STUDIES ON THE TRYPANOCIDAL DRUG, HOMIDIUM; DEVELOPMENT AND USE OF ELISA FOR ITS DETECTION AND QUANTIFICATION IN CATTLE

by

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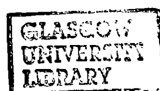

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DEDICATION

This this hiis thesis is dedicated to:

My husband, Stephen Murilla

Children : Lynette, Erick, Angella and Kennedy

for ther torr their love, perseverance and understanding

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ACRONYMS

α	Alpha
β	Beta
γ	Gamma
AUC	Area under curve
AUMC	Area under moments curve
BSA	Bovine serum albumin
Clb	Clearance, body
Cp0	Concentration at time 0 (extrapolated)
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESG	EDTA saline glucose
FAO	Food Agricultural Organisation
GLC	Gas liquid chromatography
GLC-MS	Gas liquid chromatography-mass spectrometry
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
ILRAD	International Laboratory for Research on Animal Diseases
ILRI	International Livestock Research Institute
IR	Infrared
KETRI	Kenya Trypanosomiasis Research Institute
kg	Kilogramme
KLH	Keyhole limpet haemocyanin
MAT	Mean absorption time
mg	milligramme(s)
ml	millilitre(s)

mM	Millimolar
MRT	Mean residence time
ng	nanogramme(s)
OD	Optical density
ODS	Octadecylsilane
PBS	Phosphate buffered saline
PCV	Packed cell volume
RIA	Radioimmunoassay(s)
RP	Reversed phase
SPE	Solid phase extraction
TMB	Tetramethybenzidine
UV	Ultraviolet
VAT	Variable antigen types
Vc	Volume of the central compartment
WHO	World Health Organisation

SUMMARY

This thesis concerns the development, validation and use of enzyme-linked immunosorbent assays (ELISA) to determine homidium concentrations in sera of treated cattle.

Previously published work with particular emphasis on control and specifically, chemotherapy of animal trypanosomiases are reviewed in Chapter One. This includes the development and use of trypanocidal drugs detailing previous analytical techniques used in the determination of drug levels in plasma/serum of treated cattle.

Chapter 2 describes the general materials and methods used in the experiments reported in the later Chapters of this thesis.

Chapter 3 describes the development of two highly sensitive homidium ELISA methods (detection limit 0.1 ng ml^{-1}); Assay 1 an indirect competition assay and the Assay 2 a direct competition assay. Validation of the assays was carried out on serum samples obtained from treated Friesian calves. Using Assay 2, the serum homidium concentrations obtained following treatment of calves showed less variations between individual animals when compared to Assay 1. It was thereafter adopted for use in all subsequent experiments.

Following the development and validation of the ELISA method, several experiments were carried out in cattle using homidium bromide at 1 mg kg^{-1} b.w. to establish baseline data on serum homidium concentrations and pharmacokinetics in cattle. Serum homidium concentrations and pharmacokinetics were determined following i.v. treatment of Friesian calves. No drug was detectable after approximately 17 days of treatment in four out of five and in 21 days in the remaining calf showing rapid elimination of the drug.

Following the establishment of homidium pharmacokinetics in the Friesian cattle in work carried out in Scotland, the studies were extended to Boran (*Bos indicus*) cattle, a breed of cattle which is commonly kept in the

trypanosomiasis endemic areas, in Kenya. Following i.m. treatment with homidium bromide at 1 mg kg^{-1}, serum homidium concentrations were determined. The results showed a wide variation in serum homidium concentrations between individual Boran cattle when compared to the Friesian. However, both groups showed similar the serum homidium concentration-versus-time profiles.

The results of investigations into homidium as a chemotherapeutic drug are reported in Chapter 5. Two groups of five animals were inoculated with two different populations of *T. congolense*; one drug-sensitive (IL 1180) and the other drug-resistant (IL 3330). The animals were treated with homidium bromide at 1 mg kg^{-1} seven days following detection of trypanosomes. No trypanosomes were detected in the cattle infected with the drug-sensitive trypanosome population within 24 hours in four and 48 hours in of five cattle following treatment. The animals remained aparasitaemic up to the end of the observation period of 90 days post-treatment. Whist trypanosomes did not disappear from the circulation following treatment of cattle infected with drug-resistant trypanosome population, low serum homidium concentrations of between 0.1 and 0.3 ng ml^{-1} remained in the circulation for over 10 weeks following treatment. Both groups showed an increase in the rate of drug elimination within the first week of treatment which reverted back to normal following disappearance of trypanosomes from circulation of cattle treated with drug-sensitive trypanosome population. This accelerated rate of drug elimination was maintained in the presence of drug-resistant trypanosomes until no drug was detectable within 10 days of treatment.

Investigations into homidium as a chemoprophylactic drug under controlled conditions are reported in Chapter 6. Following monthly trypanosome challenge of homidium-treated cattle using the same *T. congolense* populations mentioned above, trypanosomes were detected in blood of the five cattle challenged with the drug-sensitive trypanosome

population after 120, 134, 137, 143 and 144 days following treatment. Homidium concentrations of between 0.1 and 0.3 ng ml^{-1} were detectable for over 10 weeks in circulation in four out of five cattle challenged with drug-sensitive trypanosome population. However, trypanosomes were detected in the circulation of all the five cattle challenged with the drug-resistant trypanosome population eight to nine days following the first challenge at 30 days post-treatment. Serum homidium concentrations were undetectable within 13 days of challenge.

Reported in Chapter 7 is a field study on homidium prophylaxis carried out on Galana Ranch, Kenya, an area of endemic trypanosomiasis, to determine serum homidium concentrations in cattle under prophylactic cover exposed to natural trypanosome challenge and to investigate possible indications of drug resistance. Over 90% of exposed animals were protected for a period of 14 weeks following treatment. The results also demonstrated that serum homidium concentrations could be determined successfully in ranch cattle where large numbers of animals were involved. There was evidence of drug resistance.

Homidium pharmacokinetics in Boran cattle using both ELISA and radiometric methods are compared in Chapter 8. Extensive extravascular distribution of the drug was demonstrated using both methods. However, following i.v. injection, no drug was detectable within 17 days of treatment using the ELISA method whilst concentrations of approximately 4 ng ml^{-1} were detectable up to 4 weeks using the radiometric method. This suggested presence of metabolites. For periods after 24 hours following i.m. treatment, concentrations were ten-fold higher using the radiometric method when compared with those obtained by use of the ELISA method.

In a comparative study reported in Chapter 9 between 6-^{14}C homidium and 6-^{14}C isometamidium, extensive extravascular distribution of both drugs was demonstrated following i.v. treatment of Boran cattle. Following i.m.

treatment, the rate of release of homidium from the injection site depot was faster than that observed following treatment of cattle with isometamidium hence differences in prophylactic periods.

PART ONE

THE AFRICAN TRYPANOSOMIASES

Overview

The African trypanosomiases constitute a group of closely related diseases that affect man and livestock whose causative organism is the haemoprotozoan parasite, the trypanosome. The disease is known as 'nagana' in cattle, 'surra' in camels and 'sleeping sickness' in humans.

The severe epidemics of human trypanosomiases experienced by many African countries in the early decades of the 20th century led to the development and use of trypanocidal drugs to control the epidemics with little attention given to the animal trypanosomiases (Jordan, 1994). The increase in the human population following these control measures led to a demand for increased outputs from domestic livestock, thereby shifting the attention to animal trypanosomiasis. As in the case of human trypanosomiasis, control of the disease in livestock was mainly by use of drugs. However, up to the present day, the strong influence of the disease in precluding the keeping of susceptible breeds of cattle and other livestock is still apparent in many areas. Trypanocidal drugs have, however, permitted cattle and tsetse to co-exist and the heavily infested fly belts are slowly being exploited for cattle rearing by use of integrated tsetse and trypanosomiasis control measures in which tsetse populations are reduced by use of traps and targets and infected animals are treated with the available trypanocidal drugs.

In sub-Saharan Africa, domestic livestock have varied and important roles within the rural communities in which they are kept, and the benefits derived from livestock are both direct and indirect. Domestic animals provide meat, milk, transport, animal traction, manure, hides, skins, fuel and wool. Furthermore, milk and live animals are not only sold to obtain extra income for households as a direct benefit, but also to provide protein for improved

nutritional status to the population as an indirect benefit. Wealth in many communities is measured by the number of animals one possesses. Thus, the social security provided by domestic livestock to most of the rural communities who consider livestock as their major investment is also important.

Trypanosomiasis has severely limited animal production in many countries of sub-Saharan Africa, covering approximately 10 million square kilometres in 38 countries (WHO/FAO/OIE, 1963). An increase in the human population of approximately 50% between 1965 and 1992 in sub-Saharan Africa (Winrock Report, 1992) emphasises the need to increase animal production in order to be able to provide enough protein for this ever increasing population. However, the risk of trypanosomiasis precludes farmers from keeping cattle and small ruminants in many areas, and accounts for much of Africa's low livestock productivity. Current figures approximate the continent's production of animal protein per hectare at one seventieth that produced by Europe (ILRAD Report, 1990). Since many of the areas inhabited by the tsetse flies are potentially the most agriculturally productive in Africa, the impact of trypanosomiasis is even greater than these figures suggest. At the present time, 30% of Africa's cattle population, estimated at 174 million, as well as comparable numbers of small ruminants (ILRAD Report, 1990) are at risk from the disease. According to the KETRI Strategic Plan, 1990-2000 (1991), 30 million of the 174 million cattle are found in 7 million square kilometres of savannah and light woodland. FAO estimates that if the 7 million square kilometres were cleared of the disease, the cattle population would be raised by a further 120 million head.

Kenya will be used to illustrate how natural resources in many areas of Africa remain under-exploited due to trypanosomiasis. One of the many problems facing Kenya now and in the future is the generation of employment and an adequate food supply for its population. The mainstay of the Kenyan

economy is agriculture which contributes 60% of Kenya's total exports and accounts for 80% of the wage and non-wage employment in the country. Furthermore, it is estimated that by the year 2000, Kenya will need 540,000 tonnes of beef (three times the 1990 consumption) for its population taking into account the population increase. It is further estimated that the country will only be able to produce 420,000 tonnes of beef per year using the present production technologies. Reclamation of the arid and semi-arid areas, over 60% of which are tsetse infested, appears to be the only option open to the government to significantly increase beef production (KETRI Strategic Plan, 1990-2000).

Kenya's livestock population has been estimated at 20 million head of cattle, 30 million sheep and goats and 800,000 camels out of which three-quarters are in the arid and semi-arid areas. It is estimated that about 25% of the Kenya landmass cannot support livestock because of trypanosomiasis. Losses of up to 30% have been experienced where cattle have been kept in these areas despite the disease. Camels which are economically important in the arid and semi-arid areas have an estimated mortality rate of up to 70% due to the disease (KETRI Strategic Plan, 1990-2000).

Both indigenous cattle and exotic milk and beef cattle are kept on a commercial basis while sheep, goats and camels are kept on a subsistence level by the small scale farmer. The majority of beef cattle, sheep, goats and camels are found in the arid and semi-arid areas of Kenya, areas of extremely fragile ecosystems. Furthermore, most of Kenya's farmers are small scale farmers, living on land holdings of less than two hectares and carrying out mixed farming using mostly family labour. With fewer resources to support livestock production, cost-effective and sustained methods of disease control must be targeted to actively involve the local communities. The economic planning for Kenya has recognised the important role played by both crops and livestock in the economy and this has led to the introduction of

programmes such as zero-grazing dairy farming through the National Dairy Development Project (NDDP) for the small scale farmer. An assessment of the impact of tsetse and trypanosomiasis under the zero-grazing programme involving the small scale farmer in both the high and low trypanosomiasis risk areas of the coastal region of Kenya has been described by Maloo (1993). From the aforegoing, it is evident that tsetse and trypanosomiasis are of great economic importance to Kenya where many areas have remained under-exploited due to the presence of the disease.

It has been demonstrated that wildlife act as reservoirs for both human and animal-infective trypanosomes (Guedegbe et al., 1992) but because wildlife in the parks are the most important foreign exchange earner in most African countries, conservation strategies have to take this into account, because of the conflict that has emerged between man, livestock and wildlife due to pressure on the land by the expanding rural population.

Because of the other, more important communicable diseases in Africa, control programmes for human trypanosomiasis have not received due attention. WHO (Kuzoe, 1993) estimates suggest that some 50 million people are at risk of acquiring the disease which is endemic in 36 countries of sub-Saharan Africa. This estimate, may, however, be much lower since tsetse habitats have been affected over the years due to human activity. Surveillance breakdown was the major cause of the outbreaks of the disease in Uganda and Sudan in the 1980s. As has already been mentioned, agricultural activities in most of the countries affected by tsetse and trypanosomiasis are carried out by the small scale farmer, who is dependent mainly on family labour. The presence of the human disease will therefore affect agricultural production and reduce family incomes through lack of farm labour.

The impact of trypanosomiasis on livestock is difficult to assess directly, but it has been estimated that over 25 million doses of trypanocidal drugs are used annually for treatment (Holmes and Torr, 1988). The number of

of trypanocidal drugs currently on the market for the treatment of animal trypanosomiasis is limited. Apart from melarsenoxide cysteamine for treatment of the disease in camels and horses, no new drugs have been introduced for a long time and the situation is likely to remain so for some time to come due to the expenses involved in producing new drugs, and the inability of the African farmers to pay high prices for veterinary drugs. Lack of control in the use of trypanocidal drugs has led to problems such as underdosing and the resultant development of drug resistance. Ideally, application of trypanocidal drugs should be under the control of qualified veterinary authorities, but in most of sub-Saharan Africa this is not the case and livestock owners apply the drugs themselves often resulting in inappropriate drug usage. Strategic use of trypanocidal drugs under the supervision of qualified personnel could assist in increasing animal productivity, while minimising the problem of drug resistance. The recently developed enzyme-linked immunosorbent assay (ELISA) for the detection and monitoring of isometamidium in cattle blood plus the work reported in this thesis on an ELISA for homidium will enhance our understanding of the pharmacokinetics and pharmacodynamics of trypanocides in the field. The assays will also be useful in identifying suspected cases of drug resistance in the field.

To be able to work out appropriate control strategies, data is needed not only on the species of tsetse, fly populations and infection rates in both the flies and livestock but also on the type of vegetation and the prevailing climatic conditions. The use of meteorological and remotely-sensed satellite data has been used in the prediction of fly movements and when the disease incidence is likely to be high (Rogers and Williams, 1993; Rogers et al., 1994). Rogers et al. (1994) used a combination of ground-based and remotely-sensed data to describe the distribution of five species of tsetse in Togo. Their work highlighted the importance of spatial and temporal variations in risk as

important determinants of the impact of trypanosomiasis on humans, domestic animals and agricultural activities. Similar analyses in other affected countries could be used to predict occurrence and abundance of flies in order to plan tsetse control campaigns and appropriate drug treatment regimens.

PART TWO

THE AFRICAN TRYPANOSOMIASES

THE PARASITE

Trypanosomes are members of the Order *Kinetoplastida*, Family *Trypanosomatidae* and Genus *Trypanosoma*. They are divided into two groups, depending on their site of development in the insect vector and mode of disease transmission. The salivarian group, to which most of the parasites of veterinary importance belong, complete their development in the salivary glands and the proboscis of tsetse flies (Genus: *Glossina*). The stercoraria group, of which *T. cruzi* is the most important member, develop in the alimentary canal of the vector.

In the invertebrate host

When tsetse feed on an infected animal, trypanosomes are taken in with the blood meal. They develop in various organs of the fly. The life cycle stages for *T. brucei*, *T. congolense* and *T. vivax* may be identified partly by the shape of the trypanosome and partly by the relative positions of the nucleus, kinetoplast and point of origin of the flagellum. The various stages include the trypomastigotes, epimastigotes and metatrypanosomes. Only the metatrypanosomes (metacyclics or the bloodstream forms) are infective to susceptible mammals on which the infected fly feeds.

In the vertebrate host

Once infected, the trypanosomes multiply initially under the skin at the site of inoculation. This happens at the same time as the development of a swelling (chancre) at the site of the tsetse bite (Akol and Murray, 1982). The trypanosomes assume a flattened spindle shape known as the trypomastigote which has an undulating membrane bordered by a flagellum running along the

length of the body. The flagellum arises at one end of the body, the posterior end, near to a deeply staining body, the kinetoplast. After running along the length of the undulating membrane, it may continue as a free flagellum past the end of the body as in *T. vivax* or there may be no free flagellum as in *T. congolense*. Some bloodstream forms of *T. brucei* have a free flagellum whilst others do not.

THE VECTOR

The genus *Glossina* to which the tsetse fly belongs is divided into three distinct groups (subgenera) whose importance is determined by their distribution and feeding preferences (Mulligan, 1970). These are:

The fusca or forest group

Out of the twelve species contained in this group only three are of economic importance because of their role in the transmission of pathogenic trypanosomes to livestock. These are *G. brevipalpis*, *G. longipennis* and *G. fuscipleuris*.

The palpalis or riverine group

The important species in the palpalis or riverine group are *G. palpalis* and *G. fuscipes*; both are vectors of human and animal trypanosomiasis.

The morsitans or savannah group

The most important species in this group is *G. morsitans* which occurs in East, West and Central Africa, and is the major vector for the transmission of pathogenic trypanosomes to humans. It is found mainly in the savannah woodlands. The other species of significance in this group are *G. longipalpis*, *G. pallidipes* and *G. austeni*.

DISEASE TRANSMISSION

The main mode of disease transmission is by inoculation through an infective insect bite. The exception is *T. cruzi* in which infection is either by skin contamination or oral ingestion by the host. *Trypanosoma cruzi* is the causative organism of Chagas' disease, occurring mainly in Central and South America. Since this parasite is not the subject of the present study, it will not be discussed further.

The female tsetse fly mates only once in its lifetime and once infected remains infected for life. Besides the tsetse fly, several species of biting flies also appear to play an important role in parasite transmission. They are believed to be particularly important in the transmission of *Trypanosoma evansi*, the causative organism of camel trypanosomiasis, and *T. vivax.*

THE DISEASE IN CATTLE

In sub-Saharan Africa, mixed trypanosome infections are commonly observed in cattle in the field (Stephen, 1970; Mwangi, 1993), and in cattle the disease is caused mainly by *T. congolense* and *T. vivax* transmitted cyclically through the tsetse fly.

Pathology and pathogenesis

The pathology and pathogenesis of African trypanosomiasis in domestic livestock is dominated by the trypanosome's ability to undergo antigenic variation. Murray and Morrison (1980) recognised three broad areas when considering the pathology and pathogenesis of animal trypanosomiasis. These included the early events following the tsetse fly bite and the subsequent changes in the lymphoid system, anaemia and specific organ and tissue damage.

Development of chancre

Generally, in 4 to 10 days following the bite from an infective *Glossina*, a hard, painful nodule usually develops at the site of the bite and there is often heat and swelling of the surrounding tissues. This lesion is referred to as the 'trypanosomal chancre' and it is here that the metacyclic trypanosomes develop into long thin forms which multiply rapidly and ultimately invade the bloodstream and tissues via the lymph vessels and nodes. It has been shown that development of the chancre and the enlargement of the draining lymph nodes occur simultaneously (Akol and Murray, 1982). One to three weeks after the infective bite (commonly 10 days) there is often a sharp attack of fever in response to the invasion of the peripheral blood by the trypanosomes.

Pre-patent period

The period between the infective tsetse bite and the demonstration of trypanosomes in peripheral blood (pre-patent period) in the vertebrate host varies. According to Stephen (1986), 'factors governing the length of the pre-patent period in the vertebrate host are undoubtedly complex. They probably relate to a number of elements in the host such as innate resistance, antibody levels, nutritional state, age, sex, intercurrent infections, hormonal levels and others; to the life history of the trypanosome such as residence in an abnormal host, exposure to therapeutic drugs or plasma immunoglobulins, frequency of cyclical or mechanical passage, age of metatrypanosomes (metacyclics) and others; to the characteristics of the fly vector such as ability of mouth parts to probe deeply and extensively, suitability of the saliva to support cyclical development, density of metacyclics per unit volume of saliva, sex and age of the fly and the presence of mixed infections'. For one to two weeks following a tsetse fly bite, parasitaemias are often low and it can be difficult to find trypanosomes using the wet smear technique commonly employed in the field.

The sensitivity of the diagnostic method used is, therefore, important in the detection of early infections.

Clinical signs

Trypanosome infections in man and domestic animals have been associated with peaks of fever. These fever peaks usually coincide more or less with the appearance of trypanosomes in the bloodstream. These pyrexial reactions are often severe with temperatures reaching 105°F (40.5°C) in cattle, sheep and goats. With less virulent populations of trypanosomes, the pyrexial peaks and parasitaemia levels may gradually lessen until hardly noticeable. Other clinical signs include the development of anaemia, swelling of the lymph nodes and general wasting.

Pathophysiology

Following establishment of a trypanosome infection, several changes occur in the host. These include anaemia, low packed cell volume, decreased serum albumin, decreased total serum globulin levels, changes in endocrine function (Mutayoba et al., 1995) and gross anatomy.

Katunguka-Rwakishaya et al. (1995), studied the influence of energy intake on the pathophysiology of *T. congolense* in sheep. Their studies involved the measurement of intensity of parasitaemia, degree of anaemia, live body weight gains and biochemical changes in two groups of sheep, one on high protein diet and the other on a low protein diet. Their findings demonstrated that infected animals with a low energy intake had a longer mean pre-patent period (between 12 and 26 days) than the animals with a high energy intake (between 12 and 16 days). However, following the establishment of infection, low energy intake animals developed more severe anaemia and greater growth retardation compared to the animals with a high energy intake. The anaemia observed was due to destruction of red blood cells

and haemodilution. Both infected groups exhibited significant reductions in serum total lipids, phospholipids, plasma cholesterol and albumin. These changes were more severe in the low energy intake animals.

Most tissues and organs are damaged during the course of infection although some are more severely affected than others. One organ which undergoes persistent damage is the heart. Initially, lesions occur beneath the epicardium and the endorcardium and in advanced cases the entire myocardium may be involved (Murray and Morrison, 1980). This leads to distortion and degeneration of myocardial fibres. The pericardium eventually fills with fluid, probably due to increased vascular permeability. Based on the clinical and pathological findings, death in trypanosome-infected cattle is usually due to congestive heart failure caused by a combination of anaemia, micro-circulatory disturbances and myocardial damage.

The effects of trypanosome infections on reproduction have been studied in sheep, goats and cattle (Bealby et al., 1993; Okuna et al., 1993; Mutayoba et al., 1995). Most of these reproductive disorders have been reviewed in detail by Sekoni (1994). These include degeneration of the hypothalamus, pituitary glands and gonads with consequent disruptions in the secretions and plasma concentrations of the hormones necessary for the normal reproductive processes in both sexes. Reproductive disorders in male animals include delayed puberty, loss of libido and severe degenerative changes of genitalia manifested by production of very poor semen. In female animals, the disease causes severe genital lesions plus abnormal oestrus cycles. In addition, trypanosome infections induce abnormal pregnancy, abortion, premature birth, low birth weight and stillbirths. Early treatment with trypanocides has shown that some of these disorders may be prevented (Bealby et al., 1993; Okuna et al., 1993) with a corresponding increase in reproduction.

Changes have also been observed in carbohydrate and lipid

metabolism. Elevated levels of pyruvate have been reported in blood and urine of infected rabbits (Ashman and Seed (1973) cited by Tizard, 1985) draining the host's carbohydrate stores. The muscle wasting observed in chronically infected animals could be an attempt by the host through protein catabolism of muscle tissue to improve the poor energy state brought about by the excretion of large amounts of energy-rich compounds such as pyruvate. Both the pathophysiological and immunological changes observed during trypanosome infection are important in the pathogenesis of animal trypanosomiasis.

Clausen et al. (1993) in their studies on the pathology and pathogenesis of the disease in different breeds of cattle observed that in susceptible cattle the disease is associated with a marked reduction in food intake, swelling of subcutaneous glands and development of a rough hair coat which has a spiked appearance. As the disease progresses the mucous membranes become pale and the animals become weak and unable to walk. The animals also become thin and emaciated with evident muscular wasting and weight loss.

The role of other diseases

Ticks and helminths have been reported to influence the course of trypanosomiasis in cattle. Dwinger et al. (1994) in their studies on the effects of trypanosome and helminth infections on health and production parameters of N'Dama cattle in The Gambia found that there was an increased susceptibility to trypanosomiasis in animals infected with helminths.

Host nutritional status

Studies have been carried out on the effects of nutrition on the degree of anaemia in infected N'Dama cattle (Agyemang et al., 1990) in which the PCV, as a measure of anaemia, was monitored. Two nutritional groups were compared; both grazing on natural unimproved pastures but one group given supplements. Whereas both groups became naturally infected with

T. congolense while grazing, the group which received supplements recovered more rapidly from the anaemia produced by trypanosome infections. This indicates that the lack of adequate nutrition which occurs under field conditions constitutes a stressful condition that could weaken the degree of trypanotolerance of animals. In studies involving two groups of sheep infected with *T. congolense*, one on a high and the other on a low protein diet, Katunguka-Rwakishaya et al. (1993) showed that both groups developed similar degrees of anaemia. However, the mean corpuscular volumes and appearance of normoblasts in the circulation was greater in animals on a high protein diet. Gain in live weight in animals on the high protein diet was similar to the uninfected controls whereas those on low protein diet gained significantly less weight. They also found that all infected animals in the two groups recovered from anaemia following treatment with isometamidium chloride but the recovery rate was faster in the animals on the high protein diet. This showed that high protein intake enhances the rate of recovery following chemotherapy.

SOCIO-ECONOMIC IMPACT OF TSETSE AND TRYPANOSOMIASIS

There are direct and indirect consequences of tsetse and trypanosomiasis. One study carried out in Busia, Western Kenya, (Kamara and Echessah, 1994) demonstrated some of the commonly observed consequences. The direct consequences included decreased growth rate and increased mortality of cattle and other livestock. These adversely affected agricultural production due to a reduction in the number of draft animals and manure for use as fertiliser thereby affecting the crop yield; reduced incomes due to decreases in milk, live animals and other farm products for sale and fewer animals to pay dowry leading to fewer wives per husband and a reduction in family labour.

Finally, because of trypanosomiasis, livestock improvement was inhibited because local communities prefer not to cross-breed the local Zebus for fear that the animals may die of the disease. However, crossbreeds have performed well in other parts of sub-Saharan Africa where they have been introduced and their reproductive performance evaluated (Itty et al., 1995a, b).

Traditionally, tsetse-infested areas have been avoided and this leads to bush encroachment, and often further expansion of tsetse habitats. Several social activities such as fetching water from streams, visiting relatives, weeding or just relaxing outside one's house are also affected for fear of contracting the disease.

From the aforegoing, it is evident that trypanosomiasis is of great economic significance due to its impact on animal productivity and human activity in the tsetse infested countries of sub-Saharan Africa. Besides affecting humans in terms of reduced activity, the economic losses due to the disease in susceptible animals are high.

DIAGNOSTIC TECHNIQUES

Treatment of infections in the field depends on clinical diagnosis or the demonstration of parasites in the peripheral blood. However, the success of diagnosis depends on the sensitivity of the diagnostic technique used, especially in cases of chronic disease when parasites cannot often be detected by parasitological techniques (Ogwu et al., 1985).

Once animals are infected, intervention with drugs leads to recovery if the parasites are drug sensitive. However, the success of treatment also depends on when the drug is administered to infected animals; greater success in treatment is achieved when the animals are treated early in infection (Murray and Dexter, 1988). Several techniques have been used in diagnosis including clinical, parasitological, immunological and biochemical.

Clinical diagnosis

The occurrence of clinical signs may indicate cattle are suffering from trypanosomiasis but they are not associated exclusively with the disease. Thus, acute forms of trypanosomiasis may be confused with diseases such as babesiosis and rinderpest. Chronic trypanosomiasis may be difficult to distinguish clinically from malnutrition and/or severe intestinal helminthiasis. A presumptive diagnosis must, therefore, be confirmed by demonstration of trypanosomes in infected animals.

Parasitological techniques

These techniques depend on the demonstration of trypanosomes in the peripheral blood of infected animals.

Wet films

A wet blood smear consists of a small drop of fresh blood obtained from an ear or tail vein placed on a slide, covered with a coverslip and examined for trypanosomes. The parasites are detected by their movement or by the disturbance they cause among the blood cells. Examination of wet films provides a quick and easy means of diagnosis in the laboratory.

Thick films

A drop of blood is placed on a clean microscope slide and a thick film prepared by using the corner of another slide to produce a rounded smear of 1-2 cm in diameter. The slide is then rapidly air dried, placed in distilled water for five minutes to dehaemoglobinise prior to staining in 10% Giemsa and examination under the microscope (MacLennan, 1957). This technique is the most commonly used method for routine diagnosis of bovine trypanosomiasis in the field. Thick films are usually used in conjunction with thin films in which species identification can be made more confidently.

Thin films

A drop of blood is placed at one end of a clean grease-free microscope slide and a thin film prepared by spreading the drop along the length of the slide with a straight edge of another slide or rectangular coverslip, quickly air-dried and fixed for three minutes in methanol. The film is then stained in 10% Giemsa for 30 minutes followed by examination for parasites under oil immersion.

