 30,000-Da molecular weight cut-off filter. Total concentrations of diminazene in the PBS/albumin mixture and the ultrafiltrate were determined.

RESULTS

Pharmacokinetics of diminazene after i.v. and i.m. injections

Mean plasma concentration-time profiles of diminazene in the five heifers after i.v. and i.m. injections were plotted on a semi-logarithmic scale (Figs 1a and 1b,



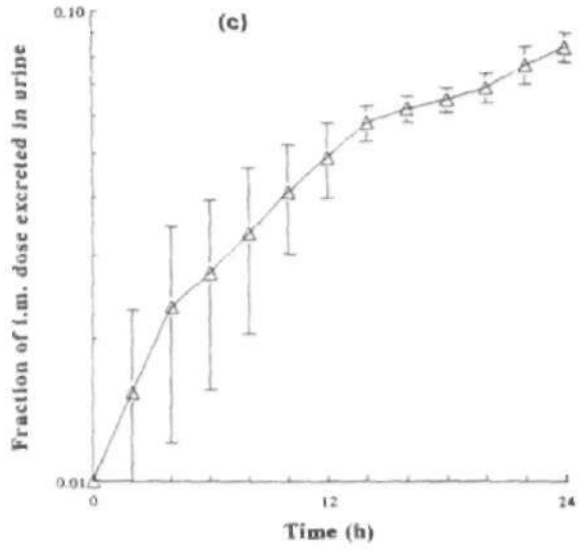


FIG. 1. Mean diminazene plasma concentrations as a function of time in five healthy Boran (*Bos indicus*) cattle following single (a) i.v. and (b) i.m. administration of the drug at a dose of 3.5 mg/kg, and (c) fraction of i.m. dose eliminated in urine.

respectively). In all five animals, the disposition kinetics of diminazene following i.v. administration was best described by a triexponential equation,

$$C_p = C_1e^{-\lambda_1 t} + C_2e^{-\lambda_2 t} + C_ze^{-\lambda_z t} \quad (2),$$

where C_p is the concentration of diminazene in plasma at time t; the coefficients C_1, C_2 and C_z are zero-time intercepts of fast and slow distribution phases, and elimination phase of the drug, respectively; the exponents λ_1 and λ_2 are the rate constants for fast and slow distribution, and λ_z is the elimination rate constant; e is the base of the natural logarithm. The coefficients and exponents (Table I) of equation 2 were used to calculate the major pharmacokinetic parameters (Table II).

Following i.m. administration of diminazene, the individual plasma disappearance curves for all five cattle were best described by equation 3,

$$C_p = C_1e^{-\lambda_1 t} + C_2e^{-\lambda_2 t} + C_ze^{-\lambda_z t} - Ae^{-K_a t} \quad (3),$$

where K_a is the absorption rate constant; A is the absorption coefficient and the other terms remain as defined above. Values of the kinetic parameters which describe the disposition (absorption, distribution and elimination) of diminazene in the cattle are given in Table II.

After i.v. administration, the high values of the biphasic distribution (λ_1 = 20.10 ± 6.41 h^{-1}; λ_2 = 1.20 ± 0.14 h^{-1}) indicated that diminazene was rapidly distributed in body fluids and tissues. This was further substantiated by the large apparent volume of distribution (V_d) at steady-state ($V_{d(ss)}$ = 1.91 ± 0.42 l/kg). The volume of the central compartment, V_c, representing the blood and well-perfused organs and tissues, was 0.12 ± 0.024 l/kg. The geometric mean distribution half-lives ($t_{1/2}\lambda_1$ and $t_{1/2}\lambda_2$) were 0.04 h and 0.58 h, respectively. Diminazene was detected at a concentration of 0.05 ± 0.02 µg/ml in the plasma of three cattle at 4

TABLE I. Pharmacokinetic parameters of diminazene in five healthy Boran *(Bos indicus)* cattle following single i.v. and i.m. doses (3.5 mg/kg).

Parameter	Units	*Intravenous treatment*		*Intramuscular treatment*	
		Mean ± SD	Range	Mean ± SD	Range
Coefficients	μg/ml				
A			—	16.52 ± 7.76	(5.57–22.12)
C_1		24.21 ± 6.10	(18.29–32.06)	14.65 ± 7.60	(3.73–20.00)
C_2		4.98 ± 0.82	(3.71–5.89)	1.19 ± 0.31	(0.72–1.52)
C_z		0.98 ± 0.23	(0.77–1.27)	0.68 ± 0.081	(0.58–0.78)
Exponents	h^{-1}				
K_a			—	6.036 ± 3.14	(4.16–11.60)
λ_1		20.10 ± 6.41	(14.23–28.71)	3.04 ± 0.66	(2.09–3.92)
λ_2		1.20 ± 0.14	(1.06–1.36)	0.19 ± 0.05	(0.14–0.27)
λ_z		0.021 ± 0.002	(0.019–0.024)	0.0051 ± 0.0024	(0.002–0.008)

SD = standard deviation

TABLE II. Absorption and disposition kinetics of diminazene in five healthy Boran *(Bos indicus)* cattle following single i.v. and i.m. doses (3.5 mg/kg).

		Intravenous treatment		*Intramuscular treatment*	
Parameter	Units	Mean ± SD	Range	Mean ± SD	Range
Absorption					
$t_{1/2}ka$*	h	NA		0.60	(0.29–1.27)
t_{max}	min	NA		14.00 ± 2.24	(10–15)
C_{max}	µg/ml	NA		4.68 ± 1.12	(2.99–6.22)
$AUC_{0\text{-}48\,h}$	µg/h/ml	35.03 ± 8.75	(27.38–44.48)	36.55 ± 2.60	(34.01–39.82)
MAT	h	NA		0.98 ± 0.56	(0.43–1.85)
F	(%)	NA		102.42 ± 7.25	(94.87–111.08)
Distribution					
$t_{1/2}\lambda_1$*	h	0.04	(0.024–0.048)	0.23	(0.17–0.33)
$t_{1/2}\lambda_2$*	h	0.58	(0.51–0.65)	3.74	(2.61–4.88)
V_c	l/kg	0.12 ± 0.024	(0.092–0.15)	0.28 ± 0.20	(0.15–0.63)
$V_{d(ss)}$	l/kg	1.91 ± 0.42	(1.44–2.29)	1.84 ± 0.12	(1.68–2.02)
Elimination					
$t_{1/2}\lambda z$*	h	31.71	(27.78–35.94)	145.48	(78.60–301.59)
MRT	h	18.26 ± 0.53	(17.56–18.84)	19.17 ± 0.68	(17.99–19.72)
Cl	ml/min/kg	1.74 ± 0.40	(1.310–2.12)	1.59 ± 0.11	(1.45–1.70)
Cl_r	ml/min/kg	ND		0.31 ± 0.32	(0.02–0.64)

SD = standard deviation

NA = not applicable

ND = not determined

* = geometric mean and range

weeks after administration of the drug. However, 2 weeks later, diminazene was not detected in plasma from any of the five animals.

Diminazene was detected in plasma from all five cattle at 5 min following i.m. administration of the drug, and peak plasma concentrations of 4.68 ± 1.12 μg/ml were attained in 10 to 15 min. Absorption half-life, $t_{1/2}k_a$, averaged 0.60 h. Systemic availability, 102.42 ± 7.25%, was apparently complete. Mean plasma concentrations (C_{mean}) of 1.58 ± 0.22 μg/ml and 0.79 ± 0.11 μg/ml were maintained for 24 h and 48 h, respectively, while minimum plasma concentrations (C_{min}) determined for the same periods were 0.63 ± 0.08 μg/ml and 0.52 ± 0.06 μg/ml, respectively. Mean plasma diminazene concentrations in the 5 animals at 1 and 8 weeks after the i.m. treatment were 0.43 ± 0.03 μg/ml and 0.26 ± 0.18 μg/ml, respectively. In three of the animals, plasma diminazene was detected at a concentration of 0.13 ± 0.06 μg/ml at, but not beyond, the tenth week of treatment.

Renal clearance (Cl_r), determined over the first 24 h following i.m. treatment, was 0.31 ± 0.32 ml/min/kg and accounted for elimination of 8.26 ± 0.79% of the administered dose and 12.40% of the total body clearance determined for the same period (see Fig. 1c).

Partition of diminazene between red blood cells and plasma

Within 30 min after the i.v. injection, diminazene established an apparent equilibrium ratio of 0.30 ± 0.15 between red blood cells and plasma (Table III).

Binding of diminazene to albumin

Results from a study which examined the binding of diminazene *in vitro* to bovine plasma albumin are presented in Table IV. Each data point represents the

TABLE III. Partition of diminazene between red blood cells and plasma in five healthy Boran *(Bos indicus)* cattle following single i.v. doses (3.5 mg diminazene/kg)

(Mean ± SD; n = 5)

Hours after intravenous injection	Conc. of diminazene in plasma (μg/ml)	Conc. of diminazene in whole blood (μg/ml)	Conc. of diminazene in rbc* (μg/ml)	C_{rbc}/C_p ratio of diminazene
0.5	6.65 ± 0.60	5.02 ± 0.27	1.93 ± 0.87	0.30 ± 0.15
1.0	4.65 ± 0.26	3.68 ± 0.21	1.78 ± 0.55	0.38 ± 0.12
12.0	1.24 ± 0.08	1.60 ± 0.07	1.99 ± 0.44	1.61 ± 0.37

Conc. = concentration
* packed cell volume (%) = 34.2 ± 0.03%
C_{rbc} = concentration of diminazene in red blood cells
C_p = concentration of diminazene in plasma
SD = standard deviation

TABLE IV. *In vitro* binding of diminazene to 1.0×10^{-4} M bovine plasma albumin in PBS, pH 7.4

(Mean ± SD; n = 3)

Initial conc. of diminazene (μg/ml) in buffer	Conc. of diminazene (μg/ml) in ultrafiltrate	Percentage of diminazene bound to albumin
0.5	0.33 ± 0.02	38.0 ± 0.3
1.0	0.22 ± 0.01	78.5 ± 0.7
2.5	0.43 ± 0.01	82.7 ± 0.0
5.0	0.44 ± 0.01	91.1 ± 0.1
10.0	1.46 ± 0.14	85.4 ± 1.4

SD = standard deviation
Conc. = concentration

mean value of three independent dupilcate analyses. The extent of binding varied between 38.01% and 91.10% at concentrations of 0.5-5.0 μg diminazene/ml PBS.

DISCUSSION

Disappearance of diminazene from plasma has previously been reported to be biphasic in cattle (Klatt & Hajdu, 1976; Kellner *et al.,* 1985). However, in the work described here, the disappearance curve of plasma diminazene was best described by a triexponential equation on the basis of Akaike and Schwartz information criteria (Yámaoka *et al.*, 1978; Schwartz, 1978). This is consistent with earlier findings in goats and sheep (Aliu *et al.,* 1984; Aliu & Odegaard, 1985).

Diminazene administered i.v. or i.m. was rapidly and extensively distributed in two phases; a fast initial phase (λ_1) and an intermediate second phase (λ_2). The geometric mean half-life values of these two parameters after the i.m. dose were 0.23 h and 3.74 h, respectively. This finding contrasts with the single phase distribution reported by Klatt & Hajdu (1976) and Kellner *et al.* (1985) in which distribution half-lives of 0.85 h and 2.7 h were described following i.m. injection. The reasons for the apparent difference between the values obtained in the earlier studies and in the work described here may be that the efficiency of recovery, high detection limit (0.5 μg/ml), the number of samples examined and intervals of sampling in the previous studies were insufficient for rigorous kinetic analysis to be conducted.

Following i.v. administration, diminazene had a large apparent volume of distribution (1.91 ± 0.42 l/kg) which was greater than the total body water of cattle (0.502–0.645 l/kg [Tasker, 1980]). Since the present study has shown that the drug binds to plasma albumin to a considerable degree (75.0 ± 21.2%), the actual extent of distribution of the unbound fraction may be considerably larger. This would therefore be consistent with the extensive distribution of diminazene in cattle reported by Kellner *et al.* (1985). In the work described here, the volume of the central compartment averaged 0.12 ± 0.024 (0.09–0.15) l/kg. This value is larger than the

plasma volume of cattle (0.028–0.037 l/kg) but less than the extracellular fluid volume (0.263–0.371 l/kg) (Tasker, 1980). Since data from the present study indicates that diminazene penetrates red blood cells (Table III) and the drug undergoes extensive distribution, the drug may also be capable of penetrating other biological membranes such as those within the brain, placenta and mammary gland.

Following i.m. administration of diminazene diaceturate, absorption was rapid with no apparent lag time between dosing and first detection of diminazene in plasma. Amongst the five animals there was little variation in the mean absorption time, peak plasma concentrations, time taken to attain the peak plasma concentrations and bioavailability of diminazene. Plasma concentrations of 1.99 ± 0.62 μg/ml were obtained at 5 min following injection of the drug and the levels peaked within 10–15 min at a concentration of 4.68 ± 1.12 μg/ml. These findings are similar to those of Klatt & Hajdu (1976) and Kellner *et al.* (1985) in which peak plasma levels in cattle were attained within 15–45 min following i.m treatment. Data from the present study also appears to indicate that absorption of i.m. diminazene was complete, 102.42 ± 7.25% (94.87–111.08%), and the rate of absorption, evaluated as $t_{1/2}k_a$, was 0.60 h. These results are comparable with those previously reported for sheep (Aliu & Odegaard, 1985), but differ markedly from those determined for goats when the commercial preparation, Berenil®, was injected i.m. (Aliu *et al.*, 1984). In the latter study, the rate and extent of absorption of diminazene in goats were considerably different from that obtained in cattle and sheep; peak plasma concentrations of 3.9–4.5 μg diminazene/ml were attained at an average time of 48 min following i.m. injection and bioavailability was 50.6 ± 11.4%.

In the present study, the half-life of the disappearance phase of diminazene following i.m. administration was 145.48 h compared with that following i.v. dosing (31.71 h). The apparently prolonged half-life following the i.m. dose may be due to the absorption and elimination processes proceeding in accordance with the `flip-flop' phenomenon (Riegelman & Collier, 1980) thus slowing elimination of the drug from

the body. Since renal clearance of diminazene in this study (0.02–0.64 ml/min/kg) was less than renal inulin clearance (1.30–2.20 ml/min/kg) of cattle (Baggot, 1977), this indicated that the total amount of diminazene excreted in the urine is probably a product of glomerular filtration and tubular reabsorption. Only 7–9% of the i.m. dose was excreted in the urine over the first 24 h following administration of the drug. However, this accounted for 12.40% of the total body clearance determined within the same period. In studies conducted with ^{14}C-diminazene, Gilbert & Newton (1982) concluded that rabbits excreted 65% of the dose (44% in urine and 21% in faeces) in the first 7 days after i.m. treatment. Similarly, in a study conducted in cattle with ^{14}C-diminazene, Kellner *et al.* (1985) demonstrated that 72% and 10% of the i.m. dose were recovered in the urine and faeces, respectively, over the first 20 days after administration of the drug.

In three of the five cattle used in the present study, trace amounts of diminazene (0.05–0.13 μg/ml) were detected in the plasma up to, but not beyond, 10 weeks after i.m. treatment; in the other two animals, the drug was detected only up to 8 weeks at a concentration of 0.13–0.28 μg/ml. This suggests possible prophylactic action and may confirm the observations of van Hoeve & Cunningham (1964) and Gilbert (1983).

Reports on plasma concentrations of diminazene which are trypanocidal are scarce. Hawking (1958) reported that, for *Trypanosoma congolense,* an *in vitro* concentration of 0.5 μg diminazene aceturate/ml culture medium was trypanocidal after 24-h incubation at 37°C. Using an *in vitro* 24-h growth inhibition assay in which bloodstream forms of *T. brucei brucei* were examined, Kaminsky *et al.* (1989) demonstrated that the 24-h effective concentration (EC_{50}) for `drug-sensitive' stocks was 0.018–0.058 μg diminazene aceturate/ml, but was 1.01–2.48 μg/ml for `drug-resistant' stocks. However, both sets of stocks died out when incubated for 2-6 days in the presence of 0.1 μg diminazene aceturate/ml.

In view of the variable susceptibility of trypanosomes to diminazene and the disposition kinetics (including bioavailability) of the drug, i.m. dosage regimens should be designed to maintain blood or tissue concentrations of diminazene at a level sufficiently high to eliminate all trypanosomes. On the basis of the pharmacokinetic parameters obtained in the present study, two i.m. doses of diminazene diaceturate at 7.0 mg/kg, administered 48 h apart, will maintain average steady-state plasma concentrations in excess of 0.3 μg/ml for 6 days. If the stocks described by Kaminsky *et al.* (1989) represent the entire spectrum of resistance in trypanosomes pathogenic for domestic livestock, and if the assay used reflects the sensitivity of the parasites *in vivo,* this therapeutic regimen would appear to be adequate to cure infections with diminazene-sensitive and diminazene-resistant trypanosomes in domestic livestock. This is currently under investigation.

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CHAPTER 4

COMPARATIVE PHARMACOKINETICS OF DIMINAZENE IN NONINFECTED BORAN *(BOS INDICUS)* CATTLE AND BORAN CATTLE INFECTED WITH *TRYPANOSOMA CONGOLENSE*

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ABSTRACT

The pharmacokinetics of diminazene were investigated in five female Boran (*Bos indicus*) cattle, sequentially, during the noninfected state, and during acute and chronic phases of experimental infections with *Trypanosoma congolense*. A 7.0% (wt/vol) solution of diminazene aceturate (Berenil) was used in all three phases of the study and administered as a single intramuscular dose of 3.5 mg diminazene base/kg body weight. There was no significant difference between values of all pharmacokinetic parameters determined in the noninfected cattle and in cattle with a chronic *T. congolense* infection. However, the C_{max} attained during the acute phase of infection, 8.25 ± 1.72 μg/ml, was significantly ($P < 0.01$) greater than that during chronic infection, 5.04 ± 0.26 μg/ml, and in the noninfected state, 4.76 ± 0.76 μg/ml. Similarly, the T_{max} when diminazene was administered during the acute phase of infection, 18.00 ± 6.71 min, was significantly ($P < 0.02$) shorter than that in noninfected cattle, 36.00 ± 8.22 min, and during chronic infection, 33.75 ± 7.50 min. The volume of distribution at steady state during acute infection (1.01 ± 0.31 liter/kg) was significantly ($P < 0.01$) smaller than that in the noninfected state (1.37 ± 0.17 liter/kg) and that in chronic infection (1.51 ± 0.24 liter/kg). Eight hours after the drug had been administered, the concentration-time data profiles for each of the three study phases were very similar. Mean concentrations of diminazene in plasma 48 h after administration of the drug were 0.43 ± 0.07 μg/ml in noninfected cattle, 0.43 ± 0.11 μg/ml during the acute phase of trypanosome infection, and 0.44 ± 0.09 μg/ml in the chronic phase of the infection. Results of the present study indicate that the area under the concentration-time curve for diminazene in trypanosome-infected cattle did not differ significantly from that for noninfected cattle. It therefore appears that the total amount of diminazene attained and maintained in the plasma of cattle is not significantly altered during infection with *T. congolense*.

INTRODUCTION

The compound 1,3-bis(4-amidinophenyl)triazene diaceturate tetrahydrate (7, 14), marketed as Berenil, Ganaseg and Veriben, is known to exert high therapeutic efficacy against a wide range of members of the *Trypanosomatidae* and *Babesidae* (10). For treatment of *Trypanosoma congolense* and *Trypanosoma vivax* infections in domestic livestock, the recommended dose is 3.5 mg of diminazene aceturate per kg body weight (b.w.), administered intramuscularly (i.m.) on one occasion. However, while the single dose is usually curative, relapses are known to occur following treatment (21, 27). These are generally attributed to either innate parasite resistance to diminazene or to re-invasion of the peripheral circulation from trypanosome populations sequestered in sites believed to be inaccessible to diminazene, such as the central nervous system (CNS) (20).

The pharmacokinetics of diminazene have been documented in cattle (2, 23, 24), rabbits (18), goats (5) and sheep (4). However, the effect of a trypanosome infection on the pharmacokinetics of diminazene has been described only in rats (38), rabbits (19) and dogs (35). Since such information would have implications for the therapeutic regime used for cattle, the present study was conducted to determine whether the pharmacokinetics of diminazene in noninfected cattle differed significantly from those in cattle with acute or chronic infection of *T. congolense*.

MATERIALS AND METHODS

Experimental design. The pharmacokinetics of diminazene were determined in five female Boran *(Bos indicus)* cows before and then during acute and chronic infections with *T. congolense*. In each study phase, a single i.m. dose of 3.5 mg of diminazene base per kg b.w. was administered as a 7.0% wt/vol solution (in normal saline) of diminazene aceturate (Berenil [Batch No. 093D574]; Hoechst AG,

Frankfurt, Germany). The interval between the first (no infection) and second (acute infection) study phases was 10 weeks, and that between the second and third (chronic infection) phases was 16 weeks. During the second study phase, cattle were infected with *T. congolense* IL 1180 and treated at the first peak of parasitaemia. At this stage of infection, the mean packed red blood cell volume percent (PCV) (28.0% ± 3.7%) was not significantly different from that before infection (34.0% ± 4.5%). During the third experimental phase, cattle were infected with *T. congolense* IL 2642. However, in contrast to the second experimental phase, infections were allowed to progress until the mean PCV had fallen to 16.2% ± 2.1% before treatment was administered. Hereafter, the terms "noninfected", "acute" and "chronic" refer to the experiments conducted with noninfected cattle, cattle infected with IL 1180 and cattle infected with IL 2642, respectively.

Experimental animals. The ages and body weights (means and standard deviations [SD] given for both) of the cattle used were 18.0 ± 1.4 months and 128.0 ± 22.9 kg before infection, 20.5 ± 1.4 months and 147.2 ± 26.6 kg during acute infection, and 24.5 ± 1.4 months and 157.6 ± 35.7 kg during chronic infection. Prior to the beginning of the experiment, serum samples from the animals were examined using an indirect immunofluorescent antibody test (22) and found to be negative for antibodies to *Trypanosoma, Theileria, Babesia* and *Anaplasma* species. Throughout the period of the study, the animals were kept under fly-proof conditions and fed hay and water *ad libitum*. They were also fed a concentrate ration (Young Stock [300 to 400 g per animal per day]; Belfast Millers, Nairobi, Kenya).

Trypanosomes and infection of cattle. *T. congolense* IL 1180 is a doubly cloned derivative (31) of an isolate collected from a lion in the Serengeti, Tanzania (15). *T. congolense* IL 2642 is a doubly cloned derivative (38) of an isolate collected from a cow in Busoga, Uganda (29). Previous studies have shown that when cattle are infected with a population of *T. congolense* and the infection is eliminated with a trypanocide, variant-specific antibody responses are elicited and confer protection

against homologous challenge for 5 months (1). Thus, *T. congolense* IL 1180 and *T. congolense* IL 2642 were used in the work described here since they are antigenically unrelated (37). In order to infect cattle, trypanosomes were expanded in sublethally irradiated (650 rads) mice. During the first peak of parasitaemia, mice were exsanguinated by cardiac puncture and the blood collected into sodium citrate (final concentration, 1.0%, wt/vol). The blood was pooled, diluted in phosphate saline glucose (pH 7.4) and the number of trypanosomes was determined with a Neubauer haemocytometer. The cattle were then infected by intravenous inoculation of 1.0 X 10^7 trypanosomes per animal. Starting a week prior to initiation of each infection and until 30 days after each treatment, the PCV was determined daily. During the same period, rectal temperature and level of parasitaemia (30) were also monitored on a daily basis.

Collection of blood and quantification of diminazene. During each phase of the study, blood was collected at various time intervals (0, 5, 10, 15, 30, 45 min, and 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 21, 24, 30, 36 and 48 h) following administration of the drug. The methods of blood collection, plasma separation and storage, paired-ion extraction of diminazene from the plasma, and quantification of diminazene in the resultant extract using high-performance liquid chromatography were previously described (2, 3). The limit of detection of the technique is 10 ng diminazene per ml. Over the concentration range consisting of 0.010, 0.05, 0.10, 0.25, 0.50, 1.00, 2.50, 5.00, 10.00 µg/ml, the response of diminazene was linear (r^2 = 0.997). Mean coefficients of variation within and between days were 4.94% and 4.64%, respectively.

Data analysis. The maximum plasma concentration of diminazene (C_{max}), as well as the time (T_{max}) taken to reach C_{max} were determined directly from the concentration-time data of samples collected from 0 to 48 h after administration of the drug. Each concentration-time data set was analysed by least squares non-linear regression analysis using computer programmes: PCNONLIN (40) and STATIS (11).

The model of best fit for each data set was selected on the basis of the Akaike (45) and Schwartz (39) information criteria. Initial values for the regression analysis were obtained from an exponential curve-stripping programme, JANA (41). The data were not weighted. The concentration-time data were also analysed by a non-compartmental approach (17) to calculate the area under the concentration-time curve (AUC) and the area under the (first) moment of the curve (AUMC); both parameters were determined from zero time to 48 h and were not extrapolated to infinity. AUC, AUMC, mean residence time (MRT), apparent total body clearance (CL) and apparent volume of distribution at steady-state (V_{ss}) were calculated in accordance with standard equations (17). Bioavailability (F) was calculated and expressed as a percentage of the ratio of AUC from 0 to 48 h determined in the present study (i.m. administration) to AUC from cattle which were treated intravenously with 3.5 mg of diminazene base per kg b.w. in a previous study (2).

Repeated-measures analysis of variance was used to test the statistical significance of differences between the means of the pharmacokinetic parameters estimated for the three phases of the study; P values less than or equal to 0.05 were considered significant. Statistically significant differences based on the analysis of variance were further evaluated by the Tukey-Kramer method to make pairwise comparisons among means. All statistical analyses were conducted using the statistical software package Minitab (28). Half-life ($t_{1/2}$) values were expressed as geometric means (with ranges); values of other parameters were expressed as arithmetic means ± SD.

RESULTS

Plots of the mean values of the concentration-time data for diminazene in plasma for each of the three phases of the study are shown in Fig. 1. The C_{max} attained when diminazene aceturate was administered to animals during the acute phase of infection (*T. congolense* IL 1180) (8.25 ± 1.72 μg/ml) was significantly (*P*

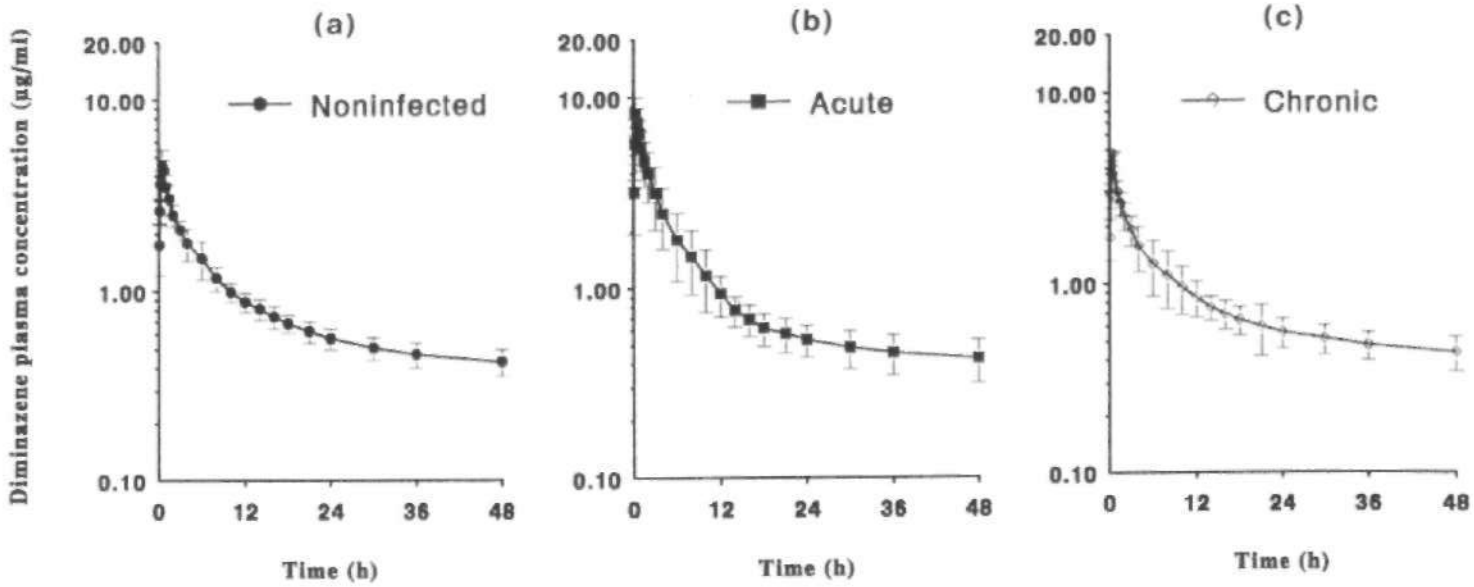


Fig. 1. Absorption and disposition of diminazene in five Boran (*B. indicus*) cows following the administration of a single i.m. dose (3.5 mg/kg of b.w.) before infection (a) and (in the same animals) during acute infection with *T. congolense* IL 1180 (b) and chronic infection with *T. congolense* IL 2642 (c).