Buffy coat dark ground illumination

An EDTA-coated capillary tube is filled with blood and centrifuged in a microhematocrit centrifuge for 10 minutes. The capillary is then cut with a diamond pencil one mm below the buffy coat to include the uppermost layer of red cells and two mm above to include plasma. The usual practice in the field, however, is that the capillary tube is cut once two mm away from the buffy-coat junction. The contents of the tube are then gently expressed onto a clean slide, mixed and covered with a 22 x 22 mm coverslip and examined under the microscope. To provide phase contrast, a combination of Phaco 2 NPL 25/0.50 objective, a Zernicke 402 condenser and a periplan NF x10 eyepieces may be used (Murray et al., 1977).

Mouse sub-inoculation

Blood from an infected animal in 0.2 ml phosphate-buffered-saline-glucose, pH 8.0, is inoculated intraperitoneally into individual mice. Wet film preparations of tail blood from these mice are then examined three times a week for at least the following 30 days for the detection of trypanosomes.

Immunological techniques

(a) Antigen enzyme linked immunosorbent assays (ELISA)

Scientists at the International Livestock Research Institute (ILRI; formerly International Laboratory for Research on Animal Diseases, ILRAD) have developed assays which can be used to detect circulating antigens of *T. congolense* and *T. vivax* (ILRAD Report, 1990; Masake and Nantulya, 1991). These are referred to as antigen-trapping ELISAs. In these assays, trypanosome species-specific monoclonal antibodies are used to capture the circulating trypanosome antigens in the serum of infected animals. The same antibody labelled with horseradish peroxidase is then introduced and binds to the free antigen epitopes of the immobilised antigen. The chromogen changes colour due to the activity of the peroxidase in the presence of substrate and is used to detect the labelled antibody. This is a sandwich ELISA.

(b) Antibody ELISA

In this technique, the circulating antibodies in the infected animals are detected rather than the antigen. The limitation of this technique is that it will not differentiate between current and past infections because antibodies may persist for long periods following treatment (Luckins et al., 1979).

A recent study was carried out to compare several diagnostic techniques using serum samples collected over a period of three years (1989-1991) from cattle maintained under conditions of varying trypanosomiasis risk in different parts of Kenya (Mwangi, 1993). The results showed that antigen ELISA detected a high proportion of infections and demonstrated the existence of a high proportion of mixed infections in comparison to the parasitological techniques. In high tsetse challenge areas, antigens were detected in over 50% of the animals compared to 20% in the low tsetse challenge areas. Following treatment of infected animals and their withdrawal

from the trypanosomiasis risk area, parasites were no longer demonstrated but both antigens and antibodies persisted for up to 7 months. The antigen-ELISA is therefore a useful diagnostic method in the detection of early infections. Even so, Nantulya (1989) showed that early in the infection, the test can give negative results due to absence or low levels of antigens in blood. He suggested that, in some situations, a combination of techniques such as the antigen-ELISA with parasitological techniques such as the microhematocrit centrifugation may be necessary. The results suggest that in epidemiological studies, a combination of techniques is necessary for complete assessment of the infection rates.

Biochemical techniques

DNA probes

DNA probes have been developed that will detect the trypanosome genes rather than the products of trypanosome genes which are the basis of antigen-ELISA diagnostic procedures. The DNA probe technique is highly specific and sensitive but its reliance on radioisotope labelling is a major drawback. The production of probes using alternative labelling will make the technique much safer to use in the field.

TSETSE AND TRYPANOSOMIASIS CONTROL

Overview

Methods employed in the control of the disease have been many and varied. These include methods directed at the vector and those directed at controlling the parasite that causes the disease. For vector control, several methods have been employed, some of which are now no longer in use such as game eradication. Others, however, have undergone tremendous improvements such as tsetse traps. The most popular and widespread method of parasite control remains the use of chemotherapy.

Parasite Control

Introduction

Control of animal trypanosomiasis by targeting the parasites has relied on three methods: (a) use of chemotherapeutic and chemoprophylactic agents (b) rearing of trypanotolerant breeds of cattle and (c) vaccination against trypanosomiasis. Each of these methods will be considered separately.

Chemotherapy

Besides the expenses involved, vector control methods alone have, almost without exception, not been effective on a long-term basis in dealing with trypanosomiasis and there continues to be extensive use of trypanocidal drugs for treatment and/or prophylaxis. Even in areas where traps and targets are in force, trypanocidal drugs are still needed to deal with infections due to the residual tsetse populations. Chemotherapy, therefore, constitutes an important method of controlling the disease in man and livestock in most African countries.

Treatment of animals in the field has relied on demonstration of parasites in the peripheral blood. As already mentioned, detection of parasites early during an infection will depend on the sensitivity of the diagnostic method used. Treatment of cattle following trypanosome infection leads to cure if the trypanosomes are drug sensitive. A brief look at the interaction between drugs, trypanosomes and cattle in the field is important in understanding drug performance under different field situations.

Natural trypanosomiasis incidence and chemotherapy

Whiteside (1962) in his work on the interactions between drugs, trypanosomes and cattle under field conditions observed that in a given area, the frequency of infective tsetse bites is proportional to the fly density and this affects the degree of protection afforded by drugs.

This observation has been confirmed by studies carried out in the coastal region of Kenya by Dolan et al. (1990, 1992) and Maloo (1993). It was demonstrated that a low number of treatments per annum afforded good protection when the fly density was low (Mwambu, 1971; Dolan et al., 1990; Maloo, 1993). However, when trypanosome challenge was high more frequent treatments were required. It appears, therefore, that the successful use of drugs is dependant on the density of infected flies, the rate of transmission to susceptible animals and the drug sensitivity of the trypanosomes being transmitted; the higher the incidence of infections and/or the less the sensitivity of trypanosomes being transmitted to the drugs, the shorter the prophylactic period.

Immune response, drugs and natural trypanosomiasis incidence

Whiteside (1962), in studies carried out at the Kenyan coast using Zebu cattle maintained on diminazene treatment, observed that there was a connection between drugs and immune response; animals receiving regular treatment and exposed to natural tsetse challenge slowly developed immunity. This was demonstrated by a reduction in the number of infections from five to just over one in a year following diminazene treatment of infected animals exposed to natural tsetse challenge.

Following infection of animals with trypanosomes, there is an activation of the immune system. Associated with this activation, some animals such as the mice suffer from severe immunosuppression (Tizard, 1985). Whilst immunosuppression during trypanosome infections in cattle is much less marked, it is significant in the laboratory animals. Holmes et al. (1979) in their studies on the immunological clearance of ^{75}Se-labelled *T. brucei* in mice demonstrated that between 97 and 99% of the radioactivity was protein bound in the parasite and spontaneous elution *in vitro* was 10% in 4 hours. The vast majority of labelled trypanosomes remained in the circulation

of normal mice but in immune mice, they rapidly disappeared from the blood.

In their review on the immune dependence of chemotherapy, Doenhoff et al. (1991) noted that immunosuppression reduces the efficacy of chemotherapy of several parasitic diseases including trypanosomiasis, schistosomiasis and malaria.

Osman et al. (1992) studied the effects of immunosuppression on the development of drug resistance by trypanosomes in mice infected with various clones of *T. evansi*. Their results demonstrated the rapid development of high levels of resistance to melarsenoxide cysteamine, diminazene and isometamidium in the clones following frequent passage in immunosuppressed mice given sub-curative drug treatments. However, drug resistance did not develop when frequent passage was undertaken in normal immunocompetent mice infected with the same clones of *T. evansi*. Their results suggested that impairment of the host immune system may lead to rapid development of resistance. An intact immune response is therefore, necessary for effective chemotherapy.

Drug resistance

Failure of trypanosomes to disappear completely from the blood following the administration of therapeutic doses of drugs could be attributed to many factors, one of which is drug resistance. Resistance has been reported to all the trypanocides currently in use, including the recently introduced melarsenoxide (Osman et al. 1992; Pospichal et al., 1994). Resistance can be either innate or acquired. Wernsdorfer (1994) in his review on resistance in *Plasmodium falciparum*, noted that natural populations of this parasite are composed of different populations of parasites with varying degrees of drug sensitivity. Sensitive populations include all organisms which will be eliminated by the minimum effective concentration. There is, however, a small proportion of parasites that is able to survive the minimum concentration. This

small group in the natural population which has not been exposed to the drug can be said to posses innate resistance. Innate resistance in *T. vivax* to homidium has been reported (Unsworth, 1954b). If a parasite population is exposed to the minimum effective drug concentrations, the sensitive population will be eliminated leaving a population that can withstand the these concentrations. This results in the selection of a new population that is less sensitive than the population prior to exposure. Further selection will take place upon renewed drug pressure, resulting in enhanced resistance (acquired).

When parasites are exposed to drug concentrations below the minimum effective concentration or to the minimum concentration but for a shorter period than is required to kill them, drug failure will occur.

Both innate and acquired resistance have been reported involving the different trypanocidal compounds (reviewed by Kinabo, 1993; Peregrine and Mamman,1993). Jones-Davies (1967,1968) in his work on tsetse fly-transmitted trypanosomes of cattle in Northern Nigeria found strains of *T. vivax* isolated from three different localities that were resistant to diminazene at 3.5 mg kg^{-1} b.w.; two of the isolates were sensitive to 7.0 mg kg^{-1} b.w. This was despite the fact that diminazene had been in use for a very short time (2 years) since the withdrawal of homidium. Mwambu and Mayende (1971) have also reported the occurrence of diminazene-resistant strains of *T. vivax* in an area of Eastern Uganda; the trypanosomes were resistant to 3.5 mg kg^{-1} b.w. and 7.0 mg kg^{-1} b.w. but sensitive to homidium at 1.0 mg kg^{-1} b.w. At the time of isolation of these trypanosomes, quinapyramine was widely used in this area, suggesting that these populations of trypanosomes may generally be resistant to quinapyramine and cross-resistant to diminazene. Both diminazene and quinapyramine are derivatives of Surfen C.

Bauer (1962) failed to induce resistance to diminazene in *T. congolense* in cattle by administering sub-curative doses of diminazene. In contrast, Whiteside (1963) produced two diminazene-resistant variants of

T. congolense. Cross-resistance tests indicated that the trypanosomes remained susceptible to the recommended curative doses of other trypanocides. Gray and Roberts (1971), investigating the stability of diminazene resistance in a *T. vivax* strain showed that diminazene resistance can be maintained by cyclical transmission to antelope for a long period of time; the *T. vivax* strain retained resistance to diminazene aceturate at a dose rate of 7.0 mg kg^{-1} b.w. and quinapyramine sulphate at 5.0 mg kg^{-1} b.w. for periods of 7 months in tsetse flies and antelope, and for a total period of 29 months in tsetse flies and cattle. More recent studies on diminazene resistance have demonstrated an 80-fold increase in diminazene resistance by the sub-curative treatment of immunosuppressed mice infected with a cloned population of *T. evansi* over a 9-week period (Osman et al., 1992)

Occurrence of trypanosomes resistant to isometamidium has been reported in several studies (Pinder and Authie, 1984; Schonefeld et al., 1987; Peregrine et al., 1991). Using mice, Pinder and Authie (1984) isolated stocks of *T. congolense* from Burkina Faso which expressed a high level of resistance to both isometamidium and diminazene. Peregrine et al. (1991) derived 9 clones from one of the *T. congolense* stocks reported by Pinder and Authie (1984) and characterised them in mice for their sensitivity to both diminazene and isometamidium. The results showed that all clones expressed high levels of resistance to isometamidium chloride and to diminazene aceturate with a 50% curative dose value in mice ranging from 1.5 to 5.1 mg kg^{-1} b.w. for isometamidium and 5.1 to 21.0 mg kg^{-1} b.w. for diminazene. When the clone expressing the highest level of resistance to isometamidium in mice was tsetse transmitted to Boran cattle, the resulting infections were shown to be resistant to 2.0 mg kg^{-1} b.w. isometamidium chloride and 14 mg kg^{-1} b.w. diminazene aceturate given by the intramuscular route.

Fairclough (1963b) compared metamidium, isometamidium, diminazene and homidium under field conditions in an area of medium to high

tsetse challenge in Nigeria, and concluded that signs of drug resistance only appeared in the homidium group. Periods between infections in the diminazene and isometamidium groups lengthened as the tsetse fly numbers decreased. In contrast, the time between infections in the homidium-treated groups continued to shorten with decreased fly numbers; an indication of the development of drug resistance.

In the experiments of Jones-Davies and Folkers (1966), *T. congolense* strains resistant to 1 mg kg^{-1} b.w. homidium bromide were isolated from cattle in Nigeria and inoculated into groups of cattle. In one group, treatment with homidium at various dose rates ranging from 0.10 to 1.0 mg kg^{-1} was carried out 24 hours after inoculation with parasites; in the other group treatment was carried out at various dose rates ranging from 1.0 to 2.0 mg kg^{-1} on the seventh day of parasitaemia. Results showed that trypanosomes in all cattle established and multiplied producing disease symptoms suggesting the presence of homidium resistant trypanosomes. The strain of *T. congolense* used appeared to be resistant to homidium bromide at 2.0 mg kg^{-1} b.w. Scott and Pegram (1974) have reported the presence of *T. congolense* strains that are resistant to homidium in Ethiopia.

Development of drug resistance by trypanosomes

In the laboratory, resistance in trypanosomes has been induced through exposure to sub-curative doses whilst in the field, underdosing could be a contributing factor to the development of drug resistance. There are several ways in which underdosing can occur in the field. These include (1) inappropriate drug use in which drugs are administered by unqualified personnel often in the absence of proper diagnosis (2) treating animals on the basis of average rather than individual weights resulting in animals being underdosed and (3) keeping cattle on prophylactic treatment in a high tsetse challenge area for long periods of time. Prophylactic regimens give good

protection throughout the year when the incidence of infections is low but this period is reduced in the presence of high tsetse challenge when incidence of infections is high (Dolan et., 1990, 1992; Stevenson et al., 1995). It has been proposed that low concentrations of prophylactic agents remain in the circulation for long periods of time and if these are sub-curative, trypanosomes are exposed to these agents, which could lead to the development of resistance. The period of exposure of trypanosomes to drug levels below the minimum effective concentration with chemoprophylactic agents would be longer than with the curative drugs such as diminazene. This may be the reason why reports of resistance to prophylactic agents are more than those due to therapeutic drugs (Whiteside, 1962; Mamman et al., 1995).

Mass drug administration has been identified as a probable cause of drug resistance because it exerts a high selection pressure especially if sub-therapeutic doses are used (Wernsdorfer, 1994). Thus, the mass treatments with homidium which followed its introduction into the field, could have contributed to the rapid development of resistance to this drug in Northern Nigeria (Jones-Davies and Folkers, 1966).

Cross-resistance

Trypanosome populations resistant to one trypanocidal drug compound have been found to be resistant to other active trypanocidal drug compounds (Whiteside, 1962). The earliest report of cross-resistance was of quinapyramine-resistant trypanosomes showing resistance to phenanthridinium compounds. Later, a diminazene-resistant population of *T. congolense* was produced using quinapyramine in the laboratory (Whiteside, 1962).

Finally, development of multiple-drug resistance involving *T. vivax* in cattle in Kenya and Somalia, and *T. congolense* in Somalia, Burkina Faso and Ethiopia has been reported (Rottcher and Schillinger, 1985; Schonefeld et al.,

1987; Ainanshe et al., 1992; Clausen et al., 1992; Codjia et al., 1993). It is possible that cross-resistance could have contributed to the multi-drug resistance observed.

Cross-resistance and chemical structure

Many of the currently available trypanocidal compounds share at least one moiety. It is therefore possible for trypanosomes resistant to one drug to be cross-resistant to the other related compound. Examples are homidium and isometamidium which share the homidium moiety; diminazene and isometamidium which share part of diminazene molecule; and diminazene and quinapyramine both of which are derivatives of Surfen C. From the aforegoing, it is possible for trypanosomes resistant to quinapyramine to be cross-resistant to diminazene and isometamidium due to structural similarities. The only two trypanocides which do not possess structural similarities are homidium and diminazene and these have been shown not to produce cross-resistant trypanosomes to each other. These were described as a 'sanative' pair by Whiteside (1962).

Control of drug resistance in the field

As mentioned above, the only drugs which do not produce trypanosome populations cross-resistant to each other are diminazene and homidium. Thus, Whiteside (1962) suggested the use of homidium in the field until signs of drug resistance appear, after which the drug should be withdrawn to be replaced by diminazene for one year followed by re-introduction of homidium. Use of diminazene alone in the field as a cure for infected animals is expensive because of the number of doses required to treat each animal per year since it does not provide long periods of protection (Mwambu, 1971). The usual practice in the field has therefore been the use of block treatment with diminazene followed by another block treatment with a

chemoprophylactic agent such as homidium or isometamidium. Any animal developing breakthrough trypanosome infections during the prophylactic cover would be treated with diminazene. This has made it possible to keep cattle economically in tsetse infested areas.

Trypanotolerant breeds of cattle

Although the wild animals of Africa harbour trypanosomes, they are generally resistant to the effects of trypanosome infections. They have become resistant (or tolerant) to the effects of trypanosome infections due to constant exposure to infection over many generations. In domestic livestock, the susceptibility to trypanosome infection varies in different breeds. Resistant (trypanotolerant) breeds of cattle include the N'Dama and the Muturu in which the disease is usually transient and terminates in recovery. In susceptible breeds such as the West African Zebu, the disease is acute or chronic and usually terminates in death if there is no intervention with drugs. Results of several studies involving trypanosome-infected cattle breeds such as the N'Dama, Baoule, Orma and Galana Boran and the Masai Zebu have shown superior performance of trypanotolerant cattle in terms of control of anaemia (measured by the packed cell volume) during infection compared to the susceptible breeds (Ismail, 1988; Kora et al., 1992; Clausen et al., 1993; Mwangi, 1993; Andrianarivo et al., 1995). The introduction of trypanotolerant breeds of cattle into areas and communities devoid of cattle has been reported (Itty et al., 1995a, b) and their productivity evaluated (Dwinger et al., 1994).

Although usually tolerant to trypanosomiasis, trypanotolerant breeds can succumb to trypanosomiasis when exposed to high challenge in a new area, or when under stress caused by factors such as malnutrition or inter-current infection.

Using the N'Dama, Trail et al. (1994) matched animal health and performance data in a two-year period of exposure of the cattle to natural

tsetse challenge in a high natural tsetse challenge situation in Zaire. Four parameters which were regarded as possible indicators of trypanotolerance, namely, species of the trypanosomes detected, length of time parasitaemic, intensity of parasitaemia and anaemic condition as estimated by the packed cell volume values were measured. The relative effects of changes in these parameters on trypanocidal drug requirements and growth were determined. It was found that all four criteria had approximately equal effects on the trypanocidal drug requirements and on the daily live weight gain. From this study it was concluded that absence of information on any of these criteria would significantly affect the accuracy of the estimate of an animal's overall trypanotolerant phenotype in a particular situation in which it is being assessed.

Programmes involving the introduction of trypanotolerant breeds of cattle in areas devoid of cattle due to high prevalence of infections have been reported. The introduction of the N'Dama in an area devoid of cattle in Zaire (Itty et al., 1995a) was reported to be successful in that rearing cattle in this area was found to be cost-effective and cattle are now established as part of the farming system. In Togo, however, the introduction of N'Dama was not as successful as that of Zaire (Itty et al., 1995b) as the scheme was found not to be profitable to the communities due to high inputs. It appears then that a thorough evaluation of health and performance and the costs incurred in introducing trypanotolerant breeds in new areas is necessary for projects involving these breeds to be successful.

Trypanotolerance has also been reported in sheep and goats (Osaer et al., 1994). Two cloned strains of *T. congolense,* of West African and East Africa origin, were used to infect female Djallonke sheep and West African Dwarf goats. Parasitaemia, PCV, body weight and clinical parameters were followed in these animals for three months in an effort to evaluate their trypanotolerant nature. The results showed that the West African strain was

more pathogenic than the East African strain. Despite these observations, both the Djallonke sheep and the West African Dwarf goats showed a high degree of trypanotolerance, as reflected in the lack of mortality and an increase in body weight during 12 weeks of observation.

In conclusion, although well controlled studies have been carried out on productivity and general performance of trypanotolerant breeds compared with non-trypanotolerant breeds, both in traditional and ranch management systems, and when exposed to different levels of risk to trypanosomiasis, it will take some time before these breeds are established and integrated in animal production systems in areas beyond West Africa.

Vaccination

Immunisation as a method of trypanosomiasis control is not foreseeable in the near future due to the substantial antigenic variation that is exhibited by trypanosome populations. When an animal becomes infected, the trypanosomes composing a parasitaemia peak population are usually a mixture of antigenic types in various proportions (Meirvenne et al., 1975). Whereas the major types are eliminated by the host's defence system, the minor variants appear to keep the infection going. The range of variable antigen types (VATs) that a single trypanosome can produce is known as the VAT repertoire; the precise number that can be produced is not known. However, Capbern et al. (1977) have reported at least 101 VATS produced from a clone of *T. equiperdum*. This has limited progress in the development of a vaccine for trypanosomiasis.

Despite the above limitation in the search for a vaccine against trypanosomiasis, recent advances have been made in this area. In a recent study (Powell, 1993) rabbits were immunised with specially prepared sub-cellular fractions of a cloned *T.b. brucei* stock. In *in vitro* studies the sera from these immunised rabbits killed different parasites of *T. brucei* which were

grown in continuous culture of salivary gland cells from *G.m. morsitans*. In another study, Grootenhuis and Olubayo (1993) isolated serum proteins from wild African buffalo which had trypanocidal activity against all species of trypanosomes. Olaho et al. (1995) have reported successful trials using flagellar pocket antigens from *T.b. rhodesience* in the vaccination of cattle against *T. congolense* and *T. vivax*. Their results showed that cattle were partially protected against natural tsetse challenge.

Destruction of wildlife reservoirs

Recent studies carried out in the Pendjari National Park, Benin, have confirmed that in this area, wild animals are reservoirs of most trypanosome species pathogenic to livestock and humans. Among them, *Adenota kob* and *Panthera leo* are carriers of *T.b. gambiense* that causes human trypanosomiasis (Guedegbe et al., 1992). Depending on the fly feeding preferences, selective destruction of game animals which act as reservoirs is the only method apart from total clearing of vegetation which would completely free the land from trypanosomiasis, by interfering with the fly's food supply. However, the success of this method of control would depend on the accuracy of the data generated from blood meal analyses. Although this control measure would lead to selective elimination of the reservoirs, it would be unacceptable for environmental reasons relating to wildlife conservation.

Vector Control

Clearing of vegetation

Clearing of vegetation is the oldest and the most effective method of eliminating *Glossina* spp. since it destroys their habitat. The clearing of vegetation can either be total or partial (Ford et al., 1970). Total clearing involves destruction of all trees and shrubs; they are cut down and, if possible, destroyed by uprooting or burning. Partial clearing involves selective,

descriminate clearing in which certain types of vegetation are targeted. This method has been successful in areas where the tsetse habitat is well defined; for example, in the control of human sleeping sickness associated with *G.f. fuscipes* in Western Kenya (Maina, 1977). With the current emphasis on the need to conserve the environment in order to prevent desertification such methods have no place in today's tsetse control programmes.

Hand-catching

Although the avoidance of the fly belt was probably the traditional method of preventing losses from tsetse transmitted trypanosomiasis, the hand catching method, although no longer in use, is one of the oldest methods of insect control, having been tried for the first time in 1913 (Glasgow and Potts, 1970). Its main advantage is that it is absolutely specific for the insect under attack. One can also obtain an exact record of the number of insects destroyed. The disadvantage of the technique is that it requires a large labour force, especially if substantial areas are to be tackled. There are also problems of re-infestation, unless one is working in an isolated area. However, this is one of the methods that is environmentally acceptable, and therefore remains suitable for tsetse. The technique has, however, been superseded by more modern technologies.

Insecticides

The first insecticide to be introduced for tsetse control was dichlorodiphenyltrichloroethane (DDT) (Burnett, 1970). Because of its stability and volatility, it was used mainly for ground spraying. When out of direct sunlight, the deposits remain lethal to tsetse for up to one year (Baldry, 1963). Dieldrin, which has similar properties of stability and volatility as DDT, soon replaced DDT after trials revealed that lethal deposits persisted better than DDT following spraying of vegetation (Glover et al., 1958).

Endosulphan, when used for residual treatment was found to be less persistent than DDT and dieldrin, and had high intrinsic toxicity and better solubility in spray solvents (Jordan, 1986). These properties made it suitable for use as a spray when dispersed as an aerosol of fine droplets from an aircraft, as demonstrated in Tanzania (Hocking et al., 1966). Synthetic pyrethroids such as deltamethrin have also been employed for impregnating traps and screens for tsetse control (Luguru et al., 1993).

Ground spraying

This involves the application of a residual insecticide to shrubs and bushes, where it remains lethal for the maximum pupal period of 60 days; the time for flies to emerge from the pupae below the ground. By the nature of the mode of application it is effective in areas that are inaccessible to insecticide from aerial spraying. Recent studies (Shereni and Pope, 1993) on the effects of residual deposits of deltamethrin applied by the ground spraying technique against tsetse populations in North-West Zimbabwe have shown that the flies (mainly *G.m. morsitans* and *G. pallidipes*) were eliminated within 4 months of treatment.

Aerial spraying

This method involves application of an aerosol of insecticide by low flying aircraft or helicopter, targeting mainly the adult flies. Very low doses are applied, killing the insects on contact. As a result, the environment has to have sufficient residual insecticide, either continuously or at intervals for the duration of the maximum pupal period, to prevent the survivors from larvipositing. However, aerial spraying affects not only the target species but also the non-target species and would be environmentally unacceptable as a control measure when compared to the improved and less expensive trap and target technologies.

Traps, targets and screens

Targets and screens were introduced after field observations on the response of tsetse flies to visual stimuli. Furthermore, several trap designs have been produced since the three-dimensional, box-shaped Harris trap of 1930 and the "animal" traps of Morris and Morris (1949). Large numbers of the Harris traps were used in an attempt to eliminate *G. pallidipes* from Zululand, and marked reductions in fly populations were achieved (Harris, 1938, reviewed by Jordan, 1986). Problems associated with traps have been difficulties in siting them so as to give consistent and comparable catches that will provide an effective method of investigating the absolute number of tsetse flies that are present.

The development of the biconical trap (Challier and Laveissiere, 1973) generally solved this problem since it could be used in areas covering various ecological zones in many African countries (reviewed by Challier, 1982). When used for tsetse control, the biconical traps are impregnated with a persistent insecticide; this modification followed observations that more flies visited and alighted on the traps than entered them (Hargrove,. 1972; Vale and Hargrove, 1979).

Impregnated traps have been used successfully on the border of Cote d'Ivoire with Burkina Faso; an area infested with *G.p. gambiensis* and *G. tachinoides*, where a 98% reduction in the fly populations was achieved and maintained for 4 months (Reviewed by Jordan, 1986). Monoconical traps (which are in effect targets since the attracted insects are not retained) impregnated with deltamethrin have been used in the Republic of Congo where they were found to be more effective than the biconical trap in controlling *G.f. quanzensis* (Lancien, 1981; reviewed by Jordan, 1985). Simple screens of blue material (120 x 90 cm) impregnated with deltamethrin have been used along the borders of Cote d'Ivoire and Burkina Faso, as in the above

experiment with traps (reviewed by Jordan, 1985). Finally, results of work done in Zimbabwe (Hargrove, 1972; Vale, 1974; Vale and Hargrove, 1979) have indicated that even with the best current traps, the trapping efficiency is about 50% compared with an incomplete ring of electrified nets (around the trap to be evaluated) which capture 95% of tsetse that collide with them. Using this method of evaluating trap efficiency, the efficiency of the Morris and Morris trap was only 10%. Other studies have shown that even in the presence of odour, the biconical trap is relatively inefficient for *G.m. morsitans*; found mainly in the savannah woodlands. An effective trap that would be efficient for most tsetse species is yet to be designed (Hargrove, 1980; Vale, 1982).

Several odours have been identified for use in conjunction with traps, targets and screens. These include 1-octen-3-ol, p-cresol, acetone and 3-n-propylphenol (reviewed by Holmes and Torr, 1988).

Traps, targets and screens offer several advantages as compared to hand catching, ground and aerial spraying because they require less labour, and work can be carried out throughout the year. The disadvantages of the technologies are associated with maintenance of the traps and targets since they are prone to thefts, bush fires and degradation (dependant on the prevailing climatic conditions). Incorporation of a UV absorber into the insecticide formulation has been found to extend the effective life of the target and the insecticide (Opiyo et al., 1993).

Also to be mentioned is the 'pour-on' technique. This is a relatively new technique for tsetse control and involves the application of insecticide in an oily emulsion to the back of animals, thereby killing any flies which come in contact with the animal. This is in effect a moving target. The technique is not only effective on tsetse, but is also effective for control of ticks. Successful use of synthetic pyrethroids such as deltamethrin (Muguwa et al., 1993), cypermethrin (Byamungu and Mramba, 1993; Kamau et al., 1993) and alphamethrin (Kitwika and Malele, 1993) in pour-on formulations have been

reported. These studies showed significant declines in tsetse catches. However, Muguwa et al. (1993) concluded that the pour-on application cannot be sustained by peasant farmers for economic reasons. The technique, therefore, still requires extensive field evaluation in terms of its sustainability in community-based programmes.

As in the use of traps, targets and screens, with or without insecticide for tsetse control, the use of pour-ons renders the environment favourable to the fly when discontinued. The flies may, therefore, re-establish themselves. Thus, for the insecticide operations to succeed on a long-term basis, re-infestation by *Glossina* has to be prevented by barriers of some sort.

Note should be made of the fact that toxic hazards to man and other animals were not often considered in the past when choosing insecticides for tsetse control. This is not the case now due to increased awareness about the impact of insecticides on the environment. For instance, insecticide residues, and specifically DDT, have been shown to have adverse effects on some non-target species; e.g., eggshell thinning in fish eagles in Zimbabwe (Matthiessen, 1983). The use of insecticides on traps ensures that only the target species is affected.

Parasites and predators of *Glossina*

This is an area that has not been fully exploited despite the discovery of some bacteria and fungi that are pathogenic to *Glossina* spp. (Nash, 1970). Under natural conditions, predators and parasites play an important role in controlling tsetse populations. However, this is unlikely to find a place in practical control programmes due to the recent improvements in trapping techniques.

Biological control

Use of chemosterilants

The use of chemosterilants is one of the biological methods of trypanosomiasis control, besides the sterile insect technique. Suggestions have been made that it would be more efficient and probably more environmentally acceptable to interfere with the reproduction of the flies rather than kill them by the use of insecticides (Bursell, 1977; Hargrove, 1977). Use of chemosterilants would assist in increasing the effectiveness of 'traps' for the control of populations of *G.m. morsitans* and *G. pallidipes*. The method would involve the capture, sterilisation, and release of the flies. The sterilized females and males would then return to the local tsetse population where they would interfere with the mating of unsterilised populations.

A field trial employing this method has been carried out to determine the efficacy of pyriproxyfen, a juvenile hormone mimic, in combination with odour-baited biconical traps on suppression of *G. pallidipes* in Kiboko, Machakos District, Kenya (Mango et al., 1993). Results of the study indicated that with time there was a suppression of the fly population in the experimental area as assessed by the use of biconical traps plus attractants and the electrical mobile screen. The findings also indicated that the *G. pallidipes* population consisted mainly of old flies as assessed by the mean wing fray ratio of both males and females, ovarian ageing of the females and the pteridine fluorescent ratio in heads and wings. There was, therefore, a reduction in catches of young flies showing that the reproduction cycle had been interfered with, resulting in reduced fly challenge and low incidence of infections in cattle.

Sterile male technique

In this method, large numbers of male tsetse are sterilized by irradiation and released in an infested area. The numbers of such males must be high

enough to overflood the natural population of wild males (Jordan, 1985). Thus, in actual practice, the sterile males are released when the population of the wild males has been reduced considerably by other existing tsetse- control methods. For the successful use of this method (1) information is needed on tsetse population dynamics and behaviour in a given area, (2) suppression of the tsetse population has to be initially carried out using an alternative method of control, such as traps or targets, and (3) an efficient system for rearing tsetse is required since thousands or hundreds of thousands of sterile males may be required per month, depending on the area of control (Glasgow, 1970). This method has been successfully used in Burkina Faso where *G.p. gambiensis* occupying an area of 100 km^2 along a stream was eradicated within 16-24 months (reviewed by Holmes and Torr, 1988). In Tanzania, an average of 81% control of *G.m. morsitans* was achieved over an area of 195 km^2 within 15 months using the same method. Provided that re-infestation is prevented by barriers of some sort, eradication is possible in a linear habitat using the sterile insect technique, so long as it is combined with impregnated traps and targets that are either baited or unbaited to initially reduce fly populations.

Community level participation in tsetse control programmes

In most tsetse-infested countries of sub-Saharan Africa, tsetse and trypanosomiasis control has been dependant on central governments, with little or no participation from local communities. Recently, efforts have been made to involve local communities in tsetse and trypanosomiasis control activities (Mate, 1993; Swallow and Mulatu, 1993; Swallow et al., 1993; Kamara and Echessah, 1994; Itty et al., 1995a, b). In Ethiopia, Swallow et al. (1993) organised a survey in an effort to find how to stop thefts of targets following the socio-political disturbances of 1990. Results of this survey are being used to modify the programme towards greater community

participation. Kamara and Echessah (1994) in their studies on the assessment of socio-economic factors affecting implementation of community-based tsetse control in Busia, Kenya, using baited targets, found that there were several factors which determined the willingness of communities and individuals to participate. These included ownership and use of livestock, beliefs and attitudes concerning trypanosomiasis, and the length of contact with the disease and control programmes. According to the findings of this study, important issues to be considered in community-based programmes were community organisational capacity, incentives needed to initiate and sustain programmes, and the intra-community distribution of programme benefits.

Community participation in projects involving assessment of the productivity and socio-economic benefits of trypanotolerant cattle have also been reported, in Zaire (Itty et al., 1995a) and Togo (Itty et al., 1995b). In the case of Togo (Itty et al. 1995b) the study examined the economics of village production of trypanotolerant cattle recently introduced in an area previously devoid of cattle. Social-level economics and private level financial analyses were performed using a herd model. Results showed that cattle production was profitable to cattle owners but returns were especially vulnerable to alterations in costs of inputs; for example, cattle purchase and veterinary care. In this study, foreign financed subsidies did not enhance the farmer's participation in the development process and distorted the incentive structure. In Zaire (Itty et al., 1995a), the study revealed that cattle production was profitable and that a cattle lease scheme provided substantially higher returns than when the farmers had to purchase all their livestock.

Due to the dynamic nature of the tsetse populations, Rogers and Williams (1993) noted that there has been a shift in emphasis from tsetse eradication to tsetse control. The future role of central governments will remain crucial, however, in determining the areas in which different control

options are practised, in facilitating control by local communities and in protecting control areas from re-invasion by flies from other areas.

In summary, most vector control methods are expensive and tend to interfere with already fragile ecosystems since most tsetse species occur in arid and semi-arid areas of the African continent. Furthermore, there are always problems of re-infestation unless barriers are put in place to prevent immigration of flies. In this regard, regional co-operation is essential in order to achieve any long lasting sustainable impact.

TRYPANOCIDAL DRUGS

Classification of animal trypanocides

Table 1.1 summarises the characteristics of the drugs used for the treatment and prophylaxis of animal trypanosomiasis. Their chemistry is given in Appendix A.

Outline of the history of the chemotherapy of animal trypanosomiasis

Potassium antimony tartrate (tartar emetic)

Chemotherapy of animal trypanosomiasis dates as far back as the turn of the century when in 1908, Plimmer and Thompson showed potassium antimony tartrate (tartar emetic) could cure infections due to *T. brucei* and *T. evansi* in laboratory rodents. The drug was given by intravenous injection since it caused tissue reactions when administered intramuscularly. In cattle, dose rates of 1.0 - 1.5 mg kg^{-1} body weight in a 5% w/v solution were found to be effective against *T. congolense* and *T. vivax* infections but not *T. brucei* infections (Bevan, 1928; Curson, 1928). The drug remained in use for nearly 40 years.

TABLE 1.1: CHARACTERISTICS OF DRUGS USED FOR THE TREATMENT AND PROPHYLAXIS OF ANIMAL TRYPANOSOMIASIS

DRUG SHOWING CHEMICAL GROUPING	TYPE[b]	ANIMAL	FIELD USE	DOSE[c]	TOXIC EFFECTS	COMMENTS
Suramin sodium (sulfonated naphthylamine)	C	camels	*T.evansi*	Approx. 10 i.v	-	Suramin resistant infections can be treated with quinapyramine
Quinapyramine dimethosulphate	C	cattle, small ruminats, pigs, dogs	*T.brucei, T. congolense, T. vivax,*	5 s.c	Effects resembling those of curare, aggrviated by stress; kidney damage	
	C	equines	*T. brucei, T. evansi, T. equiperdum*	3.5 s.c	Equine animals are more sensitive than bovine	The dose should be administered in halves separated by a 6-hour interval.
	C	camels	*T.evansi*	3.5 s.c	-	The drug is active against suramin-resistant strains
Quinapyramine dimethosulphate chloride 3:2 w/w	P	cattle	*T.congolense, T.vivax*	7.4 s.c.	-	Prophylaxis lasts about 2 months, depending on the severity of the challenge
Homidium bromide and homidium chloride (phenanhtridine)	C	cattle, small ruminants, horses, pigs	*T. congolense, T. vivax*	1.0 i.m.	Local tissue damage if given s.c.; liver damage when given high doses	The bromide is soluble only in hot water, whereas the chloride is soluble in cold water. Either salt can be used in alternation with diminazene aceturate to reduce the incidence of drug resistance
Diminazene aceturate (aromatic diamidine)	C	cattle, small ruminants, dogs	*T. congolense, T. vivax*	3.5 i.m.	-	The drug is more rapidly excreted than other trypanocides and is active against homidium-resistant infections; it is inactive against T. simiae
Isometamidium chloride (phenanthridine-aromatic amidine)	P	cattle	*T. congolense, T. vivax*	0.5-2.0 i.m.	Similar to those of homidium	The drug is less toxic than homidium. It is soluble in cold water and is heat sensitiv Prophylaxis lasts 2 to 4 months, dependoing on challenge. The dose should be determined by the intensity of challenge
	C	cattle	*T. congolense, T. vivax*	0.25-1.0 i.m.		Higher doses may be given for drug resistant infections
Melarsen oxide cysteamine	C	camels, horses	*T. evansi T. eqiperdum*	0.25-0.50 i.m	-	Therapeutic activity demonstrated with doses ranging from 0.2-1.2 mg/kg in camels Systemic reactions in camels include lachrymation, drooling saliva and muscle tremors

[b]C= curative, P= prophylactic
[c]The dose is stated in mg/kg bodyweight: i.m= intramuscular, I.V.= intravenous, s.c.= subcutaeous

Suramin

Suramin came into general use in 1920 after it had been developed in Germany during the first world war. It was found to be active against *T. equiperdum* in horses and *T. evansi* infections in camels (Knowles, 1925). Because of its slow rate of excretion, Findlay in 1930 pointed out that it could confer protection for two months. Suramin has remained the drug of choice for the treatment of *T. evansi* in camels.

Suramin has been demonstrated to undergo complex formation with other active trypanocidal drugs. Several of these complexes have been prepared and their trypanocidal activity determined. These include the pentamidine-suramin complex (Guimaraes and Lourie, 1951); the toxicity of pentamidine in this complex was reduced without affecting its prophylactic activity. Other suramin complexes have been reported by Desowitz (1957) and Williamson (1957) involving pyrithidium, dimidium, homidium, quinapyramine and diminazene, but their use was limited due to reactions at the injection site. However, some of them did confer longer periods of protection to cattle than the individual constituents.

Trypaflavine

The phenanthridinium drugs in use today owe their origin to Ehrlich's work on dyestuffs based on the acridine nucleus. This led to the development of trypanosan, acridinium yellow, trypaflavine (acriflavine) and the styrylquinolines. Trypaflavine was found to be a highly active anti-bacterial agent whereas some of the styrylquinolines were found to possess powerful antiseptic properties (Browning et al. (1922) cited by Williamson, 1970).

Phenidium chloride

Further improvements in drug development led to the synthesis of phenidium chloride, a phenanthridine which was found to have a marked

effect on *T. congolense* infections in cattle (Browning et al., 1938) unlike the styrylquinolines which acted only on *T. brucei*. Other disadvantages with the styrylquinolines were that they acted slowly on trypanosomes, were systemically toxic and produced serious reactions at the injection site. However, they were retained for longer periods in the body than the drugs that were available at that time. The activity of phenidium chloride against *T. congolense* infections in cattle was confirmed in trials in Africa. However, it had low solubility and a narrow therapeutic index (Williamson, 1970).

Dimidium bromide

More research into drug development revealed an active and water soluble derivative of phenidium with two amino groups in the phenanthridine portion of the molecule. This compound, dimidium bromide, the next phenanthridine to be introduced after phenidium, was readily soluble and was shown to be active against *T. congolense* in cattle in the field by Carmichael and Bell (1944) when given subcutaneously at 1 mg kg^{-1} b.w. The drug was used extensively in East and Central Africa until drug resistance occurred by 1952 as a result of mass treatments. The use of doses higher than 1 mg kg^{-1} to overcome resistance led to severe toxicity (Randall and Beveridge, 1946; Evans, 1948).

Surfen C

In 1937 Jensch reported the synthesis of a series of new compounds: 4-aminoquinoline derivatives. In one of these compounds, two 4-aminoquinoline moieties were linked together through amino substitution in the 6-position to melamine to produce Surfen C. Surfen C was the first metal-free anti-trypanosomal drug and was found to be active against *T. congolense* infections in the field. When evaluated under field trials, however, the drug showed variable and unacceptable toxic effects (Le Roux, 1938; Van Rensberg, 1938).

Quinapyramine

Quinapyramine was synthesised following Jensch's (1937) work, by restructuring the Surfen C molecule. The compound was introduced in the field in 1950, being the first prophylactic drug for use in cattle against *T. congolense* and *T. vivax*. Quinapyramine is used either in the form of a soluble dimethosulphate salt for treatment of infections with most trypanosomes that are pathogenic to livestock (Curd and Davey 1949, 1950; Davey, 1950), or as a mixture of dimethosulphate and the chloride to form "Quinapyramine Prosalt", which is used as a prophylactic agent. Although cases of resistance to quinapyramine in *T. evansi* and *T. congolense* have been reported (Gill, 1971; Ndoutamia et al., 1993), it is still widely used in the field against *T. evansi* in camels.

Homidium bromide/chloride

In 1952, Watkins and Woolfe reported the development of homidium (given the trivial name 'ethidium bromide') by changing the nature and position of substituents on the phenanthridine nucleus of dimidium. In the synthesis of homidium the quarternizing group $-CH_3$ in dimidium was replaced by $-C_2H_5$. Homidium was found to be effective against *T. congolense* and *T. vivax* in cattle at 1 mg kg^{-1} b.w. The efficacy of this drug, plus quinapyramine, led to mass treatments of cattle which became infected in the field through contact with tsetse flies (Wilson, 1960).

Diminazene aceturate

Diminazene aceturate is a derivative of Surfen C and consists of two amidinophenyl moieties linked by a triazene bridge. It was first introduced in 1955. Bauer (1955a,b) and Fussganger (1955) reported trials with this drug in the field. It has also been used in the control of homidium-resistant cattle infections in both East and West Africa (Whiteside, 1962). A dose of 3.5 mg

kg^{-1} b.w. was claimed to cure *T. congolense* and *T. vivax* infections in cattle, but a higher dose was recommended as necessary to eliminate *T. brucei* infections. The drug did not appear to be effective against *T. simiae* but it had a considerably wider therapeutic index than the other trypanocidal drugs that were available at that time; Fairclough (1963a) reported the safe use at a dosage of 21 mg kg^{-1} b.w. when given subcutaneously. Diminazene was therefore used in place of quinapyramine or homidium in the field following the withdrawal of the latter drugs from field use in Northern Nigeria in 1963 and 1965, because of the development of drug resistance (MacLennan, 1968).

Pyrithidium bromide

The synthesis of pyrithidium bromide was reported in 1956 (Watkins and Woolfe, 1956). The molecule was produced by combining a portion of the quinapyramine molecule with the phenanthridine-resembling phenidium. It was shown to have marked therapeutic and prophylactic activity (Finelle and Lacotte, 1965). Whiteside in 1960 found that 0.2-0.4 mg kg^{-1} b.w. would cure *T. congolense* infections in cattle. Furthermore, Finelle and Lacotte (1965) were able to protect cattle for 4-6 months in an area of high tsetse challenge, and donkeys for even longer. However, the drug produced severe local reactions when given subcutaneously at doses over 25 mg kg^{-1} b.w. (Stephen, 1963). In addition, intramuscular injections of 5 mg kg^{-1} b.w. were considered to be responsible for deaths (Leach and El Karib, 1960).

Isometamidium chloride

Wragg et al. (1958) reported the synthesis of metamidium from a coupling reaction that involved m-amidinobenzenediazonium chloride and homidium chloride. A mixture of two isomers was produced; one red and the other purple. The red was more soluble and active than the purple, both therapeutically and prophylactically, against a strain of *T. congolense* in mice,

and accounted for 55% of the total mixture. The more active form was later isolated (Berg, 1960, 1963) and marketed as isometamidium. It was recommended that this drug should be given intramuscularly at a dose rate of 0.5 mg kg^{-1} b.w. for drug-sensitive infections, 1.0 mg kg^{-1} b.w. for drug-resistant infections, and 2.0 mg kg^{-1} b.w. for prophylaxis.

Melarsenoxide cysteamine

Melarsenoxide cysteamine was discovered in 1985 and is recommended for use against *T. evansi* infections in camels. Otsyula et al. (1992) have reported results of a study carried out in an area in the Eastern Province of Kenya to evaluate the efficacy of a range of doses of the drug in camels. The doses investigated were 0.2, 0.4, 0.6 and 1.2 mg kg^{-1} b.w. administered by the subcutaneous route as a single treatment. The trial demonstrated therapeutic activity of the drug against natural *T. evansi* populations in camels in Kenya at all the dose rates used. Symptoms of systemic reactions to the drug included frequent urination, drooling saliva and muscular tremors amongst others. Swellings were produced at the site of injection with doses equal, to or more than, 0.4 mg kg^{-1}; more severe swellings being observed at higher doses. Swellings disappeared within 72 hours in all animals except those treated at 1.2 mg kg^{-1}. In a study by Zhang et el. (1992) it was demonstrated that all of 14 unrelated *T. evansi* clones used were sensitive to the drug i*n vivo*, the curative doses in mice ranging from 0.25 - 4 mg kg^{-1}. Resistance to the drug has however, been reported in *T. evansi* clones and in *T. brucei brucei* populations in laboratory studies using immunosuppressed mice (Osman et al., 1992; Pospichal et al., 1994).

ECONOMICS OF DRUG USE

Until recently (see Section 1.9), the economics of trypanosomiasis and tsetse control were governed more by possible benefits to agriculture than by

the cost of control schemes (Jahnke (1974) reviewed by Holmes and Scott, 1982). As observed by Holmes and Scott (1982), logistical and financial problems are a major constraint on the effective application of chemotherapy in the field. These include the vast areas involved, most of which are inaccessible due to lack of all-weather roads, purchase and maintenance of vehicles and inaccessibility to facilities such as diagnostic laboratories.

The cost of drugs and the infrastructure required for their distribution and use is considerable and often beyond the means of the small scale farmers. When the drugs have been afforded by the farmers, preparation and administration has been carried out by themselves with little or no knowledge as to their preparation and administration, often leading to underdosing of animals.

Holmes and Scott (1982) pointed out that foreign aid and government support has been essential to date but may be reduced in future and hence the recent introduction of community participation in control programmes on a cost-sharing basis is considered essential to ensure their future.

In the study by Jahnke (1974, reviewed by Holmes and Scott, 1982), it was shown that in land with high potential and high incidence of trypanosomiasis, tsetse control constitutes the method of choice. However, on land with low cattle populations and a low incidence of trypanosomiasis, it is preferable to protect cattle with drugs instead of controlling tsetse.

In work carried out on Mkwaja ranch, Tanzania involving 20,000 cattle (Holmes and Torr, 1988), an analysis was made of matching animal health, animal productivity and trypanocidal drug treatment for a period of 10 years (1973-1982). The animals on the ranch could not survive without the use of trypanocidal drugs. The number of treatments administered to each animal was on average 4.6 isometamidium and 0.7 diminazene per year. A high level of productivity was achieved under this drug regime demonstrating that in an area of Africa where cattle, if left untreated, rapidly succumb to

trypanosomiasis, the strategic use of the trypanocidal drug isometamidium, under a high standard of management can permit cattle to survive and be productive.

Ideally, the application of trypanocidal drugs, like other veterinary drugs should be controlled by qualified personnel. When this is so as observed by Holmes and Torr (1988), trypanocidal drugs can be used effectively and economically in the field. However, in most areas of sub-Saharan Africa, application of trypanocidal drugs lies in the hands of the cattle owner who purchases the drugs over the counter and there is lack of strict control. The mushrooming of shops dispensing veterinary drugs in many of these areas, e.g. Kajiado town, Kajiado District, Kenya, is a testimony to the demand for such drugs (Mdachi, personal communication).

Usually, cattle in the field are treated following clinical signs or detection of parasites in the peripheral blood. However, most traditional cattle owners do not have access to weighing and diagnostic facilities. Weights are therefore, estimated and doses calculated based on these estimated weights. Economic pressure tends to encourage underdosing. Administration of drugs without proper diagnosis, mostly relying on clinical signs which may not necessarily be due to trypanosomiasis may result in inappropriate drug use.

The efficacy of isometamidium prophylaxis in the field has been reported in several studies in addition to the Tanzanian study referred to above (Dolan et al., 1990, 1991; Maloo, 1993; Eisler et al., 1994; Stevenson et al, 1995). Studies by Dolan et al. (1990, 1992) using homidium bromide and isometamidium chloride and Maloo (1993) using isometamidium chloride showed that prophylactic treatments were more effective in the low tsetse challenge areas or areas of low trypanosomiasis risk where the drug has shown prophylactic cover of over four months, with an increase in cattle productivity, than in the high trypanosomiasis risk areas. Reduced periods of protection following isometamidium chemoprophylactic cover were shown to occur in

the high trypanosomiasis risk areas (Maloo, 1993; Dolan et al. 1992). The isometamidium prophylactic cover afforded in high risk areas ranged between 2 and 45 days (Maloo, 1993) and averaged 28.4 days (Dolan et al. 1992). Trypanosomiasis was found to be responsible for limiting wide scale adoption of dairy farming by the small scale farmer in the high trypanosomiasis risk areas of the Kenya Coast. Studies reported by Maloo (1993) and Dolan (1992) also showed that isometamidium did not protect cattle against outbreaks of haemorrhagic *T. vivax* infections. Maloo (1993) observed that with the small number of dairy herds at the Kenya coast scattered over large areas, large scale tsetse campaigns may not be economical. He suggested a short term solution which would be dependant on early detection of the disease followed by curative therapy using diminazene. Diminazene was found to cure all parasitaemias from breakthrough trypanosome infections. No deaths were reported in the control herd not given prophylactic treatment but maintained on diminazene curative therapy (Dolan et al, 1992).
The use of homidium both as a chemotherapeutic and chemoprophylactic agent is discussed in more detail below.

Homidium

Original use

Homidium has been used primarily as a curative drug in cattle at a dose rate of 1 mg kg^{-1} b.w. following field studies carried out in Kenya, Tanzania and Nigeria to investigate its curative effects against *T. congolense* and *T. vivax* (Wilde and Robson, 1953; Wilson and Fairclough, 1953; Unsworth, 1954a, b). The manufacturers recommended that the drug be given by intramuscular injection due to local reaction at the injection site following subcutaneous administration.

Homidium as a chemotherapeutic drug

Following the introduction of homidium, the numbers of cattle treated with the drug in Northern Nigeria rose from 45,000 in 1951-2 to 641,000 in 1957-8 (Williamson, 1970). The following five years saw a decline in the numbers of treated cattle to 25,000 in 1962-63, and this was taken to be an indication of the effectiveness of homidium in the control of animal trypanosomiasis (MacGregor (1965) cited by Williamson, 1970). In Tanzania, the cattle population which stood at 6.5 million in 1946 increased to 8 million in 1954 following treatments with homidium (Ford, 1965).

In an area in Uganda where quinapyramine had been used extensively, an investigation into a block treatment regimen with homidium at 1 mg kg^{-1} b.w. was carried out involving 11,200 head of cattle in 200 herds (Mwambu (1967) cited by Williamson, 1970). Infections in these cattle were no longer responding to quinapyramine therapeutic treatment. Results obtained following homidium treatment showed no clinical cases or deaths due to trypanosomiasis and that there was great improvement in the feeding and milk yield of animals two to three months after homidium treatment.

Current use

Although originally recommended for use as a chemotherapeutic drug, homidium has been used extensively as a chemoprophylactic drug (Mwambu, 1971; Dolan et al., 1990, 1992; Stevenson et al., 1995). Due to its strong intercalation with DNA, it has also been used extensively as a biochemical probe (Newton, 1976).

As a chemoprophylactic agent

Although homidium was introduced and recommended as a therapeutic drug, reports have appeared showing that it possesses prophylactic properties as well (Mwambu, 1971; Dolan et al. 1990; Stevenson et al., 1995). The drug

treatment regimen used in the field of administering therapeutic treatment to only clinically sick or positively diagnosed animals was found to exclude healthy carriers (Mwambu, 1971) and encouraged transmission of the disease to susceptible animals. The results obtained from the use of a block treatment regimen with homidium at 1 mg kg^{-1} b.w. showed a marked reduction in disease incidence which was maintained for three months and a reduction in calf mortality. Mwambu (1971) concluded that four regular treatments in a year would sufficiently control the disease in cattle in the Uganda situation. Use of diminazene curative therapy in the same area required six treatments per year per animal making it more expensive than the block treatment regimen using homidium.

In a 12-month study on homidium prophylaxis on Galana Ranch, Coast Province of Kenya (Dolan et al., 1990), homidium was demonstrated to confer protection against infection for more than four months during a period of low disease incidence. During a similar study in the same area involving homidium and isometamidium a year later, animals in both the isometamidium and the homidium herds required 8 prophylactic treatments each during the 12 months at a dose rate of 1 mg kg^{-1} b.w. The mean period of protection for homidium was 25.4 days (range 19 - 35) while that afforded by isometamidium was 28.4 days (range 14 - 53) with a reported disease incidence in control cattle (not given the prophylactic treatment) three-fold higher than that obtained in 1990 (Dolan et al., 1990). The period of protection obtained following treatment of cattle with homidium at 1 mg kg^{-1} b.w. in the same area in 1990 (Dolan et al., 1990) was over four months. They observed that the period of protection in individual cattle treated with homidium was less variable than that observed in cattle treated with isometamidium. Similar findings of variability (2 - 45 days) in isometamidium prophylaxis have been reported by Maloo (1993).

During the first six months of a 12-month study, Dolan et al. (1992) showed that 12 out of 90 animals in the herd on isometamidium treatment had

lost weight while no weight loss was recorded in the homidium herd despite poor grazing. It was established that animals on isometamidium prophylactic treatment which required three diminazene treatments (7 mg kg^{-1} b.w.) following the development of breakthrough trypanosome infections, lost 27 kg compared with a gain of 5 kg in those which required no diminazene treatment. Weight gains were recorded in all animals on homidium treatment, the highest gain being in animals that required fewer diminazene treatments to treat breakthrough trypanosome infections. With improvement in grazing all animals gained weight despite a more intense trypanosome challenge. The homidium group showed the highest weight gain, followed by the control herd which was maintained on diminazene curative therapy and the lowest in the isometamidium herd. No deaths occurred in either the homidium or the control herds in the 12-month period whereas mortality was 67% in the cattle on isometamidium prophylactic treatment in which diminazene was used to treat breakthrough trypanosome infections. These deaths were attributed to liver toxicity as a result of frequent sometamidium/diminazene alternate treatments.

A recent study on homidium prophylaxis was carried out at Nguruman, Kenya (Stevenson et al., 1995) covering the period between Febuary 1990 to February 1991. A high incidence of infection was observed between weeks 2 and 23 of the study period (March to July). Eleven homidium group treatments were required during the year. A total of 38 new infections of which 60% were *T. congolense* were detected in a group of 30 animals during the experimental period. During the same period, 15 infections did not respond to homidium bromide treatment and were considered to be drug resistant. Thirteen animals required diminazene treatment because of persisting infections, low PCVs or poor clinical condition.

Treatment of parasitaemic animals in the control group with diminazene at a dose rate of 7 mg kg^{-1} b.w. on individual basis failed to produce effective control. Twenty five percent of animals in this group either

died or were removed from the trial due to their extremely weak condition. It was observed that homidium bromide at 1 mg kg^{-1} b.w. was more sensitive against *T. vivax* than against *T. congolense.*

Because of the high incidence of infection and the appearance of drug resistant populations of *T. congolense*, the use of homidium as a chemoprophylactic was rather complicated. However, when compared to maintenance of a similar group of cattle on isometamidium prophylaxis under similar conditions, the use of homidium was approximately four times less expensive.

MECHANISM(S) OF DRUG ACTION

The anti-trypanosomal activity observed with diminazene, homidium and isometamidium is attributed to the primary mode of action of blocking nucleic acid synthesis through binding to kinetoplast DNA. With the phenanthridinium compounds, the binding is by intercalation between base pairs (Newton, 1976). With diminazene, however, the binding does not occur by intercalation but via specific interaction with sites rich in adenine-thymine (A-T) base pairs (Newton, 1972). Thus, diminazene has been shown to selectively fragment kinetoplast DNA (MacAdam and Williamson; 1972).