< 0.01) greater than that which occurred when the drug was administered during the chronic stage of infection (*T. congolense* IL 2642) (5.04 ± 0.26 μg/ml) and that before infection (4.76 ± 0.76 μg/ml) (Table 1). Similarly, the T_{max} during the acute phase of infection (18.00 ± 6.71 min) was significantly ($P < 0.02$) shorter than that in noninfected cattle (36.00 ± 8.22 min) and that during chronic infection (33.75 ± 7.50 min) (Table 1). Thus, C_{max} and T_{max} did not significantly differ between noninfected cattle and those with a chronic *T. congolense* infection. There were also no significant differences between noninfected cattle and those chronically infected with *T. congolense* in the values of all absorption, distribution and elimination pharmacokinetic parameters that were determined (Tables 1 to 3). Furthermore, 8 h after the drug had been administered, the mean concentration-time data profiles for each of the three study phases were very similar (Fig. 1). Mean concentrations of diminazene in plasma 48 h after administration of the drug were 0.43 ± 0.07 μg/ml in noninfected cattle, 0.43 ± 0.11 μg/ml in the acute phase of trypanosome infection, and 0.44 ± 0.09 μg/ml in the chronic phase of infection.

Values of the pharmacokinetic parameters estimated through compartmental and non-compartmental analyses are summarized in Tables 1 to 3. Of these parameters, V_{ss} during acute infection (1.01 ± 0.31 liter/kg) was significantly ($P <$ 0.01) smaller than that in the noninfected state (1.37 ± 0.17 liter/kg) and that during chronic infection (1.51 ± 0.24 liter/kg). The volume of the central compartment did not differ significantly between the noninfected and trypanosome-infected states. The zero time intercept of the second distribution phase during acute infection (4.97 ± 2.28 μg/ml) was significantly ($P < 0.05$) greater than that in the chronic state (2.06 ± 0.29 μg/ml) and that in the noninfected state (2.26 ± 0.50 μg/ml). In addition, the transfer rate from the shallow peripheral compartment to the central compartment in noninfected cattle (0.39 ± 0.10 h^{-1}) was significantly ($P < 0.05$) slower than that during acute infection, (0.77 ± 0.29 h^{-1}). Finally, there were no significant

TABLE 1. Values of non-compartmental pharmacokinetic parameters following single i.m. administration of 3.5 mg of diminazene per kg of b.w. to five Boran cows before and after infection with *T. congolense*[a]

Infection	C_{max} (μg/ml)	T_{max} (min)	AUC (μg.h/ml)	AUMC (μg.h²/ml)	MRT (h)	CL (ml.min/kg)	V_{ss} (liters/kg)	F (%)
None	4.76 ± 0.76*	36.00 ± 8.22*	40.81 ± 4.34	648.34 ± 79.7	15.89 ± 1.02	1.44 ± 0.15	1.37 ± 0.17*	123.50 ± 37.47
Acute	8.25 ± 1.72*‡	18.00 ± 6.71*‡	49.05 ± 9.43	658.93 ± 130.3	13.56 ± 2.56	1.22 ± 0.22	1.01 ± 0.31*‡	141.86 ± 12.74
Chronic	5.04 ± 0.26‡	33.75 ± 7.50‡	38.89 ± 7.10	639.40 ± 120.8	16.43 ± 0.74	1.53 ± 0.24	1.51 ± 0.24‡	120.48 ± 11.09

[a] The data are means ± SD. Values marked with the same symbols (* or ‡) are significantly different ($P < 0.05$). MRT, mean residence time; CL, total body clearance; F, Bioavailability.

TABLE 2. Pharmacokinetic parameters describing absorption and disposition of diminazene following a single i.m. administration of 3.5 mg diminazene per kg of b.w to five cows before and after infection with *T. congolense*[a]

Infection	Zero time intercept (μg/ml)			K_a (h^{-1})	λ_1 (h^{-1})	λ_2 (h^{-1})	λ_z (h^{-1})
	C_1	C_2	C_z				
None	18.58 ± 4.06	2.26 ± 0.50*	0.68 ± 0.14	3.56 ± 1.33	1.85 ± 0.66	0.15 ± 0.09	0.0099 ± 0.006
Acute	20.02 ± 0.16	4.97 ± 2.28*‡	0.58 ± 0.17	4.51 ± 0.86	2.70 ± 0.56	0.24 ± 0.08	0.0066 ± 0.005
Chronic	19.97 ± 0.05	2.06 ± 0.29‡	0.64 ± 0.10	3.31 ± 0.66	2.33 ± 0.44	0.20 ± 0.08	0.0083 ± 0.006

[a] The data are means ± SD. Values marked with the same symbols (* or ‡) are significantly different ($P < 0.05$). λ_1 and λ_2, rapid and slow exponents, respectively, of the distribution pha C_1 and C_2, zero time intercepts of the rapid and slow distribution phases, respectively. C_z, zero time intercept of the terminal elimination phase.

TABLE 3. Values of three-compartmental model parameters following single i.m. administration of 3.5 mg of diminazene per kg b.w. to five Boran cows before and after infection with *T. congolense*[a]

Infection	$t_{1/2}$				K_{el} (h^{-1})	K_{12} (h^{-1})	K_{13} (h^{-1})	K_{31} (h^{-1})	K_{21} (h^{-1})	V_C (litres/kg)
	k_a (min)	λ_1 (h)	λ_2 (h)	λ_z (h)						
None	12.22 (7.15-16.24)	0.39 (0.25-0.69)	3.94 (2.65-4.77)	86.50 (47.3-354.1)	0.22 ± 0.11	0.85 ± 0.37	0.55 ± 0.23	0.03 ± 0.01	0.39 ± 0.10*	0.17 ± 0.042
Acute	9.35 (7.52-12.87)	0.26 (0.20-0.35)	3.05 (2.16-3.97)	141.60 (57.6-408.0)	0.21 ± 0.13	1.34 ± 0.47	0.59 ± 0.21	0.03 ± 0.02	0.77 ± 0.29*	0.14 ± 0.012
Chronic	12.74 (10.29-16.91)	0.30 (0.24-0.39)	3.67 (2.22-6.20)	107.40 (48.2-388.6)	0.22 ± 0.13	1.09 ± 0.07	0.76 ± 0.24	0.04 ± 0.021	0.43 ± 0.11	0.15 ± 0.002

[a] The data for $t_{1/2}$ are geometric means, with ranges in parenthesis. All other data are means ± SD. Values marked with an asterisk are significantly different ($P < 0.05$). $t_{1/2}\lambda_1$ and $t_{1/2}\lambda_2$, rapid and slow distribution $t_{1/2}$, respectively; K_{el}, elimination constant from central compartment; K_{12} and K_{13}, transfer rate constants from central to shallow and deep peripheral compartments, respectively; K_{31} and K_{21}, transfer rate constants from deep and shallow peripheral compartments, respectively, to the central compartment; V_c, volume of the central compartment.

differences between the data of the three phases with respect to AUC, AUMC, MRT and CL (Table 1).

The temperature at the time of treatment during acute infection (40.2 ± 0.4°C) was significantly ($P < 0.05$) greater than that in the noninfected cattle (38.6 ± 0.2°C) and that in cattle at the time of treatment during chronic infection (38.2 ± 0.5°C). Following treatment of acute and chronic infections, all cattle became aparasitaemic within 24 h of drug administration and remained aparasitaemic throughout the 30-day observation period following treatment.

DISCUSSION

In the work described here, noninfected Boran (*Bos indicus*) cattle were administered diminazene i.m. as the commercial preparation Berenil. Kinetic parameters obtained in this study included plasma C_{max} (4.76 ± 0.76 μg of diminazene per ml), T_{max} (36.00 ± 8.22 min), $t_{1/2}k_a$ (12.22 min) and terminal elimination $t_{1/2}$ ($t_{1/2}\lambda_Z$) (86.50 h). These values are similar to the C_{max} (3.2 to 5.9 μg of diminazene per ml), T_{max} (15 to 30 min) and $t_{1/2}\lambda_2$ (40 to 195 h) that were observed for noninfected Holstein-Freisian (*Bos taurus*) cattle that were also administered diminazene via the i.m. route (as Berenil) (23, 24). Despite their similarity to those in previous studies, however, values of parameters determined in the present study were associated with rather wide confidence intervals. But since diminazene has a long elimination $t_{1/2}$ (23, 24), it would appear that some of these parameters may be more precisely estimated by analysis of concentration-time datum points beyond the 48 h used in the present study.

In a previous study (2), it was reported that i.m. administration of diminazene as the diaceturate salt to noninfected cattle (the same breed and sex as those used in the present study) was followed by rapid absorption of the drug and showed a similar plasma C_{max} (4.68 ± 1.12 μg of diminazene per ml), prolonged $t_{1/2}\lambda_Z$ (145.48 h) and significantly different T_{max} (14.00 ± 2.20 min) and $t_{1/2}k_a$ (36.00 min). Similar

differences in the pharmacokinetics of the two preparations of diminazene were also reported in studies with goats (5) and in sheep (4). The factors responsible for these differences are unknown but may be due to altered elimination of diminazene as a result of inclusion of antipyrine, a chemical stabilizer which comprises 55.5% of the commercial preparation Berenil (13). However, although it is established that cattle oxidatively metabolise antipyrine into several metabolites which are excreted via urine (44), metabolites of diminazene in animals remain unknown. To date, only unchanged diminazene has been recovered from the liver, feces, urine, and milk of cattle that have been treated with diminazene as the diaceturate salt or as Berenil (2, 23, 24).

In the present study, C_{max} and T_{max} in the acute stage of a *T. congolense* infection differed significantly from corresponding values for noninfected cattle and cattle chronically infected with *T. congolense*. The pathophysiological and circulatory changes that accompany trypanosome infections (8, 25) may account for the significantly ($P < 0.01$) higher C_{max} and earlier T_{max} when diminazene was administered to cattle during the acute stage of a trypanosome infection. Furthermore, the possible effects of PCV and parasitaemia on the observed differences in the C_{max} could not be excluded since the analytical technique used for quantifying diminazene quantifies intact diminazene only in the plasma. However, since the PCVs in the noninfected and acute-infection groups were not significantly different at the time of treatment, this would indicate that the difference in C_{max} between these two groups was not due to differences in PCV. Finally, the higher C_{max} in acute infection would appear to result from the significantly lower V_{ss} observed for this state compared to those for the noninfected and chronic-infection states (Table 1).

Earlier studies have shown that in noninfected cattle diminazene penetrates erythrocytes, binds to plasma proteins (2, 4, 6) and accumulates in the liver (19, 23). The compound is then eliminated from the body via the urine and feces (2, 19, 23). It is not known whether the biochemical and haematological changes which occur when

cattle are infected with trypanosomes (32, 42, 43) affect the normal distribution and redistribution of diminazene between the blood and tissues. In the animals used in this study, the V_{ss} in noninfected cattle (1.37 ± 0.17 liter/kg) and that during chronic infection (1.51 ± 0.24 liter/kg) were significantly ($P < 0.05$) greater than that during acute infection (1.01 ± 0.31 liter/kg). Changes in V_{ss} may be associated with changes in drug binding (16). For instance, decreased plasma protein binding of diphenylhydantoin in human uremic patients was associated with significant changes in the the drug's V_{ss} (34). It is therefore possible that differences in diminazene's V_{ss} values for different pathophysiological states are associated with a similar phenomenon. Whether this is the case was not investigated in the present study.

Since the trypanocidal activity of diminazene is dependent on uptake of the molecule by trypanosomes (9, 26, 33), it is possible that the presence of a trypanosome infection at the time of treatment may deplete the drug in plasma (12), thereby resulting in an apparent reduction in the elimination $t_{1/2}$ of the drug. However, results from the present study indicate that the AUC for diminazene did not differ significantly between noninfected and trypanosome-infected cattle. Similarly, in studies with rabbits (19) and dogs (35), the concentration-time data for diminazene in plasma did not differ significantly between noninfected animals and animals infected with *T. congolense*. It would, therefore, appear that the concentration of diminazene that is attained and maintained in treated animals is dependent neither on the presence or absence of a trypanosome infection at the time of treatment, nor on the stage of infection when the drug is administered.

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CHAPTER 5

RESPONSE OF *TRYPANOSOMA CONGOLENSE* IN GOATS TO SINGLE AND DOUBLE TREATMENT WITH DIMINAZENE ACETURATE

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Summary

Diminazene aceturate (Berenil) is one of a limited number of compounds currently marketed for treatment of trypanosomiasis in cattle, sheep and goats. The pharmacokinetics of the compound in goats suggest that double treatment with diminazene aceturate might enhance the compound's therapeutic activity. A study was therefore conducted in goats using two clones of *Trypanosoma congolense*, IL 3274 and IL 1180, which were previously shown to be resistant and sensitive, respectively, to single treatment with diminazene aceturate. The results indicated that, as compared to single treatment, double treatment with diminazene aceturate at a dose of 7.2 mg kg^{-1} bodyweight, at either eight or 24 hour intervals, did not greatly enhance the therapeutic activity of the drug. Furthermore, treatment with the same drug dose eliminated infections with *T congolense* IL 3274 when treatment was administered 24 hours after infected *Glossina morsitans centralis* had fed, but failed to do so if treatment was delayed until after goats were detected parasitaemic. This suggests that failure of *T congolense* IL 3274 to respond to treatment with diminazene may not be due to drug resistance *per se*.

Introduction

CHEMOTHERAPY is an important means of control of trypanosomiasis in domestic livestock across Africa (Leach and Roberts 1981) and in cattle, sheep and goats relies upon the salts of three compounds: the phenanthridine, homidium; the phenanthridine aromatic amidine, isometamidium, and the aromatic diamidine, diminazene. Despite the proven efficacy of these compounds in the field over the past 30 years, chemotherapy of trypanosomiasis is beset with a number of problems: a limited range of compounds, the occurrence of drug resistance and drug toxicity (Williamson 1976, Leach and Roberts 1981).

Diminazene aceturate (Berenil, Hoechst; Veriben, Sanofi Animal Health) was introduced in 1955 as a therapeutic agent for the treatment of *Trypanosoma*

congolense, *T vivax* and *T brucei* infections in cattle, sheep and goats. The manufacturers of the compound recommend that it should be administered to animals via the intramuscular route at doses of either 3.5 or 7 mg kg^{-1} bodyweight, and for some time after its introduction use of the compound was rarely associated with the development of drug resistance (Leach and Roberts 1981). However, subsequently, there have been reports of resistance to diminazene from parts of Africa (Jones-Davies 1967, Kupper and Wolters 1983, Mbwambo et al 1988), though whether the resistance described in these reports was true resistance to diminazene or cross resistance as a result of using other compounds is unclear (Leach and Roberts 1981).

Under ideal circumstances, when resistance to diminazene develops in the field, it is recommended that use of the compound should be terminated and that it is replaced with a compound that is chemically unrelated (that is, homidium or isometamidium [Whiteside 1958]). However, this is often not possible due to logistical and financial factors (Holmes and Scott 1982).

Studies in goats have shown that following intramuscular administration of diminazene aceturate at a dose of 3.5 mg kg^{-1} bodyweight, a maximal plasma concentration of 4.2 µg diminazene ml^{-1} is attained 48 minutes after administration, and that the plasma fall-off is tri-exponential with an elimination half-life of 14 to 29 hours (Aliu et al 1984). Baggot (1978) has suggested that if a drug has a half-life in excess of nine hours, initiation of therapy with a priming dose and maintenance of blood levels by administering a second dose at an appropriate time interval is likely to be more successful than treatment with a single large dose. Studies on asinine and canine babesiosis have demonstrated that if diminazene aceturate is administered in two doses, 24 hours apart, it is safer and more efficacious than a single large dose (Singh et al 1980, Farwell et al 1982). Since the plasma concentration of diminazene in goats declines rapidly following administration, single dose therapy with diminazene aceturate, the recommended regime for treatment of African animal trypanosomiasis, may be responsible for the apparent lack of efficacy observed with diminazene in some

areas. The aim of this study was therefore to determine whether double treatment with diminazene aceturate would enhance the therapeutic activity of the compound in goats as compared to single treatment.

Materials and Methods

Animals

Adult castrated male East African cross Galla goats weighing 20 to 44 kg were obtained from a tsetse-free area of Kenya. The housing and management of these animals were as described by Whitelaw et al (1986).

Outbred Swiss white mice, weighing 25 to 35 g were obtained from the ILRAD breeding colony. This colony was derived from an OLAC (Oxfordshire Laboratory Animal Colony) 1976 strain.

Trypanosomes

Two populations of *T congolense* were used in this study: IL 3274 and IL 1180.

T congolense IL 3274 is a cloned population and is a derivative of field isolate Banankeledaga/83/CRTA/67, collected from a cow in Burkina Faso in 1983 (Pinder and Authie 1984). The derivation of clone IL 3274 from the field isolate was described by Peregrine et al (1991). In goats, IL 3274 was shown to be refractory to intramuscular treatment with diminazene aceturate at a dose of 7 mg kg^{-1} bodyweight when treatment was administered after trypanosomes were detected in jugular blood (A.S. Peregrine, unpublished data). In mice, IL 3274 has a diminazene aceturate 50% curative dose (CD_{50}) value of 12.4 mg kg^{-1} bodyweight (Peregrine et al 1991).

T congolense IL 1180 is a double-cloned derivative of isolate Serengeti/71/STIB/212. The derivation of IL 1180 from this isolate has been described by Nantulya et al (1984). Previous studies have shown that this clone

expresses a high level of sensitivity to diminazene in mice (CD_{50} = 2.3 mg kg^{-1} bodyweight [Peregrine et al 1991]) and in cattle (A.S. Peregrine, unpublished data).

Characterisation of T congolense *IL 3274 and* T congolense *IL 1180* in vitro

The sensitivity of *T congolense* IL 3274 and *T congolense* IL 1180 to diminazene was determined in vitro (M.A. Gray, personal communication). Briefly, bloodstream forms collected from infected mice were adapted to in vitro cultivation and grown as epimastigote forms. Once established as epimastigote cultures they transformed to metacyclic trypanosomes (Gray et al 1987). Subsequently, aliquots of media containing 2.0×10^5 metacyclic trypanosomes ml^{-1} and various concentrations of diminazene aceturate (0.05 to 50.0 $\mu g\ ml^{-1}$), were incubated at 35°C in 5 per cent carbon dioxide in air for 48 hours. Aliquots (200 µl) of each drug dilution containing trypanosomes were then placed on a confluent monolayer of bovine aortic-endothelial cells. A complete medium change, with medium free of drug was made two hours later and thereafter at 48 hour intervals. The sensitivity of the two *T congolense* clones was subsequently determined on the basis of whether or not, after five days incubation in medium free of drug, trypanosomes remained intact and viable. Previous work has indicated that viable trypanosomes present on day 5 are infective for mice (M.A. Gray, personal communication).

Tsetse flies

Teneral male *Glossina morsitans centralis*, obtained from the ILRAD breeding colony, were infected with *T congolense* IL 3274 or *T congolense* IL 1180 by allowing them to feed on goats infected with either of the two populations. The techniques for producing infected flies, for ascertaining their infectivity and for using them to infect experimental cattle have been described by Whitelaw et al (1986). Goats were infected by allowing five infected tsetse flies to feed on the left flank of each animal.

Treatment with diminazene aceturate

Diminazene aceturate (Berenil; Hoechst, lot Number 093D574) was freshly prepared by dissolving in sterile distilled water, at room temperature, to make a 7 per cent w/v solution. Doses of 7.2 mg kg^{-1} bodyweight were injected either singly or on two occasions, eight or 24 hours apart, deep into the right musculus semitendinosus.

Clinical observations

Skin thickness at the site where each tsetse was to be fed, and the breadth of the prefemoral lymph node draining the site of tsetse bites, were measured for three days before infection, and daily for 30 days following infection. Measurements were conducted with vernier callipers.

The rectal temperature of each animal was monitored daily for 30 days following infection and then three times weekly for a further 60 days.

The packed red blood cell volume percentage (PCV) was monitored daily until 90 days after infection, using a microhaematocrit centrifuge. Subsequently, the PCV was determined three times a week for a further 30 days.

Bodyweights were determined weekly throughout the experiment and when required for calculating drug doses. Observations for pain at the site of injection and signs of systemic toxicity were made immediately after treatment, then daily for seven days.

Monitoring of goat infections

Jugular blood samples were collected daily for 90 days following infection, into vacutainer tubes coated with potassium ethylenediamine tetra-acetate (EDTA). Thereafter, for a further 30 days, blood samples were collected three times a week. Blood samples were examined for the presence of trypanosomes using the buffy-coat phase-contrast technique (Murray et al 1977) and the level of parasitaemia quantified.

In an attempt to detect levels of parasitaemia below the lower limit of detection of the buffy-coat phase-contrast technique, 0.5 ml aliquots of jugular blood were inoculated intraperitoneally into sublethally irradiated (600 rads) mice every two weeks following infection of the goats. Mice were then monitored three times a week, for 60 days, for the presence of parasites, by examining wet films of tail blood.

Experimental design

Twenty-three goats were weight-matched into four groups of five animals each (Groups A, B, C and D) and one group of three animals (Group E). Each goat was challenged with five infective tsetse flies and treated intramuscularly with 7.2 mg diminazene aceturate kg^{-1} bodyweight as detailed in Table 1. Animals in Group A received a single dose of diminazene aceturate three days after all the animals had been detected parasitaemic to confirm that IL 3274 was resistant to this dosage regimen. Animals in Groups B and C were also treated three days after all the animals in each group had been detected parasitaemic. However, they were then retreated with the same drug dosage eight or 24 hours later, respectively; intervals which were selected on the basis of previous pharmacokinetic studies (Aliu et al 1984) and which were deemed to be practical in the field. Since Jennings et al (1979) have demonstrated that, in mice, populations of *T brucei* may invade the central nervous system (CNS) within 14 days of infection, and that diminazene aceturate will not eliminate these infections, it was possible that appearance of trypanosomes in animals in Groups A, B and C after treatment was not due to drug resistance, but to invasion of a site, or sites, within the host which was inaccessible to diminazene. Thus, animals in Group D were treated 24 hours after infection (at a time when trypanosomes still appear to be localised in the skin [Emery and Moloo 1981] and have not had access to sites within the body which are inaccessible to

TABLE 1: Experimental design, level of parasitaemia in experimental goats and response to treatment

	A	B	C	D	E
T congolense infection*	IL 3274	IL 3274	IL 3274	IL 3274	IL 1180
Prepatent period (days)	13.4 ± 0.5	13.6 ± 1.1	14.0 ± 1.4	NTD	12.0 ± 1.0
Day of treatment	19	19	19 + 20	1	19
Treatment with diminazene aceturate	7.2 mg kg^{-1} x 1	7.2 mg kg^{-1} x 2 8 h apart	7.2 mg kg^{-1} x 2 24 h apart	7.2 mg kg^{-1} x 1 24 h after infection	7.2 mg kg^{-1} x 1
Level of parasitaemia when treated	5.0×10^5 ml^{-1}	5.0×10^5 ml^{-1}	5.0×10^5 ml^{-1}	0.0 ml^{-1}	1.0×10^4 ml^{-1}
No. relapsed/No. treated	5/5	3/5	4/5	0/5	0/3
Relapse period (days)	9.4 ± 2.5	11.7 ± 2.1	14.3 ± 1.0	NTD	NTD

* Five infected *Glossina morsitans centralis* used to infect each animal
× 1 Treated once
× 2 Treated twice
h Hours
NTD No trypanosomes detected

diminazene) to demonstrate whether IL 3274 was truly resistant to the drug dosage used. Animals in Group E were infected with *T congolense* IL 1180 and treated after all the animals in the group had been detected parasitaemic to confirm the efficacy of a single dose of diminazene aceturate against a sensitive population.

High-performance liquid chromatography

At one, eight, nine, 24, 25 and 48 hours after administration of diminazene aceturate to the goats in Groups B and C, 10 ml whole blood samples were drawn from the left jugular vein of each animal into EDTA-containing evacuated tubes. The samples were centrifuged at 1230 *g* and plasma subsequently separated and stored at –70°C until analysed. Intact diminazene in the plasma was extracted by an ion-pair technique and quantified by high-performance liquid chromatography (Aliu and Odegaard 1983).

Results

Development of parasitaemia

All animals except those in Group D became parasitaemic following feeding of infective tsetse. The mean pre-patent periods were 13.6 ± 1.4 days for animals infected with *T congolense* IL 3274 and 12 ± 1 days for animals infected with *T congolense* IL 1180 (Table 1). In all animals, once trypanosomes were detected following infection they continued to be detected on every occasion until the day of treatment. At the time of treatment, animals in Groups A, B and C, infected with *T congolense* IL 3274, had a mean level of parasitaemia of 5×10^5 trypanosomes ml^{-1} of jugular blood, while animals in Group E, infected with *T congolense* IL 1180, had a mean level of parasitaemia of 1×10^4 ml^{-1}. All animals in Groups A, B, C and E were treated on day 19 after infection. Animals in Group D, treated 24 hours following infection, were aparasitaemic at the time of treatment.

Following treatment, animals infected with *T congolense* IL 3274 became aparasitaemic within an average interval of 48 hours. In contrast, animals infected with *T congolense* IL 1180 became aparasitaemic within an interval of 24 hours (data not given). In all animals in Group A (which received treatment with a single dose of diminazene aceturate), trypanosomes reappeared following treatment within an interval of 9.4 ± 2.5 days. Similarly, in three of the five animals in Group B and four of the five animals in Group C, which received repeat treatment with diminazene aceturate, parasites reappeared following treatment within mean intervals of 11.7 ± 2.1 and 14.3 ± 1.0 days, respectively. In contrast, all five animals in Group D, treated 24 hours after infection, were never subsequently detected parasitaemic. Similarly, all three animals in Group E, infected with *T congolense* IL 1180 and treated with diminazene aceturate following detection of trypanosomes in jugular blood, were never detected parasitaemic following treatment.

All animals in which trypanosomes reappeared following treatment were detected parasitaemic on every occasion a blood sample was collected until day 47 after infection, when they were removed from the experiment because their PCVs had fallen below 18 per cent. Animals in which trypanosomes could not be detected on day 47 (two in Group B, one in Group C, all five in Group D and all three in Group E) remained aparasitaemic for the entire observation period (120 days) following infection, both by phase-contrast examination of the buffy coat and by inoculation of blood into mice.

PCV

Following infection there was a progressive reduction in PCV in all animals, except those in Group D, until treatment was administered on day 19 following infection. All animals in Group D, which were treated 24 hours after infection with *T congolense* IL 3274, were never detected parasitaemic and maintained their PCV above 25 per cent.

Skin-fold thickness

Significant increases in skin-fold thickness occurred at the site of tsetse bites in all animals except those in Group D (treated 24 hours following infection) (Table 2). The maximum skin-fold thickness was attained on days 12 to 13 in all animals infected with *T congolense* IL 3274 and on day 9 in animals infected with *T congolense* IL 1180. Infections with *T congolense* IL 1180 (Group E) resulted in a significantly larger increase in skin-fold thickness than that which occurred in goats following infection with *T congolense* IL 3274 (Groups A, B and C).

Breadth of draining prefemoral lymph node

Significant increases in the breadth of the prefemoral lymph node draining the site of tsetse bites occurred in all experimental animals (Table 2). This increase in breadth was detected as early as three days following infection (data not given) and was at a maximum on approximately day 13 following infection for animals in Groups A, B, C and D, infected with *T congolense* IL 3274, and on day 12 for animals in Group E, infected with *T congolense* IL 1180.

Characterisation of T congolense *IL 3274 and* T congolense *IL 1180* in vitro

Using a culture system in which metacyclic trypanosomes were incubated in the presence of various drug concentrations for 48 hours, *T congolense* IL 3274 was shown to be resistant to 10 μg diminazene aceturate ml^{-1} media, but sensitive to 50 μg ml^{-1}. In contrast, *T congolense* IL 1180 was resistant to 1 μg ml^{-1} but sensitive to 5 μg ml^{-1}.

High-performance liquid chromatography

Analysis of plasma samples from goats in Groups B and C demonstrated that the mean plasma concentration of diminazene from one to 48 hours following treatment was 1.52 ± 0.11 μg ml^{-1} (data not shown).

TABLE 2: Mean skin-fold thickness and prefemoral lymph node breadth in animals infected with *T congolense* IL 3274 or *T congolense* IL 1180

	Group A	B	C	D	E
T congolense infection	IL 3274	IL 3274	IL 3274	IL 3274	IL 1180
Skin fold thickness					
Days to maximum size	11.8 ± 1.1	12.0 ± 1.0	13.4 ± 1.5	-	9.7 ± 0.6
% increase in size	160	210	160	-	260
Prefemoral lymph node breadth					
Days to maximum size	12.6 ± 1.1	12.4 ± 0.9	12.6 ± 0.5	16.8 ± 4.7	11.0 ± 1.0
% increase in size	200	310	270	220	400

- No increase in thickness

Discussion

This study has demonstrated that both the time of treatment in relation to the stage of infection with *T congolense*, and the number of treatments, influence the therapeutic activity of diminazene in goats.

In the work described here, infections with *T congolense* IL 3274 were not eliminated in any of the animals in Group A. In contrast, infections were eliminated in two animals in Group B and in one animal in Group C. Thus, repeat treatment with diminazene aceturate at a dose of 7.2 mg kg^{-1} bodyweight eliminated the infection in three out of 10 animals. Furthermore, in the other seven animals reappearance of trypanosomes following treatment was significantly delayed compared to animals in Group A, treated with diminazene aceturate on only one occasion. Since the mean plasma concentration of diminazene over the first 48 hours following treatment was 1.52 ± 0.11 μg ml^{-1} for animals in Groups B and C, and metacyclic trypanosomes of *T congolense* IL 3274 remained viable after 48 hours incubation in vitro in media containing diminazene aceturate at a concentration of 10 μg ml^{-1}, the inability of double treatment with diminazene aceturate to eliminate infections with IL 3274 in seven out of 10 animals was not surprising. It would therefore appear that when animals are infected with trypanosomes that express a high level of resistance to diminazene, double treatment with diminazene aceturate, at either eight or 24-hour intervals, does not greatly enhance the therapeutic activity of the drug.

Whether *T congolense* IL 3274 is truly resistant to diminazene in goats is open to question as a result of findings with animals in Group D. These animals were infected with IL 3274 via the bites of infected tsetse and treated 24 hours later with diminazene aceturate at a dose of 7.2 mg kg^{-1} bodyweight. However, in contrast to animals in Group A, trypanosomes were not detected in any of the five animals following treatment. Thus, *T congolense* IL 3274 appeared to be sensitive to diminazene when goats were treated 24 hours after feeding of infected tsetse (Group D), but resistant when treated on day 19 (Group A). There is, therefore, an apparent

difference in sensitivity of IL 3274 to diminazene between day 1 and day 19 following tsetse fly-transmitted infection. The basis of this phenomenon is unclear.

The development of local skin reactions, or chancres, at the site of inoculation of metacyclic trypanosomes by tsetse is a well-recognised phenomenon in man (Fairbairn and Godfrey 1957). A similar lesion has also been observed in goats following tsetse transmission of *T congolense* (Emery and Moloo 1981) and was produced in the study described here by both *T congolense* IL 3274 and *T congolense* IL 1180. Fluid expressed from these lesions has been shown to contain trypanosomes several days before they can be detected in the peripheral blood (Roberts et al 1969). Furthermore, microscopic examination of such lesions seven days after tsetse infected with *T congolense* have fed on rabbits, has demonstrated the presence of trypanosomes in the dermal connective tissue (Luckins and Gray 1978) which have been termed `local-reaction' or `lesion' forms (Roberts et al 1969). It is, therefore, possible that trypanosomes would be present in this extravascular site 24 hours after tsetse have fed and that the apparent sensitivity of IL 3274 to diminazene on day 1 is because the life-cycle stage of IL 3274 present in the goat on day 1 differs in its sensitivity to diminazene from that present on day 19 (that is, bloodstream forms). Trypanosomes which are morphologically similar to lesion forms of *T congolense* have been observed in vitro (Gray et al 1979). However, in vitro, this life-cycle stage does not appear to differ from metacyclic forms in its sensitivity to diminazene (M.A. Gray, unpublished data).

A second factor that might be responsible for the apparent difference in drug sensitivity of *T congolense* IL 3274 between day 1 and day 19 could be a difference in the pharmacokinetics of diminazene between the blood and the dermis. Aliu et al (1984) have described the pharmacokinetics of diminazene in the blood of goats but no data exist for the dermis. It is possible that significantly higher concentrations of diminazene are attained in the dermis than in blood and this possibility is currently under investigation.

Invasion of a site, or sites, within the goat which is, or are, inaccessible to diminazene is a third factor that could be responsible for the apparent drug resistance expressed by IL 3274 on day 19. Such a phenomenon has been described in mice infected with *T brucei* (Jennings et al 1977, 1979). Treatment with diminazene aceturate eliminated infections if the drug was administered seven days after infection. However, trypanosomes reappeared following administration of the drug if treatment was delayed until 14 days after infection. The appearance of trypanosomes was subsequently shown to be associated with trypanosomes that had invaded the CNS. While *T brucei* has been demonstrated to occur within the CNS of cattle (Masake et al 1984) and *T vivax* has been detected in the aqueous humor of goats (Whitelaw et al 1988), *T congolense* has only been demonstrated within the CNS of ruminants when animals were infected simultaneously with both *T congolense* and *T brucei* (Masake et al 1984).

One further possible explanation for the apparent difference in sensitivity of IL 3274 between day 1 and day 19 of infection is that within this period the level of drug resistance expressed by the trypanosomes had altered. Peregrine et al (1991) have conducted experiments in mice which demonstrate that resistance to isometamidium is not a stable phenotype in *T congolense*. It is possible that the same holds true for diminazene.

Finally, there were clear differences between *T congolense* IL 3274 and *T congolense* IL 1180 in terms of the chancre development elicited and the reaction that occurred in the prefemoral lymph node draining the site of tsetse bites. Infection with *T congolense* IL 1180 resulted in an increase in skin-fold thickness that was larger, and occurred earlier, than that elicited by *T congolense* IL 3274. The same was true for the reaction elicited in the draining prefemoral node. For both IL 1180 and IL 3274, the maximum increase in skin-fold thickness occurred 1-2 days before detection of trypanosomes in the blood. Furthermore, enlargement of the draining lymph node accompanied the chancre reaction in all animals in Groups A, B, C and E. This is

consistent with the results of previous studies in goats (Emery and Moloo 1981). However, unlike the aforementioned studies, in the study described here, enlargement of the draining lymph node was not necessarily indicative of ensuing parasitaemia: goats in Group D, treated 24 hours after infection, exhibited significant increases in the breadth of the lymph node draining the site of tsetse bites but were not subsequently detected parasitaemic. In a similar manner, studies in rabbits have demonstrated that development of a chancre is not always indicative of subsequent systemic infection (Silayo 1984).

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CHAPTER 6

VARIATION IN SENSITIVITY OF *TRYPANOSOMA CONGOLENSE* TO DIMINAZENE DURING THE EARLY PHASE OF TSETSE-TRANSMITTED INFECTION IN GOATS

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ABSTRACT

Mamman, M., Katende, J., Moloo, S.K. and Peregrine, A.S. Variation in sensitivity of *Trypanosoma congolense* to diminazene during the early phase of tsetse-transmitted infection in goats. *Vet. Parasitol.*, **50**, 1-14 (1993).

Twenty-five goats were randomly allocated to five groups of five animals each and infected with *Trypanosoma congolense* IL 3274 via the bites of infected *Glossina morsitans centralis*. At intervals of 1, 4, 8, 12 or 19 days following infection, each group of five animals was treated intramuscularly with diminazene aceturate at a dose of 7.0 mg kg^{-1} body weight (b.w.). Whilst treatment on Day 1 eliminated infections in all five goats, treatment on Day 19 did not cure any of the animals; in groups treated 4, 8 or 12 days following infection, two out of five goats in each group were cured. Since the alteration in apparent resistance of *T. congolense* IL 3274 between Day 1 and Day 19 could have been due to alteration in expression of drug resistance by trypanosomes as the population expanded, the experiment was repeated using trypanosomes that reappeared in the animals that had been treated with diminazene aceturate on Day 19. On Day 36, when all five animals were parasitaemic, five groups of teneral *G. m. centralis*, each containing 160 flies, were fed on one occasion on each of the five goats (one group of tsetse flies per goat). Thereafter, each group of tsetse flies was maintained on clean rabbits. When infective, five flies from each group were allowed to feed on two naive goats each (i.e., two goats per group of tsetse flies). One animal in each pair was treated 24 h after infection with diminazene aceturate at a dose of 7.0 mg kg^{-1} b.w., the other was treated on Day 19, when parasitaemic, with the same drug dosage. As before, treatment 24 h following infection eliminated infections in all animals, but when treatment was delayed until Day 19, trypanosomes in all animals were refractory to treatment. Thus, although tsetse flies were infected with trypanosomes that had arisen in infected goats

following treatment with diminazene aceturate at a dose of 7.0 mg kg^{-1} b.w., when the same flies were allowed to feed on clean goats, the resultant infections were sensitive to treatment with the same drug dosage when administered 24 h following infection. These data therefore indicate that there is a significant alteration in diminazene sensitivity of IL 3274 between Day 1 and Day 19 and that this is associated with an alteration in resistance phenotype of trypanosomes.

INTRODUCTION

Diminazene aceturate (Bauer, 1955) is the most commonly used agent for treatment of trypanosomosis in domestic livestock and is marketed as a commercial preparation (Berenil®, Veriben®) consisting of 44.5% diminazene aceturate and 55.5% of the chemical stabilizer antipyrine. While reports of resistance to diminazene in the field continue to be made (Jones-Davies, 1967; MacLennan and Na'Isa, 1970; Kupper and Wolters, 1983; Mbwambo et al., 1988), the mechanisms of resistance in trypanosomes are poorly understood. Studies in vitro have demonstrated pronounced differences among trypanosome populations concerning their sensitivity to diminazene (Brun and Kunz, 1989; Kaminsky et al., 1989; Silayo et al., 1992), thus showing that innate differences in drug sensitivity may play a role. However, since the central nervous system is believed to be inaccessible to diminazene (Gutteridge and Coombs, 1977) and may be a source of relapse infections following treatment of mice infected with *Trypanosoma brucei* (Jennings et al., 1979) and goats infected with *T. vivax* (Whitelaw et al., 1988), the ability of a trypanosome population to be refractory to treatment may also be dependent upon the ability of trypanosomes to gain entry to such sites.

In preliminary work, Silayo et al. (1992) reported a study that was carried out in goats infected with a clone of *T. congolense*: IL 3274. Infections were initiated in goats via the bites of infected *Glossina morsitans centralis* and animals were cured if

treated 24 h following challenge with a single intramuscular (i.m.) dose of 7.0 mg diminazene aceturate kg^{-1} body weight (b.w.). However, if treatment with the same drug dosage was delayed until 19 days following infection, trypanosomes reappeared in all goats within 7-12 days following treatment. The work described here was carried out to further elucidate the mechanism(s) behind these observations.

MATERIALS AND METHODS

Experiment animals

Adult castrated male East African Galla cross-bred goats were obtained from an area of Kenya known to be free of tsetse flies. The goats weighed 15-28 kg and were maintained as described previously (Whitelaw et al., 1986). Prior to the experiment, sera were collected from all goats and examined for the presence of trypanosome antigens (Nantulya and Lindqvist, 1989) and anti-trypanosome antibodies (see below), using enzyme-linked immunosorbent assays (ELISAs).

Outbred $C_3H.He$ mice weighing 20–35 g were obtained from the ILRAD breeding colony (originally derived from Ola:MF1 and hairy progeny MF1-hr/Ola). Mice were allowed access to water and a pelleted ration (Unga Feeds Ltd., Nairobi) on an ad libitum basis.

Trypanosomes

Trypanosoma congolense clone IL 3274 (Peregrine et al., 1991) is a derivative of stock Banankeledaga/83/CRTA/67, which was isolated from a bull in Burkina Faso (Pinder and Authie, 1984). Earlier work has shown that this stock expresses high levels of resistance to both diminazene aceturate and isometamidium chloride (Authie, 1984; Moloo and Kutuza, 1990). In goats, *T. congolense* IL 3274 has previously been shown to be resistant to single i.m. treatment with diminazene aceturate at a

dose of 7.0 mg kg^{-1} b.w. when treatment was administered approximately 3 days after first detection of parasitaemia (Silayo et al., 1992). In mice, the diminazene aceturate CD_{50} value (dose of drug required to cure 50% of infected animals) for *T. congolense* IL 3274 is 12.4 mg kg^{-1} b.w. (Peregrine et al., 1991).

T. congolense IL 1180 is a doubly cloned derivative of stock Serengeti/77/STIB/212 which was isolated from a lion in Tanzania (Geigy and Kauffmann, 1973). The derivation of *T. congolense* IL 1180 from this isolate has been described previously (Nantulya et al., 1984). In mice, the diminazene aceturate CD_{50} value of *T. congolense* IL 1180 is 2.3 mg kg^{-1} b.w. (Peregrine et al., 1991).

Tsetse flies

Teneral male *G. m. centralis* flies were infected with either *T. congolense* IL 3274 or *T. congolense* IL 1180 by allowing them to feed on infected goats. The methods used to infect flies, to identify those with mature infections and to infect animals, have been described previously (Whitelaw et al., 1986). Goats were infected by allowing 5 infected tsetse flies to engorge on the left flank of each animal.

Treatment of goats with diminazene aceturate

At different time intervals following tsetse infection (see Table 1), goats in each experimental group were treated with diminazene aceturate (Berenil®, Hoechst AG, Germany) at a dose of 7.0 mg kg^{-1} b.w., administered deep into the right vastus medialis muscle. A fresh 7.0% (w/v) solution of the drug was prepared in sterile

TABLE 1

Experiment design and development of infection in goats treated with a single intramuscular dose of 7.0 mg diminazene aceturate kg^{-1} b.w. on days 1, 4, 8, 12 or 19 after challenge with tsetse flies infected with *Trypanosoma congolense* IL 3274 or IL 1180

Group	No. of goats infected	*T. congolense* challenge[a]	Time of treatment (days following infection)	Treatment with diminazene aceturate (mg kg^{-1}, i.m.)	No. cured/ No. treated	First day detected parasitaemic following infection X ± SD (Range)
A	5	IL 3274	1	7.0	5/5	-[b]
B	5	IL 3274	4	7.0	2/5	47 ± 25 (30-76)
C	5	IL 3274	8	7.0	2/5	25 ± 3 (22-28)
D	5	IL 3274	12	7.0	2/5	26 ± 4 (23-31)
E	5	IL 3274	19	7.0	0/5	17 ± 2 (15-19)
F	5	IL 1180	19	7.0	5/5	12 ± 1 (11-13)

[a]Five infective *Glossina morsitans centralis* per goat.
[b]Parasitaemia was never detected in any of the five goats in this group.

distilled water on the day of administration. Berenil® batch Number 093D574 was used throughout this work.

Monitoring goat infections

For 7 days prior to infection and for 30 days following the infection, blood samples were collected daily from the left jugular vein of each goat into 10 ml evacuated tubes containing dipotassium ethylenediamine tetra-acetate. Blood samples were collected in a similar manner, three times a week, for a further 152 days. The packed red blood cell volume per cent (PCV) of each sample was determined prior to examination of the buffy coat for the presence of trypanosomes (Murray et al., 1977). The level of parasitaemia was scored in accordance with the method of Paris et al. (1982). Throughout the experiment, sera were collected from each goat once a week and stored at –70°C until analysed for the presence of trypanosome antigens or anti-trypanosome antibodies.

The body weight of each goat was determined weekly throughout the study and whenever required for calculation of the drug dose for treatment.

Detection of low levels of parasitaemia

At 3 day intervals, from Day 7 to Day 22 following infection, 0.2 ml aliquots of blood (collected from each goat as described above) were inoculated intraperitoneally (i.p.) into sublethally irradiated (600 rads) mice (three mice per goat per day). Subsequently, the mice were monitored three times a week for 70 days for the presence of trypanosomes by microscopic examination (× 250) of wet films of tail blood.

Detection of anti-trypanosome antibodies and trypanosome antigens

Sera obtained from goats were examined for the presence of *T. congolense*-specific antibodies using a modified version of the technique described by Vos et al. (1988) to detect antibodies to common antigens of *T. vivax*. Ten sublethally irradiated (600 rads) rats were infected with *T. congolense* ILNat 2.1 (Majiwa et al., 1985). At the first peak of parasitaemia, the rats were exsanguinated and the blood collected into sodium citrate (final concentration 0.8% w/v). The blood was centrifuged at 400 × *g* for 15 min and trypanosomes in the buffy coat separated from blood cellular components using a diethylaminoethyl-cellulose (DE 52, Beckman, USA) column, in accordance with the method of Lanham and Godfrey (1970). A lysate was then prepared by freezing and thawing the trypanosomes on one occasion, followed by sonication on ice for 5 min. The lysate was then centrifuged at 18 000 × *g* for 1 h and the supernatant concentrated by ultrafiltration overnight at 4°C through a PM 10 membrane (Amicon, Massachussets, USA). The resultant concentrate was then centrifuged at 1230 × *g* for 30 min and the supernatant fractionated on a DE 52 column (Beckman, USA). The absorbence of each fraction was determined at 260 nm and 280 nm on a DU-50 spectrophotometer (Beckman, USA) to determine the protein concentration. Each fraction was then stored at –70°C until screened for its ability to detect serum anti-trypanosome antibodies. A selected fraction with optimal activity was prepared in Dulbecco's phosphate buffered saline (DPBS), pH 7.4, and used to coat 96-well flat-bottomed microtitre plates (Nunc-Immuno Plate, Polysorp, Denmark); 20 µg of protein, in 100 µl, was added to each well and incubated overnight at 4°C. The method of McGuire et al. (1979) was used to prepare rabbit anti-goat IgG/IgM. This reagent was subsequently conjugated to horseradish peroxidase (Wilson and Nakane, 1978). In experiments to screen sera for anti-trypanosome antibodies, sera collected from goats (as described above) were screened at an initial dilution of 1:250 (v/v) in bovine lymphocyte lysate (BLL) (Katende et al.,

1987) containing 0.01% (v/v) Tween 20 (Sigma Chemical Co., USA). Further dilutions of sera were made in BLL diluted 1:2 (v/v) with the buffer (DPBS + 0.01% (v/v) Tween 20) that was used for washing the microtitre plates; diluted sera were added to ELISA plates and incubated at room temperature for 30 min. The horseradish peroxidase-conjugate was then added and used at a dilution of 1:2000 (v/v) in the same buffer that was used for dilutions of sera and incubated for 1 h at 37°C. After washing the plates, a solution containing sodium citrate buffer (pH 4.0), 1.0% (v/v) hydrogen peroxide and the chromogen 2,2'-azinobis (3-ethyl-benzthiazoline-6-sulphonic acid) (ABTS, Serva, USA) was added to each plate. The ELISA plates were then kept in the dark at room temperature for 1 h to allow colour development (Katende et al., 1990). Thereafter, absorbance was measured at 414 nm in a Titertek Multiskan MCC/340 (Flow Laboratories, Finland).

Sera from goats were also examined for the presence of *T. congolense* antigen using an antigen-capture ELISA described by Nantulya and Lindqvist (1989).

Experiment designs

Experiment 1

The design of Experiment 1 is shown in Table 1. Twenty-five goats were allocated to five groups of five animals each (Groups A, B, C, D and E) and infected with *T. congolense* IL 3274 via the bites of infective *G. m. centralis* (5 tsetse flies per goat). A further five goats, constituting drug-sensitivity controls (Group F), were similarly infected but with flies infected with *T. congolense* IL 1180. Subsequent to the infection, a single dose of 7.0 mg diminazene aceturate kg^{-1} b.w. was administered i.m. to each goat in Groups A, B, C and D, on Days 1, 4, 8 and 12, respectively. Goats in Groups E and F were also treated i.m. with diminazene aceturate at a dose of 7.0 mg kg^{-1} b.w., however treatment was administered on Day 19, approximately 3 days after all goats in these two groups were first detected parasitaemic.

Experiment 2

In Experiment 1, tsetse flies were infected by allowing them to feed on a goat that had been inoculated with a stabilate of *T. congolense* IL 3274. In order to determine whether the different diminazene sensitivity results observed in goats in Groups A and E (treated on Day 1 and Day 19, respectively, following infection with *T. congolense* IL 3274) were due to an alteration in the trypanosome populations' sensitivity to diminazene between Day 1 and Day 19, Experiment 1 was repeated with tsetse flies that were fed on goats in Group E after trypanosomes reappeared following treatment with diminazene aceturate. Briefly, groups of clean *G. m. centralis* were fed on each of the goats in Group E (160 flies per goat) on Day 36 following infection (17 days following treatment). Thereafter, each batch of flies was maintained on separate rabbits and allowed to feed everyday, except at weekends, for 30 days. Those with mature infections were then identified as previously described (Dwinger et al., 1988), and infective flies from each group were fed on two normal goats each (five tsetse flies/goat). Twenty-four hours after the challenge, one goat in each pair received a single i.m. dose of 7.0 mg diminazene aceturate kg^{-1} b.w.; the other goat served as an infectivity control and was not treated until Day 19 following challenge. The five goats that were treated on Day 1 comprised Group Et(1). The five goats that were not treated until Day 19 are referred to as Group Et(19).

RESULTS

Experiment 1

Development of infection

Following infection of animals with *T. congolense* IL 1180 (Group F), trypanosomes were first detected by the buffy-coat phase-contrast technique on Days 11-13 following infection. In contrast, the interval between infection and first detection of trypanosomes was significantly longer (15–19 days) in animals infected with *T. congolense* IL 3274 (Group E; Table 1). Unlike Groups A-D, animals in Groups E and F were not treated until after detection of trypanosomes. The responses of the animals in each experiment group to treatment are given in Table 1. While infections were eliminated in all animals in Groups A and F, treatment eliminated infections in only two out of five animals in each of Groups B, C and D. Furthermore, infections in all five animals in Group E were refractory to treatment. The mean (± SD) and range of parasitaemia pre-patent periods observed in those animals that were detected parasitaemic following challenge in Groups B, C, D and E are given in Table 1.

Detection of low level parasitaemia by mouse inoculation

Trypanosomes were not detected in any of the mice that were inoculated with blood samples collected from goats in Groups A, B and D (Table 2). This was also true for four of the five goats in Group C, in which case trypanosomes were detected in mice inoculated with blood collected from one animal on Day 22 following infection (14 days following treatment). With animals in Group E, one of the five goats was shown to be parasitaemic by mouse inoculation on Day 10 following infection; on Days 16 and 19 all five goats were shown to be parasitaemic by this method. Although treatment on Day 19 with diminazene aceturate failed to

TABLE 2

Detection of trypanosomes in goats by inoculation of blood into mice

Group	*T. congolense* challenge[a]	Day of treatment[b] after challenge	Day of blood collection following challenge[c]					
			7	10	13	16	19	22
A	IL 3274	1	0/5[d]	0/5	0/5	0/5	0/5	0/5
B	IL 3274	4	0/5	0/5	0/5	0/5	0/5	0/5
C	IL 3274	8	0/5	0/5	0/5	0/5	0/5	1/5
D	IL 3274	12	0/5	0/5	0/5	0/5	0/5	0/5
E	IL 3274	19	0/5	1/5	2/5	5/5	5/5	1/5
F	IL 1180	19	1/5	4/5	5/5	5/5	5/5	0/5

[a]Five infective *Glossina morsitans centralis* per goat.

[b]Intramuscular treatment with 7.0 mg diminazene aceturate kg^{-1} body weight.

[c]0.2 ml of each blood sample inoculated into three mice each.

[d]Value indicates number of goats in group shown to be parasitaemic by inoculation of blood into mice.

eliminate infections in the five animals (see Table 1) only one of the five animals was demonstrated to be parasitaemic by mouse inoculation on Day 22 (Table 2). In Group F, one of the five animals was shown to be parasitaemic by mouse inoculation on Day 7. This increased to five of five animals on Days 13, 16 and 19 following infection. Treatment of animals in this group on Day 19 eliminated infections in all five animals (Table 1); none of the animals were demonstrated parasitaemic by mouse inoculation on Day 22 (Table 2). In general, inoculation of goat blood into mice resulted in earlier detection of trypanosomes than was achieved using the buffy-coat phase-contrast technique.

Antigen and antibody ELISAs

Table 3 shows the parasitaemia, serum trypanosome antigen and serum anti-trypanosome antibody profiles in all Experiment 1 animals for the first 35 days following infection. Of the five groups of goats infected with *T. congolense* IL 3274, the length of time between infection and treatment with diminazene aceturate was longest in Group E: 19 days. On Days 7 and 14 following feeding of tsetse flies, sera from goats in Group E were negative for anti-trypanosome antibody; trypanosome antigens were only detected in one animal on Day 14 (see Table 3). However, on Day 21, antibody and antigen were detected in four of five, and three of five goats, respectively. In contrast, neither antigen nor antibody were detected in sera from the five goats in Group A, and in two animals in each of Groups B, C and D throughout the entire 182-day sampling period (data not given); the same animals were never detected parasitaemic. Treatment therefore appeared to eliminate infections in all these animals. By contrast, in animals in Groups B, C and D that were detected parasitaemic following treatment, low anti-trypanosome antibody titres were detected from Day 14 following the challenge; detection of anti-trypanosome antibody was generally coincident with first detection of trypanosomes and/or antigenaemia in a given animal (Table 3). In goats infected with IL 1180

TABLE 3

Levels of parasitaemia, serum trypanosomal antigen and serum anti-trypanosomal antibody in goats within Experiment 1: Days 0-35

Group	*T. congolense* challenge[a]	Day of treatment[b] after challenge		Days after challenge with infected tsetse					
				0	7	14	21	28	35
A	IL 3274	1	Parasitaemia[c]	0/5	0/5	0/5	0/5	0/5	0/5
			Serum antigen[d]	0/5	0/5	0/5	0/5	0/5	0/5
			Serum antibody[d]	0/5	0/5	0/5	0/5	0/5	0/5
B	IL 3274	4	Parasitaemia	0/5	0/5	0/5	0/5	0/5	1/5
			Serum antigen	0/5	0/5	0/5	1/5	0/5	0/5
			Serum antibody	0/5	0/5	0/5	0/5	0/5	1/5
C	IL 3274	8	Parasitaemia	0/5	0/5	0/5	0/5	2/5	3/5
			Serum antigen	0/5	0/5	1/5	1/5	2/5	2/5
			Serum antibody	0/5	0/5	2/5	2/5	2/5	2/5
D	IL 3274	12	Parasitaemia	0/5	0/5	0/5	0/5	1/5	3/5
			Serum antigen	0/5	0/5	1/5	1/5	0/5	1/5
			Serum antibody	0/5	0/5	1/5	1/5	0/5	2/5
E	IL 3274	19	Parasitaemia	0/5	0/5	0/5	1/5	1/5	5/5
			Serum antigen	0/5	0/5	1/5	3/5	1/5	0/5
			Serum antibody	0/5	0/5	0/5	4/5	4/5	4/5
F	IL 1180	19	Parasitaemia	0/5	0/5	5/5	0/5	0/5	0/5
			Serum antigen	0/5	0/5	4/5	2/5	4/5	1/5
			Serum antibody	0/5	0/5	2/5	2/5	3/5	2/5

[a]Five infective *Glossina morsitans centralis* per goat.

[b]Intramuscular treatment with 7.0 mg diminazene aceturate kg^{-1} body weight.

[c]No. of goats detected parasitaemic/No. goats treated, using the buffy-coat phase-contrast technique (Murray et al., 1977).

[d]No. of sera positive/No. of goat sera tested, using ELISAs.

(Group F), in which treatment eliminated infections, both trypanosome antigen and anti-trypanosome antibody were detectable from Day 14 following the infection (Table 3). In some animals, anti-trypanosome antibody and/or trypanosome antigen were detected until Day 126 following the infection (data not shown).

Experiment 2

The five goats in Group Et(1), treated with diminazene aceturate 24 h following infection, were never detected parasitaemic by the buffy-coat phase-contrast technique during the entire 152-day observation period following treatment. In contrast, all five goats in Group Et(19) were detected parasitaemic 15-22 days following infection; trypanosomes were detected in 4 goats by Day 19; these goats received diminazene aceturate at a single dose of 7.0 mg kg^{-1} b.w. i.m. on Day 19. The same treatment was given to the other goat in this group on Day 22, the same day it was first detected parasitaemic. Infections in all 5 goats relapsed 7-12 days after the treatment.

DISCUSSION

Trypanosoma congolense clone IL 3274 was transmitted to goats via the bites of infective *G. m. centralis* and animals were treated with diminazene aceturate at a dose of 7.0 mg kg^{-1} b.w. at various intervals between Day 1 and Day 19 following infection. While infections were eliminated in all goats that received treatment on Day 1, infections relapsed in all goats that were treated on Day 19 (after detection of trypanosomes). There was, therefore, a significant alteration in the sensitivity of *T. congolense* IL 3274 to diminazene between Day 1 and Day 19 following tsetse-transmitted infections, confirming an earlier study (Silayo et al., 1992). In order to ascertain when the alteration in sensitivity between Day 1 and Day 19 occurred, groups of five goats each were infected in a similar manner and treated on Days 4, 8

or 12 following infection; infections in three out of five goats in each group were refractory to treatment. Thus, although the trypanosome population was fully sensitive to diminazene on Day 1, by Day 4 the population expressed significant resistance to diminazene.

In the above experiment, tsetse flies were infected by allowing them to feed on a goat that had been infected with a stabilate of *T. congolense* IL 3274. One possible explanation for the apparent alteration in resistance to diminazene between Day 1 and Day 19 following infection is that there was a spontaneous increase in the level of drug resistance of the trypanosomes between these 2 days. This was investigated by repeating the aforementioned experiment, but using tsetse flies that were infected with a subpopulation of *T. congolense* IL 3274 that reappeared in goats following treatment with diminazene aceturate at a dose 7.0 mg kg^{-1} b.w.; batches of tsetse flies were infected by allowing them to feed on each of the five animals in the first experiment in which trypanosomes had reappeared following treatment with diminazene aceturate on Day 19. Infective flies were then used to infect ten naive goats. Five of the goats received diminazene aceturate at a dose of 7.0 mg kg^{-1} b.w. on Day 1 following challenge; infections in all five animals were eliminated. The other five goats were not treated until Day 19; infections in all 5 animals were refractory to treatment. These results therefore provide further evidence that the sensitivity of *T. congolense* IL 3274 to diminazene is dependent on the duration of infection at the time treatment is instituted.