TOXICITY

While diminazene's toxicity in cattle has not been documented, toxicity to this drug in dogs and camels has been reported (Losos and Crockett, 1969; Homeida et al., 1981). Doses of 30-35 mg kg^{-1} b.w. in dogs cause spastic paralysis, involuntary movements, vomiting, and death in two or three days.

Most phenanthridinium compounds have been reported to be toxic (Randall and Beveridge, 1946; Unsworth, 1954a; Fairclough, 1958; Dolan et al., 1992). The toxic effects include local tissue reactions at the site of injection, involving swelling and oedema, and are more pronounced in animals

given doses that are higher than the recommended therapeutic dose. The results of studies reported by Dolan et al. (1992) have shown that liver toxicity occurs in animals given frequent isometamidium prophylactic treatments alternating with diminazene therapy for animals which develop breakthrough infections during the prophylactic cover and particularly so in the presence of poor grazing in the field.

EVALUATION OF THE EFFICACY OF THE DRUGS UNDER FIELD CONDITIONS

A thorough understanding of the pharmacokinetics and pharmacodynamics of current trypanocidal drugs is required to be able to understand their interaction with the host in the course of treatment. This understanding would be useful in designing treatment regimens that could be more appropriate for each of the drugs. Availability of sensitive analytical techniques have made it possible for studies to be carried out on diminazene and isometamidium. However, a sensitive method is yet to be developed for homidium which would allow a similar evaluation of the drug to be made under field conditions. These field evaluation studies are needed to be able to understand the following:

(a) how animals can be maintained in medium to high tsetse challenge areas following drug administration without the development of drug resistance.

(b) the contribution of the injection drug depot and the secondary tissue depots to circulating drug concentrations and how these could be exploited in terms of maintaining the minimum effective concentrations for longer periods through slow release formulations.

(c) the nature and extent of resistance in the field. This would be done

through determination of drug concentrations in treated cattle in the field and isolating trypanosomes that persist at various drug concentrations for further characterisation.

TISSUE DRUG RESIDUES AND METABOLITES

The subject of drug residues and metabolites is discussed within the sections that discuss the various methodologies used to determine the drug levels in tissues and biological fluids. Although extensive use has been made of these drugs, more work still needs to be done in the area of residues and metabolites and the role they play, if any, in the prophylaxis of animal trypanosomiasis. Furthermore, apart from diminazene, it is still unclear whether homidium or isometamidium remain in the same unchanged form or exist as active metabolites in the body.

REVIEW OF THE CURRENT ANALYTICAL METHODS

Ultraviolet/Visible Spectrophotometry

According to Clarke's isolation and identification of drugs (1969), homidium has two absorption wavelength maxima in dilute acid; one at 242 nm and the other at 283 nm (E1%,1cm = 785). Similarly, diminazene has absorption wavelength maxima in dilute acid at 257 nm (E1%,1cm = 305) and in dilute alkali at 247 nm (broad peak, E1%,1cm = 239). Although these absorption wavelength maxima have not been used, the information was the basis of the work carried out by Phillips et al. (1967) who described two methods for the quantification of isometamidium in plasma, tissues, urine and faeces. In the first method, isometamidium was extracted with ether from a basic solution containing the drug. Briefly, homogenates of tissue (10%w/v) and faeces (2.5%w/v) were prepared in water. Urine and plasma were also diluted in water. To 4 ml of each of the various preparations were added 2 g sodium chloride, 25 ml ether and 1 ml of 30%w/v sodium hydroxide. The

mixtures were then centrifuged and the two phases separated. The drug was extracted from 20 ml of the ether into 4 ml 0.1M acetate buffer, pH 4.7, shaken for 10 minutes, and the absorbance of the buffer determined at 378 nm; the absorption wavelength maximum for isometamidium. An absorbance of 1 unit was obtained with a solution containing 21.2 μg in 0.1M acetate buffer, pH 4.7, and this was used to calculate isometamidium concentrations in the various materials analysed. Recoveries from aqueous solutions containing isometamidium were 80.0±1.6%. Recoveries from rat liver, spleen, kidney, heart, small intestine and plasma that had been spiked with isometamidium were 100-103%. Finally, recovery from brain and faecal homogenates and urine spiked with the drug was 80-86%. Limits of detection for the various tissues and body fluids were as follows: plasma, 0.7 μg ml^{-1}; tissue, 3.3 μg g^{-1}; faeces, 13 μg g^{-1}; urine, 13 μg ml^{-1} per 24 hour collection.
The second method of Phillips et al. (1967) was an improvement to Method 1, mainly to solve the problem of frothing which had been observed when the samples were shaken. In this method, isopropanol, ether and sodium hydroxide were added to the homogenates, while the rest of the procedure remained as in Method 1. The sensitivity was very much improved with this method. The limits of detection were as follows: blood, 1.3 μg ml^{-1}; kidney, 1.3 μg g^{-1}; and liver, 2.5 μg g^{-1}.

This second method has been used by several workers interested in carrying out studies on isometamidium residues in cattle after treatment (Eghianruwa and Uduebholo, 1979; Braide and Eghianruwa, 1980; Shetty, 1986).

Lastly, Raether et al. (1972) described a colorimetric procedure for diminazene in which diminazene was split into 4-amidinophenyldiazonium chloride and 4-aminobenzamidine. After converting the 4-aminobenzamidine to a diazonium salt, both components were coupled to an azo dye (reaction according to Bratton and Marshall [1939]). The optical density of the colour

produced by the reaction was determined at 545 nm. Since this reaction was light sensitive, light was avoided. The limit of detection of the assay was 0.5 μ g ml^{-1} serum.

Fluorescence microscopy

Homidium and isometamidium are highly fluorescent in UV light. This property has therefore been used to demonstrate the distribution of the drugs in various organs and tissues of animals after treatment with the drugs (Hill and MacFadzean, 1963; Phillips et al., 1967).

Hill and McFadzean (1963) in their work on isometamidium used fluorescence microscopy to study the role of drug depots in mice and rats in prophylaxis against *T. congolense.* The technique involved fixation of tissues for 1 hour in formol saline, followed by cutting of 10 μm frozen sections which were examined immediately. The organs examined were liver, spleen, kidney, gut, lung, skin, muscle, bone-marrow and the brain. In initial work, serum taken from a mouse at 1, 3 and 6 hours after sub-cutaneous (s.c.) injection of 0.16 mg (cation) g^{-1} b.w. fluoresced when spotted on paper. No red or orange fluorescence was observed in the organs from untreated animals. However, orange fluorescence was detected in the liver, kidneys, and at the injection site, of the animals given s.c. or intravenous (i.v.) injection of 0.0006 mg (cation) g^{-1} b.w. Cellular level distribution could not be determined under these conditions. This technique, however, was found to be more sensitive and simpler than the other available methods.

Phillips et al. (1967), using fluorescence microscopy, also examined organs of animals treated with isometamidium. Their results showed that all the tissues contained isometamidium.

Paper chromatography

Phillips et al. (1967) used the paper chromatographic technique to look at faecal extracts for the presence of metabolites of isometamidium. Sodium hydroxide and sodium chloride were added to aqueous solutions of isometamidium, homidium and to faecal extracts. These mixtures were then extracted with ether. The extracts were then evaporated, the residues dissolved in acetone, spotted on Whatman No. 3 paper, and dried. The dried spots were then developed using ascending chromatography with the following systems:

Dimethylformamide:water 1:1 (v/v)

Triethylamine:water:dimethylformamide 1:5:14 (v/v/v)

Acetic acid:water:dimethylformamide 1:5:14 (v/v/v)

The dried chromatograms were then viewed under UV light for orange-pink fluorescent spots. These were not seen with the faecal extracts from untreated animals. However, small pink zones were seen at the origin of the chromatograms of spots of faecal extracts of treated rats showing the presence of isometamidium. The technique is only suitable for qualitative work because of the difficulties in quantifying the spots.

Thin Layer Chromatography (TLC)

This technique, though simple and fast, is mainly for qualitative and sometimes semi-quantitative work. The materials usually used for the stationary phase are silica and alumina which are coated on a sheet of glass as a support. After spotting the plate with samples and reference standards, the plates are developed in an equilibrated tank containing an appropriate solvent system. Various compounds migrate differently depending on their size and polarity. Detection is achieved by viewing under UV light for fluorescent substances; the spots can be quantified using a scanning densitometer. TLC is a technique usually employed for general screening of samples, followed by

more sensitive techniques. Moffat (1986) has described several solvent systems which have been established for general screening of basic nitrogenous drugs, which include diminazene, homidium and isometamidium. TLC was used by Kellner et al. (1985) to determine the presence of metabolites in urine from diminazene-treated cattle. Their findings were that the metabolite pattern in the urine of the first day after treatment was different in two animals. In one calf, three radioactive peaks were detected; the largest of the peaks, which accounted for 74% of the total radioactivity, was the original compound diminazene. The next smaller peak (Rf = 0.53) was identified as p-aminobenzamidine and accounted for 22% of the radioactivity. The third peak (Rf = 0.63) was identified as p-aminobenzamine and accounted for only 4%. Interestingly, these two latter peaks were undetected in the second calf, showing only the presence of diminazene.

Mass Spectrometry

Diminazene has an established mass spectrum with principal peaks at *m/z* 30, 43, 72, 102, 73, 42, 118 and 99 (Moffat, 1986). There is no established analytical method that has made use of this technique on its own, except one in which gas liquid chromatography has been coupled to a mass spectrophotometer.

Gas Liquid Chromatography- Mass Spectrometry (GLC-MS)

All the trypanocidal drugs currently in use are polar compounds. Determination of such polar compounds by gas liquid chromatography is impossible since the technique requires that compounds must be non-polar, volatile and thermally stable. The procedure for diminazene required the conversion of the drug to a non-polar compund before extraction and clean-up, and determination by GLC-MS.

Infra-red (IR) Spectrometry

Using a potassium bromide disk, the principal peaks of the trypanocidal compounds shown below have been established at the following wavenumbers:

Homidium - 1628, 1492, 1260, 1312, 836, 1077

Diminazene - 1610, 1635, 1588, 1171, 1267, 1198

The IR spectra of these drugs have not been exploited in terms of developing procedures for detection and quantification of the drugs in biological materials. At the moment the technique is only suitable for pure drug compounds or for determination of the active ingredient in formulations.

Radiometric

Gilbert and Newton (1982) studied the pharmacokinetics of homidium in *T. congolense*-infected and non-infected rabbits and calves; ^{14}C-labelled homidium bromide was administered at dose rates of 1 or 10 mg kg^{-1} b.w. to the rabbits and at 1 mg kg^{-1} b.w. to calves. In non-infected rabbits homidium concentrations reached a maximum of 180 ng ml^{-1} in blood, and 50 ng ml^{-1} tissue fluid, within one hour following drug administration. After this there was a rapid fall to less than 10 ng ml^{-1} in blood at 96 hours. In non-infected calves the levels in blood after one hour were 120-170 ng ml^{-1}. Concentrations then fell rapidly within the first 24 hours and then slowly to 15 ng ml^{-1} within the next 8 days. It was also observed that 80-90% of the total dose given was excreted within 96 hours; one-third in urine and two-thirds in faeces. Between 63 and 79% of the drug was found bound to trypanosomes separated from ear vein blood obtained one hour following intramuscular drug administration to calves.

Nine days after drug administration to rabbits, it was estimated that only 2 - 3% of the total dose given was present in tissues; the highest levels of 0.44 and 0.42 μg g^{-1} wet tissue were in liver and kidneys, respectively. In

normal calves, ten days following treatment, these levels were 1.54 and 1.02 μg g^{-1} wet tissue and in infected calves they were 1.47 and 0.48 μg g^{-1} wet tissue, respectively.

Using both thin-layer and gel chromatography, all radioactivity in serum from non-infected and infected rabbits and calves treated with ^{14}C homidium was found to chromatograph in the same position as homidium. Gel chromatography of urine and bile, however, showed two components eluting before homidium, representing up to 46% of the total radioactivity eluted. One of the components carried about 38% of the total radioactivity. Neither of the components was the monoacetyl or the diacetyl conjugates of homidium. Acid extraction of urine indicated that all activity in the extract was due to the presence of homidium when chromatographed. This indicated that the two components mentioned above were not recovered in the acid extract.

Results from the above studies indicate that homidium is excreted very rapidly. It is therefore difficult to relate these results to field observations on the prophylactic activity of homidium; of 8 to 17 weeks (Dolan et al., 1990).

In 1983, Gilbert used radiolabelled diminazene aceturate to study its pharmacokinetics in rabbits. After intramuscular administration of bis-phenyl-U-^{14}C-labelled diminazene aceturate at a dose rate of 3.5 mg kg^{-1} b.w., radioactivity was determined in blood, tissue fluid, urine, faeces and tissues. One hour after treatment, the drug level obtained in blood was 1.1 μg ml^{-1} and that of tissue fluid was 0.2 μg ml^{-1}. The level of the drug in the blood 7 days later was 25 ng ml^{-1}. Sixty-five percent of the dose given was excreted within 7 days, 44% through urine.

Kellner et al. (1985) administered radiolabelled diminazene aceturate (bis-phenyl-U-^{14}C) to two calves at a dose rate of 3.5 mg kg^{-1} b.w. by intramuscular injection. Radioactivity was determined in blood, plasma, urine, faeces and edible tissues. The maximum drug levels, obtained 15 minutes after drug administration, were 4.6 and 4.7 μg ml^{-1} blood. The values for the two

individual animals were 0.62 and 0.36 μg ml^{-1} after 7 and 20 days, respectively. The disappearance of the drug from plasma was bi-phasic. The total amount of drug excreted through urine and faeces within the first 7 days after drug administration was 47.1% and 7.6%, respectively of the total dose administered. These values were 72.2% and 10.3%, respectively, after 20 days.

High Performance Liquid Chromatography (HPLC)

The term chromatography embraces a family of closely related methods. Three of these methods, namely paper chromatography, thin layer chromatography and gas chromatography, have already been discussed. The other method is high performance liquid chromatography (HPLC). The distinguishing feature of this method is that two mutually immiscible phases are brought into contact; one phase is stationary and the other mobile. The sample mixture is introduced into the mobile phase and undergoes a series of interactions between the stationary and the mobile phases as it is carried through the system by the mobile phase. Differences between the physical and chemical properties of the components of the sample govern the rate of migration of the individual components.

Various compounds differing in both physical and chemical properties can be analysed by HPLC, so long as they are soluble in a solvent of some sort, aqueous or non-aqueous. In the normal HPLC, the stationary phase employed is polar (possessing amino or cyano functional groups) with a non-polar mobile phase. In the reverse-phase HPLC the stationary phase is non-polar and the mobile phase polar. It is the reverse of normal HPLC, and hence its name. Reverse phase chromatography utilises a hydrophobic bonded phase packing usually possessing octadecylsilane (C_{18}; ODS) or octylsilane (C_8; RP-8) functional groups, and a polar mobile phase. In this type of separation, the polar compounds prefer the polar mobile phase and elute first. Retention is

usually longer for non-polar compounds since they prefer the hydrophobic stationary phase. The simplicity and reproducibility of reversed phase chromatography on bonded phases make this method particularly attractive in clinical chemistry. Because the water content of the mobile phase can range from 100% to very low percentages, or none at all, a broad spectrum of biomolecules can be chromatographed; lipophilic, ionic, small or large. Bonded phases tend to be unstable in the presence of strong acids and bases. Strong acidic and basic systems cannot be handled by ordinary liquid chromatographic techniques involving bonded phases. However, by forming an ion-pair (association of two ions of opposite electrical charge) with a suitable counterion, ionic or ionisable compounds can be converted to eletrically neutral compounds which can then be chromatographed. A large organic counterion added to the mobile phase forms a reversible ion-pair complex with the ionised sample; this complex behaves as an electrically neutral and non-polar (lipoliphic) compound. The extent to which the ionised sample and counterion form an ion-pair complex affects the degree to which the retention is increased. This in turn depends on the pH of the solvent.

In the procedure of Fauda (1977), diminazene was first converted to 4-aminobenzamidine which was then extracted and analysed by ion-pair reverse-phase liquid chromatography; 3,4-dimethoxybenzamidine was used as an internal standard. The analytical column used was a C_8 column; the effluent was monitored at 310 nm. The mobile phase consisted of methanol, water and glacial acetic acid in the ratio 7:3:0.05 to which was added 8 mg 1-pentane sulphonic acid as a counter ion in every 100 ml of solution. The flow-rate was 1.2 ml per minute with a pressure of 1850 pounds per square inch (psi). With this procedure, diminazene levels as low as 0.1 $\mu g\ ml^{-1}$ were detected.

The introduction of solid-phase extraction (SPE) columns has revolutionised HPLC techniques in terms of sample clean-up before analysis. A number of such methods have been described for the trypanocidal drugs

diminazene (Aliu and Odegaard, 1983; Murilla and Kratzer, 1989), and homidium, isometamidium and quinapyramine (Perschke and Vollner, 1985), resulting in much improved limits of detection.

Aliu and Odegaard (1983) used paired-ion extraction and HPLC to determine diminazene concentrations in plasma. The SPE columns for sample clean-up were C_{18}. The analytical column was a cyano-propyl column; the effluent was monitored at 254 nm. The mobile phase was acetonitrile:methanol in the ratio 50:50, plus 2% v/v triethylamine, at a flow-rate of 0.8 ml min^{-1}. The internal standard was imidocarb [(3,3'-di(2-imidazolin-2-yl) carbanilide)], and the counter-ion was 1-heptane-sulphonic acid. Linearity was established with diminazene over the range 0.05-5.0 μg ml^{-1}. Recovery from plasma, without any concentration step, was 92.0±7.8% with a limit of quantification of 50 ng ml^{-1}.

Murilla and Kratzer (1989) described a reversed-phase HPLC procedure for diminazene in which the sample clean-up was done using silica-gel SPE columns. The analytical column used was a reversed-phase C_{18} column, and the mobile phase consisted of methanol:water:acetic acid in the ratio 20:80:0.03 pumped at 1.5 ml min^{-1}. The column effluent was monitored at 370 nm and the limit of detection was 25 ng ml^{-1} serum or plasma, and 100 ng g^{-1} tissue.

In the method of Perschke and Vollner (1985), concentrations of homidium, isometamidium and quinapyramine were determined after sample clean-up on disposable silica-gel columns. Isometamidium was cleaved and determined as homidium. The analytical column was C_{18} and the mobile phase (pumped at a flow-rate of 2 ml min^{-1}) was acetonitrile:water:perchloric acid in the ratio 40:60:0.03 (v/v/v) for homidium and 20:80:0.03 (v/v/v) for quinapyramine. Both homidium and the cleaved isometamidium were detected using a UV detector at a wavelength of 292 nm, whereas quinapyramine was determined using a fluorescence detector with an excitation wavelength of 296

nm and an emission wavelength of 405 nm. The detection limits achieved by this procedure were 50 ng of homidium, 100 ng of isometamidium and 30 ng of quinapyramine per ml serum when 1 ml serum was processed; and 5 ng of homidium, 20 ng of isometamidium and 3 ng of quinapyramine per ml when up to 10 ml of serum was processed. Finally, the different procedures were developed using spiked samples and were not validated with samples from treated animals.

Kinabo and Bogan (1988a) established a solid-phase extraction and ion-pair reversed-phase HPLC technique with fluorescence detection for isometamidium in bovine serum and tissues. The procedure utilises enzyme hydrolysis to release the drug bound to protein before the clean up and analysis. However, the long extraction procedure reduces the sensitivity of the method, making it unsuitable for routine analysis. Thus, the method has only been used to detect and monitor isometamidium concentrations in serum during the first 24 hours after drug administration. It has also been used to investigate the absorption and distribution patterns of isometamidium in tissues of cattle (Kinabo and Bogan, 1988b). Finally, the method has also facilitated an examination of isometamidium's pharmacokinetics in lactating goats (Kinabo and McKellar, 1990) and in pigs (Kinabo and McKellar, 1991). The procedure is, however, not sensitive enough to detect and monitor the drug levels for periods longer than 24 hours in serum of treated cattle.

Biological assays

In vivo

In vivo drug assays for the different trypanocidal drugs currently in use have been reported. These include methods of Hill and McFadzean (1963), Cunningham et al. (1964), Hill (1965) and Goodwin and Tierney (1977).

In the procedure of Hill and McFadzean (1963), groups of mice were given a sub-cutaneous injection of isometamidium. Heart blood was then taken

at various times post-treatment and sera prepared. The sera were then injected i.v. into other mice immediately after they had been inoculated i.p. with *T. conglonse*. In all experiments, there were untreated infected controls and infected controls given normal mouse serum. Some infected mice were given known doses of isometamidium in aqueous solution. The level of parasitaemia of all mice was monitored several times per week for 4 weeks after inoculation, and the pre-patent period compared to the controls. Mice which remained negative at the end of the 4 weeks were considered to be fully protected. The results indicated that there was a slight elongation of the parasitaemia pre-patent period by serum taken 3, 24, 48 hours and 7 days after the s.c. injection of 0.04 mg (cation) g^{-1} b.w. In order to be able to detect maximum activity, 0.16 mg g^{-1} b.w. was used, which was the largest dose that would not cause toxicity. Prolongation of the onset of parasitaemia to various degrees was observed with sera taken after 1, 3 hours and 7 days following treatment from mice which the elevated dose. One mouse was protected completely by the serum taken 1 hour after drug administration, but serum taken 14 days later had no effect at all. These responses were compared to the effect of the standard aqueous solutions of isometamidium. The comparison indicated that the bio-assay of serum from mice given 0.16 mg isometamidium (cation) g^{-1} b.w. showed it contained approximately 0.0004 mg ml^{-1}, 1 or 3 hours after treatment, and approximately 0.0002 mg ml^{-1}, 24 hours later. Lastly, the serum fluoresced up to 6 hours after treatment, but no fluorescence could be seen 24 hours later.

The bio-assay for diminazene described by Cunningham et al. (1964) was different from that of Hill and McFadzean (1963) for isometamidium. In this method, estimates of drug levels in cow serum after treatment were made by comparing the infectivity of trypanosomes after *in vitro* exposure to serum from a cow before, and at intervals after treatment, with the infectivity of trypanosomes after *in vitro*-exposure to serum containing known

concentrations of diminazene.

In the method of Hill (1965), three drugs were investigated in mice; namely, homidium, isometamidium and pyrithidium The protocol followed was similar to that of Hill and McFadzean (1963) except that higher dose rates were used. The results indicated that none of the mice inoculated with serum taken 24 hours after treatment with homidium at 7.5 mg kg^{-1} b.w. were cured. The donor mice, however, were cured with homidium bromide doses as low as 0.75 mg kg^{-1} b.w. The CD_{50} in mice of the *T. congolense* used was 0.2 mg kg^{-1} b.w.

Finally, Goodwin and Tierney (1977) used a biological assay to determine the trypanocidal activity of body fluids from rabbits treated with homidium bromide at 10 mg kg^{-1} b.w., suramin at 100 mg kg^{-1} b.w., diminazene aceturate at 15 mg kg^{-1} b.w., and isometamidium chloride at 10 mg kg^{-1} b.w. In this bioassay the serum, or tissue fluid collected from tissue cages implanted s.c. in treated rabbits, was serially diluted in a 96-well microtest plate and incubated at 35°C in the presence of 50,000 trypanosomes of *T. brucei* in basic medium. The highest dilution in which trypanosomes could still be seen moving was taken as the end point and was recorded after 24 and 48 hours incubation, or longer in some experiments. Controls, to which normal rabbit serum and tissue fluid were added, were used on all the plates, together with serial dilutions of the appropriate trypanocidal drug in basic medium containing Medium 199 with Earle's Salts, 25mM HEPES buffer, L-glutamine, glucose, sodium penicillin G, and streptomycin. For homidium bromide, the approximate dilutions of drug in microtest plates required to kill 100% of the trypanosomes were $1:1.5\times10^4$ after a 24-hour and $1:5.0\times10^6$ after a 48-hour incubation at 35°C. Exposure for 48 hours to body fluids from rabbits treated with phenanthridinium derivatives did not show much increase over the 24-hour figure. Lastly, results for diminazene indicated that after i.m. injection, significant trypanocidal activity appeared in tissue fluid before it

reached the plasma.

In vitro

When tsetse ingest blood from infected animals, trypanosomes are taken in with the bloodmeal and immediately lose their infectivity to mammals. These trypanosomes develop into different forms in various organs of the fly. The mammalian infective forms are the metatrypanosomes (metacyclics). Non-infective procyclic trypanosomes have been grown in culture since 1903, but *in vitro* assays to assess drug sensitivities of trypanosomes using this stage of the life cycle have been unsuccessful as results do not correlate with those obtained from animal experiments (Hawking, 1963; Elrayah and Kaminsky, 1991). Elrayah and Kaminsky (1991) examined the effect of diminazene and isometamidium on cultured procyclic forms of drug-susceptible and drug-resistant *T. congolense* and demonstrated that it was not possible to distinguish between drug-resistant and drug-susceptible stocks or clones using growth inhibition or death of the trypanosomes.

Efforts directed towards improvement of the techniques for *in vitro* propagation of mammalian infective, bloodstream form, trypanosomes have been successful for *T. brucei* (Cunningham and Honigberg, 1977; Hirumi et al., 1977), *T. congolense* (Gray et al., 1981; Hirumi and Hirumi, 1984) and *T. vivax* (Brun and Moloo, 1982). These culture systems have thereafter been developed and used successfully in screening for drug sensitivity in *T. congolense* and *T. brucei* trypanosome species. Gray et al. (1993) used *in vitro*-derived metacyclic trypanosomes to determine the drug sensitivity of 7 populations of *T. congolense* collected from cattle and tsetse flies. In this assay, the metacyclic trypanosomes were incubated for 48 hours at 35°C with concentrations of homidium bromide, homidium chloride, isometamidium chloride and diminazene aceturate ranging from 0.5 ng to 50 μg ml^{-1}. The

trypanosomes were then tested for their ability to survive on a bovine aortic endothelial monolayer over a period of 5 days in the absence of drugs. With the stocks of *T. congolense* used, the results obtained correlated well with field observations; that is, the stocks which showed resistance in the field to the drugs showed resistance *in vitro.*

Radioimmunoassays

Kinabo and Bogan (1988c) have described a radioimmunoassay technique for isometamidium which has not found much application in studies on isometamidium. This is probably because it has been superseded by easier and safer enzyme-linked immunosorbent assays. In the development of the radioimmunoassay procedure, sheep were immunised using an isometamidium-glutaraldehyde-human serum albumin conjugate. The antiserum obtained after 18 weeks of immunisation was used in the radioimmunoassay procedure. About 50% of the radioligand was bound at a final dilution of 1:500 in the absence of unlabelled isometamidium. The limit of detection of the assay was 38.3 ng ml^{-1}. When serum samples from calves treated with isometamidium chloride at 0.5 mg kg^{-1} b.w. were tested, the highest concentration measured was 69.3 ng ml^{-1}, attained 30 minutes following treatment. Thereafter, within 4 hours, it declined rapidly to below the detection limit.

A SUMMARY OF THE LIMITATIONS OF THE CURRENT METHODS FOR MONITORING OF TRYPANOCIDAL DRUGS IN TREATED ANIMALS

In summary, colorimetric methods lack both sensitivity and specificity. For example, certain samples such as plasma and urine may naturally give colour reactions that will interfere with the compound of interest. Although fluorescence methodologies are by nature more sensitive than the

colorimetric methods, any substances in the solution that quench the emitted light will interfere with the sensitivity of the method. Thus, using HPLC with a fluorescence detection method, Kinabo and Bogan (1988a) were unable to detect isometamidium in serum of treated cattle for more than 24 hours after drug administration. The fluorescence method on its own has been useful in examining the distribution of drugs, especially phenanthridines, in tissues.

Paper and thin-layer chromatography are not sensitive enough to determine sub-microgramme levels of the drug, especially in serum or plasma following administration of low doses. Thin-layer chromatography is useful as a screening method since it is cheap, simple, well adapted to routine use and multiple samples can be run on one plate. However, the disadvantage with this technique is that it is difficult to quantify and therefore not suitable as an analytical method.

Gas liquid chromatographic methods are sensitive but require that samples must be volatile and thermally stable. All the trypanocidal drugs are polar compounds and therefore require derivatisation to non-polar compounds before they can be chromatographed. This is time consuming as it requires long and tedious procedures of derivatisation, extraction and clean-up before analysis. Furthermore, only one sample can be analysed at a time, making it unsuitable for routine analysis.

Radiometric methods, although sensitive, lack specificity because they determine the total radioactive components, some of which could be metabolites.

Use of *in vitro* methods for drug-sensitivity tests has been successful, but the time taken in adapting trypanosomes to culture could be as long as three months, making the time taken to get any results too long. Extrapolation of data to different animal species is also often difficult.

As a result of the aforementioned problems and limitations of the analytical methods available for the anti-trypanosomal compounds, it was

necessary to develop methods with better sensitivity and specificity for the detection and monitoring of the drugs in animals following treatment. Lack of a sensitive and specific analytical method for evaluating levels of homidium in serum of treated cattle, and, until recently, isometamidium, in the field has been one of the factors limiting our knowledge on the relationship between the drugs' therapeutic and prophylactic activity and their pharmacokinetic properties.