In work carried out with a stock of *T. congolense* in mice, Sones and Holmes (1992) demonstrated that the sensitivity of the population to isometamidium was dependent on the size of the population used to characterise the sensitivity; as the population size was increased, the minimum curative dose for the population also increased. Such a population size-dependent effect may have been responsible for the observations in the experiments described here. However, the biological basis for the

apparent alteration in resistance phenotype with population size is unclear, particularly since a cloned population was used.

Results from the first experiment demonstrated that when *T. congolense* IL 3274 was transmitted to goats via infective tsetse flies, the trypanosomes were fully sensitive to treatment with diminazene on Day 1, but expressed significant resistance to diminazene in some animals by Day 4. Several studies have indicated that when tsetse flies infected with *T. congolense* are allowed to feed on ruminants, the flies infect the animals by depositing metacyclic trypanosomes within the dermis of the skin (Luckins and Gray, 1978; Emery and Moloo, 1981; Akol and Murray, 1982). Four days later, the trypanosomes access the afferent lymph draining the dermis (Dwinger et al., 1990). Furthermore, it is not until 2–3 weeks following infection that trypanosomes are first detected in the blood (Luckins and Gray, 1978; Emery and Moloo, 1981; Akol and Murray, 1982; Dwinger et al., 1988, 1990). Thus, while the trypanosomes would appear to be localised in the dermis at the time an infection is initiated, by Day 4 they have begun to leave this site. When located in the skin the trypanosomes exist in a distinct morphological form. As a result of this location and/or various ultrastructural features they have been referred to as `extravascular forms' (Gray et al., 1985), `intracutaneous forms' (Dwinger et al., 1988) or `intermediate forms' (Gray et al., 1985; Dwinger et al., 1988). Intermediate forms have ultrastructural features such as a small mitochondrion, a well differentiated secretory reticulum and a lack of large lipid inclusions (Dwinger et al., 1988), which distinguish them from both metacyclic and bloodstream-form trypanosomes. One possible explanation for the apparent alteration in sensitivity to diminazene which occurs between Day 1 and Day 19 is that this life-cycle stage differs in sensitivity to diminazene compared to life-cycle stages which occur in the host once the trypanosomes migrate away from the dermis. In recent work, it has been shown that compounds closely related to diminazene, such as pentamidine, exert growth-retarding effects on human peripheral lymphocytes, particularly in the G_0 phase of the

cell-division cycle (Stauffert et al., 1990). It remains to be established whether the susceptibility of trypanosomes to diminazene is greatest when cells are in G_0, and whether on Day 1 following tsetse challenge *T. congolense* in the restricted environment of the dermis exist in a quiescent, non-proliferating state characteristic of the G_0 phase of the cell-division cycle (Pardee, 1989).

An alternative explanation for the apparent alteration in sensitivity to diminazene during the early stages of infection is that during this period the trypanosomes access sites within the host which are inaccessible to diminazene; when the systemic drug levels decline they are then able to re-emerge from such sites. Such a phenomenon occurs in mice when infected with *T. brucei* (Jennings et al., 1979). In order to determine whether the same phenomenon played a role in the observations described here, we have in other work collected cerebrospinal fluid from seven goats on a daily basis from Day 1 to Day 19 following tsetse-transmitted infection with *T. congolense* IL 3274; all cerebrospinal fluid samples were negative for trypanosome antigen (Nantulya and Lindqvist, 1989) (M. Mamman, unpublished data). The results indicate that invasion of the central nervous system between Days 1 and 19 is unlikely and therefore probably does not explain the alteration in sensitivity of *T. congolense* IL 3274 between Day 1 and Day 19 following infection.

In view of the potential threat that drug resistance poses to the control of trypanosomiasis in domestic livestock it is important that the biological basis for the results described herein is fully elucidated. Such information may lead to the design of intervention strategies capable of overcoming apparent drug resistance, thereby maximising the useful life of current trypanocidal compounds.

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CHAPTER 7

A SIMPLE METHOD FOR REPEATED SAMPLING OF LUMBAR CEREBROSPINAL FLUID IN GOATS

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Running title: Sampling lumbar cerebrospinal fluid in goats

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Summary

A technique has been developed for repeated sampling of cerebrospinal fluid from conscious goats by means of a catheter placed surgically in the subarachnoid space between the sixth and seventh lumbar vertebrae. Uncontaminated cerebrospinal fluid in excess of 1.0 ml could be obtained readily from the cannulated goats several times daily for up to 6 weeks. Cannulated goats did not appear stressed during, and in between, sampling periods.

Key words: Sampling method; Cerebrospinal fluid; Goats

Introduction

The cerebrospinal pathway comprises the ventricles of the brain, the central canal of the spinal cord, the subarachnoid space and perivascular extensions of this space (Swenson, 1977; Coles, 1980b). Contained within this pathway is cerebrospinal fluid (CSF), which has the function of protecting, nourishing and maintaining the homeostasis of the brain and spinal cord (Swenson, 1977; Coles, 1980b). In some nervous disorders, examination of CSF may provide information that is essential for accurate diagnosis, prognosis, therapeutic and surgical intervention (Wilson, 1977; Pandey & Nayak, 1982; Duncan & Prasse, 1986; Sorjonen, 1987). Furthermore, introduction of radiopaque dyes or air into the cerebral ventricles or subarachnoid space may occasionally be indicated to determine the location and extent of pathology of certain CSF–containing systems (Coles, 1980b). Characterisation of CSF from normal and diseased animals has also enhanced the state of knowledge on the pathophysiology of nervous diseases (Coppins *et al.*, 1977; Mayhew *et al.*, 1977; Coles, 1980a; Tsai *et al.*, 1982; Collis & Kimberlin, 1983; Strain *et al.*, 1984; Allsop & Pauli, 1985; Rand *et al.*, 1990a,b).

Although CSF can be sampled with little difficulty, such procedures are not routine and are only indicated under special circumstances. In animals, single or repeated sampling of CSF can be achieved through a puncture made into the cerebral

ventricles, or at one of two levels of the subarachnoid space: the cisterna magna, or in the lumbar region at a point beyond the termination of the spinal cord (Feldman, 1989). Regardless of the method used, the process of obtaining CSF must be undertaken with great care and must be carried out with physical restraint, chemical sedation or local anaesthesia (Mayhew, 1975; Coles, 1980b; Brewer, 1987). General anaesthesia is usually essential, especially in procedures which require surgical placement of indwelling cannulae for long-term sampling of CSF from either the cerebral ventricles (Mayhew, 1975; Beal & Bligh, 1977; Hedlund *et al.*, 1977; Allsop & Pauli, 1985) or the cisterna magna (Falconer *et al.*, 1985). In the absence of adequate restraint, or when a faulty technique is employed, the nervous system may be traumatised and may result in contamination of CSF (Mayhew, 1975; Coles, 1980b; Allsop & Pauli, 1985). In addition, infection may be introduced into the nervous system if the CSF is sampled under septic conditions. It is therefore contraindicated to sample CSF through any area of skin with an existing infection (Coles, 1980b). Because of some of these potential problems, experiments requiring repeated sampling of CSF for short or long-term durations are rarely carried out in animals.

With the hope of circumventing such problems, and to simplify the methodology for CSF cannulation, we have developed a safe, rapid and reliable surgical technique for cannulating the subarachnoid space between the sixth and seventh lumbar vertebrae in the goat. The technique permits repeated sampling of CSF in conscious animals and will therefore be useful for studies in this laboratory on the neuropathology of trypanosome infections in small ruminants.

Materials and methods

Animals

Twenty-two adult, male, castrated East African × Galla goats were used in the study. The animals weighed 18.3 ± 2.0 (15–22) kg and were fed a concentrate ration (Young

Stock, Belfast Millers, Nairobi; 200 g/animal/day) throughout the experimental work. The animals were allowed free access to hay and water.

Cannulation apparatus

The epidural apparatus was the commercially available Minipack® (Portex, Kent, England; Lot No. 90G 18 AA01) which comprises a 10.5 cm long 18 G Tuohy needle (outer diameter 1.3 mm, inner diameter 1.0 mm x 80 mm) with graduations at the 3, 5 and 7 cm levels. The lumen of the needle was guarded by a plastic probe. A detachable plastic wing for ease of handling of the needle during operation was also supplied. The catheter was made of a 90 cm long radio–opaque nylon (outer diameter 0.9 mm) which was open only at one of its two ends. The closed end, marked with 3 lateral "eyes", was used for catheterisation of the subarachnoid space between the sixth and seventh lumbar vertebrae. The pack also included a transparent tube for guiding the catheter into the needle, and a luer–lock connector consisting of upper and lower halves. The lumen from the lower half of the connector was guarded by an insert tube and a cover was included for closing the upper half of the connector (Plate 1).

Presurgical care

The goats were starved for 24 hours prior to beginning the surgical procedure. A rectangular area (40 cm × 15 cm) was then shaved and clipped in the lumbosacral region, and cleaned using a cotton swab, with the animal in the standing position. The shaved area included the areas immediately cranial and caudal to the median sacral crest and the tubera coxae. The longitudinal axis along the lumbosacral spine region was then marked with a straight interrupted line, using a marker pen (Pentel Co., Japan). Subsequent to anaesthesia and placement of the animal in left lateral recumbency, the line aided in placement of the skin in its normal position in relation to the spinal longitudinal axis.

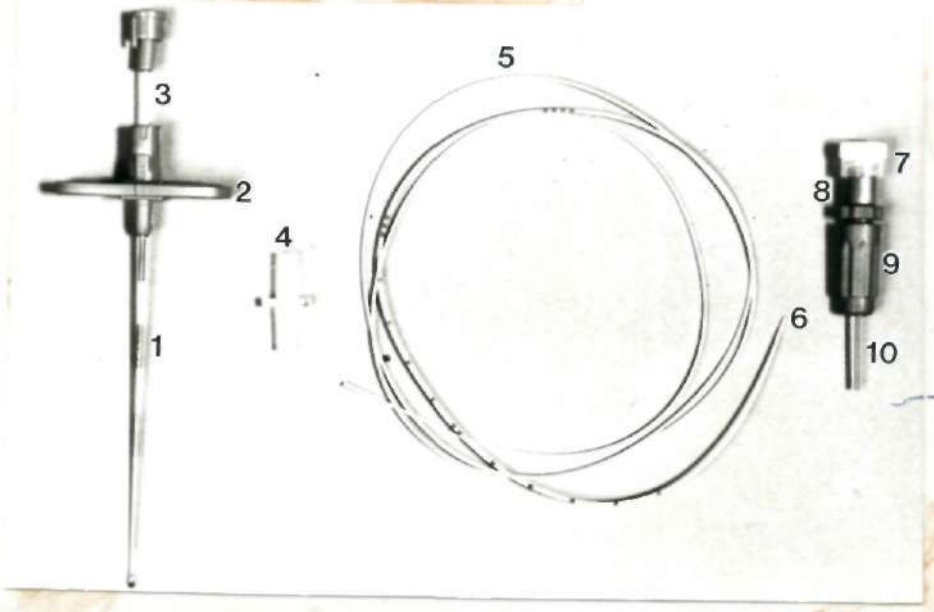


Plate I. Epidural Minipack® (Portex, Kent, England). Components are shown here after removal from the sterile plastic pack. Key: 1, 18 G Tuohy needle; 2, detachable needle wing; 3, plastic probe in the lumen; 4, catheter guide; 5, graduated radio-opaque catheter; 6, closed end of the catheter; 7, cover; 8, 9, upper and lower halves, respectively, of the connector assembly; 10, insert tube.

Routinely, each goat was administered with 0.2 ml of 2.0% xylazine (Chanazine®, Chanelle, Galway, Ireland) intravenously (i.v.) to ease induction of anaesthesia. Following premedication, the goat was placed in left lateral recumbency on the surgical table, then anaesthetised using a 5% solution of thiopentone sodium (Intraval®, May & Baker, Dagenham, England) administered dose-to-effect via the right jugular vein. Immediately after induction of anaesthesia the goat was intubated with a size 8 endotracheal tube (Rosch, Germany). After connection to an anaesthetic machine (Komesaroff®, Medical Developments Machine PTY Ltd, Sydney, Australia), goats were then maintained in surgical anaesthesia using an oxygen-methoxyfluorane-halothane mixture. The four legs of the animals were then tied together to ensure the spinal column was in a convex position. The shaved area of skin above the sixth and seventh lumbar vertebrae was then washed with a 1% solution of chlorhexidine gluconate (Savlon®, I.C.I. Pharmaceuticals, Cheshire, England) and cleaned with a cotton swab. Sterile drapes were clipped to the trunk of the animal, leaving the surgical area exposed, and strict asepsis was maintained throughout the surgery.

Surgical procedure

Immediately prior to surgery, the epidural Minipack® was unpacked and the different parts identified and assembled. A 5 mm incision was then made into the skin overlying the subarachnoid space between the sixth and seventh lumbar vertebrae (Plate II). The Tuohy needle, containing the lumen guard, was then inserted into the incision with the curved end orientated in a cranial direction. The winged outer end of the needle was then used to slowly advance the needle in a ventral direction through the tissues. When the 3 cm mark on the needle was at the level of the skin incision, the probe guarding the lumen of the needle was withdrawn. Gradually, the needle was advanced deeper until access into the subarachnoid space was achieved. If anaesthesia was not sufficiently deep, entry into the space was



Plate II. Incision (arrow) into the skin overlying in the subarachnoid space between the sixth and seventh lumbar vertebrae. Note interrupted line marked along the longitudinal axis of the spinal column.

accompanied by short-lasting opisthotonos. The appearance of clear normal CSF at the outer end of the needle indicated a successful cannulation (Plate III). Immediately after appearance of the fluid, the catheter was inserted rapidly through the needle into the subarachnoid space (Plate IV). Care was taken to ensure that the correct end of the catheter was used for the catheterisation. After approximately 7 cm of the catheter had been introduced into the space, the needle was carefully withdrawn, leaving the catheter in place. A pair of scissors was then used to cut off the excess portion of the catheter so that approximately 8 cm remained outside the incision site. The outer tip of the catheter was then positioned in the connector assembly, to a level just above the stop washer in the connector's lumen. The upper and lower halves of the connector were then twisted securely, but not too tightly, to fasten the catheter. At this stage, a sterile 1.0 ml Plastipak® syringe (Becton Dickinson, Dublin, Ireland) was used to obtain a reference CSF sample (Plate V). The white screw-cover was then used to close the outer end of the upper half of the connector.

In order that the catheter set-up should not be accidentally pulled or damaged by the goat when conscious, a rectangular sheet of elastoplast (Leukoplast®, Beiersdorf AG, Hamburg, Germany), measuring approximately 8 x 6 cm, was placed on the shaved skin, immediately cranial to the cannula insertion site. The connector was then placed on the elastoplast in a cranial orientation, and another, but shorter (6 × 4 cm), piece of elastoplast used to affix the connector to the underlying elastoplast (Plate VI). The two sheets of elastoplast were then jointly sutured to the skin area using approximately six silk 2–0 (Supramid®, B. Braun, Melsungen AG, Germany) sutures. In this way, the connector was fixed semi-rigidly in position.

For each goat, the total time taken for the entire cannulation procedure varied between 30 and 60 min. At completion of the procedure, each goat was administered 2.0 ml of oxytetracycline (Duphaycline®, Duphar, B.V., Amsterdam, Holland) intramuscularly. An oxytetracycline spray (Terramycin®, Pfizer, Sandwich,



Plate III. Drops of CSF (arrowed) from Tuohy needle after insertion of needle into subarachnoid space.

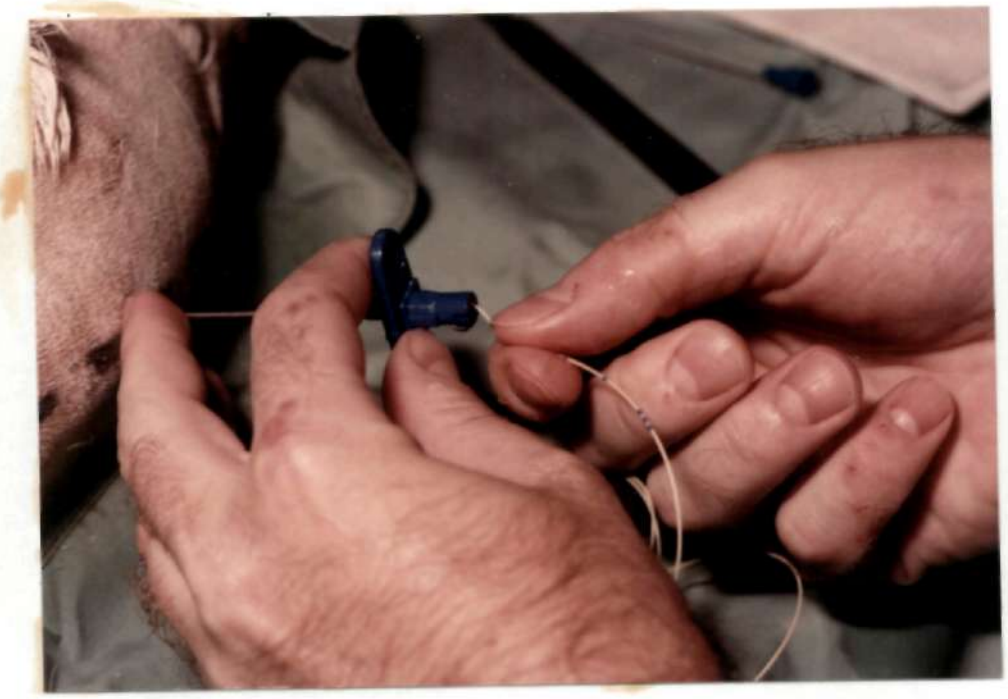


Plate IV. Insertion of the catheter via the needle into the subarachnoid space.



Plate V. Sampling CSF through the connector-catheter (arrowed) set-up.



Plate VI. Connector-catheter set-up fixed in position between elastoplast sheets.

England) was also applied to the incision area. Generally, following some degree of ataxia, goats were able to stand and resumed feeding within one hour of the operation (Plate VII).

Sampling procedure

Sampling was carried out with the aid of an assistant restraining the goat in a standing position. The operator's hands were then pre-cleaned with a solution of 70% alcohol, before removal of the connector cover. Using a sterile 1.0 ml Plastipak® syringe (Becton Dickinson, Dublin, Ireland) tightly fitted to the mouth of the connector, the desired volume of CSF was then gradually aspirated. When the flow of CSF was compromised, infusion of 0.2 ml of 200 iu/ml of heparin solution (Novo Nordisk, A/S Bagsvaerd, Denmark) in sterile distilled water at 27°C into the catheter was found to restore the flow. At completion of the sampling, the connector cover was returned into position, but without overtightening.

At the termination of an experiment, goats were administered 0.2 ml of 2.0% xylazine i.v. 3–5 min prior to the removal of the cannula. With the goat restrained in a standing position, the cannula was easily removed from the intervertebral space by a single rapid movement.

Results

Eighteen out of the 22 goats were cannulated successfully using the procedure described. One of the four other goats died immediately after induction of anaesthesia. In two others, clots of blood occluded the lumen of the Tuohy needle and further attempts to gain access to the subarachnoid space using new needles were futile. In a fourth goat, the cannulation was of limited success because flow of the CSF ceased when the goat was still on the surgical table.

Cerebrospinal fluid samples in volumes of 0.5–1.0 ml (or more) were readily obtained for up to 42 days from goats when sampled once daily. Similar volumes could be obtained several times within a day when an interval of 5–10 min was



Plate VII. A group of cannulated goats.

allowed between sampling. Cannulated goats appeared and behaved normally and did not show signs of discomfort.

Although rarely observed, the main problem encountered in the study was cessation in the flow of CSF. Often, however, this was prevented by infusion of 0.2 ml of 200 iu/ml of heparin solution through the catheter, just prior to sampling. In some cases, the flow was intermittent with cessation of flow spanning a few days. When catheters from such animals were removed and examined, no visible damage or obstruction could be demonstrated. Occasionally, cytological examination of CSF from freshly cannulated animals indicated the presence of erythrocytes. This, however, was usually confined to the first few days following surgery.

Discussion

The technique described in the present study permits collection of CSF from conscious goats by means of a catheter placed surgically in the subarachnoid space between the sixth and seventh lumbar vertebrae. Once in place, CSF in volumes of 0.5–1.0 ml (or more) could be collected once or several times daily, for 3–6 weeks. The catheterisation procedure utilises an inexpensive commercially available epidural cannulation pack and is completed within 1 hour. It is therefore considerably easier than earlier-described techniques for collection of CSF which require expensive equipment, and take several hours to cannulate the cisterna magna (Pappenheimer *et al.*, 1962; Falconer *et al.*, 1985). Convulsions and leg paralysis are also problems that have been associated with previously described cisternal or lumbar cannulation methods (Allsop and Pauli, 1985; Falconer *et al.*, 1985). Furthermore, due to formation of extensive subarachnoid fibrous adhesions, such techniques have proved unsuitable for long-term studies (Pappenheimer *et al.*, 1962). Such side effects were not associated with the present technique, and sampling of CSF was carried out for up to 6 weeks without causing any apparent stress to the animal. This is almost twice as long as that reported by Falconer *et al.* (1985) for a cisterna magna cannulation technique in sheep. Finally, the technique described here has also circumvented the

problem of CSF contamination with blood, which is frequently encountered with other methods (Strain *et al.*, 1984; Allsop and Pauli, 1985).

The described technique is particularly suitable for studying concentrations of endogenous substances, or therapeutic compounds (Baggot, 1977; Booth & McDonald, 1988), in the lumbar CSF of healthy and diseased animals. To this end, it was used successfully to determine the pharmacokinetics of the trypanocide diminazene in the CSF of 5 cannulated goats; each animal was sampled 9–16 times within the first 48 hours following treatment and, thereafter, once daily for 4 weeks (M.M., unpublished data). In another study, the technique was used to examine the CSF from 7 goats infected with *Trypanosoma congolense* for the presence of antigen; each animal was sampled once daily for 3–6 weeks (M.M., unpublished data). Successful application of the technique in these studies was due largely to implementation of two measures to maintain flow of the CSF until termination of the experiments. First, the catheter was protected from any accidental damage, malpositioning or loss, by fixing the catheter–connector assembly in position between two sheets of elastoplast and suturing the elastoplast to the skin. In so doing, the cannulae were prevented from damage and remained in position until completion of the studies. Secondly, in some animals, flow of CSF was occasionally observed to decrease gradually or cease over time. In such cases, infusion of 0.2 ml of 200 iu/ml of heparin solution in sterile distilled water through the catheter was usually sufficient to restore the flow. While the cause of the impairment of CSF flow was not determined, catheters from such animals were without any visible damage or obstruction on physical examination. However, restriction in flow may have been due to the presence, at the end of the catheter, of fibrous adhesions induced during the process of entry into the dural and subarachnoidal spaces (Mayhew, 1975; Falconer *et al.* 1985).

In view of the comparative advantages of the cannulation procedure described, the technique could have many applications for studies on the CSF in small ruminants.

Since the catheterisation apparatus is available in many sizes, the technique could also readily be applied to other animal species.

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CHAPTER 8

EXAMINATION OF THE CEREBROSPINAL FLUID OF GOATS FOR *TRYPANOSOMA CONGOLENSE* ANTIGEN USING AN ENZYME–LINKED IMMUNOSORBENT ASSAY (ELISA)

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Introduction

The reappearance of trypanosomes in infected animals following treatment, when re-infection and drug underdosage are excluded, is often attributed to drug resistance or the ability of parasites to survive in cryptic foci that are poorly accessible to trypanocidal drugs (Williamson, 1980). The latter phenomenon has been associated with some strains of *Trypanosoma brucei* in laboratory mice and rats (Jennings *et al.*, 1979; Abolarin *et al.*, 1982; Jennings and Gray, 1983), and *T. vivax* in goats (Whitelaw *et al.*, 1988), and is thought to be due to invasion of sites such as the central nervous system (CNS).

We recently reported studies in which goats were infected by the feeding of *Glossina morsitans centralis* infected with a cloned population of *T. congolense* (IL 3274) and, thereafter, treated at various intervals following infection with diminazene aceturate at a single intramuscular (i.m.) dose of 7.0 mg/kg body weight (b.w.) (Silayo *et al.*, 1992; Mamman *et al.*, 1993). It was observed that when treatment was administered on day 1 of infection, all the goats were cured. In contrast, infections were refractory to treatment when diminazene aceturate was administered on day 19 of infection, approximately 3 days after detection of trypanosomes in the peripheral blood. Since the parasitaemia profiles obtained from the latter group of animals mimic those observed with parasites which relapse from drug-inaccessible foci, the present experiment was carried out to determine whether invasion of the CNS contributed to the apparent resistance phenotype of *T. congolense* IL 3274 on day 19 of infection. The experimental protocols used by Silayo *et al.* (1992) and Mamman *et al.* (1993) were modified in the present study to include examination of the cerebrospinal fluid (CSF) for the presence of trypanosomes.

Materials and Methods

Seven adult, male, castrated East African X Galla goats were used. Sera were collected from these animals at the beginning of the experiment and were shown to be negative for *Trypanosoma* spp. antigen and anti-trypanosomal antibodies using

enzyme-linked immunosorbent assays (Nantulya and Lindqvist, 1989; Mamman *et al.*, 1993). Three days prior to initiation of infections, an epidural cannula (Minipack®, Portex, Kent, England) was placed in the lumbar subarachnoid space of each goat to permit collection of CSF, in accordance with a previously developed technique (Peregrine and Mamman, unpubl. obs.). The goats were then infected with *T. congolense* IL 3274 by feeding 5 infected *G. m. centralis*, as described by Mamman *et al.* (1993). Infections in the goats were monitored by determination of the level of parasitaemia and the packed red blood cell volume percent (Murray *et al.*, 1977; Paris *et al.*, 1982). Trypanosomes were first detected on days 12–16 after infection. On day 19, a single i.m. dose (7.0 mg/kg b.w.) of diminazene aceturate was administered to each goat. All goats showed temporary remission of parasitaemia within 48 hours of the treatment. However, infections in all animals relapsed 6–8 days after drug treatment.

Lumbar CSF was collected daily from each goat from the day of cannulation until 3 days after trypanosomes had reappeared following treatment (days 25–27 following infection). The cytology and presence of trypanosomes in each CSF sample was determined in 3 ways. First, within 10 minutes of collection, a 20 µl aliquot of each sample was dispensed onto a Neubauer haemocytometer and examined microscopically (400 ×). When CSF cell counts were very low, 500 µl of each sample was centrifuged at 590 *g* for 3 minutes at 4°C in a Model J-6B centrifuge (Beckman, California, U.S.A.) and then examined microscopically. Second, 200 µl aliquots were centrifuged at 42 *g* for 5 min in a Cytospin (Shandon Inc., Cheshire, England); slides were prepared from each sample, dried at room temperature (27°C), stained with Diff Quick® (Baxter Health Care, Miami, U.S.A.) and examined microscopically (1000 ×). Third, each sample was analyzed for the presence of trypanosomal antigen using an enzyme-linked immunosorbent assay (Nantulya and Lindqvist, 1989). In this assay, each sample was analyzed undiluted, and at dilutions of 1:10 and 1:50.

Results and Discussion

None of the three techniques used to examine CSF revealed the presence of trypanosomes or trypanosomal antigen in any of the CSF samples collected from the goats, either prior to or after infection. The results therefore suggest that reappearance of *T. congolense* IL 3274 following treatment with diminazene aceturate on day 19 of infection is not associated with trypanosomes that have entered the CNS. However, the findings do not exclude the possibility that the parasites could survive treatment in such sites as the microcirculation, lymph nodes, spleen, heart or bone marrow (Maxie and Losos 1977; Banks 1978; WHO, 1986; Kalu *et al.*, 1988) where the bioavailability of diminazene may be compromised.

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CHAPTER 9

PHARMACOKINETICS OF DIMINAZENE IN PLASMA AND CEREBROSPINAL FLUID OF GOATS

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Research in Veterinary Science, 57, p. 253–255 (1994).

Summary

An investigation was carried out to compare the pharmacokinetics of diminazene in the cerebrospinal fluid (CSF) and plasma of five non-infected goats treated with single intramuscular doses (3.5 mg diminazene base/kg body weight). Diminazene in CSF and plasma was determined by high-performance liquid chromatography. Concentrations of the drug in CSF were 3 to 4 times lower than in plasma. Kinetics of the drug in CSF and plasma was found to differ significantly with respect to C_{max}, t_{max}, $AUC_{0\text{-}48\ h}$, $AUMC_{0\text{-}48\ h}$, *MRT*, *Cl*, and $V_{d(ss)}$. The data indicate that diminazene enters the central nervous system, but that it attains concentrations in CSF that are significantly lower than in plasma.

Introduction

AFRICAN trypanosomiasis is a vector-borne disease of man and animals caused by extracellular protozoan parasites of the genus *Trypanosoma*. In man, the disease is characterised by non-specific symptoms in the early stage of infection. Thereafter, a specific neurological syndrome occurs when the parasites invade the central nervous system (CNS); if left untreated, patients will die within a few months to approximately 3 years (WHO 1986). The disease also poses a significant constraint to livestock production in sub-Saharan Africa (Murray and Gray 1984, Morrison et al 1985). In the absence of a vaccine, control of the disease currently depends on vector control and treatment with anti-trypanosomal drugs (Murray and Gray 1984).

Diminazene aceturate (Berenil®, Veriben®) is an aromatic diamidine that has been in use as a chemotherapeutic agent against animal trypanosomiasis since its introduction over three decades ago (Jensch 1955, Williamson 1980). In general, the drug is effective when administered as a single intramuscular (i.m.) dose of 3.5–7.0 mg/kg body weight (b.w.). However, treatment with diminazene aceturate is not effective against trypanosomes that have invaded the CNS (Jennings et al 1979, Abolarin et al 1982, Jennings and Gray 1983, Whitelaw et al 1988). Since the

parasites which re-emerge from the CNS after such treatment do not appear to be drug resistant (Jennings et al 1979, Whitelaw et al 1988), it is generally believed that the poor response to treatment with diminazene aceturate results from restricted entry of the drug into the nervous system, across the blood-brain-barrier (Williamson 1970). Furthermore, this appears to be the case since only low amounts of diminazene have been detected in the CNS of animals that were treated with single doses of the drug (Kellner et al 1985, Onyeyili and Anika 1991). However, rigorous investigations into the pharmacokinetics of the drug in the CNS have not been carried out because the techniques to obtain sufficient quantities of cerebrospinal fluid (CSF), and numbers of samples, for such a study were not available. Recently, a suitable technique has been developed (Peregrine and Mamman, unpublished) and has been used in the present study to determine, and compare, the disposition of diminazene in the CSF and plasma of 5 non-infected East African × Galla goats.

Materials and Methods

The lumbar subarachnoid space between the sixth and seventh lumbar vertebrae in each goat was catheterised using an epidural apparatus (Minipack®, Portex, Kent, England), as described previously (Peregrine and Mamman, unpublished). Twenty–four hours after insertion of the cannula, each goat was administered a single i.m. dose of 3.5 mg diminazene base/kg b.w., using a freshly prepared 7.0% (w/v) solution of diminazene aceturate (Berenil®, Hoechst, Frankfurt, Germany) in sterile distilled water. Samples of CSF and jugular blood were obtained simultaneously from each animal at 0, 10, 15, 30, 45 min, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 21, 24 and 48 h after drug administration. Thereafter, the samples were collected once daily for a further 4 weeks. Blood samples were collected into 5 ml evacuated tubes (Monoject®, Sherwood Medical, N. Ireland) containing potassium ethylenediamine tetra-acetate. Plasma aliquots were separated from the blood samples following centrifugation at 400 *g* for 30 min. Plasma and CSF samples were then stored at –20°C until analysis. Diminazene in each sample was extracted by an

ion-pair technique and quantified using high-performance liquid chromatography (Aliu and Ødegaard 1983, Aliu et al in press). The concentration–time data were then analysed by a non-compartmental approach (Gibaldi and Perrier 1982).

Results and discusions

Semilogarithmic plots of the disposition of diminazene in the CSF and plasma of the goats are given in Fig 1. In general, concentrations of the drug in the CSF were 3 to 4 times lower than those determined in plasma at the same time points. The values of C_{max}, t_{max}, $AUC_{0\text{-}48\ h}$, $AUMC_{0\text{-}48\ h}$, MRT, Cl and $V_{d(ss)}$ were found to differ significantly between the CSF and plasma (Table 1). The peak concentrations (C_{max}) averaged 1.04 ± 0.81 µg/ml in the CSF, as compared to 4.31 ± 0.22 µg/ml in the plasma. The corresponding times (t_{max}) at which C_{max} occurred in the CSF and plasma were 2.00 ± 0.09 h and 0.41 ± 0.29 h, respectively. The differences in C_{max} and t_{max} between CSF and plasma may be due to marked ionization of the drug at blood pH, resulting in restricted and delayed passage into the CSF (Williamson 1970). In addition, significant differences in the distribution and clearance of the drug between the two fluids may also explain the lower concentrations observed in the CSF. In the CSF, $V_{d(ss)}$ and Cl were 3.74 ± 0.46 L/kg and 3.07 ± 0.43 ml/min/kg, respectively. The corresponding values of these parameters in the plasma were 1.14 ± 0.11 L/kg and 1.16 ± 0.02 ml/min/kg, respectively. Compared to the plasma, therefore, higher distribution and more rapid clearance limit concentrations of the drug attained in the CSF.

The present findings, together with previous observations from studies with diminazene aceturate in cattle and dogs (Kellner et al 1985, Onyeyili and Anika 1991), and pentamidine, a structural analogue of diminazene, in humans (Bronner et al 1991), indicate that aromatic diamidines are distributed to the CNS, but in concentrations that are significantly lower than in the plasma. Although the anti-trypanosomal significance of these low concentrations remains to be established, it

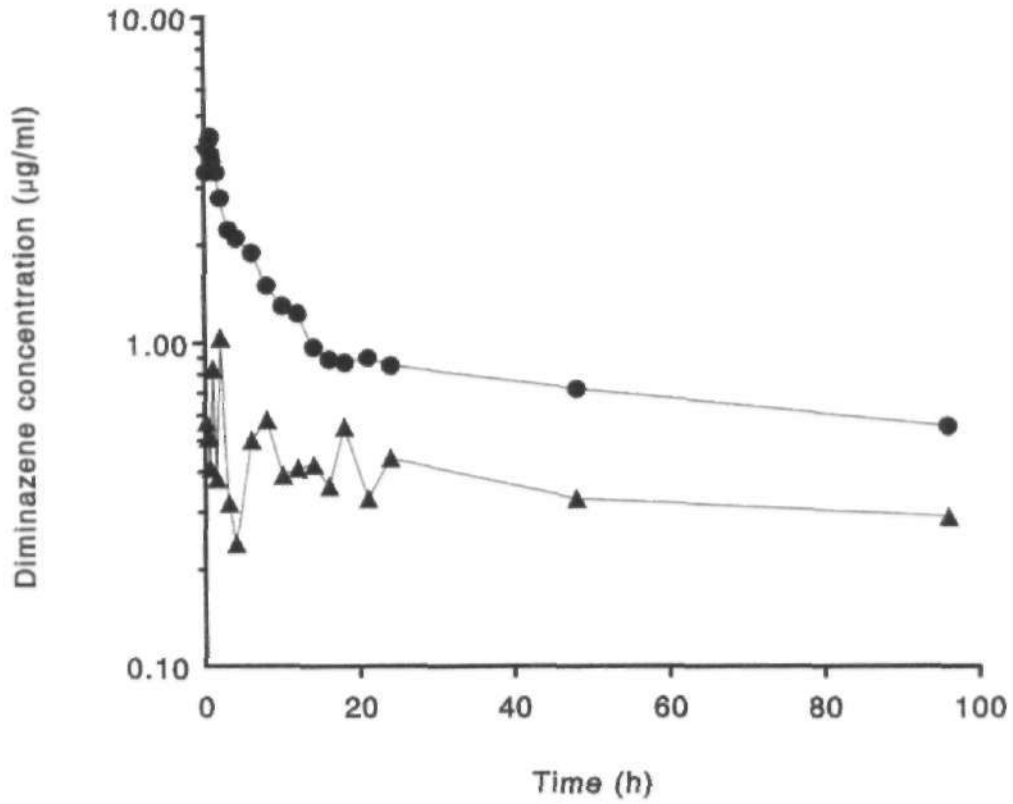


FIG 1: Mean concentrations of diminazene in plasma (●) and cerebrospinal fluid (▲) of goats following intramuscular administration of the drug at a dose of 3.5 mg diminazene base/kg body weight.

TABLE 1: Disposition parameters of diminazene in plasma and cerebrospinal fluid (CSF) of goats following single intramuscular administration of the drug at a dose of 3.5 mg diminazene base/kg body weight (n = 5)

Parameter	Units	Plasma	CSF
C_{max}	µg/ml	4.31 ± 0.22*	1.04 ± 0.81 *
t_{max}	h	0.41 ± 0.29*	2.00 ± 0.09*
$AUC_{0\text{-}48\,h}$	µg.h/ml	50.30 ± 1.07*	19.76 ± 2.71*
$AUMC_{0\text{-}48\,h}$	µg.h²/ml	830.53 ± 55.54*	391.98 ± 76.38*
MRT	h	16.33 ± 1.41*	20.60 ± 1.42*
Cl	ml/min/kg	1.16 ± 0.02*	3.07 ± 0.43*
$V_{d(ss)}$	L/kg	1.14 ± 0.11*	3.74 ± 0.46*

* Values with the same symbol are significantly different ($P < 0.05$)

would appear that if trypanosomes did enter the CNS, they would be less likely to be eliminated than if located in the systemic blood circulation.

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CHAPTER 10

PHARMACOKINETICS OF DIMINAZENE IN PLASMA AND LYMPH OF GOATS

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ABSTRACT

Mamman, M., McKeever, D.J., Aliu, Y.O. and Peregrine, A.S. Pharmacokinetics of diminazene in plasma and lymph of goats. *Veterinary Research Communications*,

The pharmacokinetics of the anti–trypanosomal drug diminazene aceturate (Berenil®) were determined in peripheral lymph of three goats by cannulation of the efferent prescapular lymphatic duct 8 weeks after surgical removal of the node. Values of the pharmacokinetic parameters were compared with those in blood plasma of the same goats and three intact control animals. The animals were each administered a single intramuscular dose of 3.5 mg diminazene base/kg body weight. Concentrations of diminazene in plasma and afferent lymph were determined by high–performance liquid chromatography. Ten minutes following treatment, concentrations of diminazene were 3.27 ± 0.98 μg/ml in plasma of non–cannulated goats, and 3.38 ± 0.83 μg/ml and 0.35 ± 0.22 μg/ml in the plasma and lymph, respectively, of cannulated goats. The maximum concentrations (C_{max}) of diminazene in plasma of non–cannulated goats, 4.53 ± 0.41 μg/ml, and cannulated goats, 3.64 ± 0.61 μg/ml, were both significantly higher than in lymph, 1.21 ± 0.58 μg/ml. T_{max}, the time at which C_{max} occurred, was 1.11 ± 0.77 h in lymph and was delayed compared to that in the plasma of non–cannulated goats, 0.44 ± 0.09 h, and cannulated goats, 0.38 ± 0.09 h. Furthermore, over the first 24 h following drug administration, concentrations of the drug in plasma of non–cannulated goats were generally higher than in lymph. Thereafter, concentrations of the drug in plasma of the non–cannulated goats were similar to that in lymph. However, due to the diminazene that was removed via the lymph, concentrations of the drug that occurred in the plasma of the cannulated goats after 16 h of treatment were significantly lower than in both the plasma of the non–cannulated goats and lymph. Because of extensive distribution and slow clearance, the elimination half–life of the drug in lymph was significantly prolonged compared to that in plasma of both non–cannulated and cannulated goats. The findings therefore

indicate that disposition of diminazene in treated goats is characterised by the presence of higher concentrations of the drug in blood than in lymph. However, the drug persists longer in lymph than in blood.

INTRODUCTION

Diminazene aceturate (Berenil®; Hoechst, Germany) is one of a limited number of drugs currently being used against the tsetse-transmitted protozoal disease, African animal trypanosomiasis. Efficacy of the drug at the recommended single intramuscular (i.m.) doses of 3.5 and 7.0 mg/kg body weight (b.w.) is widely acknowledged (Williamson, 1980; Leach and Roberts, 1981). However, resistance to the drug at these dosages has been reported (Jones–Davies, 1967a,b; Gray and Roberts, 1971; Clausen *et al.*, 1992; Mohamed-Ahmed *et al.*, 1992). Although the mechanisms of resistance to diminazene are poorly understood, field and experimental data indicate that it may occur naturally (Jones–Davies, 1968), through administration of subcurative doses (Hawking, 1963; Whiteside, 1963; Osman *et al.*, 1992), or as a result of cross–resistance (Gray and Roberts, 1971; Mwambu and Mayende, 1971; Ndoutamia *et al.*, 1993). Evidence from other experimental studies indicates that there are additional mechanisms by which trypanosomes may resist treatment with diminazene aceturate. For instance, some populations of *Trypanosoma brucei* (Jennings *et al.*, 1979) and *T. vivax* (Whitelaw *et al.*, 1988) are refractory to treatment due to their ability to invade the central nervous system (CNS); a site that is believed to be poorly accessible to diminazene (Williamson, 1970). Furthermore, in recent studies carried out in goats, it has been demonstrated that the ability of *T. congolense* IL 3274 to survive treatment with diminazene depends on the stage of infection at which treatment is administered (Silayo *et al.*, 1992; Mamman *et al.*, in press); populations of the parasite reappeared in animals that were treated on day 19 following tsetse challenge, whereas all goats were cured when treated on day 1 of infection. Since trypanosomes are confined to the skin on day 1 following infection

(Dwinger *et al.*, 1988), it is possible that the efficacy of the treatment at this stage was due to exposure of the small number of parasites to higher concentrations of drug than those attained in blood. This possibility was investigated in the present study.

MATERIALS AND METHODS

Experiment animals

Six adult, male, castrated East Africa × Galla goats weighing 25.3 ± 6.6 (18–33) kg were used in the study. Afferent lymph was collected by cannulation of the right efferent prescapular lymphatic ducts of three of the animals, 2 months after surgical removal of the lymph node, essentially as described by McKeever *et al.* (1991).

Drug administration and sample collection

A freshly prepared 7.0% w/v solution of Berenil® in sterile distilled water was used to treat each animal at a dose of 3.5 mg diminazene base/kg b.w. The drug was administered in the left *vastus medialis* muscle. Samples of blood from the right jugular vein of all goats, and afferent lymph (hereafter referred to as lymph) from the cannulated goats, were obtained at 0, 10, 20, 30, 40, 50 min, and 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 21, 24 and 48 h after drug administration. In order to prevent clotting, the blood samples were drawn into sterile heparinised tubes (Vacutainer®, Becton Dickinson, England). Similarly, the lymph was collected in 5 ml vials containing 100 i.u. of heparin (Novo Nordisk, A/S Bagsvaerd, Denmark) in 0.2 ml of distilled water. Plasma was separated from the blood samples by centrifugation. Plasma and lymph samples were stored at –20°C until analysed. Concentrations of diminazene in the samples were determined by reversed-phase high-performance liquid chromatography (Aliu *et al.*, in press) following ion-pair extraction (Aliu and Ødegaard, 1983).

Since changes in protein concentrations of blood and other body fluids may influence the concentrations of drugs, such as diminazene, in these fluids (Baggot, 1977; Aliu and Ødegaard, 1985; Alvi *et al.*, 1985; Aliu *et al.*, in press), total protein concentrations in the plasma and lymph samples were determined using Coomassie Protein Assay Reagent® (Pierce Chemical Company, Illinois, USA). Samples were analysed in triplicates on Immulon™ microtiter plates (Dynatech Labs Inc., Virginia, USA). Each plate included standard solutions of bovine serum albumin (BSA) at concentrations of 0.0625, 0.125, 0.25, 0.5, 1.0 and 2.0 mg/ml which were prepared in sterile distilled water from a stock of BSA provided with the kit. Results were read at a wavelength of 620 nm on a Titertek Multiskan® MCC/340 (LabSystems, Helsinki, Finland). Mean values of the standard BSA preparations were analysed by least squares linear regression to obtain equations that were then used to determine unknown protein concentrations in the plasma and lymph samples.

Data analyses

Diminazene concentration–versus–time data sets were analysed by compartmental methods using JANA (S.C.I. Inc., Kentucky, USA) for curve-stripping, and NONLIN (S.C.I., Inc., Kentucky, USA) and STATIS 3 (Clydesoft, Glasgow, UK) for curve-fitting. The models of best fit were selected on the basis of the Akaike (Yamaoka *et al.*, 1978) and Schwartz (Schwartz, 1978) information criteria. Peak drug concentrations (C_{max}) in plasma and lymph, and the corresponding time (T_{max}) taken to attain C_{max} were determined directly from the concentration–versus–time data profiles. Non–compartmental parameters, including the area under the concentration–versus–time curve ($AUC_{0-\infty}$) and its first moment ($AUMC_{0-\infty}$) calculated from time 0 to infinity, mean residence time (MRT), apparent volume of distribution at steady-state ($V_{d(ss)}$), and total body clearance (Cl), were determined by standard procedures (Gibaldi and Perrier, 1982; Riviere, 1988). Symbols of the pharmacokinetic parameters used in this paper are as previously defined (Allen *et al.*, 1982; Gibaldi and

Perrier, 1982; Riviere, 1988). Differences between values of pharmacokinetic parameters determined in plasma and lymph were compared using one–way analysis of variance (ANOVA).

Differences between groups, as well as group × time interactions with respect to the total protein concentrations determined in the plasma of non–cannulated goats, and lymph and plasma of cannulated goats, were analysed using ANOVA. Comparisons between plasma and lymph values were made using a three–factor repeated measures design that took account of data being derived from the same animals. Comparisons involving different groups of animals were undertaken using a two–factor repeated measures design. In both cases, the animal factor was regarded as random.

In all analyses, significant levels of 5% were used. Half-life values are expressed as geometric means; values of other parameters are presented as arithmetic means (± SD).

RESULTS

Over the first 48 h following drug administration, the mean total protein concentration in the plasma of non–cannulated goats, 6.96 ± 0.27 g/dl, was not significantly different from that in plasma of cannulated goats, 6.97 ± 0.47 g/dl (Table I). However, both values were significantly ($P < 10^{-6}$) higher than the mean total protein concentration determined in lymph over the same period, 3.40 ± 0.77 g/dl. The protein concentrations in plasma of non–cannulated goats, and plasma and lymph of the cannulated goats, did not vary significantly with time over the first 48 h of treatment.

Semi-logarithmic plots of the mean (± SD) concentrations of diminazene in the plasma of non–cannulated goats, and in plasma and lymph of cannulated goats,

Table I

Mean (± SD) total protein concentrations in plasma and lymph of goats

Time (h)	Total protein concentration (g/dl)		
	Plasma[N]	Plasma©	Lymph©
0.50	7.06 ± 0.16	6.94 ± 0.23	2.99 ± 0.94
1.00	6.92 ± 0.35	6.85 ± 0.19	3.48 ± 1.04
2.00	6.87 ± 0.33	6.90 ± 0.30	3.16 ± 0.95
10.00	6.87 ± 0.29	6.66 ± 0.36	3.56 ± 0.87
12.00	7.03 ± 0.27	7.34 ± 0.60	3.55 ± 0.65
14.00	6.81 ± 0.08	7.32 ± 0.45	3.58 ± 0.70
18.00	7.29 ± 0.04	7.16 ± 0.49	3.45 ± 0.76
21.00	6.93 ± 0.08	6.86 ± 0.27	3.56 ± 0.94
24.00	6.98 ± 0.19	6.98 ± 0.35	3.27 ± 1.07
48.00	6.83 ± 0.57	6.77 ± 1.14	3.42 ± 0.97

N Non-cannulated goats (n = 3)

© Goats cannulated via the efferent prescapular lymphatic duct (n = 3)

are shown in Figure 1. The derived compartmental and non–compartmental pharmacokinetic data are summarised in Tables II, III and IV.

Following intramuscular administration, the drug was rapidly absorbed and was detected in both plasma and lymph within 10 min of treatment. C_{max} in the plasma of non–cannulated goats, 4.53 ± 0.41 μg/ml, and in cannulated goats, 3.64 ± 0.61 μg/ml, were significantly ($P < 0.01$) higher than in lymph, 1.21 ± 0.58 μg/ml. Values of T_{max} and K_a did not differ significantly between plasma and lymph. However, values of plasma $AUC_{0-\infty}$, $AUMC_{0-\infty}$ and MRT in non–cannulated goats were significantly greater than in cannulated goats ($P < 0.01$). With respect to lymph, values for each of the three parameters varied widely and were not significantly different from those in plasma of both non–cannulated and cannulated goats. Total clearance (Cl) of the drug from the body was greatest from the plasma of cannulated goats, 1.76 ± 0.11 ml/min/kg, intermediate in plasma of non–cannulated goats, 0.69 ± 0.11 ml/min/kg, and lowest in lymph, 0.13 ± 0.11 ml/min/kg; $P < 0.005$ for all comparisons. The elimination half–life ($t_{1/2}\lambda_z$) of the drug in plasma of non–cannulated goats, 47.86 h, was significantly prolonged compared to that determined in plasma of cannulated goats, 11.77 h; both values were significantly shorter than the value of the same parameter ($t_{1/2}\lambda_z$) determined for lymph, 926.11 h. The $V_{d(ss)}$ in plasma of non–cannulated goats, 2.35 ± 0.38 L/kg, did not differ significantly from that in plasma, 1.90 ± 0.79 L/kg, or lymph, 6.51 ± 2.90 L/kg, of cannulated goats. However, $V_{d(ss)}$ in lymph was significantly higher than in the plasma of cannulated goats.

As shown in Figure 1, concentrations of diminazene in lymph were consistently lower than in plasma of the non–cannulated goats over the first 24 h of drug administration. Thereafter, however, concentrations of the drug in lymph were essentially the same as in plasma of the non–cannulated goats. Concentrations of diminazene in plasma of the cannulated goats which occurred after 16 h of treatment

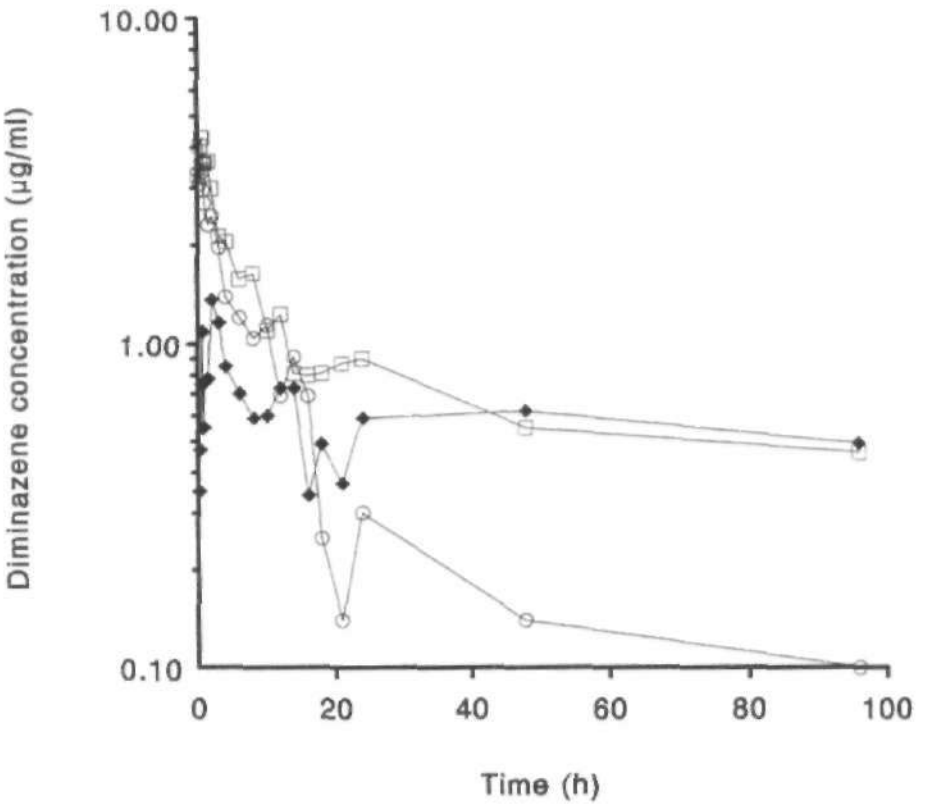


Figure 1. Absorption and disposition of diminazene in plasma of non–cannulated goats (□), and in plasma (○) and lymph (♦) of cannulated goats following intramuscular administration of 3.5 mg diminazene base/kg body weight. The lymph was collected from goats that were cannulated via the right efferent prescapular lymphatic duct

Table II

Coefficients and exponents describing disposition of diminazene in plasma and lymph of goats administered single intramuscular doses of 3.5 mg diminazene base/kg b.w.

Parameter	Units	Plasma[N]	Plasma©	Lymph©
C_2	μg/ml	4.23 ± 1.25*	1.98 ± 1.15	0.53 ± 0.56*
C_1	μg/ml	0.78 ± 0.56	0.96 ± 0.40	0.50 ± 0.31
K_a	h	6.21 ± 3.16	12.28 ± 11.15	1.30 ± 0.23
λ_1	h^{-1}	4.41 ± 1.26*	7.52 ± 7.80	0.55 ± 0.55*
λ_2	h^{-1}	0.34 ± 0.27	0.16 ± 0.12	0.24 ± 0.04
λ_z	h^{-1}	0.014 ± 0.003	0.065 ± 0.033*	0.005 ± 0.007*

N Non-cannulated goats (n = 3)
© Goats cannulated via the efferent prescapular lymphatic duct (n = 3)
* and ‡ Values with the same symbol are significantly different ($P < 0.05$)

Table III

Pharmacokinetic parameters of diminazene in plasma and lymph of goats following single intramuscular administration of 3.5 mg diminazene base/kg b.w.

Parameter	Units	Plasma[N]	Plasma[©]	Lymph[©]
$t_{1/2}\lambda_1$	h	0.16* (0.13-0.23)	0.13‡(0.042-0.29)	1.71*‡(0.58-3.0)
$t_{1/2}\lambda_2$	h	2.57 (1.06-6.75)	5.24 (2.25-9.59)	2.81 (2.34-3.37)
$t_{1/2}\lambda_z$	h	47.86* (36.68-58.37)	11.77* (7.33-24.08)	926.11* (396.91-4159.4)
$t_{1/2}K_{el}$	h	8.44* (4.68-12.38)	3.52* (3.34-3.82)	354.81* (138.58-1450.85)
$t_{1/2}K_a$	h	0.12* (0.07-0.18)	0.07‡(0.02-0.13)	0.54*‡(0.43-0.59)
K_{el}	h^{-1}	0.08 ± 0.05*	0.19 ± 0.01*	0.003 ± 0.002*
K_{12}	h^{-1}	1.23 ± 0.90	3.14 ± 3.04	0.49 ± 0.83
K_{13}	h^{-1}	0.19 ± 0.17	0.08 ± 0.14	0.11 ± 0.04
K_{31}	h^{-1}	0.06 ± 0.03	0.07 ± 0.01	0.05 ± 0.01
K_{21}	h^{-1}	4.21 ± 2.22	4.24 ± 4.72	1.11 ± 1.40
V_c	L/kg	0.58 ± 0.03	0.59 ± 0.09	3.38 ± 2.07

N Non-cannulated goats (n = 3)
© Goats cannulated via the efferent prescapular lymphatic duct (n = 3)
* and ‡ Values with the same symbol are significantly different ($P < 0.05$)
Geometric mean (range) values are given for $t_{1/2}K_a$, $t_{1/2}\lambda_1$, $t_{1/2}\lambda_2$, $t_{1/2}\lambda_z$ and $t_{1/2}K_{el}$

Table IV

Values of non-compartmental pharmacokinetic parameters of diminazene in plasma and lymph of goats following single intramuscular administration of 3.5 mg diminazene base/kg b.w.

Parameter	Units	Plasma[N]	Plasma©	Lymph©
C_{max}	µg/ml	4.53 ± 0.41*	3.64 ± 0.61‡	1.21 ± 0.58*‡
T_{max}	h	0.44 ± 0.09	0.38 ± 0.09	1.11 ± 0.77
$AUC_{0-\infty}$	µg.h/ml	83.65 ± 12.07*	31.89 ± 3.17*	2319.93 ± 3531.65
$AUMC_{0-\infty}$	µg.h²/ml	4924.69 ± 1956.65*	567.40 ± 291.57*	13526403.0 ± 21477057.0
MRT	h	57.27 ± 16.49*	17.51 ± 7.92*	2432.7 ± 3080.39
Cl	ml/min/kg	0.69 ± 0.11*	1.76 ± 0.11*	0.13 ± 0.11*
$V_{d(ss)}$	L/kg	2.35 ± 0.38	1.90 ± 0.79*	6.51 ± 2.90*

N Non-cannulated goats (n = 3)

© Goats cannulated via the efferent prescapular lymphatic duct (n = 3)

* and ‡ Values with the same symbol are significantly different ($P < 0.05$)

were generally significantly lower than in the plasma of the non–cannulated goats and lymph of the cannulated goats.

DISCUSSION

The present study has investigated the pharmacokinetics of diminazene in blood plasma and afferent lymph of goats treated i.m. with single doses of diminazene aceturate (3.5 mg diminazene base/kg b.w.). Since diminazene was detected in both plasma and lymph at 10 min following treatment, this indicated that there was rapid absorption of the drug. However, while maximum concentrations in the plasma were attained at 20–30 min, maximum concentrations in lymph occurred at 40 min–2.0 h. Furthermore, for the first 24 h following drug administration, concentrations of diminazene in plasma of non–cannulated goats were higher than in lymph. However, the elimination half–life of the drug in lymph was prolonged significantly compared to that in plasma. Thus, compared to plasma, the drug attained significantly lower concentrations, but persisted longer, in peripheral tissues that drain into the afferent lymph.