Of the three drugs currently recommended for use in cattle (that is, diminazene, homidium and isometamidium), diminazene, which is purely a curative drug, can be investigated adequately at the present time with the available HPLC analytical methods since the drug can be monitored up to 28 days after administration of the recommended doses. Similarly, isometamidium, a widely used chemoprophylactic drug can be investigated adequately with the use of the recently established ELISA method (Eisler et al., 1993). However, homidium which was originally introduced as a chemotherapeutic drug and has been demonstrated to possess prophylactic properties cannot be investigated due to lack of sensitive analytical methods.

PART THREE

INTRODUCTION TO THE PRESENT STUDY

Sensitivities of most drug assays that involve sample extraction and clean-up are usually lowered (1) because drug is lost through the many steps involved, and (2) co-extracted proteinous material interferes with the detection of the drug of interest. Thus, direct methods not involving extractions, one of which is the enzyme-linked immunosorbent assay, are now the method of choice in many clinical analyses.

Enzyme-linked immunosorbent assays

Many drugs are administered at low dose rates e.g. mg kg^{-1} b.w. and it might be expected that concentrations in the blood will be low, frequently at nanogram levels, and therefore, difficult to detect. Therefore, suitable tests for the determination of low drug concentrations in biological fluids would have to be of high sensitivity and specificity. They should also be:

- devoid of extraction steps
- easy to perform
- suitable for analysis of a wide range of samples
- permit analysis large numbers of samples
- relatively cheap

Radioimmunoassays (RIA) utilising radiolabelled antigen and later, enzyme-linked immunoassays (ELISA) utilising enzyme-labelled antigen fulfilled many of these criteria and have made a significant contribution in the area of drug analysis.

Since drugs are small molecular weight molecules (haptens) not capable of inducing the formation of antibodies, they are usually coupled to a carrier protein to form an antigen. The most common carriers are serum albumin of various animal species, keyhole limpet haemocyanin, and

thyroglobulin.

The determination of a substance in a biological fluid consists of two steps: reaction and detection. Both RIA and ELISA are binding assays and involve antigen and antibody reactions. In most immunoassay reactions, the reagents are permitted to react for long enough for equilibrium to be reached.

At equilibrium: $Ag + Ab \Leftrightarrow AgAb$

The distribution of antigen between bound and free phases is directly related to the amount of antigen present. This provides a means of quantifying the antigen. Therefore, the principle of all binding assays is: 'Given a fixed amount of antibody, the ratio of bound to free antigen at equilibrium will be quantitatively related to the total amount of antigen present'.

Based on the above principle, methods have been developed for the determination of various substances in biological fluids. RIA was first introduced by Yalow and Berson (1960). Later, Engvall and Perlmann (1971,1972) introduced the idea of using enzymes conjugated with antibody or with antigen to detect and measure antigens and antibodies respectively. Rubenstein et al. (1972) described an ELISA method for morphine in which as little as 1×10^{-9} M morphine could be detected. Elliot et al. (1995) have described a method that is a modification of the conventional ELISA in which extremely low concentrations of β-agonist residues have been determined in bovine eye and hair samples. This is a simple qualitative screening test suitable for use on farms or in slaughterhouses in residue control programmes.

In the field of trypanosomiasis, Kinabo and Bogan (1988c) developed a radioimmunoassay method for the determination of isometamidium in serum of treated cattle which had a limit of detection of 38 ng ml^{-1}. This method had several disadvantages which included (a) lack of sensitivity (b) use of radiolabelled drug which is expensive and required special equipment and

special handling facilities (c) application was limited to laboratory use and (d) physical separation of the labelled from the unlabelled drug before the determination of radioactivity.

The use of enzymes as labels which are relatively cheap and require no special facilities and physical separation of samples is rapidly replacing radioisotopes. Using enzyme immunoassays, very low drug concentrations can be determined in biological fluids as will be demonstrated later.

Use of enzyme immunoassays in the study of parasitic diseases had previously been limited mainly to disease diagnosis. The technique is however, the method of choice in many clinical analyses in which detection of low levels of drugs is required. All drug ELISA methods are competition assays. Competition may be direct or indirect. In a direct competition assay, the solid phase is coated with antibody followed by addition of test serum diluted in a solution containing enzyme-labelled antigen. The free drug in solution (from the test sera) competes directly with the enzyme-labelled antigen for the antibody. Detection is by spectrophotometric method after addition of enzyme substrate and chromogen. The higher the concentration of free drug in test sera, the less the enzyme-labelled antigen will bind and the less the intensity of colour.

In an indirect competition ELISA, the solid phase is coated with antigen. The test serum, diluted in antibody solution is added and incubated for some time (any specific antibody attaches to the antigen). Washing removes unreacted components as well as any antibody attached to free antigen (from the test sera). A conjugate of enzyme-labelled antiglobulin is then added and incubated. This is followed by the addition of substrate and chromogen as in the direct method described above.

In the ELISA of Eisler et al. (1993) for isometamidium, anti-isometamidium antibody was produced in sheep after immunisation with an isometamidium-bovine serum albumin conjugate. High antibody titres were

obtained using this procedure. The antibody was purified using the ammonium sulphate precipitation method. Thereafter, it was biotinylated. For the assay, isometamidium-ovalbumin conjugate was adsorbed onto the wells of 96-well microtitre plates. The purified, biotinylated, antibody was then added, followed by streptavidin-horseradish peroxidase enzyme conjugate (streptavidin has a high affinity for biotin). After addition of substrate and chromogen, the optical density of the resulting colour change was determined in an ELISA reader. In order to analyse serum samples from treated animals for the drug, the serum was mixed with the biotinylated antibody in a micronic tube before addition to the wells on a microtitre plate. The ELISA is, therefore, an indirect competition ELISA for isometamidium in which isometamidium is detected via the biotin-streptavidin bridge. The method has been used to study the pharmacokinetics and the pharmacodynamics of isometamidium in cattle in the field (Eisler et al., 1994).

Investigations into the circulating concentrations of homidium in serum of treated animals in the field have not been carried out for reasons already mentioned, such as the lack of sensitive analytical techniques. The purpose of this study, therefore, was to develop a sufficiently sensitive enzyme-linked immunosorbent assay that would be used to determine the levels of homidium in serum of treated animals in the field, and to relate these levels to the curative and prophylactic activity of homidium observed in the field.

THE STUDY OBJECTIVES

In view of the lack of a sensitive and specific method for the detection and monitoring of homidium in serum of treated animals, the objectives of this work were to:

1. Develop an enzyme-linked immunosorbent assay that is sufficiently specific and sensitive for the assay of homidium in plasma, urine, tissues and tissue fluid.

2. Validate the method using serum samples from homidium-treated cattle.

3. Use the validated method to:

(a) collect baseline data in laboratory studies on:

(i) serum concentrations in normal cattle treated with homidium at the recommended therapeutic dose of 1 mg kg^{-1}.

(ii) serum homidium concentrations following treatment of cattle infected with *Trypanosoma congolense* populations with varying sensitivities to homidium.

(iii) the relationship between the serum homidium concentrations and the prophylactic period following monthly trypanosome challenge of homidium-treated cattle with *T. congolense* populations of varying sensitivities to the recommended therapeutic dose of homidium.

(b) carry out a pharmacodynamic study of homidium in the field.

(c) compare values obtained for homidium levels in serum of treated animals

with those obtained by use of a radiometric method.

(d) carry out a metabolite study in serum and, if possible, determine the role of metabolites in chemotherapy and prophylaxis of animal trypanosomiasis. This would be possible with the use of a combination of techniques, for example, the radiometric and ELISA techniques.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

Homidium bromide

Homidium bromide was obtained from two different sources (Camco Animal Health, Cambridge, U.K; Laprovet, Toure, France) was used. Details including the batch numbers is provided in the relevant Chapters. A 2.5%w/v solution of the drug was prepared in sterile water freshly and administered at a dose rate of 1 mg kg^{-1} b.w. It was given as a single bolus by either intravenous (i.v.) or deep intramuscular (i.m.) injection into the muscles of the neck using 1½″ x 18 gauge needle.

Animals

Boran cattle

The animals used in the experiments in Kenya were Boran (*Bos indicus*) weaner male castrates aged between six and nine months and weighing between 120 and 180 kg purchased from areas of Kenya free from endemic trypanosomiasis. These animals had already been vaccinated by the farmers against babesiosis and anthrax at the time of purchase.
On arrival at the Kenya Trypanosomiasis Research Institute (KETRI) laboratories, the steers were ear-tagged. They were also sprayed twice at an interval of three days with a solution containing 12.5%w/v amitraz (Triatix®, Coopers (K) Ltd.) against ticks within the first week of their arrival after which they were housed in a fly-proof barn. Blood smears were prepared on clean microscopic slides, stained with Giemsa and examined microscopically for theileriosis, babesiosis and anaplasmosis. Faecal samples were examined for nematode infection. The animals were orally given a drug formulation containing 3.0% oxyclozanide BP and 1.5% levamisole hydrochloride BP (Nilzan® Plus with Cobalt, Cooper Kenya Ltd.) at the dose rate of 0.5ml per kg body weight (b.w.) recommended by the manufacturer against worms. Two weeks later the animals faecal samples were re-examined to ascertain that they were worm-free. The animals were maintained on hay, protein diet supplement

(Ranch cubes, Unga Ltd., Kenya) at 250 g per animal per day plus mineral supplements. During the experiments the animals were weighed once a week.

Friesian cattle

Healthy Friesian cattle aged approximately six months, purchased from two different farms in the UK, were used in the studies in Scotland. On arrival at the Veterinary Hospital, University of Glasgow, the animals were housed in a closed barn, ear-tagged and maintained on hay plus concentrate supplements, and had free access to water. The experiments were initiated after the animals were acclimatised to the laboratory conditions.

Sheep

Scottish Blackface aged six months, obtained from a commercial farm were used in the production of hyperimmune serum. They were fed on hay plus concentrate supplements and had free access to water.

Preparation of antisera

Preparation of homidium-keyhole limpet hemocyanin (homidium-KLH) conjugate

This conjugate was prepared through a diazotisation procedure. To 50 mg homidium bromide dissolved in 1 ml 0.5M hydrochloric acid on ice, was added 1 ml of ice-cold 0.127M sodium nitrite. The mixture was stirred on ice for 10 minutes in the absence of light. Four hundred microlitres of this mixture equivalent to 10 mg homidium was added to 96 mg KLH dissolved in 2 ml 0.1M sodium bicarbonate, pH 8.3. The mixture was stored at +4°C until required.

Immunisation of sheep and collection of hyperimmune sera

Three sheep were immunised with homidium-KLH conjugate to raise antibodies against homidium using the method described by Whitelaw et al. (1991). Three dilutions of the conjugate containing 2 mg, 1 mg and 500 μg KLH per ml were prepared. Four doses were given to each sheep by subcutaneous injection.

The primary immunisation consisted of 2 mg KLH in 3 ml of water-in-oil emulsion containing conjugate and Freund's Complete Adjuvant (FCA) mixed in the ratio 1:2 v:v conjugate:FCA. Each sheep received 1.5 ml of this emulsion in each hind leg. Four weeks later each sheep received a booster immunisation containing 1 mg KLH also in 3 ml of an emulsion containing conjugate plus Incomplete Freund's Adjuvant (IFA) mixed in the ratio 1:2 conjugate:IFA. Ten ml blood was collected 10 days after this second booster immunisation, serum separated and the protein concentration determined by measuring the OD at 280 nm in a UV/Visible spectrophotometer (Pye Unicam, SP 800; OD of 1.0 ≡ 0.75 mg protein per ml).

Second and third booster immunisations given also at 4 weekly intervals consisted of 500μg KLH in 3 ml of an emulsifiable suspension prepared as described above in Incomplete Freund's Adjuvant. Similarly, ten ml samples of blood were collected after each of these booster immunisations, serum separated and protein concentration determined. The sheep were bled out under terminal anaesthesia ten days after the third booster immunisation. Serum was prepared, aliquoted and stored at -20°C.

Purification of anti-homidium IgG

In a 250 ml glass beaker, one volume anti-serum was added to two volumes of 0.06M acetate buffer, pH 4.0, to give a serum-buffer solution of pH 4.8. On a high speed magnetic stirrer, 3.20 ml caprylic acid was added dropwise for every 100 ml serum to the acetate-serum mixture. Serum albumin

was precipitated leaving the globulins in solution. The mixture was stirred for 30 minutes after which it was then aliquoted into 25 ml universal bottles and centrifuged at 3000 rpm for 10 minutes. The supernatant was separated and the pH adjusted to 5.7 with 1M sodium hydroxide solution. The salt content of the supernatant was lowered by a 24 hour dialysis against 0.015M acetate buffer, pH 5.7.

After this overnight dialysis, the antibody containing solution was centrifuged since it appeared cloudy after the dialysis. The optical density was measured at 280 nm and the protein concentration calculated (OD of 1.0 ≡ 0.75 mg ml^{-1} protein solution at 280 nm measured in 1 cm cuvette). The remaining contaminants, if any, were removed by batch adsorption of the globulin-containing solution using the Whatman DE-52 medium. The DE 52 medium (50 g for every 2 ml supernatant) was equilibrated by stirring in 0.1M acetate buffer, pH 5.7.

The particles were allowed to settle (approximately 10 minutes) and the buffer decanted. This was repeated three times but using 0.015M acetate buffer instead of the 0.1M. This medium was left overnight in 500 ml of the 0.015M buffer at +4°C. After this equilibration, two different protocols were followed in further purification of the antibody.

(a) One fraction was loaded onto a column packed with pre-equilibrated DE 52. The globulins were desorbed using a gradient elution with different molar concentrations of sodium chloride starting at 0.1M and ending with 2M solutions. During the gradient elution, five millilitre fractions of the column effluent were collected during the elution and their optical densities (ODs) determined. Fractions representing each of the various peaks were pooled separately. The various pooled solutions were concentrated using an immersible-CX low binding filter unit, 10 000 (Millipore (U.K.) Ltd., Edinburgh, Scotland). The protein concentration was determined as above

from the ODs. The volume of the solutions was adjusted such that each solution contained 2 mg protein /ml. The solutions were stored at -20°C.

Finally, the antibody activity of the two peaks collected separately in the effluent of the DE-52 column were titrated by ELISA using doubling dilutions (See section on assay optimisation).

(b) In the second DE-52 protocol the DE 52 medium in 0.015M acetate buffer, pH 5.7 was filtered through an All Glass Filter Holder, 47 mm (Millipore (U.K) Ltd., Edinburgh, Scotland) with a funnel capacity of 300 ml. This was done under gentle suction taking care not to let the gel compress. The suction was removed as soon as the buffer was at the level of the gel. To the wet DE 52 in a glass beaker was added the caprylic acid purified antibody (2g DE 52 per 1 ml of antibody solution). This was stirred with a glass rod and left on the bench for 2 hours (with occasional stirring). The slurry was aliquoted, in 25 ml universal bottles and centrifuged at 3000 rpm for 40 minutes. The supernatant was separated, the OD measured and the protein concentration calculated. The solution was then dialysed against PBS overnight, the OD determined and the protein calculated. After the overnight dialysis, the solution was concentrated using the immersible-CX low binding filter unit, 10,000 (Millipore (U.K.) Ltd) and the protein adjusted to 2 mg ml^{-1} and tested for antibody activity using titrations. This purified antibody solution was, thereafter, biotinylated. The determination of protein after every step in the purification was necessary so that the recovery could be monitored.

Sample collection

Pre-treatment sera

Fifty millilitres of blood were collected aseptically from each animal by jugular venipuncture before the homidium treatment. The time at which the pre-treatment sera was collected and the number of animals used is described in the appropriate Chapters. The blood samples were incubated for 4 hours at

37°C, overnight at +4°C and centrifuged at 1500 g for 20 minutes and the sera separated. These pre-treatment samples served as negative control sera from which a pool was prepared, aliquoted and stored at -20°C until required.

Blood samples of cattle for parasitological examination

Ear-vein blood samples were collected in heparinised capillary tubes daily during the first four weeks following infection and thereafter three times a week to the end of the observation period specified under the various Chapters.

Blood samples for biochemical and drug analysis

Ten millilitre blood samples were collected from each animal as described above immediately before treatment and at pre-determined intervals following homidium treatment. The interval at which samples were collected is described in the appropriate Chapters. These samples were used to determine total serum protein, serum albumin levels and serum homidium concentrations.

Enzyme-linked immunosorbent assay (ELISA) reagents

Purification of homidium-horseradish peroxidase (homidium-HRP) conjugate

A 30 cm dialysis tubing (size 2, inflated diameter 14.3 mm, BDH, Poole, England) was placed in a beaker of water and boiled for 15 minutes. One end of the tube was tightly clipped and using a glass dropper, the conjugate solution was added to the tubing. After removal of air from the tubing, another clip was used to close the other end. The tubing plus contents were then placed in a beaker containing 2 litres of the sodium chloride solution and dialysed for 5 hours with constant stirring. The dialysis solution was replaced with that which had been freshly prepared and the dialysis

continued for a further 16 hours. After the dialysis, one end of the tubing was cut, purified conjugate removed and placed in a glass bijou. A volume of 500µl of the double strength charcoal was added and vortex mixed. The contents were further mixed on an end-over-end mixer for 15 minutes after which they were transferred to mini-centrifugation tubes (Eppendorf) and centrifuged at 12,000 g for 10 minutes. The supernatant was removed, aliquoted into 500µl fractions, mixed with an equal volume of glycerol and stored at -20°C.

ELISA substrate and chromogen solution

A 42 mM stock solution of 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma Chemical Ltd., Poole, England) was prepared by dissolving 100 mg of TMB in 10 ml of dimethyl sulphoxide (DMSO). This solution was stored at +4°C until required. A 3%v/v solution of hydrogen peroxide was also prepared in water. One ml of the TMB in DMSO solution was slowly added to 100 ml 0.1M sodium acetate/citrate buffer pH 6.0 followed by 145.8 µl of the diluted hydrogen peroxide to give a final concentration of 1.3mM per litre. This TMB/H_2O_2 solution was prepared just prior to use. It was also allowed to warm up to 37°C just before addition to the plate.

Buffers

Unless where otherwise specified, all chemicals used in reagent preparation were supplied by Sigma Chemical Co. Ltd., Poole. England. Water was de-ionised and distilled.

Acetic acid, 5% (v/v)

This solution was prepared by adding 50 ml glacial acetic acid (BDH, Poole, England) to water and adjusting the volume to 1 litre.

Acetate buffer, 0.015M, pH 5.7

Solution A was made by dissolving 4.88g of anhydrous sodium acetate in 4 litres of distilled water and solution B by adding 0.85 ml of glacial acetic acid to water and adjusting the volume to 1 litre. A volume of 380 ml of solution B was added to solution A and the pH adjusted with acetic acid.

Acetate buffer, 0.06M, pH 4.0

Solution A was made by dissolving 2.46g sodium acetate in water and adjusting the volume to 500 ml. Solution B was made by adding 1.72 ml glacial acetic acid to water in a 500 ml volumetric flask and adjusting to volume. Solution A was mixed with solution B and the pH adjusted accordingly using acetic acid.

Acetate buffer, 0.1M, pH 5.7

Solution A was made by dissolving 8.203g of sodium acetate in water and adjusting the volume to 1 litre. Solution B was prepared by adding 5.73 ml glacial acetic acid to water in a 1 litre volumetric flask and making up to volume. A volume of 95 ml of solution B was mixed with solution A and the pH adjusted accordingly using acetic acid.

Acetate/citrate buffer, 0.1M, pH 6.0

A solution of 0.1M sodium acetate was prepared by dissolving 8.203g in water and adjusting the volume to 1 litre. The pH of this solution was adjusted to 6.0 by the addition of small amounts of 0.1M citric acid.

Hydrochloric acid, 0.5M

This solution was prepared by adding 15.5 ml of concentrated hydrochloric acid (BDH, Poole, England) to water and adjusting the volume to 1 litre.

Sodium nitrite, 0.1M

This solution was prepared by dissolving 6.95 g sodium nitrite in water and the volume adjusted to 1 litre.

Sodium bicarbonate buffer, 0.1M, pH 8.5

This buffer was prepared by dissolving 8.4g sodium bicarbonate in 1 litre of water.

Sodium carbonate/bicarbonate buffer, 0.1M, pH 9.2

Solution A was made by dissolving 8.4g sodium bicarbonate in water and making to 1 litre. Solution B was prepared by dissolving 2.65g sodium carbonate in water and adjusting the volume to 250 ml. The pH of 900 ml of A was adjusted using B. The volume was adjusted to 1 litre with water.

Phosphate buffer, 0.1M, pH 7.4

Solution A was made by dissolving 57.52g di-sodium hydrogen phosphate in 2025 ml of water. Solution B was made by dissolving 31.21g sodium dihydrogen phosphate in 1 litre of water. A volume of 420 ml of solution B was added to solution A and the pH adjusted by adding small quantities of B. The final volume was made up to 5 litres.

Phosphate buffered saline (PBS)

Phosphate buffered saline was prepared by dissolving 16g sodium chloride, 2.96g di-sodium hydrogen phosphate and 0.86g potassium di-hydrogen phosphate in water and adjusting the volume to 1 litre.

EDTA saline glucose (ESG; pH 8.0)

A solution of ESG containing 8mM EDTA, 2.7mM potassium di-hydrogen phosphate, 0.14M sodium chloride and 11mM glucose was prepared in water and the pH adjusted to 8.0.

Phosphate buffered saline plus glucose, pH 7.4 (PSG)

A solution of PSG was prepared by adding 1.5 g glucose to every 100 ml PBS.

Phosphate buffered saline plus Tween 20 (PBST)

Phosphate buffered saline plus Tween 20 was made by adding 500µl of Tween 20 (BDH, Poole, England) to every litre of the buffer solution.

Washing buffer

A 5-fold dilution was made from PBS after which 1 ml Tween 20 was added for every 2 litres of solution.

Activated charcoal

This reagent was prepared by weighing out 2.5g activated charcoal (BDH, Poole, England) and 0.25g dextran adding 1 litre of distilled water , mixing and storing at +4°C until required.

Trypanosomes

Two populations of *Trypanosoma congolense* with differing sensitivity to homidium were used, one sensitive and the other resistant to the recommended therapeutic dose of homidium of 1 mg kg^{-1} b.w. Stabilates of these trypanosome populations had been maintained in microcapillary tubes in liquid nitrogen in the Kenya Trypanosomiasis Research Institute (KETRI) trypanosome bank. One population was *T. congolense* ILRAD 1180 first

described by Nantulya et al. (1984) and reported to be sensitive to diminazene and isometamidium at CD_{50} values of 2.3 mg kg^{-1} b.w., and 0.018 mg kg^{-1} b.w respectively in mice (Peregrine et al., 1991). The other population was *T. congolense* IL 3330 which was isolated from the Ghibe area of Ethiopia (Codjia et al., 1993) and has been reported to be multi-resistant to therapeutic doses of the three most commonly prescribed trypanocides. Gray et al., 1993 found the population to be resistant to diminazene (>30 mg kg^{-1} b.w.), homidium (>20 mg kg^{-1} b.w) and isometamidium (>20 mg kg^{-1} b.w) *in vitro*. The procedures followed in the preparation of the inoculum and the size of inoculum given are described in detail in the relevant chapters.

To prepare the inoculum for infection of cattle, trypanosome stabilates were inoculated intraperitoneally into irradiated mice. At the first peak of parasitaemia, the mice were bled out by cardiac puncture under chloroform anaesthesia, using EDTA as an anticoagulant. The trypanosome density in the pooled blood from the infected mice was determined with a haemocytometer and subsequently the blood was diluted in ESG to give a density of $1x10^6$ trypanosomes per millilitre. The stabilates were passaged twice in mice before inoculation into cattle.

Detection of anti-trypanosome antibodies

The indirect fluorescent antibody test (IFAT) of Katende et al. (1987) was used for the detection of anti-trypanosome antibodies in serum of treated and untreated cattle. A group of 10 male Swiss white mice were infected intraperitoneally with *T. congolense* IL 1180. The inoculum given from the stabilate was 1 x 10^6 trypanosomes per mouse. The mice were bled out into EDTA-containing tubes at the first peak parasitaemia and the blood centrifuged at 12000g for 15 minutes at +4°C. The trypanosome-rich buffy coat was then re-suspended in phosphate-buffered saline plus glucose (PSG), pH 8.0, separated on DE 52 and washed three times with cold PSG.

Thereafter, the trypanosomes were re-suspended in cold physiological saline to give a concentration of 1 x 10^6 trypanosomes per ml, then fixed in a mixture of 80% acetone and 0.25% saline in the ratio 1:2 trypanosome suspension:fixative. After overnight fixation at -20°C, the trypanosomes were centrifuged at 450g for 15 minutes at +4°C, washed 3 times with physiological saline, then re-suspended in PBS containing 0.2% bovine serum albumin with no azide to yield a final concentration of 1 x 10^5 trypanosomes per millilitre. The fixed trypanosomes were stored at -20°C in aliquots of 0.5 ml.

Elimination of non-specific fluorescence commonly associated with this assay was carried out by prior absorption of the test sera with normal bovine lymphocyte lysate. This lysate was prepared according to the method described by Goddeeris et al. (1982). In brief, a splenectomised, four-month old calf was maintained under tick and tsetse-free conditions. Blood smears were examined for parasites (after Giemsa staining) weekly for a period of four weeks inorder to exclude any possibility of latent infections. All accessible lymph nodes were removed at the time of slaughter and sliced in cold PBS, pH 7.2 in the presence of anticoagulant (EDTA).

Cells were separated from tissue debris by passing through a sieve. The cells were then washed three times with PBS-EDTA by centrifugation at 200 g for 20 minutes at +4°C. The washed cells were re-suspended in PBS without EDTA to give a final concentration of 5 x 10^7 cells per ml. The solution containing the cells was sonicated in 100 ml aliquots on ice at one minute intervals using the 3/8 probe (Virsonic 16 850, Virtis Company, Gardiner, NY). The sonicated material was centrifuged at 400 g for 30 minutes at +4°C and the supernatant containing 5 mg protein per ml stored at -70°C in four ml aliquots.

Sheep anti-bovine immunoglobulins were prepared following the procedure described by McGuire (1979) cited by Katende et al., 1987). In brief, ten ml sheep anti-bovine IgG containing 10 mg ml^{-1} total protein were

dialysed against 100 ml of 0.05M carbonate/bicarbonate buffer, pH 9.5, containing 1 mg fluorescein isothiocyanate (FITC) and 0.025% sodium azide while continuously stirring, at 4°C for 24 hours. Un-conjugated FITC was removed by gel filtration through sephadex G-50 (Pharmacia Fine Chemicals, Sweden) while the optimally conjugated protein was separated from the over-conjugated and under-conjugated protein on a diethylaminoethyl (DEAE) cellulose 52 column.

The fixed trypanosomes were thawed out at room temperature and the antigens were distributed onto the wells of the Teflon-coated multitest slides (Cooper Wellcome, Erembodegem, Belgium) using a 200 µl Eppendorf pipette. The antigens were dispensed and the trypanosome suspension immediately sucked leaving a thin layer of trypanosomes on each well. The slides were dried at 37°C for 10 minutes. A 1/20 dilution of test sera was made in the lymphocyte lysate (10 µl of test sera plus 190 µl lysate) and incubated at room temperature for 30 minutes. Twenty-five µl of the diluted test sera was then added onto the slides bearing the antigens. On each slide negative and positive controls were included diluted in a similar manner to the test sera.

After the addition of the test sera to the antigen, the slides were incubated at room temperature in a moist chamber for 30 minutes. The slides were washed twice with PBS for 30 minutes at 15 minute intervals.

Ten microlitres of sheep anti-bovine immunoglobulin conjugate, in which was incorporated Evan's blue as a counter stain, were applied onto each well. The slides were incubated for 30 minutes at room temperature in a moist chamber, washed with PBS as before and mounted in 50% glycerol in PBS, pH 8.0. The slides were thereafter viewed under a Leitz Ortholux 11 microscope equipped with epi-ploem illumination (100W mercury lamp), UV filter block, 6.3 x eyepieces and a Phaco FL 40/1.3 oil objective.

Bright yellowish green flourescence from the fixed the trypanosome antigens indicated the presence of anti-trypanosome antibodies in the test sera

Blood parameters

Parasitological examination

After the determination of the PCV, the buffy coat was examined for trypanosomes. Trypanosomes were detected by the darkground/buffy coat method of Murray et al. (1977). The intensities of parasitaemia were graded using the scoring system shown below (Table 3, Magnification = x 400).

Table 2.1: Parasitaemia estimation

No. of trypanosomes per field	Estimated parasitaemia tryps/ml
1 - 3 per film	10^2-10^3
4 - 10 per film	10^3-10^4
1 per field	5 x 10^3-5x10^4
2-10 per field	5 x 10^4-5x10^5
> 10 per field	>5 x 10^5
> 100 per field	>6 x 10^6

Haematological examination

Packed cell volume (PCV)

The collection of blood samples was as described in Section 2.3.1.2. The blood filled-capillary tubes were centrifuged in a haematocrit centrifuge at 12,000 g for 10 minutes after which they were read with a microhaematocrit reader (Hawksley micro-haematocrit reader, Hawksley, England).