The therapeutic significance of these concentration–time profiles remain unclear since the amount of diminazene required to eliminate trypanosomes *in vivo* is unknown. However, if *in vivo* susceptibility of trypanosomes to diminazene depends on the total amount of the drug to which the parasites are exposed within the first 24 h of treatment, these findings would suggest that trypanosome populations are less likely to be eliminated by the drug when confined to the skin than if located in blood. However, if the susceptibility is dependent upon the total amount of drug to which the trypanosomes are exposed, trypanosomes would appear to be more likely to be eliminated in the skin than in the blood. Although it remains to be established whether prolonged exposure to low concentrations of diminazene is trypanocidal *in vivo*, studies *in vitro*, using a diminazene-resistant population, *T. congolense* IL 3338, have indicated the importance of such exposure (Kaminsky *et al.*, 1993). In those *in vitro*

studies, the parasites were eliminated following 10 days exposure to 500 ng diminazene aceturate/ml medium; a concentration that was significantly lower than that, 13.02 μg/ml, required to produce 24–h growth inhibition of the same parasite population. It is unclear, however, whether the low concentrations of diminazene in lymph would account for previous observations in which diminazene aceturate eliminated infections with *T. congolense* IL 3274 in goats when administered 24 hours following tsetse challenge, but failed to eliminate infections if treatment was delayed until day 19 of infection (Silayo *et al.*, 1992; Mamman *et al.*, in press). Differences in drug sensitivity between day 1 and day 19 of infection may also have been due to differences in drug sensitivity or numbers of the different parasite life–cycle stages occurring in animals at these two stages of infection (Silayo *et al.*, 1992).

As indicated above, concentrations of diminazene in afferent lymph were lower than in plasma of the non–cannulated goats for the first 24 h following treatment. Similarly, over the first 16 h following drug administration, concentrations of the drug in the lymph were lower than in the plasma of the cannulated goats. However, owing to more rapid clearance of diminazene from plasma than lymph, concentrations of the drug in lymph thereafter approximated those in plasma of the non–cannulated goats and exceeded those in plasma of the cannulated goats. Similar disposition patterns were also observed in blood and interstitial fluid of rabbits treated i.m. with single doses (3.5–15.0 mg/kg b.w.) of diminazene aceturate (Goodwin and Tierney, 1977; Gilbert and Newton, 1982b). Such differences in drug disposition between plasma and tissue fluids have also been observed with homidium bromide in rabbits and calves (Gilbert and Newton, 1982a), oxytetracycline, penicillin G and sulphadimidine in calves (Bengtsson *et al.*, 1989), and ceftazidime in sheep (Rule *et al.*, 1991). Since such diverse compounds exhibit very similar disposition kinetics, it would appear that a common mechanism may be involved in the distribution of the drugs between plasma and extravascular tissues. In practice, several factors appear to influence the transfer of drugs between plasma and tissues. For instance, suramin, a

sulphonated naphthylamine, has very limited extravascular distribution due to extensive binding to plasma proteins (Rollo, 1975). In contrast, the organic base tylosin attains higher concentrations in tissues than in plasma; undergoes extensive distribution in the body, and is concentrated in milk, due to low levels of binding to plasma proteins (Ziv and Sulman, 1973; Baggot and Gingerich, 1976; Atef *et al.*, 1991). Other factors that may contribute to differences in drug kinetics between plasma and extravascular tissues include the physiological status of animals, drug molecular sizes, lipid solubility of drugs, and concurrent administration of other drugs (Klatt and Hajdu, 1976; Baggot, 1977; Gibaldi, 1984).

In general, values of plasma pharmacokinetic parameters determined in goats used in this study are similar to those reported in earlier work (Aliu *et al.*, 1984). However, the findings of Aliu *et al.* (1984) indicated a shorter $t_{1/2}\lambda_z$ (14.00–30.00 h), lower $V_{d(ss)}$ (0.90 ± 0.27 L/kg) and lower $AUC_{0-\infty}$ (47.20 ± 8.30 µg.h/ml) than the data presented here. Since the dosage regimen used in both studies were the same, these differences may have occurred because the investigations of Aliu *et al.* (1984) involved dairy goats in which a considerable proportion of the drug was excreted via milk. In the work reported here, some of the pharmacokinetic parameters for diminazene in plasma were also found to differ significantly between non–cannulated and cannulated goats. For instance, significantly slower Cl, prolonged $t_{1/2}\lambda_z$ and extended $V_{d(ss)}$ were observed in the cannulated goats due to drug that was lost from the body via the cannulated lymph.

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CHAPTER 11

APPARENT LOW FREQUENCY OF DIMINAZENE–RESISTANT TRYPANOSOMES IN GOATS INFECTED WITH A DIMINAZENE–RESISTANT POPULATION OF *TRYPANOSOMA CONGOLENSE*

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Summary

Experiments were carried out in goats to determine the frequency with which diminazene-resistant trypanosomes occur in parasite populations prior to, and after, intramuscular treatment with diminazene aceturate. *Trypanosoma congolense* IL 3274, a diminazene-resistant clone, was used to initiate infections in 3 groups of 5 goats each. In 1 of the 3 groups, animals were treated with diminazene aceturate at a dose of 7.0 mg/kg body weight within 10 seconds of infection; 1 out of 5 goats was cured. In the second group, which received no treatment, all 5 animals were detected parasitaemic. In the third group, goats were administered the same drug dosage as for the first group, 3 days after all the goats in the group were first detected parasitaemic; trypanosomes reappeared in all 5 goats following treatment. The frequency of trypanosomes resistant to the drug dosage in this last group of animals was estimated to be less than 1 in 10^3. The parasites which reappeared following treatment in the third group of goats were used to infect 2 additional groups of 5 goats each intravenously. In one of the 2 groups, treatment with the same drug dosage as before, within 10 seconds of infection, cured all 5 goats. In contrast, all 5 goats in the second, untreated, group were detected parasitaemic. It therefore appeared that there was a low frequency of trypanosomes resistant to this drug dosage in the parasite inoculum used to infect these animals. Finally, when the goats in which infections had relapsed were re-treated with diminazene aceturate at the same dosage, the level of parasitaemia temporarily decreased by at least 10^3 trypanosomes/ml. Remission of parasitaemia following each treatment did not result from variable surface glycoprotein–specific antibody responses. However, since the total amount of diminazene that the trypanosomes were exposed to was greater following the second treatment, this may have contributed to the remission of parasitaemia that was observed following the second treatment. Collectively, however, these findings suggest that diminazene-resistant *T. congolense* occur at low

levels in trypanosome populations despite attempts to select for a population which was resistant to the drug dosage used.

Introduction

Diminazene aceturate (Berenil®, Ganaseg®, Veriben®) is an aromatic diamidine that was introduced as a chemotherapeutic agent for animal trypanosomiasis more than three decades ago (Bauer 1955, Fussgänger and Bauer 1958). Unlike other trypanocidal drugs, resistance to diminazene was initially rarely reported and slow to develop (Hawking 1963, Williamson 1976). However, in later years, there have been a number of reports describing resistance to diminazene in parts of the African continent (Jones-Davies 1967, 1968, MacLennan and Na'Isa 1970, Küpper and Wolters 1983, Mbwambo et al 1988, Clausen et al 1992, Mohamed-Ahmed et al 1992). While resistance to diminazene in many field isolates can be demonstrated both in vivo and in vitro, the underlying mechanisms which lead to the emergence of drug resistance are poorly understood (Hawking 1963, Williamson 1980, Leach and Roberts 1981). Furthermore, the in vivo bio-dynamics of drug-resistant trypanosomes, and the frequency with which drug-resistant trypanosomes occur in populations prior to drug treatment, and in relapse populations following treatment, are poorly understood.

In this study we have investigated the diminazene-resistance phenotype of trypanosomes in goats and attempted to derive an estimate for the frequency of trypanosomes in trypanosome populations that are refractory to treatment, both before and after treatment.

Short running title: Frequency of diminazene-resistant *Trypanosoma congolense* in goats.

Materials and methods

Experimental animals

Thirty-five adult, male, castrated East African × Galla goats were obtained from a tsetse-free area of Kenya. At the beginning of the experiment, sera were collected from all goats and found to be negative for trypanosomal antigen and anti-trypanosomal antibodies when examined by ELISA techniques (Nantulya and Lindqvist 1989, Mamman et al in press). Throughout the study the animals were kept in fly-proof housing, fed concentrates (200 g/goat) daily and allowed free access to hay and water.

C3H/He mice were obtained from the ILRAD breeding colony, weighed 20–35 g and were fed water and a pelleted ration (Unga Feeds Ltd., Nairobi) ad libitum.

Trypanosomes

Peregrine et al (1991) have described the derivation of *Trypanosoma congolense* clone IL 3274 from a stock which was originally isolated from a bull in Burkina Faso (Pinder and Authie 1984). In goats, *T. congolense* IL 3274 is resistant to intramuscular (i.m.) treatment with diminazene aceturate at a dose of 7.0 mg/kg body weight (b.w.) when administered after detection of trypanosomes (Silayo et al 1992, Mamman et al in press).

Infection and treatment of animals

A freshly prepared 7.0% (w/v) solution of diminazene aceturate (Berenil®; Hoechst, Germany, batch No. 093D574) in sterile distilled water was used to treat goats at a dose of 7.0 mg/kg b.w. The drug was administered i.m. in the right *vastus medialis* muscle.

Monitoring of goat infections

Three ml aliquots of jugular blood were drawn from each goat into evacuated tubes containing dipotassium ethylenediamine tetra-acetate (EDTA) on a daily basis, beginning 7 days prior to infection and continuing for the first 30 days following infection. Thereafter, the blood was collected once weekly for a further 5 months. At each sampling, the packed red blood cell volume percent (PCV) was also determined using the haematocrit centrifugation technique, followed by examination of the buffy coat for trypanosomes (Murray et al 1977). The method of Paris et al (1982) was used to score the level of parasitaemia. The minimum number of parasites detected using this method is 10^2/ml blood.

Experimental design

Thirty goats were randomly divided into 6 groups (A, B, C, D, E and F) of 5 animals each (Table 1). Goats in Group A were infected intravenously (i.v.) with *T. congolense* IL 3274, prepared in phosphate-saline-glucose (PSG). After detection of trypanosomes, aliquots of blood from each of the 5 goats were used to infect 3 goats i.v.; one in each of Groups B, C and D at an inoculum of 1.0 X 10^5 trypanosomes/goat. Within 10 seconds of infection, diminazene aceturate was administered at a single i.m. dose of 7.0 mg/kg b.w. to each animal in Group B. Goats in Group C were not treated and served as infectivity controls for Group B.

During the rising phase of the first peak of parasitaemia, each goat in Group D was treated i.m. with diminazene aceturate at a dose of 7.0 mg/kg b.w. Following the treatment, all 5 goats became aparasitaemic but relapsed within 9 days of the treatment. On day 15 following treatment, trypanosomes from each of the 5 goats were inoculated i.v. (1.0 X 10^5 trypanosomes/goat) into 2 goats; one in each of Groups E and F. Within 10 seconds of infection, each goat in Group E was treated i.m. with diminazene aceturate at a dose of 7.0 mg/kg b.w. The animals in Group F were not treated and functioned as infectivity controls for animals in Group E.

TABLE 1: Experiment design and response to treatment

Group	No. of goats infected	*Trypanosoma congolense* clone*	Treatment with diminazene aceturate			No. cured/ No. infected	Parasitaemia prepatent period (days)	
			Dose (mg/kg b.w.)	Route	Time of treatment		Mean value ± SD	(range)
A	5	IL 3274‡	——	——	——	0/5	19.4 ± 3.1	(16—23)
B	5	IL 3274@	7.0	i.m.	IAI	1/5	20.0 ± 4.7	(13—23)
C	5	IL 3274@	——	——	——	0/5	5.2 ± 1.8	(4—8)
D	5	IL 3274@	7.0	i.m.	10 dfi	0/5	5.6 ± 1.5	(3—7)
D	5	IL 3274*f*	7.0	i.m.	29 dfi	0/5	6.2 ± 1.8	(3—7)
E	5	IL 3274ℜ	7.0	i.m.	IAI	5/5	§	
F	5	IL 3274ℜ	——	——	——	0/5	6.6 ± 1.5	(5—8)

* 1.0×10^5 trypanosomes/goat inoculated via the left jugular vein; b.w. body weight; ‡ *infection in this group was initiated from a stabilate of the parasite in PSG;* —— *treatment* not given; @ infections were initiated with trypanosomes occurring in Group A on day 28 of infection; i.m. intramuscular treatment via the right *vastus medialis* muscle; IAI immediately (within 10 seconds) after infection; dfi days following infection; *f* infections in these goats that had relapsed following the first treatment; ℜ infection in this group was initiated with trypanosomes that had reappeared in Group D 14 days following the first treatment; § trypanosomes were never detected in any of the 5 goats in this group

Finally, 19 days following the first treatment, all 5 goats in Group D were re-administered a single dose (7.0 mg/kg b.w.) of diminazene aceturate; a 19–day intertreatment interval was utilised since it maximised drug elimination following the first treatment, but resulted in goats having PCV values above 15% at the time of the second treatment.

Determination of variable surface glycoprotein (VSG) antibody responses by fluorescence activated cell sorter (FACS) analysis

In animals in Group D, experiments were carried out to determine the contribution of the host immune response to the disappearance of trypanosomes following each of the 2 treatments. Sera were collected from the goats weekly throughout the experiment and stored at –70°C until analysis. Trypanosome stabilates were prepared from each goat on each of the 2 occasions that the goats were treated with diminazene aceturate, shortly before drug administration, and cryopreserved in liquid nitrogen (Dar et al 1972). At a later date, the stabilates were thawed, diluted in PSG (pH 7.4), and used to infect groups of sublethally irradiated (650 rads) mice. During the rising phase of the first peak of parasitaemia (day 10), the mice were exsanguinated into sodium citrate (1.0% [w/v] final concentration). The blood was centrifuged at 400 *g* for 15 min, and the trypanosomes in the buffy coat were separated from other cellular elements on a diethylaminoethyl-cellulose (DE 52, Beckman, U.S.A.) column using the method of Lanham and Godfrey (1970). Trypanosomes were then maintained on ice at 4°C.

Using half-log dilutions, goat sera in phosphate-buffered-saline (PBS) were dispensed into V-bottomed 96-well microtitre plates (Costar®, U.S.A.) to final concentrations of 1:10, 1:32, 1:100, 1:320, 1:1000 and 1:3200. Trypanosomes, freshly harvested from mice, were then added at a concentration of 10^6 trypanosomes/100 µl PBS/well of the microtitre plates. The plates were covered, mixed, and incubated on ice for 30 min. At the end of the incubation period, the

plates were spun at 1000 rpm for 10 min at 4°C in a Model J-6B centrifuge (Beckman, USA). The supernatant was then carefully aspirated leaving the trypanosome pellet at the bottom of each well. Subsequently, the wells were washed once. In the washing procedure, 200 µl of PSG (pH 7.4) was dispensed into each well. The plates were then covered, mixed, and spun at 495 *g* for 10 min at 4°C. Following centrifugation, the supernatant from each well was aspirated and discarded, the trypanosomes were resuspended and 50 µl of a 1:250 dilution of fluorescein isothiocyanate–rabbit antigoat immunoglobulin (Mamman et al in press), prepared in PSG, was dispensed into each well. Thereafter, the plates were covered, mixed and incubated on ice for 30 min. At the end of the incubation period, the contents of each well were transferred into tubes for FACS analysis using a FACScan (Becton Dickinson, U.S.A.), with an emission photometer fitted with a 520 nm filter, and an Argon-ion Laser (Cyonics, U.S.A.) which produced the 488 nm beam that was used to excite the cells. For each sample, 10^4 cells were analysed.

Two sets of analyses were carried out. First, parasites from the stabilates collected from each goat on days 10 and 29 after infection were expanded in sublethally irradiated mice and tested against sera from the same goat collected weekly, 0 to 42 days following infection. Second, antisera was raised against each of the parasite stabilates (collected from the 5 goats at the time of the first and second treatments) according to the method of Barry and Gathuo (1984). Briefly, intact mice were infected with each parasite population. Six days later, the mice were treated intraperitoneally with diminazene aceturate at a dose of 15 mg/kg b.w. The mice were exsanguinated after 2 days and sera were collected, aliquoted and stored at –20°C. The battery of antisera were then used to determine the variable antigen types (VATs) present in the parasite populations at the time of the first (day 10) and second (day 29) treatments with diminazene aceturate, both within individual goats and between goats.

Comparative pharmacokinetics of diminazene in plasma

Goats in Group D were treated with diminazene aceturate on days 10 and 29 of infection. At 0, 5, 10, 15, 30, 45 min, and 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 21, 24, 30, 36 and 48 h following each drug treatment, 5.0 ml blood samples were collected from each animal into EDTA-containing Monoject® tubes (Sherwood Medical, N. Ireland). Using similar time intervals, blood samples were also collected from an additional 5 non-infected goats which were each treated i.v. with diminazene aceturate at 7.0 mg/kg b.w.; the i.v. data were used in the calculation of values of some pharmacokinetic parameters in the goats that were treated i.m. Following centrifugation of the samples, plasma aliquots were obtained and stored at –20°C until assayed. Diminazene in the samples was extracted by an ion-pair technique, then quantified by high-performance liquid chromatography in accordance with previously described techniques (Aliu and Ødegaard 1983, Aliu et al in press). The concentration–versus–time data were analysed using compartmental and noncompartmental approaches. In the compartmental analysis, use was made of the curve-stripping programme JANA (SCI, U.S.A.), and the polyexponential nonlinear regression analysis software packages NONLIN Version 3.0 (SCI, U.S.A.) and STATIS 3.0 (Clydesoft, U.K.). Selection of the model of best fit for each concentration-time data set was based on the Akaike and Schwartz information criteria (Schwartz 1978, Yamaoka et al 1978). Derivation, and symbols, of the absorption, distribution and elimination parameters used in this paper are as previously described (Allen et al 1982, Gibaldi and Perrier 1982, Riviere 1988, Kinabo and McKellar 1989). Noncompartmental parameters, including the area under the plasma concentration-time curve ($AUC_{0-\infty}$), area under the (first) moment curve ($AUMC_{0-\infty}$) determined from time 0 to infinity, mean residence time (MRT), apparent total body clearance (Cl), apparent volume of distribution at steady-state ($V_{d(ss)}$) and mean plasma concentration of diminazene maintained over the first 48 h following treatment (C_{mean}) were calculated by standard equations (Gibaldi and Perrier 1982,

Aliu and Ødegaard 1985, Riviere 1988). Values of the pharmacokinetic parameters were compared using a one-way analysis of variance at the 5% significance level. All values are expressed as arithmetic means (±SD), except for half-life parameters which are given as geometric means.

Results

The parasitological results obtained in the 6 groups of goats are summarized in Table 1. Of the 5 goats in Group B, each of which was administered diminazene aceturate within 10 seconds following infection, 4 were detected parasitaemic 13–23 days after the treatment; the other goat appeared to be cured since it was not detected parasitaemic during an observation period of 180 days following infection. In comparison, all 5 untreated goats (Group C) were detected parasitaemic 4–8 days after initiation of the infection.

In a similar manner to goats in Group C, all 5 goats in Group D were detected parasitaemic within 3–7 days after initiation of infections. Following treatment with diminazene aceturate on day 10 of infection, a period of aparasitaemia was observed in all 5 goats. However, trypanosomes reappeared in all 5 goats within 5–9 days of the treatment. When treatment with diminazene aceturate was re-administered to the goats on day 29, temporary aparasitaemia occurred in all the animals. However, all 5 animals were again detected parasitaemic within 7 days of the treatment. Following the second treatment, the level of parasitaemia decreased from at least 10^5 trypanosomes/ml blood, in all animals, to less than 10^2 trypanosomes/ml.

Goats in Group E were infected with parasites that reappeared in Group D animals following the first treatment with diminazene aceturate. All the 5 goats in this group, however, were cured by treatment with a single dose of diminazene aceturate when administered within 10 seconds following infection; the goats were never detected parasitaemic during a 150-day observation period following treatment. In contrast, all untreated control goats (Group F) which were similarly infected with

relapse trypanosomes, were detected parasitaemic within 5–8 days following initiation of infections.

Determination of variable surface glycoprotein antibody responses by FACS analysis

The results of the FACS analysis showed that the sera collected from Group D goats on day 7 after infection (3 days prior to the first treatment) contained very low titres of specific antibodies against the VATs that were present on day 10 after infection (Fig 1). However, there was an increase in antibody titre 4 days after treatment. Thereafter, titres continued to rise, reaching a peak on day 28 post infection. Between day 0 and day 28 after infection, there were relatively low levels of specific antibodies to the VATs that were present in the goats on the day of the second treatment (day 29 after infection).

Table 2 summarises the antigenic relationships determined between VATs present within the same, and between different, goats on the days of the first and second treatments. In 1 of the 5 goats (C 123) the antiserum prepared against the day 10 parasites cross-reacted with parasites collected on day 29, suggesting that the same variants were present on day 10 and day 29 in this goat. However, in the other 4 goats, different populations were expressed on days 10 and 29. The results also showed that variants present in goats CD 75, C 177 and CD 189 on day 10 were expressed in goat C 123 on day 29 and that variants present in C 123 and CD 189 on day 10 were found in C 177 on day 29.

Comparative pharmacokinetics of diminazene in plasma

Following administration of diminazene aceturate (Berenil®) to animals in Group D, a series of blood samples were collected from the goats to determine whether disappearance of trypanosomes following the second treatment was

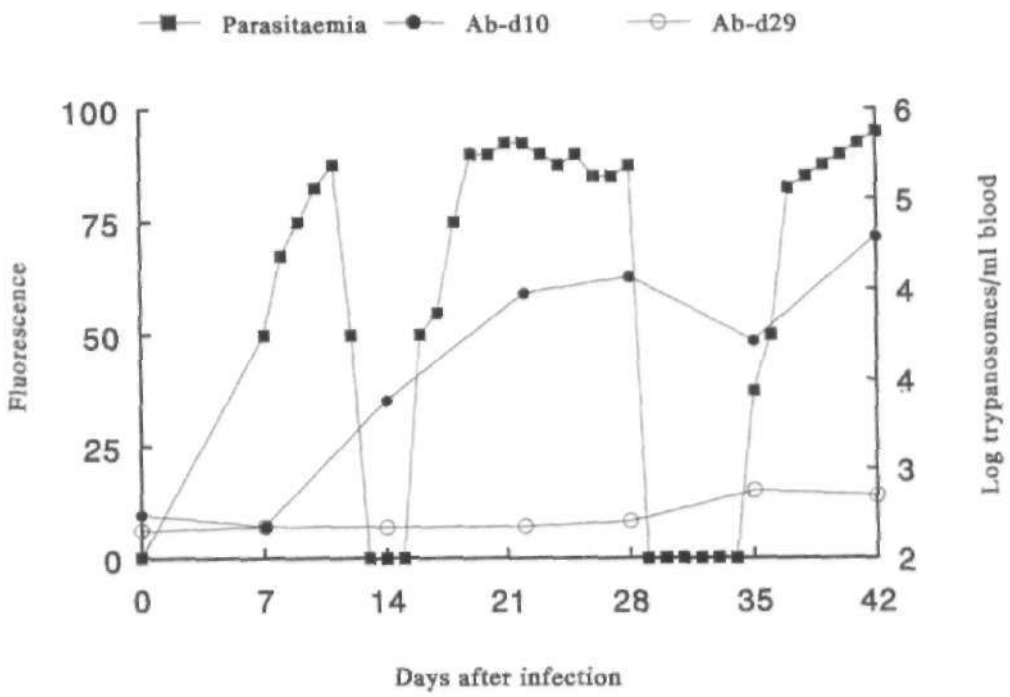


FIG 1: Mean level of parasitaemia and antibody prior to, and after, i.m. administration of single doses (7.0 mg/kg b.w.) of diminazene aceturate to goats in Group D (n = 5). Arrows indicate times of drug treatment. The goats were infected via the jugular vein with bloodstream forms of *T. congolense* IL 3274 (10^5 trypanosomes/goat) obtained from goats in Group A on day 28 of infection. Stabilates were prepared from the goats in Group D on day 10 (day of the first treatment) and day 29 (day of the second treatment) of infection. The parasites were expanded in sublethally irradiated mice. Serum samples which were collected weekly from the goats from day 0 to day 42 of infection were analysed, at a dilution of 1:100, using FACS to determine relative amount of fluorescence bound to each population of parasites present on day 10 (Ab-d10) and day 29 (Ab-d29).

TABLE 2: Inter-relationships between antigenic types of *Trypanosoma congolense* IL 3274 which occurred prior to treatment in infected goats, and in populations of the parasite which relapsed following i.m. administration of diminazene aceturate at a dose of 7.0 mg/kg b.w.

Parasites②	Antisera① (Goat No.)				
	CD 74	CD 75	C 123	C 177	CD 189
Goat No. CD 74	—	—	—	—	—
„ CD 75	—	—	—	—	—
„ C 123	—	✓	✓	✓	✓
„ C 177	—	—	✓	—	✓
„ CD 189	—	—	—	—	—

① Anti–sera were raised in non–irradiated mice infected with parasite populations collected from each goat prior to the first treatment (day 10 after infection)

② Parasite populations were collected from each goat 29 days after infection

— No binding of antibody to parasite populations

✓ > 50% of parasite populations measured positive by FACS analysis

associated with higher plasma concentrations of diminazene as a result of residual amounts remaining following the first treatment.

Values of the pharmacokinetic parameters determined following i.v. treatment in noninfected goats, and following the first and second i.m. doses in infected goats (Group D), are presented in Tables 3, 4 and 5.

In the goats treated i.m. (Group D), peak plasma concentrations (C_{max}) following the first treatment, 7.48 ± 2.81 µg/ml, and that following the second dose, 9.58 ± 6.34 µg/ml, did not differ significantly. Similarly, the time (T_{max}) at which C_{max} occurred following the first treatment, 0.21 ± 0.04 h, did not differ significantly from that determined following the second treatment, 0.36 ± 0.18 h. However, the $AUC_{0-\infty}$ following the second treatment, 136.09 ± 56.42 µg.h/ml was significantly greater than that after the first dose, 29.70 ± 3.42 µg.h/ml. In addition, values of the *MRT*, *Cl*, $V_{d(ss)}$, terminal elimination half-life ($t_{1/2}\lambda_z$) and the constant for elimination of the drug from the central compartment (K_{el}) also differed significantly between the 2 treatments. The mean plasma concentration of diminazene maintained over the first 48 h (C_{mean}) following the first treatment, 0.16 ± 0.10 µg/ml, was significantly lower than that, 2.16 ± 1.66 µg/ml, after the second treatment. Values of C_{mean} following the i.m. treatments were significantly higher than that determined following the i.v. treatment, 0.08 ± 0.03 µg/ml. Similarly, the values of several pharmacokinetic parameters including $t_{1/2}\lambda_z$, V_c, $AUC_{0-\infty}$, *MRT*, *Cl* and $V_{d(ss)}$ differed significantly between the i.v. and i.m. treatments (Tables 3, 4 and 5).

Discussion

This study has provided preliminary information on the diminazene susceptibility of *T. congolense* IL 3274 in goats and in subpopulations of the clone which reappeared following treatment with diminazene aceturate. *Trypanosoma congolense* IL 3274 was initially expanded in 5 goats. Prior to the first peak of parasitaemia, parasites in the jugular blood were collected and used to initiate

TABLE 3: Coefficients and exponents describing disposition of diminazene in plasma of noninfected and *Trypanosoma congolense*–infected goats administered a single dose of diminazene aceturate (7.0 mg/kg) intravenously or intramuscularly

Parameter	Units	Route of administration		
		Intravenous[N]	Intramuscular[①]	Intramuscular[②]
C_1	µg/ml	79.15 ± 110.06	4.51 ± 2.38	6.55 ± 6.40
C_2	µg/ml	6.55 ± 1.50*‡	1.97 ± 1.05*	2.60 ± 0.37‡
C_z	µg/ml	1.22 ± 0.26*	0.99 ± 0.40‡	0.42 ± 0.14*‡
K_a	h	NA	21.33 ± 13.08	8.67 ± 8.07
λ_1	h^{-1}	19.33 ± 11.23*	9.81 ± 11.76	2.93 ± 1.11*
λ_2	h^{-1}	1.30 ± 0.31‡	0.35 ± 0.16*	0.20 ± 0.01‡
λ_z	h^{-1}	0.058 ± 0.010*	0.042 ± 0.014‡	0.0055 ± 0.0025*‡

N Noninfected goats (n = 5)

① Group D animals; first treatment administered on day 10 of infection, during the rising phase of the first peak of parasitaemia (n = 5)

② Group D animals; second treatment administered 19 days after the first treatment, during the rising phase of relapse parasitaemia (n = 5)

NA Not applicable

* and ‡ Values with the same symbol are significantly different ($P < 0.05$)

TABLE 4: Pharmacokinetic parameters of diminazene in noninfected and *Trypanosoma congolense*–infected goats following single dose (7.0 mg/kg) administration by the intravenous or intramuscular routes

Parameter	Units	Route of administration		
		Intravenous[N]	Intramuscular[①]	Intramuscular[②]
C_{max}	µg/ml	86.94 ± 111.49	7.48 ± 2.81	9.58 ± 6.34
T_{max}	h	NA	0.21 ± 0.04	0.36 ± 0.18
$t_{1/2}\lambda_1$	h	0.043 (0.025–0.099)*	0.14 (0.023–1.20)	0.25 (0.15–0.36)*
$t_{1/2}\lambda_2$	h	0.54 (0.40–0.72)*‡	2.23 (1.33–8.08)*	3.37 (3.02–3.63)‡
$t_{1/2}\lambda_z$	h	11.74 (9.84–13.66)*	16.67 (10.7–30.03)‡	137.40 (74.3–332.06)*‡
$t_{1/2}K_{el}$	h	0.44 (0.096–1.15)*	4.24 (3.47–5.39)*	14.92 (9.57–36.32)*
$t_{1/2}K_a$	h	NA	0.037 (0.016–0.076)*	0.11 (0.03–0.21)*
K_{el}	h^{-1}	2.54 ± 2.76	0.16 ± 0.03*	0.05 ± 0.022*
K_{12}	h^{-1}	9.30 ± 6.75*	2.79 ± 3.32	0.79 ± 0.93*
K_{13}	h^{-1}	4.57 ± 3.65*‡	0.22 ± 0.11*	0.21 ± 0.08‡
K_{31}	h^{-1}	0.20 ± 0.07*	0.13 ± 0.08‡	0.029 ± 0.009*‡
K_{21}	h^{-1}	4.05 ± 1.81	6.88 ± 8.44	2.04 ± 0.76
V_c	L/kg	0.12 ± 0.11*‡	0.73 ± 0.15*	0.75 ± 0.31‡

N Noninfected goats (n = 5); ① Group D animals; First treatment administered on day 10 of infection, during the rising phase of the first peak of parasitaemia (n = 5)

② Group D animals; second treatment administered 19 days after the first treatment, during the rising phase of relapse parasitaemia (n = 5); NA Not applicable

* and ‡ Values with the same symbol are significantly different ($P < 0.05$); Geometric mean (range) values are given for $t_{1/2}\lambda_1$, $t_{1/2}\lambda_2$, $t_{1/2}\lambda_z$, $t_{1/2}K_{el}$ and $t_{1/2}k_a$

TABLE 5: Values of non-compartmental pharmacokinetic parameters for diminazene *in* noninfected and *Trypanosoma congolense*–infected goats following a single dose (7.0 mg/kg) administered intravenously or intramuscularly

Parameter	Units	Route of administration		
		Intravenous[N]	Intramuscular[①]	Intramuscular[②]
$AUC_{0-\infty}$	µg.h/ml	33.60 ± 3.23*	29.70 ± 3.42‡	136.09 ± 56.42*‡
$AUMC_{0-\infty}$	µg.h²/ml	709.92 ± 115.9	617.93 ± 253.04	34518.9 ± 37935.2
MRT	h	21.10 ± 2.59*	20.48 ± 6.82‡	210.05 ± 134.40*‡
Cl	ml/min/kg	1.74 ± 0.16*	1.97 ± 0.22‡	0.48 ± 0.17*‡
$V_{d(ss)}$	L/kg	2.20 ± 0.31*	2.39 ± 0.66‡	5.10 ± 1.04*‡

N Noninfected goats ($n = 5$)

① Group D animals; first treatment administered on day 10 of infection, during the rising phase of the first peak of parasitaemia (n = 5)

② Group D animals; second treatment administered 19 days after the first treatment, during the rising phase of relapse parasitaemia (n = 5)

* and ‡ Values with the same symbol are significantly different ($P < 0.05$)

infections (10^5 trypanosomes/goat) by i.v. inoculation to three groups of 5 goats each. Animals in the first group were each administered a single i.m. dose (7.0 mg/kg b.w.) of diminazene aceturate within 10 seconds of infection. The early treatment was carried out to minimise the chances of the parasites invading sites that may be poorly accessible to the drug (Jennings et al 1979, Whitelaw et al 1988). Although treatment was administered at this early stage of infection, 4 of the 5 goats were subsequently detected parasitaemic. All the 5 goats in the second group, which functioned as untreated controls, were also detected parasitaemic. Thus, resistance to diminazene aceturate at a dose of 7.0 mg/kg b.w. was expressed at an inoculum size of 10^5 trypanosomes/goat. This is in contrast to earlier observations in which 5 out of 5 goats infected with the same parasite clone, via the bites of 5 tsetse flies, were cured using the same drug dosage, provided that the treatment was administered 24 h following initiation of infection (Silayo et al 1992, Mamman et al in press). However, the numbers of trypanosomes present in these animals at the time of treatment would have been considerably fewer than 10^5 in total since tsetse flies infected with *T. congolense* extrude approximately 100 metacyclic trypanosomes at each feed (Whitelaw et al 1986). As a result, the infections would have been expected to be more sensitive to treatment (Sones and Holmes 1992).

Animals in the third group were used in a set of studies to determine the frequency of parasites in a trypanosome population that are able to withstand a particular drug dosage in vivo. Three days after each of the goats in this group were first detected parasitaemic each animal was treated with diminazene aceturate; after temporary remission of parasitaemia, trypanosomes were detected in each of the 5 goats. Since the level of parasitaemia declined from greater than 10^5 trypanosomes/ml to less than 10^2 trypanosomes/ml (the minimum limit of detection of the diagnostic technique used [Paris et al 1982]) following treatment, this would indicate that at the time of the treatment less than 0.1% of the trypanosome population was resistant to the drug dosage used.

In the second part of the study, the trypanosomes which reappeared in these animals following the first treatment were used to infect 2 other groups of 5 goats each; each goat was inoculated i.v. with 10^5 trypanosomes. Goats in one of the two groups were administered diminazene aceturate within 10 seconds of infection. This was carried out, as before, to determine the sensitivity of the parasites when present only in the cardiovascular system, prior to possible invasion of drug-inaccessible sites. Surprisingly, all the 5 goats were cured. In contrast, trypanosomes were detected in all the 5 goats in the second, untreated, control group.

Assuming a high level of infectivity of the trypanosomes (demonstrated in other studies [Mamman M., unpublished data]), these results suggest that the frequency of trypanosomes able to survive this drug dosage was less than 1 in 10^5 in the parasite inoculum that was used to infect the 2 groups of goats. It therefore appears that the majority of the parasites in the relapse population were in fact sensitive to the dose of diminazene aceturate that was used to treat the goats. In order to confirm this, the group of goats in which the parasites had relapsed following treatment on day 10 of infection was retreated with diminazene aceturate at the same dose on day 29. If all the trypanosomes that had reappeared in these goats following the first treatment were resistant to the drug dosage that had been used, the second treatment would be expected to have no effect on the level of parasitaemia. However, the second treatment resulted in the parasitaemia decreasing from a level in excess of 10^5 trypanosomes/ml, to below the detection limit of 10^2 trypanosomes/ml; trypanosomes reappeared in all goats within 7 days of treatment.

The decrease in parasitaemia following the second treatment may have been due to some event other than administration of drug. For instance, antibody responses to VSG are known to clear parasites from the bloodstream of infected hosts (Morrison et al 1982). Thus, titres of anti-VSG antibodies were measured prior to the first, and the second drug treatment. The results indicated that serum levels of VAT-specific antibodies present 3 days before the first treatment, and a day preceding

the second treatment, were low and therefore would appear not to account for the decline in parasitaemia observed following each treatment. Also in 4 of the 5 goats, the second peak of parasitaemia appeared to comprise VATs antigenically unrelated to those in the first peak. Thus, the remission of the second wave of parasitaemia was unlikely to be due to antibodies against the first wave of parasitaemia which were still present in the serum at the time of the second treatment. It would appear, therefore, that the remission in parasitaemia following the second treatment resulted from exposure of the parasites to the drug.

High-performance liquid chromatographic analyses indicated that the area under the concentration-time curve ($AUC_{0-\infty}$), mean plasma concentration at 48 h (C_{mean}), elimination half life, and steady-state volume of distribution of the drug were significantly greater following the second treatment. Thus, although the observed C_{max} and T_{max} did not differ significantly, a greater concentration of diminazene was maintained in the plasma following the second treatment. The higher concentration following the second dose may, as indicated by the C_{mean} values, be due to residual drug remaining following the first dose. However, these differences may also be secondary to effects of the infection, as was observed in previous studies with diminazene aceturate in *T. congolense*-infected dogs (Onyeyili and Anika 1989) and imidocarb, a closely related aromatic diamidine, in *T. evansi*-infected goats (Abdullah and Baggot 1986). As a result of the differences in the kinetics of the drug between the 2 treatments, and since it is not clear which pharmacokinetic parameters are responsible for elimination of trypanosomes, the factors responsible for the decline in parasitaemia following the second treatment must be interpreted with caution. Accordingly, if only C_{max} and T_{max} are essential for elimination of trypanosomes, then the frequency of trypanosomes in the parasite population which were resistant to the drug dosage used at the time of the second treatment would appear to be less than 0.1%, as was determined following the first treatment. If, however, elimination of parasitaemia is significantly influenced by other parameters, such as $AUC_{0-\infty}$ and

C_{mean}, it would then be impossible, using the present data, to derive such an estimate for the parasite population that was present at the time of the second treatment.

Finally, in the first part of this study, a stabilate of *T. congolense* IL 3274 was inoculated into 5 goats. Prior to the first peak of parasitaemia, trypanosomes were collected from each of these animals and inoculated i.v. into 5 additional goats (10^5/animal) which were treated within 10 seconds of infection with diminazene aceturate; infections in 4 of the 5 animals were refractory to treatment. However, when the same experimental protocol was used with a subpopulation of *T. congolense* IL 3274 that had reappeared in goats following treatment with diminazene aceturate at the same dosage, infections in 5 out of 5 goats were eliminated. Thus, drug pressure did not increase the frequency of "diminazene-resistant" trypanosomes within the parasite population, but surprisingly resulted in an apparent decrease in the frequency of resistant trypanosomes. It is possible that the parasites accumulated diminazene following their first exposure to the drug and that this sensitised them to further treatments. However, since repeat treatments with diminazene did not eliminate infections with the same trypanosome clone in animals in Group D, this would dispute this hypothesis. Alternatively, the FACS analyses described suggest that *T. congolense* IL 3274 underwent antigenic variation during the course of infection. This would suggest that different VATs were present in the 2 groups of experimental animals at the time of treatment. Thus, if different degrees of drug sensitivity are associated with different VATs, this could account for the difference in drug sensitivity observed between the 2 experimental groups. Such a hypothesis would, however, be difficult to evaluate and would require large numbers of experimental animals.

In summary, the diminazene sensitivity of *T. congolense* clone IL 3274 has been determined in infected goats both before and after treatment with diminazene aceturate. The findings suggest that diminazene-resistant trypanosomes occur at low frequencies in such populations. However, some of the findings are based on

assumptions concerning the infectivity and diminazene-sensitivity of the parasite populations. More rigorous determinations of the frequency with which diminazene-resistant trypanosomes occur in vivo require experiments that utilise larger numbers of experimental animals.

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CHAPTER 12

FREQUENCY OF DIMINAZENE–RESISTANT TRYPANOSOMES IN POPULATIONS OF *TRYPANOSOMA CONGOLENSE* ARISING IN INFECTED ANIMALS FOLLOWING TREATMENT WITH DIMINAZENE

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SUMMARY

The frequency of trypanosomes resistant to diminazene aceturate at a dose of 25 mg/kg body weight was investigated in populations of *Trypanosoma congolense* IL 3274 which reappeared in infected mice following intraperitoneal (i.p.) treatment with diminazene aceturate at the same dosage. At inoculum sizes of 10^2, 10^3, 10^4, 10^5 and 10^6 trypanosomes/mouse, the relapse populations were used to initiate infections in 5 groups of 100 mice each by the intravenous route. Immediately after infection, 50 mice in each group were treated i.p. with diminazene aceturate at the aforementioned dosage; the other 50 mice functioned as untreated controls. Thereafter, all animals were monitored for 100 days for the presence of trypanosomes. In each group, trypanosomes were detected in 50 out of 50 control mice, indicating 100% infectivity for all 5 inoculum sizes. In contrast, in the groups of 50 mice infected with 10^2, 10^3, 10^4, 10^5 or 10^6 trypanosomes and treated with diminazene aceturate, trypanosomes were detected in 4/50, 11/50, 13/50, 28/50 and 39/50 mice, respectively. Using logistic regression, a good fit was found between the number of mice detected parasitaemic and the inoculum sizes. Maximum likelihood estimates for the proportions resistant to diminazene aceturate at 25 mg/kg body weight in the inoculum sizes 10^2, 10^3, 10^4, 10^5 and 10^6, were 8.335×10^{-4}, 2.485×10^{-4}, 3.02×10^{-5}, 8.3×10^{-6} and 1.6×10^{-6}, respectively. These findings indicate that the majority of the relapse trypanosomes were sensitive to the drug dosage used for selecting the population and that, surprisingly, the calculated proportion of organisms which survived drug exposure varied inversely with the inoculum sizes. Further experiments in mice indicated that the inverse relationship did not result from alterations in the pharmacokinetics of the drug with different inoculum sizes. The data therefore suggest that parasite inoculum size and drug dosage are important factors in estimating the apparent frequency of diminazene–resistant trypanosomes in populations of *T. congolense* occurring *in vivo*.

INTRODUCTION

African trypanosomiasis is a disease complex caused by unicellular protozoan parasites belonging to the genus *Trypanosoma*. Pathogenic trypanosomes cause clinical disease in humans and are an important factor limiting development of domestic livestock in Africa (Murray & Gray, 1984).

Diminazene aceturate (Berenil®, Veriben®) is an aromatic diamidine that was introduced in 1955 as a therapeutic agent for treatment of animal trypanosomiasis. In trypanosomatids, the drug appears to selectively block replication of kDNA (Newton & Le Page, 1967; MacAdam & Williamson, 1972). However, the primary target(s) for diminazene's trypanocidal activity remain unknown.

Experimental studies in rodents indicate that the number of parasites used to initiate an infection has a significant influence on the subsequent growth of trypanosomes *in vivo* (Diffley *et al.* 1980; Miller & Turner, 1981; Bungener, 1982; Diffley & Scott, 1984; Diffley *et al.* 1987); increasing the inoculum size shortened the parasitaemia pre–patent period and the time to reach the first peak of parasitaemia. However, it also resulted in an increased density of trypanosomes at the first peak of parasitaemia (Diffley *et al.* 1980; Bungener, 1984). Such differences in density appear to inhibit trypanosome proliferation *in vivo* in a density–dependent fashion, characteristic of the parasite strain (Bungener, 1982, 1984). For example, Diffley *et al.* (1987) have shown that at levels of parasitaemia exceeding 10^8 trypanosomes/ml of blood, a strain of *T. brucei gambiense* exhibited a significantly prolonged generation time compared to lower levels of parasitaemia with the same strain.

Growth and differentiation of trypanosomes appear to be regulated by both host (Black, Jack & Morrison, 1983; Black *et al.* 1983; Olsson *et al.* 1993) and parasite factors (Bungener, 1982; Olsson *et al.* 1991, 1992; Bakhiet *et al.* 1993). There is also evidence that cyclic adenosine monophosphate (cAMP) is directly involved in such regulation, since high levels of parasitaemia have been shown to be associated with elevation in intracellular levels of cAMP and prolongation of the

doubling time in *T. b. brucei* (Mancini & Patton, 1981). The dynamics of growth of mixed populations of trypanosomes within the same host has received considerable interest and it appears that rapidly–growing strains competitively outgrow, and dominate, slow–growing subpopulations (Seed, 1978). There is also evidence to suggest that some drug–sensitive trypanosomes grow at relatively faster rates than drug–resistant strains, which may explain why, in the absence of chemotherapy, drug–sensitive populations appear to overgrow drug–resistant populations *in vivo* (Oehler, 1914; Cantrell, 1956; Hawking, 1963; Sones, Holmes & Urquhart, 1989). While the frequencies with which drug–sensitive and drug–resistant trypanosomes occur *in vivo* are unknown, we have previously shown in goats that the majority of trypanosomes which relapse following treatment with diminazene aceturate appear to remain sensitive to the drug dosage that was used (Mamman *et al.* submitted). However, in that study, the proportions of the populations which survived drug treatment were not determined. In this report, we present estimates of these proportions based on studies carried out in mice that simulate the dose–response relationship previously observed in goats.

MATERIALS AND METHODS

Experimental animals

Swiss white mice (25–38g), obtained from the ILRAD breeding colony, were used throughout the study. They were allowed free access to both water and a pelleted ration (Unga Feeds Ltd., Nairobi).

Trypanosomes

Trypanosoma congolense IL 3274 (Peregrine *et al.* 1991) is a cloned population which was derived from a stock, Banankeledaga/83/CRTA/67, that was isolated from a bull in Burkina Fàso (Pinder & Authie, 1984). In goats, infections with this parasite are refractory to treatment with diminazene aceturate at an intramuscular (i.m.) dose of 7.0 mg/kg body weight (b.w.) when treatment is administered on day 19 of infection (Silayo *et al.* 1992).

Treatment

Berenil® (Hoechst AG, Frankfurt, Germany), freshly prepared in sterile distilled water, was used for intraperitoneal (i.p.) treatment of mice with diminazene aceturate. Calculations for the different doses of diminazene aceturate used in the study were corrected such that the required amounts of drug were contained in a volume of 0.2 ml of sterile distilled water. The same volume of sterile distilled water was administered i.p. to infected, but untreated, control mice.

Study design

Trypanosoma congolense IL 3274 was initially expanded in sublethally (650 rad.) irradiated mice. On day 9 of infection, the resultant population was used to infect non-irradiated mice i.p. at a dose of 10^7 trypanosomes/mouse. After detection of trypanosomes, the mice were treated i.p. with diminazene aceturate at a dose of 25 mg/kg b.w. This dosage was used since it resulted in a parasitaemia profile that was similar to that seen in goats treated i.m. with diminazene aceturate at a dose of 7.0 mg/kg b.w.; treatment caused temporary remission of parasitaemia which was subsequently followed by reappearance of trypanosomes. At this stage the mice were anaesthetised with diethyl ether, exsanguinated by cardiac puncture and the blood collected into sodium citrate (final concentration 1.0% w/v). The number of

trypanosomes/ml in the pooled blood was quantified using a Neubauer haemocytometer. Thereafter, the blood was diluted in phosphate saline glucose (PSG) to prepare 5 different trypanosome inoculum sizes. Using an inoculum volume of 0.2 ml, mice were infected intravenously (i.v.), via the tail vein, with 10^2, 10^3, 10^4, 10^5 or 10^6 trypanosomes. Each inoculum size was used to infect a group of 100 non-irradiated mice. Within a minute of infection mice were either treated i.p. with diminazene aceturate at a dose of 25 mg/kg b.w. or administered 0.2 ml distilled water i.p. Administration of diminazene aceturate and distilled water was carried out in an alternate manner; i.e., mice 1, 3, 5, 7, etc., were administered diminazene aceturate; mice 2, 4, 6, 8, etc., were administered only distilled water. Immediate treatment following infection was carried out in order to minimise the chances of parasites invading drug–inaccessible sites. In this way, 50 mice in each group were administered diminazene aceturate; the other 50 mice were administered only sterile distilled water, and functioned as infectivity controls. Following infection, development of parasitaemia was monitored in all mice for 100 days by examining wet films of tail blood at × 250 magnification; any mouse detected parasitaemic was removed from the experiment.

Data analysis

The relationship between the fraction of the mice detected parasitaemic following treatment with diminazene aceturate and the trypanosome inoculum sizes was investigated using logistic regression. Parameter estimation was undertaken using maximum likelihood methods with GLIM Version 3.77 (Royal Statistical Society, 1985).

Characterisation of the diminazene sensitivity of T. congolense *IL 3274 that reappeared in mice following treatment*

In a similar manner to that described above, the subpopulation of *T. congolense* IL 3274 which reappeared in mice following treatment with diminazene aceturate at a dose of 25 mg/kg b.w. was diluted in PSG to prepare inoculum sizes of 10^3 and 10^6 trypanosomes in 0.2 ml. Each inoculum size was used to i.v. infect 6 groups of 25 mice each. Thereafter, mice in each group were treated immediately with diminazene aceturate i.p. at doses of 0, 10, 20, 30, 40 or 50 mg/kg b.w. For 100 days following treatment, all mice were monitored for development of parasitaemia by examining wet films of tail blood at × 250 magnification; mice detected parasitaemic were removed from the experiment.

Values of the doses of diminazene aceturate required to cure 50% of infected mice (CD_{50}) were determined using logit analysis and the minimum chi–square method (Hewlett & Plackett, 1979).

Characterisation of the diminazene sensitivity of clones derived from T. congolense *IL 3274*

Experiments were carried out to determine the diminazene sensitivity of clones of *T. congolense* IL 3274 that arose in mice prior to, or following, treatment with diminazene aceturate. Bloodstream forms of the parasite were used to infect a group of non-irradiated mice i.p. (10^7 trypanosomes/mouse). Following detection of trypanosomes, on day 3 of infection, the mice were each treated i.p. with diminazene aceturate at a dose of 25 mg/kg b.w. Subsequently, all mice exhibited temporary remission of parasitaemia followed by reappearance of trypanosomes. Trypanosome populations occurring in these animals were cloned on days 2 and 3 of infection (prior to treatment) according to the method of Barry & Gathuo (1984). They were also cloned on days 18, 19 and 20 of infection, when trypanosomes had reappeared following treatment. Each clone was thereafter expanded in sublethally irradiated

(650 rad.) mice. The resultant populations were then used to infect groups of normal mice at an inoculum size of 10^7 trypanosomes/mouse. On day 3 of infection, the mice were treated i.p. with diminazene aceturate at a dose of 25 mg/kg b.w. All mice were then monitored for 100 days for the development of parasitaemia, as described above.

Pharmacokinetics of diminazene in mice

In the first mouse study, all 5 groups of mice were treated with diminazene aceturate using a dose of 25 mg/kg b.w. In order to determine whether differences in the disposition of diminazene occurred as the trypanosome inoculum size was increased, the pharmacokinetics of the drug were determined in three groups of mice. Group A comprised non-infected mice which were treated i.p. with diminazene aceturate at a dose of 25 mg/kg b.w. Mice in Group B were infected i.v. (10^6 trypanosomes/mouse) with a population of *T. congolense* IL 3274 that had reappeared in infected mice following treatment with diminazene aceturate at a dose of 25 mg/kg b.w. Immediately after infection, the mice were treated i.p. with diminazene aceturate at a dose of 25 mg/kg b.w. Mice in Group C were not infected but were treated i.v. with diminazene aceturate at a dose of 15 mg/kg b.w. This last group was included to provide data required to determine values of a number of pharmacokinetic parameters.

Following treatment, blood samples were obtained from mice in each of Groups A, B and C at 5, 10, 15, 30, 40, 45 minutes, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 21, 24 and 48 hours. Since ion–pair extraction of diminazene from blood (Aliu & Ødegaard, 1983) requires a volume of 1 ml, samples from 2 treated mice were pooled in a 3 ml potassium ethylenediamine tetra–acetate–containing Vacutainer® tube for each time point. Furthermore, at each sampling time–point, 10 mice were used to obtain 5 replicate samples. The methods used for plasma separation, storage and analysis of diminazene by high–performance liquid chromatography were as previously described (Aliu & Ødegaard, 1983; Aliu,

Mamman & Peregrine, in press). Plasma concentration–time data sets were modelled using NONLIN Version 3.0 (Statistical Consultants Inc., USA) and STATIS Version 3.0 (ClydeSoft, UK), using JANA (SCI, USA) to determine initial parameter estimates. The Akaike and Schwartz information criteria (Schwartz, 1978; Yamaoka, Nakagawa & Uno, 1978) were used for selection of the compartmental model that best fitted each concentration–time data set. Symbols of compartmental parameters used in this paper are as commonly defined (Allen *et al.* 1982; Gibaldi & Perrier, 1982; Riviere, 1988; Kinabo & McKellar, 1989). Noncompartmental parameters, such as the area under the concentration–time curve ($AUC_{0-\infty}$) and the area under the (first) moment curve ($AUMC_{0-\infty}$) determined by the trapezoidal rule from time 0 to infinity, mean residence time (MRT), apparent total body clearance (Cl) and apparent volume of distribution at steady–state ($V_{d(ss)}$), were calculated in accordance with standard equations (Gibaldi & Perrier, 1982). Bioavailability (F) represents the percentage ratio of $AUC_{0-\infty}$(i.p.) to $AUC_{0-\infty}$(i.v.). All statistical analyses were carried out using Minitab Version 8.2 (Minitab Inc., USA). Geometric means (range) are given for half–life parameters. Values of other parameters are expressed as arithmetic mean ± standard deviation. A single classification analysis of variance (ANOVA) was used to test differences between values of pharmacokinetic parameters determined in the three groups at the 5% significance level.

RESULTS

Relationship between inoculum size and diminazene sensitivity

Five groups of 100 mice each were infected i.v. with a population of *T. congolense* IL 3274 that relapsed in infected mice following i.p. treatment with diminazene aceturate at a dose of 25 mg/kg b.w. The results obtained in each of the 5 groups are

Table 1 Proportions of mice with diminazene-resistant trypanosomes after infection with relapse* populations of *Trypanosoma congolense* IL 3274, and estimated proportions of diminazene-resistant trypanosomes in each inoculum size

No. trypanosomes/ mouse	No. observed parasitaemic/ No. treated	No. predicted parasitaemic§ /No. treated	Estimate for proportion resistant
10^2	4/50	3.9/50	8.335×10^{-4}
10^3	11/50	8.6/50	2.485×10^{-4}
10^4	13/50	16.9/50	3.020×10^{-5}
10^5	28/50	27.8/50	8.300×10^{-6}
10^6	39/50	37.8/50	1.600×10^{-6}

* The population had relapsed in infected mice following intraperitoneal treatment with 25 mg diminazene aceturate/kg body weight.

§ Estimated using a logistic model (see text).

presented in Table 1. In each group, 50 out of 50 control mice were detected parasitaemic, thus indicating 100% infectivity for all 5 inoculum sizes. In contrast, in the groups of mice inoculated with 10^2, 10^3, 10^4, 10^5 or 10^6 trypanosomes and treated with diminazene aceturate at a dose of 25 mg/kg b.w., trypanosomes were detected in 4/50, 11/50, 13/50, 28/50 and 39/50 mice, respectively. Blood from the mice that remained aparasitaemic was not infective to irradiated mice (data not shown). In addition, sera from the same negative mice were negative for *T. congolense* antigen when examined with an enzyme–linked immunosorbent assay (Nantulya & Lindqvist, 1989) (data not shown).

Estimation of the proportion of diminazene sensitive and diminazene–resistant trypanosomes

The data presented in Table 1 shows that not all mice infected with *T. congolense* IL 3274 and treated with diminazene aceturate became parasitaemic. Furthermore, mice receiving lower inoculum sizes appeared less likely to become parasitaemic than mice receiving higher inoculum sizes. Logistic regression was used to investigate the relationship between the probability of a mouse being parasitaemic and the size of the inoculum. A relationship of the form

$$\text{Log}\,(p/1-p) = -4.27 + 0.90\log(IS)$$

where p = the proportion of mice detected parasitaemic following treatment with diminazene aceturate,

and IS = the trypanosome inoculum size,

was found to be a good fit. The 95% confidence intervals for the intercept and slope were (–5.38, –3.16) and (0.66, 1.14), respectively. Table 1 shows the closeness of fit between the observed number of mice that were detected parasitaemic and the number that were predicted parasitaemic using the logistic equation.

The possibility that each inoculum contained a mixture of sensitive and resistant trypanosomes, and that sampling variation resulted in some inocula not containing any resistant trypanosomes, was investigated as a possible explanation for the relationship between the inoculum size and the proportion of mice that became parasitaemic. For an inoculum of size n trypanosomes containing a proportion θ of resistant trypanosomes, the probability of a mouse receiving at least one resistant trypanosome is $1 - (1-\theta)^n$. Using the observed proportion of animals detected parasitaemic (Table 1) as an estimate of this proportion, it was possible to obtain an estimate of θ for each inoculum size. For inocula of sizes 10^2, 10^3, 10^4, 10^5 and 10^6 trypanosomes, the maximum likelihood estimates for θ were 8.335×10^{-4}, 2.485×10^{-4}, 3.02×10^{-5}, 8.3×10^{-6} and 1.6×10^{-6}, respectively. Likelihood ratio testing confirmed that the estimates were significantly different.

Diminazene sensitivity of relapse populations of T. congolense *IL 3274*

Table 2 summarizes the diminazene sensitivity data for *T. congolense* IL 3274 that appeared in mice following treatment with diminazene aceturate at a dose of 25 mg/kg b.w. As shown in Table 2, regardless of the inoculum size, trypanosomes were detected in all mice that served as untreated controls and in those treated with diminazene aceturate at a dose of 10 mg/kg b.w. Similarly, trypanosomes were detected in all mice which were treated with diminazene aceturate (20 mg/kg b.w.) after infection with 10^6 trypanosomes. In other treated groups the number of mice that were detected parasitaemic decreased with increasing doses, 20 – 50 mg/kg b.w., of diminazene aceturate. Based on these data, the diminazene aceturate CD_{50} value determined for mice infected with 10^6 trypanosomes was 29.88 mg/kg b.w., and was significantly ($p < 0.001$) greater than that determined for mice infected with 10^3 trypanosomes (CD_{50} = 22.28 mg/kg b.w.).