Blood biochemistry

Total serum protein

Total serum protein was determined using the Coomassie® Plus Protein Assay Reagent (Pierce, Rockford, IL U.S.A.). The Micro Assay Procedure was followed for determining protein concentrations in the range of 1-25 $\mu g\ ml^{-1}$. This assay is based on the absorbance shift from 465 to 595 nm which occurs when Coomassie® Blue G-250 (Pierce, Rockford, IL U.S.A.) binds to proteins in an acidic medium, with the resulting colour change from reddish brown to blue. The blue-coloured complex formed is stable up to 90 minutes.

A protein concentration series of 1, 5, 10, 15 and 25 $\mu g\ ml^{-1}$ was prepared by diluting a stock of bovine serum albumin (BSA) (Sigma Chemical Co. Ltd., Poole, England) in normal saline (0.9% w/v sodium chloride). To 1 ml of each of the diluted standard solutions in a test tube was added 1 ml of the protein assay reagent, mixed and the absorbance determined against water at 595 nm. A blank was prepared by adding one ml of the protein reagent to 1 ml of the normal saline in a test tube. The absorbances of all samples were measured against this reagent blank. All the determinations were carried out in duplicate. A standard curve was plotted from the mean absorbances of the above standards. Test serum samples were treated in exactly the same way as the standards. Using the standard curve, the protein concentration of each of the unknown protein samples was then determined. The dilutions made for each of the test serum samples are described in the relevant chapters.

Total serum albumin

For the determination of total serum albumin, a quantitative, colorimetric method of determination (Procedure No. 631, Sigma Diagnostics) was used. In this method, albumin binds to bromocresol green to produce a green-coloured complex with an absorbance maximum at 628 nm; the

magnitude of which was directly proportional to the albumin concentration in the sample.

A series of test tubes for reagent blank, standard and test samples were prepared. Into each of these test tubes was placed 1.0 ml Albumin Reagent (bromocresol green). At timed intervals, 10 μl of deionised water was added to the reagent blank test tube, 10 μl of the standard to the standard test tube and 10 μl of the test serum sample to the test sample test tube respectively. The absorbances of the above solutions were read at 628 nm exactly 30 seconds after the addition of bromocresol green. Since the coloured complex was extremely unstable, the absorbances were therefore read within 30 seconds after either the standards or the test samples were added. In order to ensure reproducibility and consistency in the readings all absorbances of the blanks, standards and test samples were determined at exactly 30 seconds after the addition of the albumin reagent.

Calculation of the albumin concentrations was as follows:

Sample albumin concentration (g/100 ml) =

$$\frac{\text{absorbance of the test sample}}{\text{absorbance of the albumin standard}} \times \text{concentration of albumin standard}$$

Serum globulin levels

The total serum globulin levels were determined as the difference between the total serum protein and the total serum albumin levels.

Statistical evaluation

The serum homidium concentrations, total serum protein levels, serum albumin levels, total globulin levels, PCV and body weights are presented as means ±standard deviation (±sd). The PCV, the live weight, total serum proteins, serum albumin and globulin data was analysed on the basis of change

from their respective pre-infection values. Comparison of pharmacokinetic parameters between assays and between various treatment groups were subjected to the student's t test to test for significance at both 95 and 99% confidence limits.

Handling of drug ELISA data

An automated data handling system was used for the enzyme-linked immunosorbent assays (ELISA) in which the microtitre plate reader was interfaced with a computer, such that there was direct input of data from the reader to the computer. Using the appropriate software (Eiaquik, Lotus 123 Release 4 for windows) similar to that used by Eisler et al.(1993) the standard deviation and coefficient of variation of sample replicates, and the intra- and inter-assay, and the intra- and inter-sample, coefficients of variation (CVs) were calculated.

Typical immunoassay data usually gives a linear dose-response curve. However, as reagents age, the assay tends to lose sensitivity. To normalise this effect, B/B_0 as a response parameter was plotted against dose. Because the curve still remains curve linear, an accurate fit was obtained by plotting B/B_0 against logarithm of the dose transforming the curve into an S shape. Further transformation of the curve to a linear shape was obtained by plotting the logit of the response versus the logarithim of the dose (Chan and Perlstein, 1987; Eisler et al., 1993).

$$\text{logit} = \log \frac{B/B_0}{1 - B/B_0}$$

All graphs were fitted using the SlideWrite Plus computer package. The lines which appear in the drug profiles following i.v. treatment are predicted concentrations obtained using the compartmental modelling using

PCNONLIN. Those which appear in the drug profiles following i.m. treatment are predicted concentrations obtained using log curve-fit.

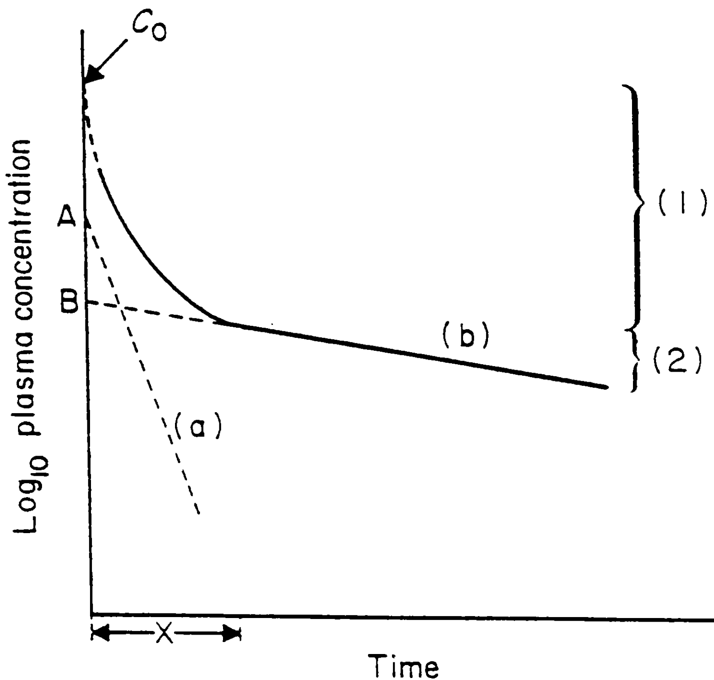
Pharmacokinetic analysis

Pharmacokinetic evaluation of the serum concentration versus time profiles was carried out using PCNONLIN computer software (Statistical Consultants, I.nc., 1992 SCI Software, Carl M Metzler and Daniel L. Weiner). Both compartmental and non-compartmental data analyses were carried out on the intravenous data. Non-compartmental model was selected arbitrarily for the intramuscular data. For the compartmental analyses, the number of exponents best describing each data set was confirmed using Akaike's Information Criteria (Yamaoka et al. 1978). Non-linear regression was used to fit the model to the experimental data. The formulae shown below for the various pharmacokinetic parameters used in the data analysis were as described by Baggot (1977), Gibaldi and Perrier (1982):

- The elimination half-life, t½, in hours = 0.693/β where β is the elimination rate constant
- The plasma concentration at time 0, Cp0, in ng ml^{-1} = sum of 0 time intercepts of the coefficients A and B (Fig. 2.1).
- The volume of the central compartment, Vc, in l kg^{-1} = Dose/Cp0
- The area under the curve, AUC, in ng.h ml^{-1} and the area under the moments curve, AUMC, in $ng.h^2$ ml^{-1} were estimated by the trapezoidal rule from the serum concentration versus time plots (Figs. 2.2 and 2.3).
- The apparent volume of distribution, Vd_{area}, in l kg^{-1}=Dose/AUC.β
- Body clearance, Clb, in ml h^{-1} kg^{-1} = Dose/AUC
- The elimination rate constant, kel, in hours = Clb/Vc
- The Mean Residence Time, MRT, in hours = AUMC/AUC

The rate constants k_1, k_{21}, k_{13} and k_{31} were determined using tri-exponential equations as described by Gibaldi and Perrier (1982).

Fig. 2.1



Shows how the intercepts A and B are derived from the plasma/serum drug concentration versus time plot (After Clark and Smith, 1986). The plasma/serum decline can be divided into two phases, 1 and 2. The first phase (1) includes the distribution of the drug from the central compartment (plasma/serum) and rapidly distributed tissues into a second compartment. After a certain time period expressed here as X, equilibrium will be attained between the two compartments when they behave essentially as one: the graph then moves into log/linear phase (2) (line (b)). This log/linear phase represents elimination from the central compartment in equilibrium now with the second compartment. The slope of line (b) is used to determine a rate constant (β). In the figure shown $C_0 = Cp0 = A + B$

Fig. 2.2

The area (A) is described as the area under the curve or AUC

$$AUC_{0-\infty} = \int_0^\infty C_P \, dt.$$

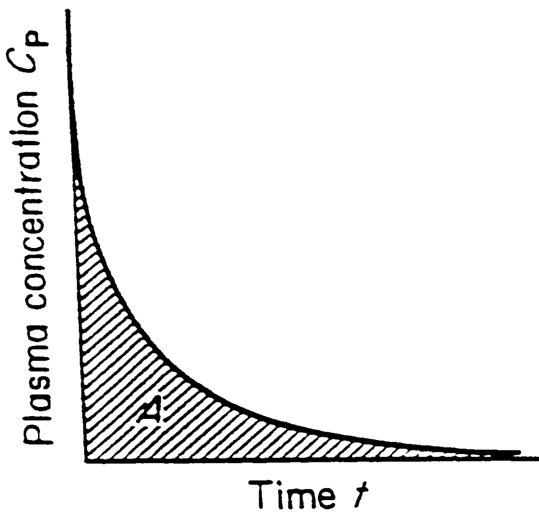
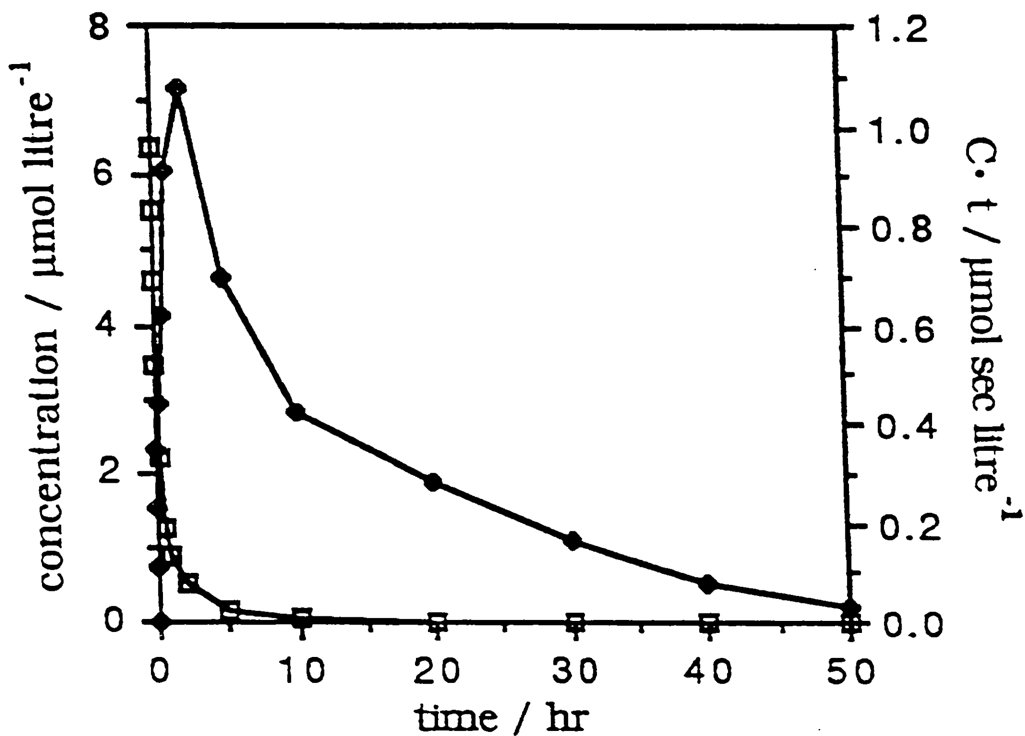


Fig. 2.3

A comparison of plots of the concentration versus time (hollow squares) used in the calculation of AUC and of the product concentration x time versus time (filled diamonds) used in the calculation of AUMC (after Hladky, 1990)



CHAPTER THREE

DEVELOPMENT AND VALIDATION OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR HOMIDIUM

INTRODUCTION

This chapter describes the development of two enzyme-linked immunosorbent assays for the determination of homidium in the serum of treated cattle. One is an indirect and the other a direct competition assay.

Homidium, a phenanthradine compound has been used in the treatment of animal trypanosomiasis since its introduction in 1952 (Watkins and Woolfe, 1952). Although widely used, many aspects of its efficacy in the field have not been possible due to lack of sensitive and specific methods for detecting and monitoring the serum drug levels. Goodwin and Tierney (1977) used a biological assay to determine the trypanocidal activity of body fluids from rabbits treated with homidium bromide; very high doses were administered so that activity could be detected in the body fluids for as long as was practically possible. Serum dilutions of $1{:}1.5\times10^4$ and $1{:}5.0\times10^5$ were able to kill *Typanosoma brucei in vitro* when collected 24 and 48 hours, respectively, after treatment. Gilbert and Newton (1982) studying the pharmacokinetics of homidium bromide in *T. congolense*-infected and non-infected rabbits, and non- infected cattle using ^{14}C labelled homidium showed that the drug was eliminated rapidly following i.m. injection. Although sensitive, these radiometric and biological procedures (Goodwin and Tierney (1977; Gilbert and Newton, 1982) lacked specificity since all the radioactive and biologically active components in solution were measured. Finally, a high performance liquid chromatographic (HPLC) procedure for quantification of the drug was described by Perschke and Vollner (1985) which used a solid-phase extraction methodology and a C18-reversed-phase analytical column with UV detection at 292 nm. This procedure, using spiked serum, had recoveries of over 99% and detection limits of 50 ng ml^{-1} when 1 ml was processed, and 5 ng ml^{-1} when 10 ml serum was processed. The high volumes of plasma or serum required to achieve the lower detection limit were impractical for use in field

samples. Furthermore, the procedure was not validated with serum samples from treated animals.

In the treatment of infected animals, low doses of homidium were recommended by the manufacturers (i.e. 1 mg kg^{-1})due to the fact that these doses were found to be effective against trypanosome infections and that high doses of phenathridinium compounds were shown to be toxic to cattle (Unsworth, 1954 a, b). Therefore, due to the low doses administered, the circulating levels in blood would be expected to be equally low. Determination of these low levels by chemical means is laborious and often with low sensitivity due to the extensive extraction and purification steps that are required.

For reasons discussed in Chapter 1 (Section Three), the development of an ELISA for the detection of homidium was judged to be a realistic and desirable objective.

The aim of the present study was to develop an ELISA for homidium which can be used on field samples to detect and monitor homidium concentrations in serum from treated cattle. Such a method has been lacking since the present methods, other than HPLC, measure activity rather than the drug of interest. Ideally, an assay should have a level of sensitivity that will allow it to detect and monitor homidium levels for the duration of the prophylactic period observed in the field (Dolan et al., 1990; 1992).

In this Chapter the development and validation of two ELISA methods for homidium detection are reported, one utilising homidium-ovalbumin conjugate (Assay 1) in which homidium was detected via a biotin-streptavidin-horseradish peroxidase system similar, in principle, to that described by Eisler et al. (1993) and the other utilising homidium-horseradish peroxidase conjugate (Assay 2).

STUDY OBJECTIVES

(a) To develop a sensitive ELISA method for the determination of homidium using spiked serum homidium standard solutions.

(b) Validate the method by determination of homidium concentrations in serum obtained from Friesian cattle following intramuscular (i.m.) treatment with homidium bromide at a dose rate of 1 mg kg^{-1} body weight (b.w.).

MATERIALS AND METHODS

Friesian cattle

Five healthy male Friesian cattle aged approximately six months, and weighing between 150 and 165 kg purchased from a commercial farm were used in the studies in Scotland. Their management before and during the experiments was as described in Chapter 2. The experiments were initiated after the animals were acclimatised to the laboratory conditions.
Two weeks after being housed, the cattle were treated with homidium.

Sheep

Scottish Blackface aged six months, obtained from a commercial farm were used in the production of hyperimmune serum. They were fed on hay plus concentrate supplements and had free access to water.

Drug treatment

Homidium bromide (Ethidium®, Camco, U.K; Lot B4B3) was used at a dose rate of 1 mg kg^{-1}. A 2.5%w/v solution was prepared in sterile water immediately prior to treatment and given by deep intramuscular (i.m) injection into the neck muscles.

Sample collection

Pre-treatment sera

One week prior to treatment, 50 ml blood samples were collected by veni-puncture into plain vacutainers from the jugular of each of the five Friesian cattle and sera prepared as previously described (Chapter 2). A pool was made out of the individual serum samples, aliquoted also as previously described (Chapter 2) and stored at -20°C until required.

Serum samples for drug analysis

Ten ml blood samples were collected from the cattle immediately prior to treatment, and at the following intervals after treatment: one, eight hours, twice daily during week 1, once a day during week 2, thrice a week during week 3, twice a week during week 4 and once a week from week 5 to the end of the observation period of 90 days. Serum prepared and stored as described in Chapter 2 until required.

Normal bovine serum

Normal bovine sera were obtained from 20 Friesian cattle in Glasgow with no history of exposure to trypanosomiasis or trypanocidal drugs.

Assay 1: utilising homidium-ovalbumin conjugate

Preparation of homidium-ovalbumin conjugate

This conjugate was prepared using a diazotisation process. One ml of a 1 in 4 dilution of concentrated hydrochloric acid in water and 1 ml of a 0.1M solution of sodium nitrite were added to 50 mg homidium bromide in a glass bijou and stirred on ice for 10 min. Ten microliters of the resulting mixture was added to 5 ml of a solution containing 4 mg chicken egg albumin (CEA) per ml 0.1M phosphate buffer, pH 7.4, (ratio 1:80 w/w homidium:CEA).

The homidium-CEA mixture was mixed on an end-over-end mixer for 2 hours in the dark at +4°C followed by an overnight dialysis against 5% acetic acid also in the dark. After the overnight dialysis, the dialysis solution was changed twice more at intervals of one hour. The dialysed homidium-ovalbumin conjugate was aliquoted in 500µl fractions, mixed with an equal volume of glycerol and stored at -20°C.

Biotinylation of anti-homidium IgG

The procedure followed for biotinylation of the purified anti-homidium IgG was as described by Eisler et al. (1993). In brief, the antiserum was dialysed against 0.1M bicarbonate buffer, pH 8.2-8.6 for 16 hours. The protein content was adjusted to 1 mg ml^{-1} with the bicarbonate buffer. The solution was aliquoted in 1 ml portions into glass bijou. A solution of N-hydroxysuccinamide ester (NHS-Biotin) was prepared in dimethyl sulphoxide (DMSO) to give a concentration of 1 mg ml^{-1}. A volume of 120 µl of NHS-Biotin in DMSO was immediately added to every 1 ml of the 1 mg ml^{-1} protein solution and left to stand at room temperature for 4 hours.

The biotinylated IgG was dialysed against PBS, pH 7.0-7.4 for 16 hours at +4°C. The buffer solution was replaced by that which had been freshly prepared and the dialysis continued for a further 15 minutes. The resulting purified and biotinylated antibody in a universal bottle was concentrated to the original volume using the immersible-CX low-binding filter unit, 10 000 (Millipore (U.K.) Ltd., Edinburgh, Scotland), with constant stirring. The protein concentration was determined and the volume of the solution adjusted with PBS to 2 mg ml^{-1}, aliquoted into 500µl fractions and stored at -20°C.

The assay

All the optimal dilutions used were obtained by chequerboard titration of the conjugate and the biotinylated anti-homidium IgG (See Section below on optimisation of assay reagents). Homidium-ovalbumin was used to coat the 96 well microtitre plates (Immulon 4, Dynatech, U.K.) by adding 100μl per well at the optimal dilution of 1/3200 in carbonate/bicarbonate buffer, pH 9.2 followed by an overnight incubation at +4°C. The plates were washed five times using washing buffer (prepared as described in Chapter 2). This was followed by addition of 100μl per well of a dilution of 1/7000 biotinylated anti-homidium IgG in PBST.

Test serum samples from treated animals were diluted ten-fold in 1/7000 biotinylated anti-homidium IgG in PBST before addition to wells of the plate (100μl per plate). This was carried out by adding 25μl of the test serum to 225μl of a dilution of 1/7000 biotinylated anti-homidium IgG in PBST in micronic tubes (Micronic Systems, Muratech Scientific, Aylesbury), mixing, and adding on to the plate. The volume of 250μl was adequate for duplicate wells.

Test serum samples containing high homidium concentrations were pre-diluted in pooled negative control sera followed by the ten-fold dilution in 1/7000 biotinylated anti-homidium IgG in PBST. Following addition of the antibody, the plates were incubated for one hour at 37°C in a shaker incubator (Verishaker, Dynatech, U.K.). The plates were then washed as before and 100 μl of 1/3000 streptavidin-peroxidase added, incubated for 45 minutes and washed five times as described above. This was followed by 100μl of the TMB/H_2O_2 (chromogen/substrate solution prepared as described in Chapter 2) and incubated for 10 minutes with shaking at 37°C. A redox reaction in which hydrogen peroxide was reduced to oxygen and water, TMB was oxidised to produce a blue coloured complex. The reaction was stopped with 100μl 2M sulphuric acid after the 10 minutes of incubation, producing a

yellow coloured complex. Optical densities of the yellow coloured complex were determined at 450 nm using an ELISA plate reader (Multiskan Plus Mk II, Labsystems Oy, Helsinki, Finland).

In every assay, duplicate determinations with coefficients of variation (CVs) above 12% were rejected and the samples re-tested. Homidium-spiked standards for assay calibration were included in duplicate in every assay on routine basis. The quality control standards were included twice in different locations on the plate to monitor both intra- and inter-assay variations. A diagrammatic representation of Assay 1 is shown in Fig 3.1.

Optimisation of assay reagents

After the preparation of the conjugate and antibody solutions, the optimal reagent dilutions were determined by chequerboard titration using doubling dilution of the antibody and conjugate reagents. End-points of the titrations were considered to be those dilutions which resulted in ODs for negative samples of around 1.0, and in 50% displacement resulting from the smallest concentration of homidium.

Whole plate CVs

Whole plate coefficient of variation values (CVs) were determined following the establishment of the optimal reagent dilutions. This was carried out by preparing a ten-fold dilution of the pooled negative control sera in a dilution of 1/7000 biotinylated IgG in PBST and adding 100μl per well of the 96-well microtitre plate. This was carried out as part of assay optimisation to assess the possible variations due to the solid phase.

Spiked homidium standard solutions

Commercial normal bovine serum (NBS) and pooled negative control sera prepared from pre-treatment sera as described in Chapter 2 was spiked

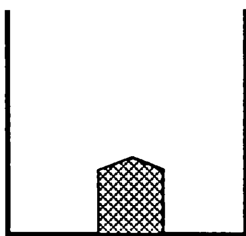
Fig. 3.1

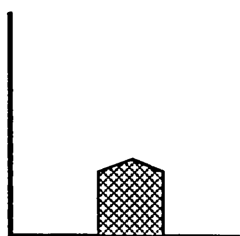
A diagrammatic representation of Assay 1

3.1 Assay 1: Indirect competition ELISA for homidium

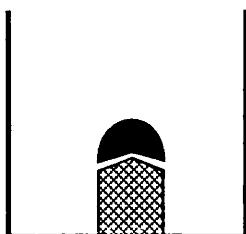
Plate coated with homidium-ovalbumin conjugate

Incubated overnight at +4°C



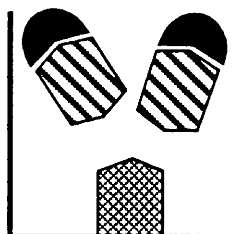


Washed 5x



Serum sample possibly containing homidium mixed together with biotinylated sheep anti-homidium IgG

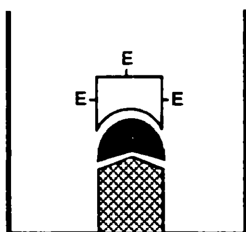
Incubated at 37°C for 1 hour



No homidium in serum sample

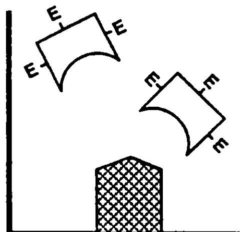
Washed 5x

Homidium in sample reacts with antibody in solution

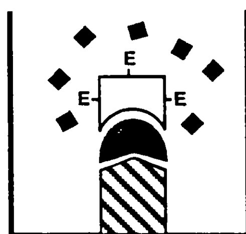


Streptavidin-horseradish peroxidase added

Incubated for 37°C for 45 minutes

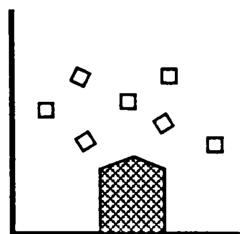


Washed 5x



Chromogen/substrate (TMB/H_2O_2) solution added

Incubated for 10 mins at 37°C



Reaction stopped with 2M sulphuric acid

Substrate conversion (blue colour) changing to yellow on addition of sulphuric acid.

High colour intensity indicates absence of homidium in sample.

Substrate conversion. Intensity of colour depends on amount of homidium present.

No colour indicates high levels of homidium in sample.

with a range of homidium concentrations. Starting with a top concentration of 250 ng ml^{-1} a series of 3-fold dilutions were made in the pool and stored at -20 °C. Only the set of standards in use was stored at +4°C for one week. Eight of the standard solutions which gave the true shape of the sigmoidal standard curve were included in each microtitre plate daily during sample testing.

The results obtained were used to construct a calibration curve from which the homidium concentrations in the test serum samples were calculated. With the assistance of appropriate statistical software, precision profiles (plot of homidium concentrations of standard solutions against CVs) were determined for every assay calibration curve, indicating the range of concentrations over which the results of the test sample would be acceptable and beyond which the results would be unacceptable depending on the CVs obtained.

Quality control (Q.C) standards

A serum pool was made consisting of an aliquot from each of the serum samples collected from all the Friesian cattle 2 days following homidium treatment. The pooled sample was diluted either in pre-treatment sera or in commercially available normal bovine serum (NBS, Gibco, Paisley, U.K.). The dilutions were made such that they represented both the highest and also the sensitive parts of the calibration curve. Also included as part of the quality control standards was a zero dose serum sample. The three quality control standards were included twice in different locations of the microtitre plate daily during sample analysis (giving a total of 12 replicates in each plate). This was carried out as part of as assessment of assay performance.

Intra-assay variation

The intra-assay CVs were determined on the QC standards tested daily in duplicate (n = 10) at the same time as the test sera as described above. The

mean ODs were determined for the duplicate samples in each assay and concentrations calculated with the aid of the calibration curve. The mean variance of the duplicate QC standards were also calculated.

Inter-assay variation

The inter-assay CVs were determined from the mean concentrations of duplicate determinations of the QC standards.

Assay detection limit

This was determined by assaying zero dose sera in duplicate (n = 20). The Mean ODs, B/B_0 (B_0 = OD obtained from the calibration curve at zero dose; B = ODs of individual animal sera) values and the standard deviation (SD) among negative animals were determined. A concentration corresponding to the B/B_0 value of Mean minus 3SD read from the calibration curve was taken as the least detectable dose.

Parallelism

A pooled serum sample collected on Day 2 following treatment of the Friesian cattle was diluted serially at 1/4, 1/8, 1/16 and 1/32 in the pooled negative control sera. Similarly, the same Day 2 pooled serum sample was diluted in commercially available normal bovine serum (NBS, Gibco, Paisley, Scotland). Homidium concentrations in the diluted sera were determined using Assay 1. The final concentrations were calculated by multiplying by the appropriate dilutions and the results plotted against the dilution of the sample. A parallel response was inferred from a horizontal line.

Cross-reactivity

Cross-reactivity of the Assay 1 reagents was investigated with other trypanocides commonly used in cattle; namely, diminazene aceturate (Berenil ®, Hoechst, Germany) and isometamidium chloride (Samorin®, May and

Baker). This was carried out by assaying a range of diminazene and isometamidium-spiked control sera along with the homidium-spiked serum. Cross-reactivity was expressed as the dose of diminazene or isometamidium required to reduce by 50% the maximum optical density obtained in the absence of the drug (concentration at 50% B/B_0).

$$\text{Cross-reactivity} = \frac{\text{mass of competitor at 50\% displacement}}{\text{mass of homidium at 50\% displacement}} \times 100$$

where competitor = diminazene or isometamidium

Assay validation

Serum samples from animals treated with homidium were tested in a competition assay in which the free drug in the serum competed with the homidium in the homidium-ovalbumin conjugate for the biotinylated antibody. On every plate were included a set of homidium standard solutions prepared as already described for assay calibration. These were also diluted in antibody solution using the micronic tubes before addition onto the plate, similar to the test serum samples.

Assay 2: utilising homidium-horseradish peroxidase (homidium-HRP) conjugate

Preparation of homidium-horseradish peroxidase (homidium-HRP) conjugate

Homidium was conjugated to horseradish peroxidase (Boeringer Mannheim, GmbH, Germany) in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Boeringer Mannheim GmbH, Germany). Ten milligrammes of horseradish peroxidase were weighed, dissolved in 1 ml water in a glass bijou and vortex mixed. This solution was placed at +4°C until required.