Table 2 Diminazene sensitivity of *Trypanosoma congolense* IL 3274 that reappeared in infected mice following intraperitoneal treatment with 25 mg diminazene aceturate/kg b.w.

Dose of diminazene aceturate (mg/kg b.w.)	Inoculum size: No. of trypanosomes/ mouse	
	10^3	10^6
	No. detected parasitaemic/No. treated	
0.0	25/25	25/25
10.0	15/25	25/25
20.0	2/25	13/25
30.0	4/25	4/25
40.0	2/25	2/25
50.0		

b.w. Body weight

Table 3 Coefficients and exponents describing disposition of diminazene following administration of diminazene aceturate at single doses of 15.0 mg/kg b.w. intravenously, or 25.0 mg/kg b.w. intraperitoneally in non-infected and *T. congolense*-infected mice

Parameter	Units	Route of administration of diminazene aceturate		
		Intraperitoneal *(non-infected)*	Intraperitoneal *(infected)*	Intravenous *(non-infected)*
C_1	μg/ml	14.24 ± 10.37*	13.21 ± 8.23‡	2.01 ± 1.44*‡
C_2	μg/ml	2.79 ± 0.43*	1.42 ± 1.77	0.93 ± 0.40*
C_z	μg/ml	0.46 ± 0.45	0.28 ± 0.17	0.24 ± 0.08
λ_1	h^{-1}	23.30 ± 17.10*	15.17 ± 16.45	5.36 ± 1.67*
λ_2	h^{-1}	1.31 ± 0.64*	0.83 ± 0.96	0.45 ± 0.10*
λ_z	h^{-1}	0.07 ± 0.05	0.03 ± 0.03	0.04 ± 0.01

* and ‡ Values with the same symbol are significantly different ($P < 0.05$).

Table 4 Pharmacokinetic parameters of diminazene in non-infected and *T. congolense*-infected mice following administration of diminazene aceturate at doses of 15.0 mg/kg b.w. intravenously, and 25.0 mg/kg b.w. intraperitoneally

Parameter	Units	Route of administration of diminazene aceturate		
		Intraperitoneal *(non-infected)*	Intraperitoneal *(infected)*	Intravenous *(non-infected)*
$t_{1/2}\lambda_1$	h	0.036 (0.013-0.086)*	0.074 (0.016-0.28)	0.13 (0.096-0.25)*
$t_{1/2}\lambda_2$	h	0.57 (0.29-0.84)*	1.86 (0.28-19.23)	1.51 (1.23-2.27)*
$t_{1/2}\lambda_z$	h	10.71 (4.11-24.54)	25.70 (8.07-105.92)	17.37 (12.59-31.80)
$t_{1/2}K_{el}$	h	0.38* (0.17-0.68)	0.45‡ (0.15-0.79)	1.94*‡ (1.26-3.20)
K_{el}	h^{-1}	2.00 ± 1.18*	1.88 ± 1.46‡	0.37 ± 0.12*‡
K_{12}	h^{-1}	14.59 ± 14.78	8.64 ± 9.86	2.45 ± 1.73
K_{13}	h^{-1}	2.88 ± 1.77*	3.60 ± 2.36‡	0.53 ± 0.26*‡
K_{31}	h^{-1}	0.25 ± 0.29	0.14 ± 0.13	0.12 ± 0.04
K_{21}	h^{-1}	4.96 ± 0.46*	2.64 ± 3.40	2.36 ± 1.27*
V_c	L/kg	1.92 ± 1.25*	2.17 ± 1.04‡	5.30 ± 2.12‡*

* and ‡ Values with the same symbol are significantly different ($P < 0.05$).
Geometric mean (range) values are given for $t_{1/2}\lambda_1$, $t_{1/2}\lambda_2$, $t_{1/2}\lambda_z$ and $t_{1/2}K_{el}$.

Table 5 Values of non-compartmental pharmacokinetic parameters of diminazene in non-infected and *T. congolense*-infected mice following administration of diminazene aceturate at single doses of 15.0 mg/kg b.w. intravenously, and 25 mg/kg b.w. intraperitoneally

Parameter	Units	Route of administration of diminazene aceturate		
		Intraperitoneal *(non-infected)*	Intraperitoneal *(infected)*	Intravenous *(non-infected)*
C_{max}	µg/ml	17.49 ± 10.25*	14.89 ± 10.05‡	3.19 ± 1.17*‡
$AUC_{0-\infty}$	µg.h/ml	9.70 ± 1.83	13.33 ± 3.83	9.14 ± 1.92
$AUMC_{0-\infty}$	µg.h²/ml	174.76 ± 67.23	785.41 ± 914.91	218.40 ± 111.29
MRT	h	17.89 ± 5.39	50.04 ± 46.24	22.97 ± 8.65
Cl	ml/min/kg	28.25 ± 5.73	28.62 ± 5.73	28.28 ± 5.73
$V_{d(ss)}$	L/kg	47.32 ± 15.24	84.29 ± 54.68	36.99 ± 10.52
F	(%)	66.16 ± 20.97	87.00 ± 15.71	NA

* and ‡ Values with the same symbol are significantly different ($P < 0.05$).
NA Not applicable.

in the non-infected group treated i.v., 5.30 ± 2.12 L/kg, was significantly greater than in both the non-infected and infected i.p. treatment groups. Similarly, the geometric mean half–life of the elimination rate constant from the central compartment ($t_{1/2}K_{el}$) in the i.v. group was significantly higher than in the i.p. treatment groups. In contrast, C_{max} in the i.v. group, 3.19 ± 1.17 μg/ml, was significantly lower than in both i.p. treatment groups. There were no significant differences between the i.v. and i.p. treatment groups with respect to $AUC_{0-\infty}$, $AUMC_{0-\infty}$, MRT, Cl and $V_{d(ss)}$.

DISCUSSION

In the work presented here, experiments were carried out to investigate the dynamics of expression of diminazene resistance amongst trypanosomes which reappear in infected mice following treatment with diminazene aceturate. *Trypanosoma congolense* IL 3274 was used for these studies. In initial work, experiments were carried out to examine the trypanosome populations which reappeared in infected mice following treatment with diminazene aceturate at a dose of 25 mg/kg b.w. Inoculum sizes of 10^2, 10^3, 10^4, 10^5, and 10^6 trypanosomes were injected i.v. into large numbers of mice. Immediately thereafter, their sensitivity to i.p. treatment with diminazene aceturate at a dose of 25 mg/kg b.w. was determined. In the absence of treatment, trypanosomes were detected in the blood of all mice that were infected. In additional work, infectivity was demonstrated at the level of individual trypanosomes (data not shown). In mice, however, which were infected and treated, different proportions of animals were cured depending on the inoculum size. The number of mice that became parasitaemic, despite treatment, progressively increased from 4/50 to 39/50 from the lowest (10^2) to the highest (10^6) inoculum sizes used. A close fit to these data was obtained when the data was modelled using logistic regression. The observed data was also analysed using a mathematical model to determine the proportions of the diminazene–resistant and diminazene–sensitive trypanosomes that were present in the relapse population. The model assumed that these proportions

were fixed constants regardless of the inoculum size. The resultant data indicated that with all 5 inoculum sizes, less than 0.1% of the trypanosomes were resistant to the drug dosage used. Thus, the majority of the populations appeared to be sensitive to diminazene aceturate at this dosage. Surprisingly, however, the proportion of the population that contained resistant trypanosomes did not remain constant, but varied inversely with the inoculum sizes. Interpretations of these results are given below.

The above data indicate that the subpopulation of *T. congolense* IL 3274 which reappeared in infected animals following treatment with diminazene aceturate at a dose of 25 mg/kg b.w. contained trypanosomes that were both resistant and sensitive to the drug dosage used. There could be several explanations for this observation. First, it has been shown that populations of *T. brucei* in mice (Jennings *et al.* 1979) and *T. vivax* in goats (Whitelaw, Gardiner & Murray, 1988) can evade treatment and establish relapse parasitaemias by invading the central nervous system (CNS). It is therefore possible that a similar phenomenon may have accounted for the presence of the drug–sensitive trypanosomes that were observed in the relapse populations described herein, although immediate treatment should have circumvented this possibility. Secondly, sites other than the CNS may serve as cryptic foci (Waitumbi *et al.* 1988). Thus, since *T. congolense* can localize in the microvasculature (Maxie & Losos, 1977; Banks, 1978; Kalu, Lawani & Omotainse, 1988), such sites may serve as cryptic foci for the parasite if they are poorly perfused with blood. A third possibility is that diminazene resistance in *T. congolense* IL 3274 is unstable, as has previously been reported for isometamidium in *T. congolense* (Peregrine *et al.* 1991). It is therefore possible that during expansion of the trypanosome population, a drug–sensitive variant with a growth rate more rapid than that of a drug–resistant population could dominate the relapse population (Sones *et al.* 1989). However, since there appeared to be little variation in diminazene sensitivity amongst clones derived from *T. congolense* IL 3274, this would appear unlikely.

The present study also demonstrated that the level of diminazene resistance of trypanosome populations varies directly with the inoculum size. However, the inoculum size varied inversely with the calculated drug–resistant proportion for each population. The reasons underlying this latter observation were investigated in two experiments. In the first, we examined whether the resistance of the trypanosome population decreased as the inoculum size was increased. This was investigated by characterising the sensitivity of two relapse population inoculum sizes (10^3 and 10^6 trypanosomes) to different doses of diminazene aceturate in mice. The results indicated that the diminazene aceturate CD_{50} value for mice infected with 10^6 trypanosomes was 29.88 mg/kg b.w., and was significantly greater than that for mice infected with 10^3 trypanosomes, 22.28 mg/kg b.w. These observations therefore indicate that there is a direct relationship between the inoculum size and the level of resistance of a population, as described by Sones & Holmes (1992). The object of the second experiment was to determine whether the inverse relationship between trypanosome inoculum size and the proportion of surviving trypanosomes occurred because the parasites were exposed to decreasing concentrations of the drug as the inoculum size was increased, due to effects of parasite numbers on drug pharmacokinetics. The resultant data indicated that the disposition of diminazene did not vary significantly with the trypanosome inoculum size.

The concentration that a trypanosome population attains in rodents is regulated both by host factors (Black, Jack & Morrison, 1983; Black *et al.* 1983) and host–independent mechanisms associated with the parasite population (Bungener, 1982). The results described here have demonstrated that the diminazene sensitivity is dependent upon the concentration of a trypanosome population *in vivo*. Other studies have shown that the concentration of a population may influence the rate of proliferation of individual trypanosomes (Bungener, 1982, 1984), thus potentially altering the proportions of the population in the different stages of the cell–division cycle. While little is known about the mechanism of action of diminazene on

trypanosomes, evidence from other systems indicates that diminazene acts on organisms at specific stages of the cell–division cycle (Kishore *et al.* 1990; Poot *et al.* 1990; Stauffert *et al.* 1990; Byres *et al.* 1991). A full explanation of the results observed in the present study requires an extensive study into the factors which influence trypanosomal cell–division and proliferation, and whether there is developmental and/or cell–division cycle control of expression of drug resistance *in vivo*.

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SUMMARY AND CONCLUSIONS

Chapter 13

SUMMARY AND CONCLUSIONS

Vector control and chemotherapy remain the only practical methods used in limiting the severe economic losses due to African animal trypanosomiasis. However, a number of problems limit full utilisation of these approaches.

The major constraints of vector control include re–invasion by tsetse flies into cleared areas (Hursey, 1972; Finelle, 1980; Itard, 1980), pollution of the environment with chemical insecticides (Koeman *et al.*, 1971), and the cost required to achieve and maintain efficient use of traps and screens in the field.

Development of resistance against all available animal anti–trypanosomal drugs is perhaps the most important problem associated with chemotherapy of animal trypanosomiasis. In addition, the number of these chemical agents has, over the years, *dwindled. Furthermore, cross–resistance limits application of structural analogues of* the existing anti–trypanosomal compounds. However, little is known about the pharmacokinetics and molecular mechanisms of action of these agents. Thus, since it is not likely that new compounds will be introduced against animal trypanosomiasis in the near future, it is becoming necessary to properly address problems associated with chemotherapy of the disease in order to make optimal use of the existing anti–trypanosomal drugs. In this dissertation, therefore, studies were carried out to define the pharmacokinetics of diminazene and the mechanisms of trypanosomal resistance operating *in vivo*. It is hoped that these studies would provide data that could be used *to enhance the therapeutic efficacy of diminazene aceturate in the chemotherapy of* animal trypanosomiasis.

Since its introduction in the late 1950s, diminazene aceturate (Berenil®) has remained the most commonly used curative agent against animal trypanosomiasis and is administered intramuscularly (i.m.) as a single dose of 3.5 or 7.0 mg/kg b.w. (Williamson, 1980; Leach and Roberts, 1981). The pharmacokinetic studies

described in this dissertation were carried out to compare disposition of diminazene in non-infected and trypanosome–infected animals, and also to supplement existing data on the kinetics of the drug in plasma with information about disposition of the compound in the cerebrospinal fluid (CSF) and lymph.

In non-infected Boran (*Bos indicus*) cattle, single i.m. administration of 3.5 mg diminazene base/kg b.w. was followed by rapid absorption; the absorption constant, K_a, was 2.56–11.60 h^{-1}. Concentrations of the drug in plasma rose from 1.12–2.71 μg/ml at 5 min of treatment to maximum levels, 2.99–6.22 μg/ml, within 10–45 min. However, they declined to 0.36–0.58 μg/ml by 48 h. Only about 8.0% of the administered dose appeared in urine over the first 24 h of treatment. Thus, although only low concentrations of the drug were detectable in plasma after the first 48 h of treatment, the greater percentage of the administered dose was retained in the body. Indeed, the large apparent volume of distribution at steady state ($V_{d(ss)}$), 1.20–2.02 L/kg, slow total body clearance (Cl), 1.24–1.70 ml/min/kg, and long elimination half–life ($t_{1/2}\lambda_z$), 86.50–145.48 h, suggested extensive systemic distribution and persistence of the drug in the body. These findings contrast with earlier suggestions that the drug is rapidly excreted from the body within 24 h of treatment (Fairclough, 1963; Leach and Roberts, 1981). However, these earlier reports were not based on detailed pharmacokinetic data since, until recently, the highly sensitive techniques required to analyse diminazene in biological fluids were not available.

In general, values of the aforementioned pharmacokinetic parameters are similar to those determined in *Bos taurus* cattle breeds (Klatt and Hajdu, 1976; Kellner *et al.*, 1985). However, due to considerable differences in experimental design and data analysis, the three–compartmental model which best characterised the disposition of the drug in our studies in cattle was in contrast to the two–compartmental model reported by Klatt and Hajdu (1976) and Kellner *et al.* (1985). Consequently, the values of some of the parameters in the earlier studies differed from those in our findings. Despite the differences, however, the general patterns of the

plasma concentration–versus–time profiles were similar and, as reported by Kellner *et al.* (1985), diminazene was also detected in plasma of cattle several weeks after treatment.

Pharmacokinetic data of chemotherapeutic agents, derived both in health and disease, form a rational basis for design of dosage regimes against diseases (Gibaldi, 1984; Berman and Fleckenstein, 1991). Subsequent to characterising the disposition of diminazene in healthy cattle we, therefore, extended our studies to examine the influence of infection with diminazene–sensitive populations of *T. congolense* (clones IL 1180 and IL 2642) on the pharmacokinetics of the drug in cattle. The studies involved 5 Boran cattle which were used in a double cross–over design comprising 3 phases; non-infected, acute infection with IL 1180, and chronic infection with IL 2642. Whereas the pharmacokinetic data determined in these animals when non-infected and during chronic infection with *T. congolense* IL 2642 did not differ significantly, values of C_{max}, t_{max}, and $V_{d(ss)}$ determined in these two states of infection were significantly different from those obtained during acute infection with *T. congolense* IL 1180.

Factors accounting for the observed differences in acute infection are not clear but may be due to altered plasma protein binding, elevated metabolic rate, tachycardia and increased cardiac output which characterise acute states (van Miert, 1985; Baggot, 1977; Swenson, 1977; Gibaldi, 1984). Significant changes in the pharmacokinetics of diminazene aceturate in *T. congolense*-infected dogs (Onyeyili and Anika, 1989) and imidocarb, a closely related aromatic diamidine, in *T. evansi*-infected goats (Abdullah and Baggot, 1986) have previously been reported.

Other noncompartmental and compartmental parameters of the drug determined from the animals were found not to differ significantly between the three phases of the study. At 48 h after treatment, mean plasma concentrations of diminazene maintained in the cattle were 0.43 ± 0.07 μg/ml in the non-infected state, 0.43 ± 0.11 μg/ml during acute phase of infection, and 0.44 ± 0.09 μg/ml in chronic

infection. Unlike previous assumptions (Davey, 1957), therefore, plasma concentration–versus–time profiles of diminazene in non-infected cattle were not significantly influenced by concurrent acute or chronic trypanosome infections. In agreement with these findings we have also examined the disposition of diminazene in mice and found no significant differences between pharmacokinetic data of the drug in non-infected and *T. congolense*–infected animals. Similar conclusions were reached from earlier studies in *T. congolense*–infected rabbits (Gilbert and Newton, 1982) and dogs infected with *T. congolense* (Onyeyili and Anika, 1989).

In the aforementioned studies, we also observed that diminazene, at a single dose of 3.5 mg/kg b.w., was curative against parasite populations that were present in the cattle during acute and chronic infections (i.e., infected with *T. congolense* IL 1180 or *T. congolense* IL 2642). The findings therefore suggested that diminazene, maintained in plasma at a mean concentration of 0.43 ± 0.11 μg/ml for 48 h, was sufficient to eliminate diminazene–sensitive *T. congolense* infections in animals. In contrast, the plasma concentrations of diminazene required to eliminate infections with diminazene–resistant trypanosomes are unknown.

However, it is clear from *in vitro* studies with *T. congolense* that elimination of diminazene–resistant trypanosomes requires higher concentrations of the drug than with diminazene–sensitive populations (Kaminsky *et al.*, 1993; Gray and Peregrine, in press). In addition, *in vitro* studies indicate that the optimal efficacy of Berenil® against both diminazene-sensitive and diminazene-resistant populations of *T. congolense* occurs only after incubation of the parasites with the drug for a minimum of 48 h (Gray and Peregrine, 1993). Similarly, Kaminsky *et al.* (1993) have reported that growth or viability of diminazene-resistant *T. congolense* can be impaired by incubating the parasites with high or relatively lower amounts of the drug for 24 h or 10 days, respectively. Collectively, these observations indicate that elimination of trypanosomes *in vitro* is dependent on both the amount and duration of drug to which parasites are exposed, and further suggest that cure of diminazene–resistant

trypanosome populations *in vivo* may be achieved, but with higher plasma concentrations of the drug maintained for a longer duration than required for diminazene–sensitive strains.

To investigate this possibility, therefore, experiments were carried out in 3 groups of 5 goats which were infected by the feeding of *Glossina morsitans centralis* infected with *T. congolense* IL 3274, a cloned population which expresses resistance to single treatment with diminazene aceturate at a dose of 7.0 mg/kg b.w. in goats. After detection of trypanosomes, the goats were treated with diminazene aceturate at a dose of 7.2 mg/kg b.w. administered once or twice (at 8 or 24 h intervals). Parasitologically, 5 out of 5 goats which were treated once relapsed after the treatment. In contrast, cures were observed in 2 out of 5 goats in which the treatment was repeated at 8 h, and in 1 out of 5 goats which received a second dose at 24 h. It is interesting that infections with the drug–resistant clone were eliminated in some animals which received a second dose within 24 h after the first treatment. Concentrations of the drug in plasma of animals that were treated twice were significantly higher than in those treated once. Furthermore, the pharmacokinetic data for the drug were found to differ significantly with the number of doses administered. In particular, the drug exhibited a significantly higher plasma concentration and longer elimination half–life in animals treated twice. The pharmacokinetic data suggested that the partial efficacy observed in animals which received the two doses resulted from exposure of the parasites to the drug at concentrations that were significantly higher than normally occur following single dose treatment. This would further suggest that elimination of diminazene–resistant trypanosomes *in vivo* is possible through modification of dosage regimes of diminazene, such that the drug concentration in the plasma is maintained above a certain therapeutic threshold. These findings suggest that a two–dose treatment is therapeutically more advantageous than single–dose treatment. However, further studies are required to determine the appropriate time interval between the two doses required for maximum

curative efficacy, and also whether the observed differences in response to the treatments are reproducible and statistically significant.

In additional studies, we observed that the sensitivity of *T. congolense* IL 3274 to diminazene aceturate depends on the time at which the drug is administered in relation to initiation of infection via tsetse challenge. In goats that were infected in such a manner, a single dose of diminazene aceturate (7.0 mg/kg b.w.) administered on day 1 after challenge eliminated infections in 5 out of 5 goats. In contrast, infections relapsed in all goats that received the same drug dosage on day 19 of infection (3 days after first detection of trypanosomes in jugular blood). When the same treatment was administered, prior to detection of parasites in peripheral blood, on days 4, 8 or 12 of infection, the number of goats that were cured compared to the number treated were 2/5, 2/5 and 2/5, respectively. Quite surprisingly, when trypanosomes which reappeared in goats following treatment on day 19 of infection were cyclically transmitted to naive goats, parasites present in the skin on day 1 of infection were again completely susceptible to a single dose of diminazene aceturate. However, as before, infections relapsed in all goats that were given the same treatment on day 19 of infection. The aetiology of this apparent time–dependent response of *T. congolense* IL 3274 to treatment with diminazene aceturate was unclear and was therefore investigated.

Firstly, we investigated whether the reappearance of *T. congolense* IL 3274 in goats following treatment with diminazene aceturate occurs as a result of invasion of the central nervous system (CNS), a site that is believed to be inaccessible to diminazene. Such a phenomenon has previously been shown to occur with *T. brucei* in mice (Jennings *et al.*, 1979) and *T. vivax* in goats (Whitelaw *et al.*, 1988). In the study described here, *T. congolense* IL 3274 infections were transmitted to 7 goats via the bites of infected tsetse flies. Following treatment with diminazene aceturate at a dose of 7.0 mg/kg b.w. on day 19 of infection, trypanosomes reappeared in all goats within 6–8 days of drug administration. For the purpose of this study, we developed

a lumbar subarachnoid space cannulation technique which permitted collection of lumbar CSF samples from the goats on a daily basis, beginning one day prior to initiation of infection, and continuing until 3 days after trypanosomes had reappeared following the treatment. Neither trypanosomal antigen (by ELISA) nor intact trypanosomes (by microscopic examination) could be demonstrated in any of the samples. The findings, therefore, indicated that *T. congolense* IL 3274 does not establish relapse parasitaemias following treatment on day 19 of infection as a result of survival in the CNS.

Secondly, since *T. congolense* is confined to the skin on day 1 following tsetse transmission (Dwinger *et al.*, 1988), a pharmacokinetic study was carried out in goats to determine whether the apparent efficacy of the treatment at this stage of infection was due to the availability of higher concentrations of the drug at the level of the skin than in plasma. The pharmacokinetics of diminazene were therefore determined in afferent lymph, the fluid draining the skin. Results from the study indicated that concentrations of the drug in blood plasma were significantly higher than in lymph draining the skin. It is notable, however, that the elimination half–life of the drug in lymph was significantly longer than in plasma. It remains to be determined whether the prolonged persistence of the drug in the lymph, compared to plasma, and/or the number of parasites present in the skin are the key factors responsible for the apparent susceptibility of the parasites to treatment on day 1 of tsetse challenge.

In a third study, we used the subarachnoid space cannulation technique described above to investigate the pharmacokinetics of diminazene in the lumbar CSF of 5 non-infected goats which were each treated i.m. with a single dose of 3.5 mg diminazene base/kg b.w. For the purpose of this study, we developed a lumbar subarachnoid space cannulation technique which permitted daily collection of CSF. As previously observed in cattle (Kellner *et al.*, 1985) and dogs (Onyeyili and Anika, 1991), the drug was found to access the CSF. However, compared to the plasma, the drug in the CSF attained significantly lower concentrations, extensively larger

distribution and higher clearance. Since *T. congolense* IL 3274 survived the higher concentrations of the drug attained in blood plasma, the data from our studies suggested that the low concentrations of the drug in CSF would be unlikely to eliminate populations of *T. congolense* IL 3274 if they invaded the CNS. However, if the clearance rate of diminazene from the CSF is altered in trypanosomiasis as occurs with penicillin when used for treatment of meningitis (Gibaldi, 1984), therapeutic concentrations of the drug may be attained in the CNS.

It is apparent from our studies that infections with *T. congolense* IL 3274 in goats show a consistent response pattern to single doses of diminazene aceturate when administered after microscopic demonstration of the parasites in peripheral blood; i.m. treatment with 7.0 mg/kg b.w. results in temporary remission of parasitaemia within 24–48 h followed by relapse parasitaemia within 7–14 days of drug administration. WE also observed a similar pattern in infected mice following intraperitoneal (i.p.) treatment with diminazene aceturate at a dose of 25 mg/kg b.w. This response pattern following treatment indicated that the parasitaemic wave is a mixture of diminazene-sensitive and diminazene-resistant subpopulations. However, the frequency of drug-resistant trypanosomes in populations occurring prior to, and after, drug treatment was unknown. Therefore, studies were carried out to determine the frequency with which diminazene–resistant trypanosomes occur *in vivo*. Identification and characterisation of markers for drug resistance from these studies may lead to the design and implementation of effective control strategies against animal trypanosomiasis.

Estimates of the frequency of diminazene–resistant trypanosomes in trypanosome populations were initially determined in groups of goats that were infected intravenously (i.v.) with bloodstream forms of *T. congolense* IL 3274. Following infection, the animals were administered diminazene aceturate at a dose of 7.0 mg/kg b.w., either immediately or subsequent to detection of trypanosomes in peripheral blood. In populations of the parasites occurring in infected goats prior to

treatment, the frequency of diminazene–resistant trypanosomes was estimated to be less than 1.0 in 10^3. Quite surprisingly, this was higher than the frequency determined in populations which reappeared after treatment (less than 1.0 in 10^5). The findings therefore suggested that diminazene–resistant trypanosomes occurred at a very low frequency *in vivo*, and that the vast majority of trypanosomes which reappeared after treatment were in fact sensitive to the therapeutic dose of diminazene aceturate that was used.

In order to obtain more rigorous estimates for the frequency at which diminazene–resistant trypanosomes occur *in vivo*, similar experiments were carried out in large numbers of mice infected with a subpopulation of *T. congolense* IL 3274 that had reappeared in infected mice following i.p. treatment with diminazene aceturate at a dose of 25 mg/kg b.w. In this study, the parasites were found to be 100% infective to mice at inoculum sizes of $10^0 - 10^6$ trypanosomes/mouse. In 5 groups of 50 mice each, which were infected with 10^2, 10^3, 10^4, 10^5 or 10^6 trypanosomes/mouse, animals were treated i.p. immediately after infection with diminazene aceturate at a dose of 25 mg/kg b.w. The subsequent responses to treatment indicated that estimates of the diminazene–resistant proportions in the inoculum size were 8.3×10^{-4}, 2.4×10^{-4}, 3.0×10^{-5}, 8.3×10^{-6} and 1.6×10^{-6}, respectively. These findings confirmed our earlier observations in goats. However, they also indicated that the proportion of diminazene–resistant trypanosomes in relapse populations did not remain constant but decreased with increasing inoculum sizes. The factors responsible for this inverse relationship are unknown.

Two experiments were therefore carried out to determine whether the observed variation occurred due to alterations in the pharmacokinetics of diminazene and/or drug–sensitivity of the parasites as the inoculum size increased. Differences in pharmacokinetics as a function of inoculum size did not appear to play a role since pharmacokinetic data for diminazene, determined in groups of mice when non-infected and during infection with *T. congolense* IL 3274, did not differ significantly.

Furthermore, diminazene aceturate CD_{50} values determined in mice infected with *T. congolense* IL 3274 increased with increasing inoculum sizes, as previously observed (Sones and Holmes, 1992). While the factors which govern the *in vivo* relationship between the frequency of diminazene–resistant trypanosomes and inoculum size remain to be established, earlier studies with *Acanthamoeba* (Kishore *et al.*, 1990; Byres *et al.*, 1991), amniotic fluid fibroblast–like cells (Poot *et al.*, 1990) and human peripheral lymphocytes (Stauffert *et al.*, 1990) suggest that the effects of diminazene, and related diamidines, are specific to certain stage(s) of the cell–division cycle. In future studies, therefore, differences in drug susceptibility of trypanosomes in the various stages of the cell–division cycle should be characterised. Such information may enhance our understanding of the mode of action of anti–trypanosomal drugs, mechanisms of drug resistance in trypanosomes, and could lead to the identification of new targets for therapeutic intervention with chemotherapeutic compounds.

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