To ten milligrammes of homidium bromide in a glass bijou, were added 250μl pyridine and 250μl water and vortex mixed. Similarly, 10 mg of 1-ethyl-3-(3-dimethlaminopropyl) carbodiimide in a glass bijou was dissolved in 250 μl and vortex mixed. With constant stirring, the carbodiimide solution was added in 50 μl aliquots to the homidium solution. Over a period of 15 minutes, the homidium-carbodiimide was added to the peroxidase solution in 200 μl amounts also with constant stirring. The homidium-carbodiimide-peroxidase mixture was incubated at 37°C for 20 hours after which it was purified by dialysis against 0.9%w/v sodium chloride following procedures described in Chapter 2.

The assay

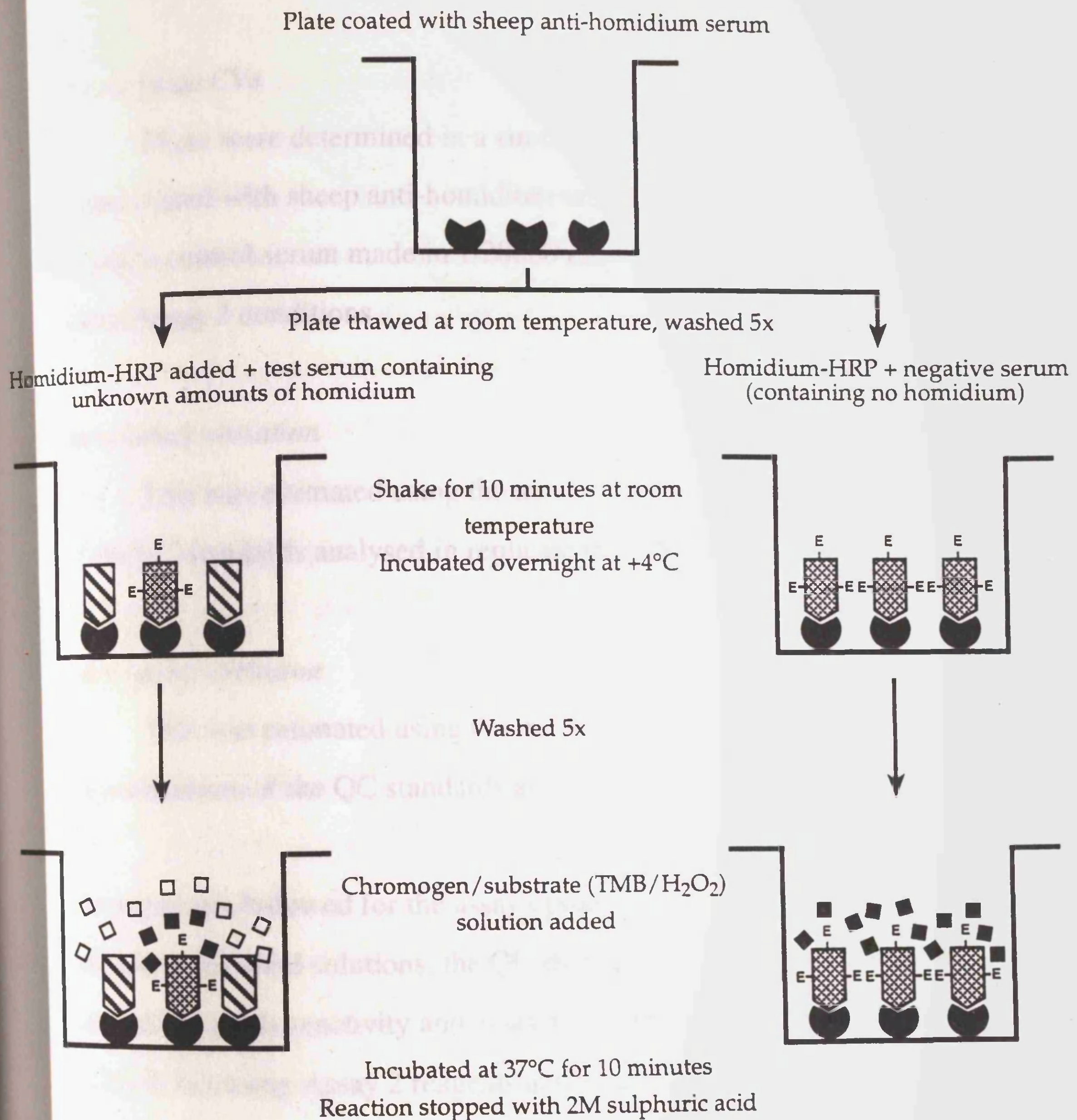
All the optimal dilutions used were obtained by chequerboard titration of the conjugate and the sheep anti-homidium serum (See Section on optimisation of assay reagents, Assay 1). The microtitre plates (Immulon 4, Dynatech, U.K.) were coated with anti-homidium antiserum at an optimal dilution of 1/20,000 in carbonate/bicarbonate buffer, pH 9.2, and incubated overnight at +4°C. The plates and the contents were frozen *in situ* and stored at -20°C. When required, the plates were thawed at room temperature and washed as in assay 1. Homidium-HRP conjugate diluted 1/20000 in PBST was then added (100μl per well). The plates were incubated overnight at +4°C, washed, developed and the ODs determined as described above.

Test serum samples from treated animals were diluted ten-fold in 1/20000 homidium-HRP in PBST before addition to wells of the plate. This was carried out by adding 25μl of the test serum to 225μl of a dilution of 1/20000 homidium-HRP in PBST in micronic tubes, mixing and adding on to the plate. The volume of 250μl was adequate for duplicate wells. Test serum samples containing high homidium concentrations were pre-diluted in pooled negative control sera followed by the ten-fold dilution in 1/20000 homidium-

Fig. 3.2

A diagrammatic representation of Assay 2

Fig. 3.2 **Assay 2: Competitive enzyme immunoassay for homidium**



Substrate conversion. Intensity of colour depends on amount of homidium present.

No colour indicates high levels of homidium in sample.

Substrate conversion (blue colour) changing to yellow on addition of sulphuric acid.

High colour intensity indicates absence of homidium in sample.

HRP in PBST. Assays were calibrated using homidium-spiked sera as in Assay 1. A diagrammatic representation of this assay is shown in Fig 3.2.

Whole plate CVs

These were determined in a similar manner as in Assay 1 but with the plates coated with sheep anti-homidium serum and the 1/10 dilution of the negative control serum made in 1/20000 dilution of homidium-HRP in PBST under Assay 2 conditions.

Intra-assay variation

This was estimated using the mean of the variances of duplicate wells of the QC standards analysed in replicate (n = 15).

Intra-assay variation

This was estimated using the mean absorbances of duplicate determinations of the QC standards analysed in replicate (n = 15).

The protocols followed for the assay optimisation, preparation of the spiked homidium standard solutions, the QC standards, the assay detection limit, parallelism, cross-reactivity and assay validation were as described under Assay 1, but using Assay 2 reagents and assay conditions.

Data evaluation

The procedures and the software used for evaluation of the results were as already described in the General Materials and Methods (Chapter 2). The 4-parameter logistic method was used in the data reduction (Eisler et al., 1993). Serum homidium concentrations of the test sera were calculated, and the intra- and inter-sample variances determined.

RESULTS

Assay 1

Reagent titrations

Following the immunisation, collection and purification of the sheep anti-homidium sera, high antibody titres were obtained. The high titres allowed small volumes of reagents to be used. The optimal dilutions obtained following the chequerboard titration were 1/3200 for homidium-ovalbumin conjugate and 1/7000 for the biotinylated anti-homidium IgG. The number of samples analysed per plate was 34 (excluding the eight homidium-spiked standards and the six QC standards). Analysis of 34 samples at a biotinylated anti-homidium IgG dilution of 1/7000 showed that one ml of the undiluted biotinylated antibody and approximately two ml of the homidium-ovalbumin conjugate would be adequate for the analysis of approximately 20,000 samples.

Calibration curves

A typical calibration curve for Assay 1 is shown in Fig. 3.3. Using the four-parameter logistic method for curve fitting, the results obtained from the response of the QC standards diluted in either pooled negative control sera or the commercially available NBS was expected.

Parallelism

Parallelism was demonstrated following determination of homidium concentrations in the pooled Day 2 serum sample diluted in either pooled negative control serum or in commercially available NBS.

Intra- and inter-sample variation

The results obtained for the intra- and inter-assay (intra- and inter-sample) variance (calculated as described by Eisler et al., [1993]) are shown in

Fig. 3.3

A typical dose response curve obtained from the mean (±SD) B/B_0 values of the spiked serum homidium standards analysed in replicate (n = 15) using either Assay 1 or Assay 2.

Table 3.1. The low variance obtained following sample pre-dilutions resulted in the overall low intra- and inter-assay CVs. Using the QC standards, the intra- and inter-assay variations were also shown to be low (Table 3.2).

Cross-reactivity

Cross-reactivity of the anti-homidium reagents with diminazene was negligible. The result showed cross-reactivity of 0.002%. There was however, significant cross-reactivity with isometamidium in which a value of 86.7% was obtained.

Assay detection limit

Using the mean and the SD of the B/B_0 of the negative sera (zero dose samples; n = 20), the serum homidium concentration, read from the standard curve corresponding to the mean minus 3SD was 0.1 ng ml^{-1}. This was taken as the least detectable dose or the assay limit of detection.

Determination of homidium in serum of treated cattle

The mean (±SD) serum homidium concentration versus time plot following determination of homidium in serum samples from treated cattle using Assay 1 is shown in Fig. 3.4. The mean drug concentration one hour following i.m. treatment was 69.78±7.92 ng ml^{-1} (range 50.6 - 88.1). This declined exponentially to 12.63±1.57 ng ml^{-1} (range 9.85 - 18.22) in 24 hours, 0.31±0.02 ng ml^{-1} (range 0.25 - 0.36) in 60 days and 0.16±0.02 ng ml^{-1} (range 0.15 - 0.20) in 90 days (limit of detection: 0.1 ng ml^{-1}). The results showed extremely low concentrations in serum over long periods of time. The first sample was collected one hour following treatment, therefore no peak concentrations were observed. A wide variation in serum concentrations between individual animals was observed using this assay. Inconsistency in the reproducibility of homidium concentrations was reflected in the wide

Table 3.1

Intra-sample (intra-assay and inter-sample (inter-assay) variance expressed as coefficients of variation, CV_w and CV_b, including the mean response of *r* replicates, CV_x, calculated for homidium-free sera from normal Friesian calves tested in duplicate at 1/10 dilution using homidium Assays 1 and 2.

		Assay 1 (n = 20)	Assay 2 (n = 20)
CV_w		4.0%	6.9%
CV_b		6.4%	11.5%
CV_x	r = 4	5.1%	9.0%
	r = 2	6.0%	10.7%
	r = 1	7.5%	13.4%

Table 3.2

Intra-sample (intra-assay and inter-sample (inter-assay) variance, expressed as coefficients of variation (%), CV_w and CV_b calculated from the concentrations obtained for the quality control standards (QC1 and QC2) tested in duplicate at a dilution of 1/10 using homidium Assays 1 and 2.

	Assay 1 n = 10		Assay 2 n = 15	
	QC1	QC2	QC1	QC2
CV_w	7.6	4.4	10.0	5.3
CV_b	14.4	14.5	18.4	12.2

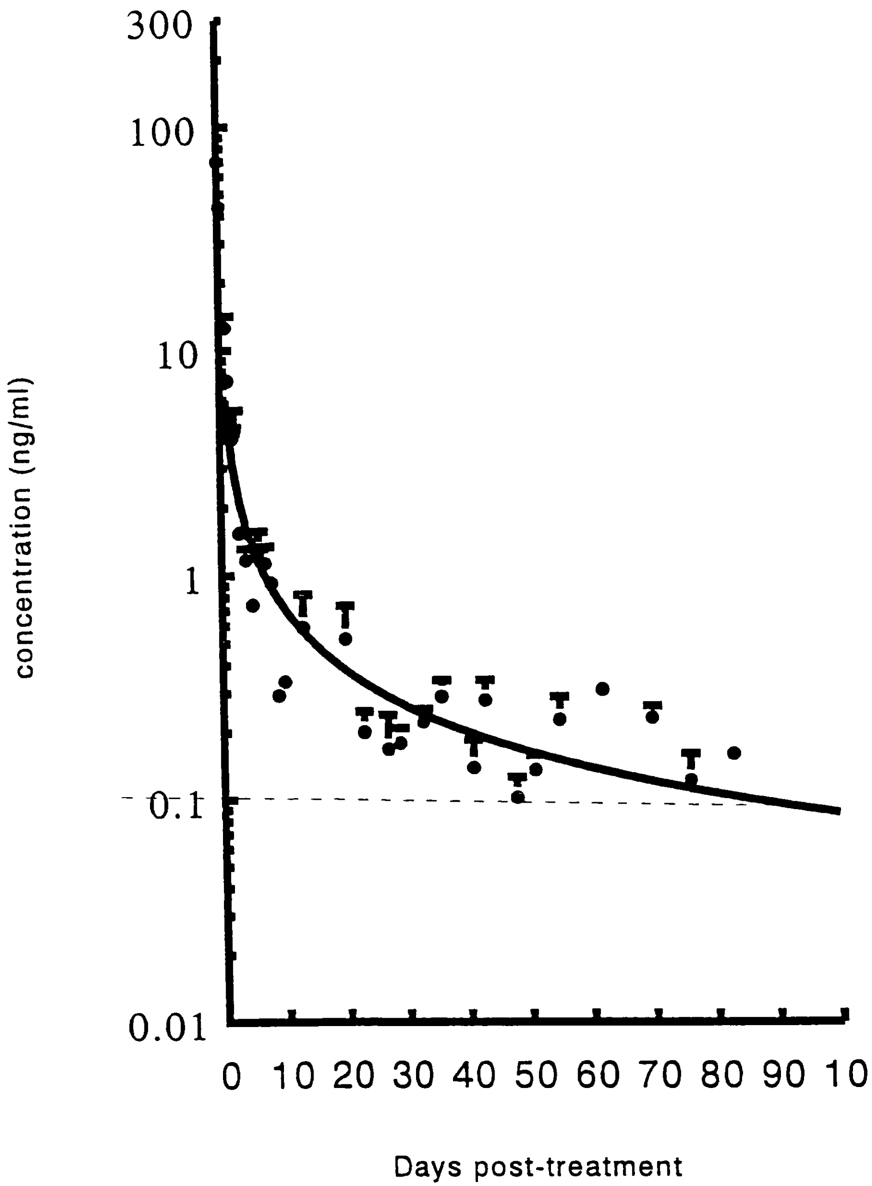


Fig. 3.4

Assay 1: Mean (±SD, n = 5) serum homidium concentrations in normal cattle treated with homidium at a dose rate of 1 mg kg^{-1}

scatter of data points along a line obtained by a log curve fit of the mean ± SD serum homidium concentration versus time plot (Fig. 3.4)

Assay 2

Reagent titrations

On the basis of the procedure described for the immunisation of sheep, high anti-homidium titres were obtained. Following the chequerboard reagent titration, the optimal dilution of 1/20000 obtained for sheep anti-homidium sera was similar to that obtained for homidium-HRP conjugate. Similar to Assay 1, the number of serum samples analysed in duplicate was 34. Analysis of 34 samples at an antibody dilution of 1/20000 showed that one ml of each of undiluted sheep anti-homidium sera and the homidium-HRP conjugate would be adequate for the analysis of approximately 50,000 samples.

Calibration curves

The calibration curve obtained using Assay 2 was markedly similar to that obtained using Assay 1 (Fig. 3.3). The four-parameter logistic method was used for data reduction and similarly showed a good response to the QC standards.

Parallelism

Parallelism was demonstrated following determination of homidium concentrations in the Day 2 serum sample diluted in pooled negative control sera. There was however, lack of parallelism in the Day 2 serum diluted in commercially available NBS.

Intra- and inter-sample variation

The results for the intra- and inter-assay variance are shown in Table 3.2. Variance due to the pre-dilutions and the solid phase were quite low

resulting in overall low CVs for the assay. The consistency in reproducibility of the low intra- and inter-sample CVs was good (Tables 3.1 and 3.2).

Assay detection limit

The least detectable dose determined from the zero dose samples gave a value for the Mean B/B_0 - 3SD read off the calibration curve as 0.1 ng ml^{-1}.

Cross-reactivity

Cross-reactivity of the anti-homidium reagents with diminazene was negligible. The value obtained was 0.001%. Cross-reactivity with isometamidium was however significant giving a value of 126%.

Determination of serum homidium concentrations treated cattle

The plot showing mean ± SD serum drug concentration versus time plot of samples analysed by Assay 2 is given in Fig. 3.5. One hour following treatment the mean serum concentration was 72.5±2.22 ng ml^{-1} (65.95 - 78.4). This declined to 9.77±1.78 ng ml^{-1} (range 7.7 - 12.05) in 24 hours, 0.22±0.02 ng ml^{-1} (range 0.14 - 0.28) in 60 days and 0.15±0.07 ng ml^{-1} (range 0.10 - 0.27) in 90 days (limit of detection: 0.1 ng ml^{-1}). No peak concentrations were observed since the first sample collection was one hour following treatment.

Comparison of Assays 1 and 2

Reagent titration

High antibody titres were obtained following the immunisation protocol used. Following antibody purification and biotinylation, the antibody titre was still high resulting in the use of an optimal dilution of purified, biotinylated IgG of 1/7000 in Assay 1. However, the optimal dilution of the unpurified sheep anti-homidium sera used in Assay 2 was 1/20000.

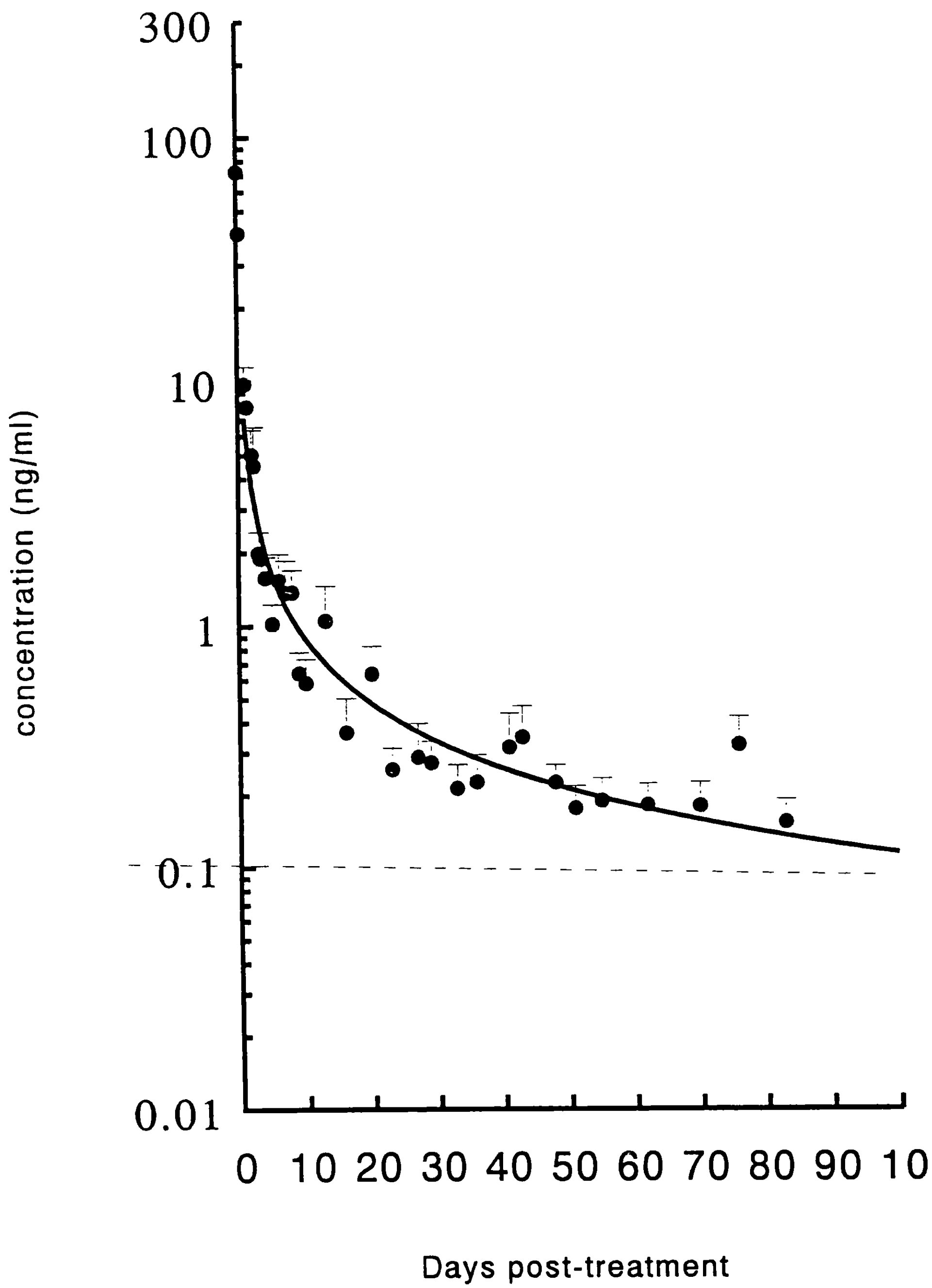


Fig. 3.5

Assay 2: Mean (±SD, n = 5) serum homidium concentrations in normal cattle treated with homidium at a dose rate of 1 mg kg^{-1} b.w.

In both Assays, the dilutions for homidium conjugates were also high and allowed the use of small volumes of reagents for large numbers of samples.

Calibration curve

There was marked similarity in the calibration curves obtained following analysis of spiked homidium standard solutions. Fig. 3.3 shows a typical calibration curve (mean $B/B_0 \pm SD$, $n = 15$) obtained from data collected following replicate determinations using either of the assays.

Intra- and inter-sample variation

Low intra-assay (intra-sample) CVs were obtained for the QC standards using either of the assays showing high within run precision (Table 3.2). Whilst QC1 and QC2 gave markedly similar inter-assay CVs using Assay 1, the same QCs gave different values using Assay 2. The concentration of QC2 fell within the most sensitive part of the dose-response curve, the concentration of QC1 fell within the less sensitive part of the curve in which small changes in response resulted in large changes in the calculated concentration.

Parallelism

Whilst parallelism was demonstrated in pooled Day 2 serum sample obtained from homidium-treated cattle diluted in either negative control sera or the commercially available NBS using Assay 1, there was lack of parallelism when the serum sample was diluted in the commercially available NBS using Assay 2. The optical densities appeared to decrease with increase in the amount of NBS added resulting in higher homidium concentrations than the expected. Parallelism was however, demonstrated when the Day 2 serum sample was diluted in the negative control sera and analysed using Assay 2.

Cross-reactivity

Using both assays, significant cross-reactivity with anti-homidium reagents was demonstrated with isometamidium. However, cross-reactivity was negligible with diminazene.

Assay detection limit

The limit of detection obtained for both assays was 0.1 ng ml^{-1} showing that both assays were highly sensitive.

Serum homidium concentrations

In comparing the two assays, no peak serum concentrations were determined since the first sampling interval was one hour following treatment. Whilst a wide variation in serum homidium concentrations between individual animals was observed using Assay 1 (Fig. 3.4), the drug disappearance curve obtained using Assay 2 was much smoother with most data points closer to line obtained by the log curve-fit of the mean (±SD) serum drug concentration-versus-time semi-log plot (Fig. 3.5).

However, the differences between the serum homidium concentrations obtained using the two assays at any given time following treatment were insignificant ($p>0.05$).

DISCUSSION

The development and validation of two sensitive enzyme competitive assays for the determination of homidium in the sera of treated cattle are reported in the present Chapter. Assay 1 was an indirect homidium competition ELISA in which the microtitre plates were coated with homidium-ovalbumin and the detection of homidium carried out via the biotin - streptavidin-horseradish peroxidase system (using biotinylated anti-homidium IgG). The homidium in the homidium-ovalbumin conjugate coated on the plate

competed with the homidium in the test sera for the biotinylated anti-homidium IgG (Fig. 3.1).

Assay 2 was a direct competition assay in which the microtitre plates were coated with sheep anti-homidium serum. The homidium in the homidium-HRP conjugate competed with the homidium in the test sera for the sheep anti-homidium serum coated on the plate. Both assays showed high sensitivity and good reliability. Both assays had a limit of detection of 0.1 ng ml^{-1} serum.

In view of the limitations of the previous methods for the detection of homidium in the blood of treated animals, the development of these ELISAs is significant. The previous methods suffered from several drawbacks. These included the relative insensitivity of biological assay (Goodwin and Tierney, 1977), the poor specificity of both the biological and radiometric techniques and the poor sensitivity and high volumes of sera required for the HPLC technique. The ELISAs described offer significant advantages over these previous methods.

The high antibody titres obtained by the immunisation procedure described allowed the use of very high dilutions of the anti-homidium reagents in the assays. This meant that very low volumes of reagents were needed. For instance, in Assay 1 the biotinylated anti-homidium IgG was used at an optimal dilution of 1/7000 suggesting that one ml of the antibody and approximately two ml of the homidium-ovalbumin conjugate would be adequate to analyse approximately 20,000 samples at 34 samples per plate (in duplicate). With Assay 2, one ml of each of the unpurified sheep anti-homidium serum and homidium-HRP conjugate at an optimal dilution of 1/20000 would be adequate to analyse approximately 50,000 samples.

Demonstration of parallelism showed that samples containing high concentrations of homidium could be diluted in the pre-treatment sera without affecting the outcome of the final result and that commercially available NBS

was found to unsuitable for use in Assay 2 due possibly, to the presence of cross-reacting substances.

The assessment of assay precision and establishment of assay working concentration range was carried out through the precision profiles (obtained by analysing in duplicate the spiked homidium standards and plotting the CV against known concentrations). It was important that the random and systematic errors were reduced to the minimum. The determination of the intra- and inter-assay variations helped in assessing these errors. Inclusion of homidium-spiked sera and QCs assisted in monitoring changes in assay conditions and the results of the test samples adjusted accordingly based on the B/B_0 values. Minor day to day variations in OD did not affect concentration determinations significantly, since calibration standards, subjected to the same variations in OD were included on every ELISA plate. The inclusion of QCs allowed this to be verified on an on-going basis.

With these assays, low levels of homidium were still detectable in serum 12 weeks after drug administration. These methods are easy to perform, require no sample extraction and approximately 120 samples could be analysed in a day once the systems are established. For every assay only 25 µl of serum was required compared to 10 ml needed for HPLC . However, Assay 1 showed a wider variability in the serum homidium concentrations between individual animals plus lack of consistency in the reproducibility of the results. This could have been due to the inherent nature of the two assays. Whilst the micro-titre plates were coated on a daily basis in Assay 1, plates were coated in batch in Assay 2 suggesting that one could coat enough plates to cover samples from one whole experiment. Also, the overnight incubation at +4°C of the plates following the addition of the homidium-horseradish peroxidase and the test sera could have contributed to the reproducibility of the results. On the basis of these findings, Assay 2 was adopted for use in the subsequent investigations on homidium pharmacokinetics.

Reaction to anti-homidium reagents by isometamidium but not diminazene was expected since homidium shares structural similarities with isometamidium but not diminazene.

Determination of homidium levels in Friesian cattle showed that the serum drug levels rapidly declined within 24 hours following drug administration. Thereafter, the drop was slow to below 1 ng ml^{-1} within 10 days and to concentrations between 0.15 and 0.3 ng ml^{-1} within 8 weeks.

From the afore-going, it is evident that the ELISA methods developed are highly sensitive to be used to determine low concentrations of homidium in sera of treated cattle for long periods of time following treatment. The assays have afforded the use of very low volumes of reagents to analyse large numbers of samples. The volume of sera required per test in duplicate was only 25 µl with neither sample extraction (cf HPLC) nor physical separation of bound drug (cf radioimmunoassays). Approximately 120 test sera could be analysed daily. The assays are easy to perform and require no expensive equipment. All the above criteria make the ELISA methods ideal for analysing large volumes of field samples.

Assay 2 was selected and later used in studies both in the laboratory and in the field. The results of these studies are reported in the subsequent Chapters (Chapters 4, 5, 6 and 7).

CHAPTER FOUR

PHARMACOKINETICS OF HOMIDIUM BROMIDE IN FRIESIAN AND BORAN CATTLE USING AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

INTRODUCTION

There has only been one previously reported study of the pharmacokinetics of homidium and that was by Gilbert and Newton (1982). Their study was conducted in uninfected and *T. congolense*-infected rabbits and cattle using ^{14}C homidium. However, a detailed pharmacokinetic evaluation of the drug was not given in the study by Gilbert and Newton (1982), although the results did show a rapid elimination of the drug in both rabbits and cattle following drug administration. Details of these studies by Gilbert and Newton are discussed in Chapter 8.

The radiometric method used in the earlier study had a number of potential disadvantages. One is that the method determines total radioactivity and this may not represent intact homidium. Morover, radiometric studies require expensive radiolabelled drugs and their use is limited to laboratory studies in relatively small numbers of animals. The development of a reliable and sensitive ELISA for the detection of homidium has several advantages over the radiometric method. These include high sensitivity, low volumes of sample and reagents required per test, suitability for analysis of large numbers of samples, the ease with which the assays are performed and the relatively low cost of equipment and reagents.

The second enzyme-linked immunosorbent assay (Assay 2) described in Chapter 3 was used in present study to determine the pharmacokinetic parameters of homidium in Friesian and Boran cattle. The study using Friesian (*Bos taurus*) cattle was carried out in the Veterinary School of the University of Glasgow whilst the study using Boran (*Bos indicus*) cattle was carried out in Kenya. The study using Boran cattle was considered to be desirable since this is one of the breeds that is indigenous to trypanosomiasis endemic areas and is widely kept on many ranches in Kenya for production of beef.

STUDY OBJECTIVES

This Chapter is divided into two Sections, one based on the studies in Friesian cattle and the other on the studies in Boran cattle.

Section One

(a) To determine homidium pharmacokinetics in Friesian cattle following intravenous (i.v.) drug administration.

(b) To determine homidium pharmacokinetics in Friesian cattle following intramuscular (i.m.) drug administration.

(c) To compare pharmacokinetic parameters obtained following i.m treatment of Friesian cattle with those obtained following i.v. injection of homidium.

Section Two

(a) To determine homidium pharmacokinetics in Boran cattle following i.m drug administration.

(b) To compare the pharmacokinetic data obtained following treatment of Boran cattle with those obtained following i.m. treatment of the Friesian cattle reported in Section One of the present Chapter.

(c) To collect baseline data to be used in the subsequent investigations on the performance of homidium both as a chemotherapeutic and a chemoprophylactic drug.

SECTION ONE

Homidium pharmacokinetics in Friesian cattle following intravenous drug administration

Materials and Methods

Experimental cattle

Five six-month old Friesian cattle, bred in Scotland and with no history of exposure to trypanosomiasis or trypanocidal drugs were used. They weighed between 185 and 210 kg. Their management before and during the experiment was as described in Chapter 2.

Drug treatment

Homidium bromide (Ethidium®, Laprovet, France; Batch No. 52072/N [NP30L]) was used. A 2.5% (w/v) solution prepared in sterile water immediately prior to use was injected by the intravenous route into the jugular on the side of the neck opposite to the cannulated jugular vein.

Sample collection

(a) Pre-treatment sera

Two days before homidium treatment, all five cattle were prepared with intra-jugular cannulae. Following cannulation, 50 ml pre-treatment blood samples were collected aseptically from the jugular vein via the cannula using a syringe and dispensed into plain vacutainers. Pre-treatment sera was prepared, pooled and stored as described in Chapter 2. The pooled pre-treatment sera were used as negative control and in the preparation of homidium-spiked and quality control standards.

(b) Serum samples for drug analysis

Ten ml of blood were collected via the jugular cannulae immediately prior to homidium treatment, and at 5, 15, 30, 45 minutes, 1, 2, 4, 6, 8 and 12 hours following treatment, and then twice a day for the next 3 days. After three days, the cannulae were removed and further blood samples were collected by venipuncture into vacutainers twice daily for days 4, 5, 6 and 7, once daily during the second week, three times a week during the third week, and twice a week during the fourth week. Thereafter the sample collection was once a week to the end of the observation period of 40 days. Serum was separated and samples stored at -20°C until required. These serum samples were used in the determination of homidium concentration.

Determination of serum homidium concentrations

Serum homidium concentrations were measured using the second homidium ELISA method (Assay 2) described in Chapter 3.

Data evaluation

Procedures followed for the evaluation of data from the drug assays were as described in Chapter 2. Statistical evaluation included determination of standard deviation and coefficient of variation of sample replicates and inter- and intra-sample variation.

Pharmacokinetic evaluation

Compartmental and non-compartmental pharmacokinetic analyses were carried out on data from all the five i.v.-treated animals. The serum concentration-versus-time data following i.v. drug administration were fitted with two-compartmental and three-compartmental models using a weighted non-linear least squares regression using PCNONLIN computer package. Akaike's Information Criterion (AIC) described by Yamaoka et al. (1978) was

used to compare the adequacy of the two-compartmental and three-compartmental models.

Previous studies have shown that serum homidium concentrations decline rapidly following i.m. treatment (Gilbert and Newton, 1982), No i.v. data was available. These results suggest that serum homidium concentrations following i.v. treatment would similarly be low due to the rapid decline. Therefore, during compartmental analysis of i.v. data, weighting was used in order to improve on the numerical stability of the data especially when dealing with very low concentrations.

The formulae used for the various pharmacokinetic parameters were as described in Chapter 2.

The results from the i.v. injection were later compared with the results obtained in similar cattle given homidium by i.m. injection (Section Two of the present Chapter).

Results

All the five cattle remained healthy throughout the experimental period, except one which developed complications during the third week after treatment. These complications included stiffness of the neck which made it difficult for the animal to turn its head. This animal was removed from the experiment and humanely destroyed during the fourth week following drug administration. A post-mortem report on the calf showed that it had contracted meningitis.

Serum drug concentrations

Semi-log plots of the serum drug concentration versus time representing animal Nos. 1 to 5 are given in Figs. 4.1(a) to 4.1(e) respectively. Five minutes following i.v. treatment, the mean drug concentration in serum was 220.1$\pm$ 40.7 ng ml^{-1}. The decline in drug concentrations was exponential and rapid.

Within one hour, the mean concentration was 31.9±2.1 ng ml^{-1}. Concentrations after 24 hours and 15 days of drug administration were 3.9± 0.4 ng ml^{-1} and 0.16±0.03 ng ml^{-1} respectively. No drug was detectable 17 days following treatment in four of the five cattle {Figs. 4.1(a) to 4.1 (d); limit of detection: 0.1 ng ml^{-1}}. The drug was undetectable in the remaining animal within 22 days {Fig. 4.1(e)}. The mean (±SD, n = 5) serum homidium concentra.ion versus time plot is shown in Fig. 4.2.

Compartmental pharmacokinetic analysis

The pharmacokinetic parameters obtained using the compartmental model are shown in Table 4.1. Using the compartmental model, the data from the cattle given the i.v. injection were best described by tri-exponential equations with half lives of 0.064±0.037 hours (range 0.048 - 0.144) for $t\frac{1}{2}\alpha$, 7.17±1.87 hours (range 5.54 - 10.70) for $t\frac{1}{2}\beta$ and 106.3±6.6 hours (range 99.50 - 119.0) for $t\frac{1}{2}\gamma$. The mean homidium serum concentration extrapolated to time 0 (Cp0) was 576.8±292.9 ng ml^{-1} (range 130.9 - 1013.0). The mean $AUC_{0\text{-last}}$ and AUMC $_{0\text{-last}}$ values were 577.3± 75.5 ng.h ml^{-1} (range 477.1 - 672.2) and 36675±7332 ng.h^2 ml^{-1} (range 29316 - 50042), respectively. The calculated MRT from these values was 63.39±7.51 hours (range 51.12 - 74.45). The rest of the pharmacokinetic parameters are shown in Table 4.1. The volume of the central compartment (Vc) was 2.81±2.45 l kg^{-1} (range 0.99 - 7.63) and that of the steady state (Vd) as 111.6±18.0 l kg^{-1} (range 80.2 - 129.6).

The results obtained for the rate constants k_{12}, k_{21}, k_{13} and k_{31} showed an approximately ten-fold difference between rate constants k_{12} and k_{21} and an approximately 50-fold difference between rate constants k_{13} and k_{31}. This demonstrated non-attainment of equilibrium of free drug concentrations between the plasma and the tissues and steady state was not achieved between the drug distribution and elimination phases. There was a strong tendency for

Fig. 4.1(a) to 4.1(e)

Serum homidium concentration in five individual Friesian cattle following i.v. treatment with homidium bromide at 1 mg kg^{-1} b.w.

Fig. 4.1(a)
Animal No. 1

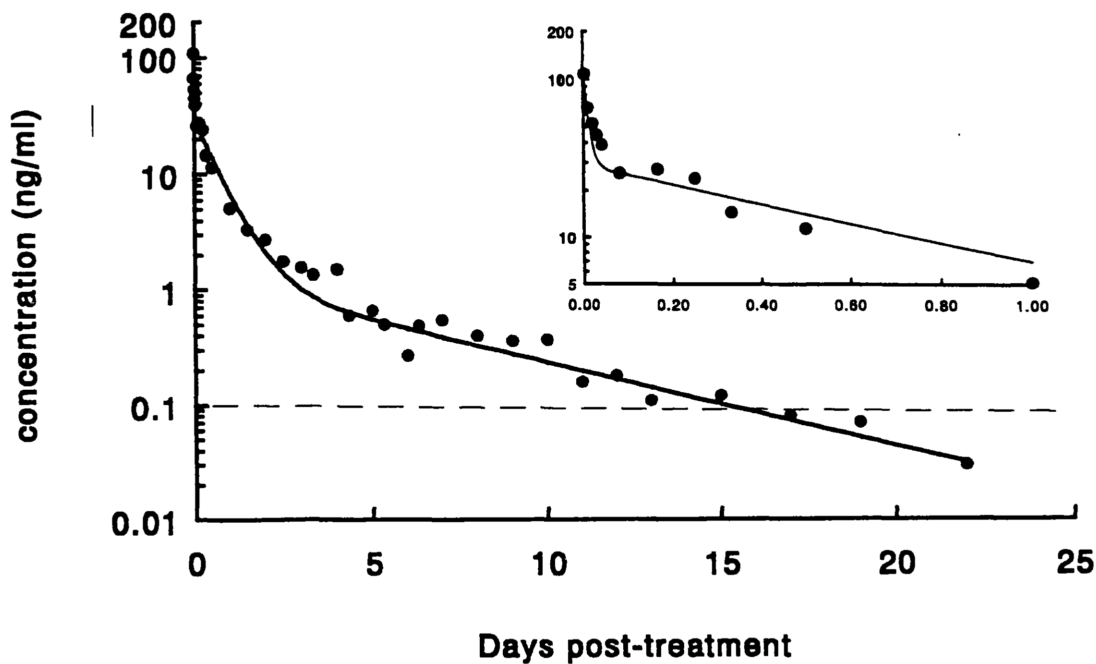


Fig. 4.1(b)
Animal No. 2

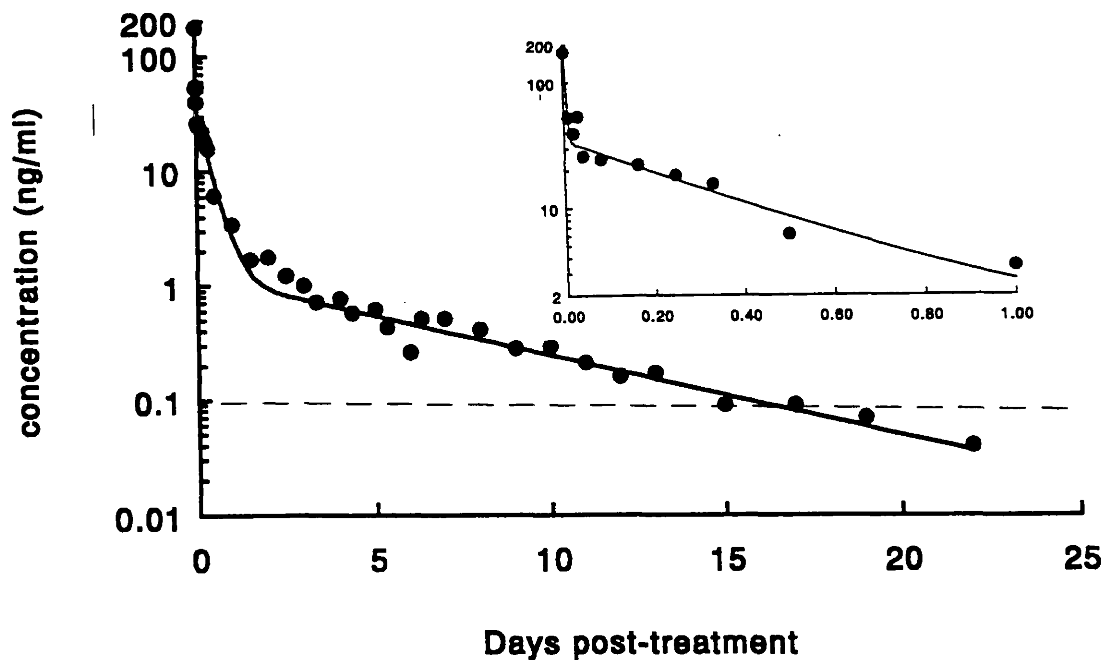


Fig. 4.1(c)
Animal No. 3

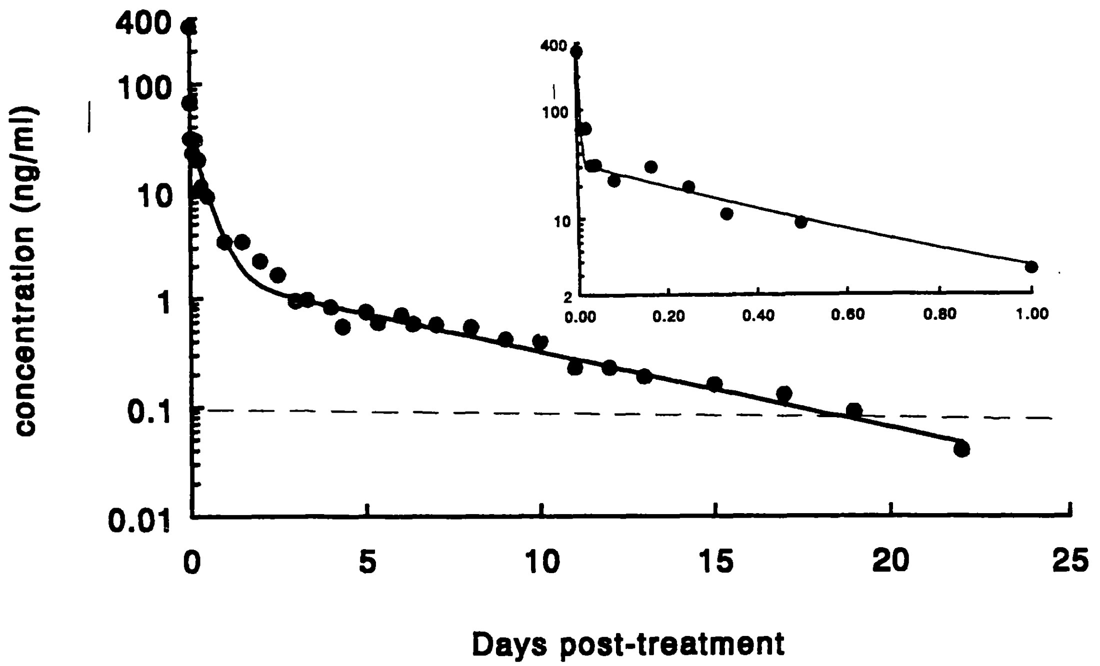


Fig. 4.1(d)
Animal No. 4

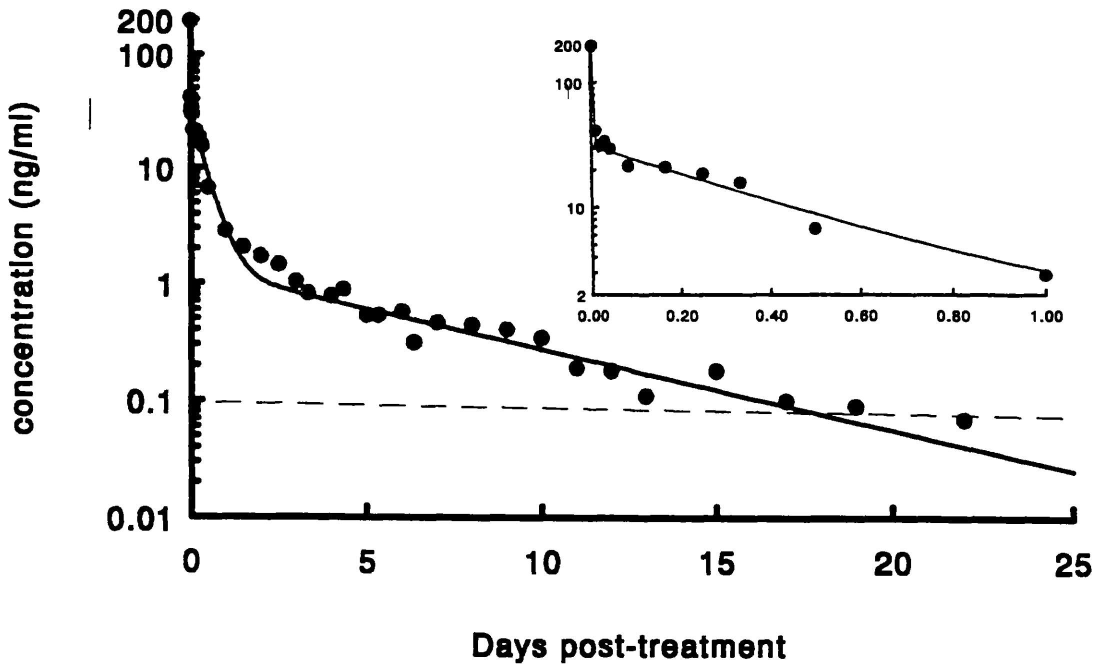


Fig. 4.1(e)
Animal No. 5

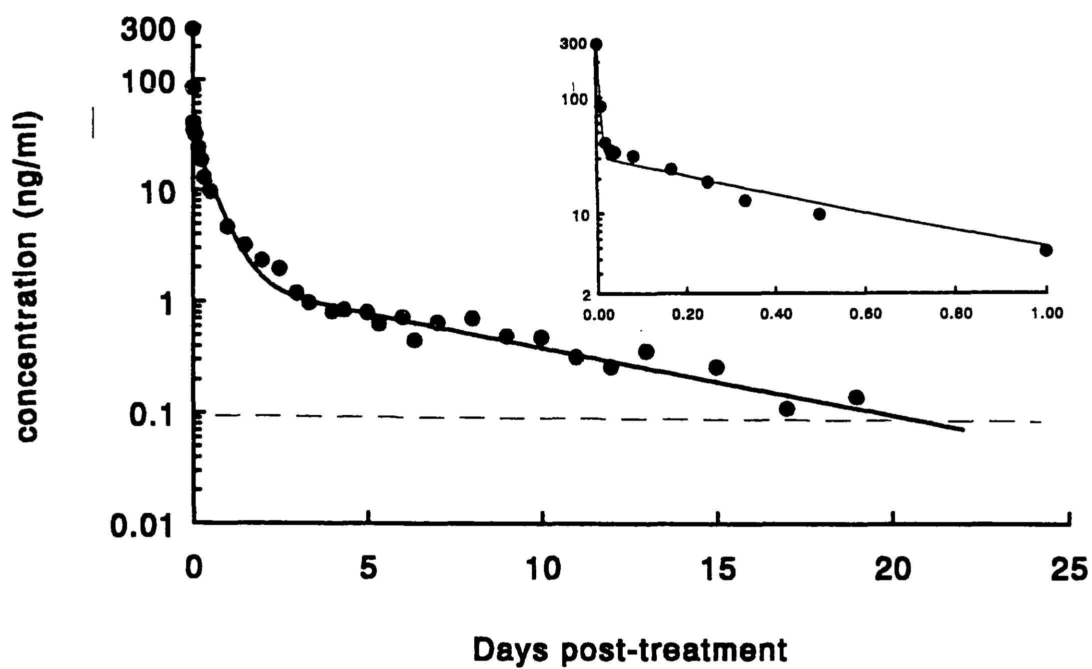


Fig. 4.2

The mean (±SD; n = 5) serum homidium concentrations in Friesian cattle following i.v. treatment with homidium bromide at 1 mg kg^{-} b.w.

Fig. 4.2

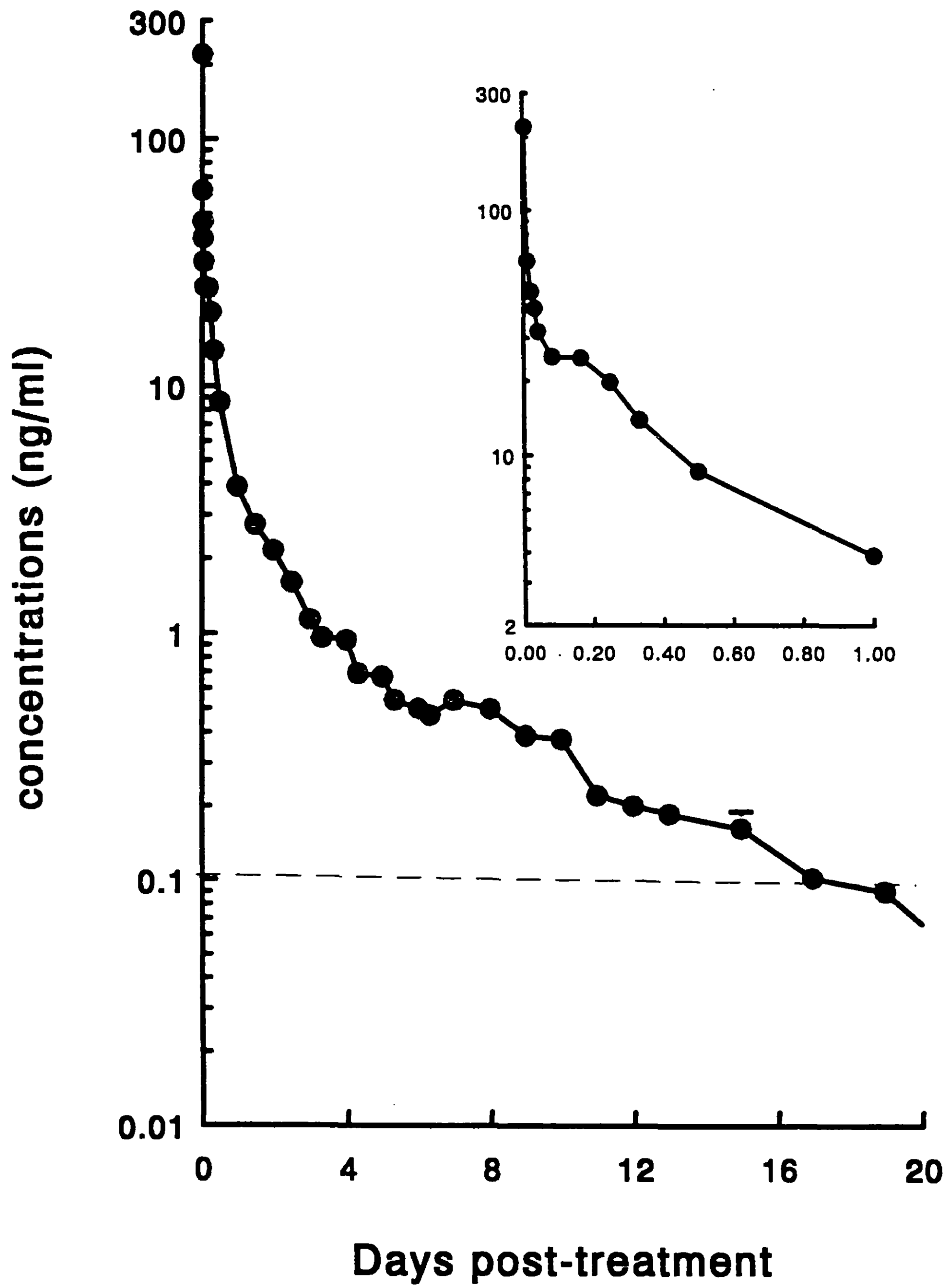


Table 4.1

Pharmacokinetic parameters of homidium in normal Friesian cattle after intravenous injection of homidium bromide at a dose rate of 1 mg kg^{-1} b.w.: Compartmental analysis

Parameter	Animal Nos. 1	2	3	4	5	Mean ± SD
A (ng ml^{-1})	101.5	398.1	980.4	682.8	561.9	545.0±292.8
B (ng ml^{-1})	28.14	33.43	31.03	29.88	29.64	30.42±1.76
C (ng ml^{-1})	1.25	1.19	1.61	1.32	1.56	1.38±0.17
α	4.563	13.25	14.20	17.54	9.92	11.89±4.39
β	0.065	0.125	0.108	0.113	0.085	0.099±0.022
γ	0.0071	0.0070	0.0070	0.0072	0.0060	0.0070±0.0003
t½α (h)	0.144	0.048	0.048	0.048	0.072	0.064±0.037
t½β (h)	10.70	5.54	6.43	6.12	8.14	7.17±1.87
t½γ (h)	99.5	104.6	103.8	105.4	119.0	106.3±6.6
Vc (l/kg)	7.63	2.31	0.99	1.40	1.69	2.81±2.45
Cp(0) (ng ml^{-1})	130.9	432.8	1013.0	714.0	593.1	576.8±292.9
AUC (ng.h ml^{-1})	637.0	477.1	597.2	503.1	672.2	577.3±75.5
AUMC(ng.h^2 ml^{-1})	32560	29316	38666	32792	50042	36675±7332
Vss (l kg^{-1})	80.2	128.8	108.4	129.6	110.7	111.6±18.0
Cl (ml h^{-1}kg^{-1})	26.16	34.93	27.91	33.13	24.79	29.39±3.96
MRT (h)	51.12	61.44	64.75	65.18	74.45	63.39±7.51
k_{12} (h^{-1})	3.287	10.82	11.09	14.57	8.047	9.56±3.76
k_{21} (h^{-1})	1.073	1.172	0.557	0.871	0.599	0.854±0.246
k_{13} (h^{-1})	0.060	0.470	0.961	0.791	0.477	0.552±0.309
k_{31} (h^{-1})	0.009	0.010	0.011	0.011	0.009	0.010±0.001
k_{10} (h^{-1})	0.206	0.907	1.696	1.419	0.882	1.022±0.512
t½k_{10}(h^{-1})	3.370	0.768	0.408	0.480	0.720	0.834±1.11

the movement of the drug into the tissues. Despite the non-achievement of a steady state in the drug distribution, the calculated values for the Vd in all animals were extremely large.

Non-compartmental pharmacokinetic analysis

The pharmacokinetic parameters obtained using the non-compartmental model are given in Table 4.2. The value for the terminal phase half-life using this model was 58.58±1.52 hours (range 56.69 - 61.07). The mean $AUC_{0-\infty}$ and $AUMC_{0-\infty}$ observed values were 585.6±67.9 ng.h ml^{-1} (range 498.6 - 657.1) and 36757±4724 $ng.h^2$ ml^{-1} (range 30064 - 44314), respectively. Using these values, the $MRT_{0-\infty}$ observed was calculated as 62.98±5.91 hours (range 53.21 - 70.18).

Homidium pharmacokinetics in Friesian cattle following intramuscular drug administration and comparison with data obtained from cattle treated by intravenous injection

Materials and Methods

Experimental cattle

Five Friesian cattle aged 6 months and weighing between 160 and 175 kg were used. Their management before and during the experiment was as described in Chapter 2 (Materials and Methods). These animals were bred in Scotland and had no history of exposure to trypanosomiasis or trypanocidal drugs.

Drug treatment

Homidium bromide (Ethidium®, Camco, U.K) was used at a dose rate of 1 mg kg^{-1} b.w. A 2.5% (w/v) solution was prepared in sterile water

Table 4.2

Pharmacokinetic parameters of homidium in normal Friesian cattle after intravenous injection of homidium bromide at a dose rate of 1 mg kg^{-1} b.w.: Non-compartmental analysis

Parameter	Animal No.					Mean ± SD
	1	**2**	**3**	**4**	**5**	
β	0.0120	0.0121	0.0125	0.0112	0.0122	0.0122±0.0003
$t_{1/2}\beta$ (h)	56.69	57.33	58.96	61.07	58.84	58.58±1.52
AUC_{0-last} (ng.h ml^{-1})	648.7	495.2	606.4	505.2	657.1	582.5±69.4
$AUC_{0-\infty}$ (observed) (ng.h ml^{-})	651.2	498.6	609.8	511.4	657.1	585.6±67.9
$AUC_{0-\infty}$ (predicted) (ng.h ml^{-})	649.6	496.1	607.7	506.6	658.5	583.7±69.5
$AUMC_{0-last}$ (ng.h^2 ml^{-1})	33151	28044	36790	32087	44314	34877±5480
$AUMC_{0-\infty}$ (obs.) (ng.h^2 ml^{-1})	34647	30064	38876	35887	44314	36757±4754
$AUMC_{0-\infty}$ (pred.) (ng.h^2 ml^{-1})	33686	28589	37582	32923	45166	35589±5575
MRT_{0-last} (h)	51.10	56.63	60.67	63.51	67.44	59.87±5.63
$MRT_{0-\infty}$ (observed) (h)	53.21	60.30	63.75	70.18	67.44	62.98±5.91
MRT0-∞(predicted) (h)	51.86	57.62	61.84	64.99	68.59	60.98±5.81

immediately prior to use and administered by deep i.m. injection as a single bolus into the muscles of the neck.

Experimental design

Following treatment, serum homidium concentrations were determined for a period of 90 days. A comparison was carried out of the pharmacokinetic parameters calculated from the serum homidium concentration- versus-time data obtained following i.m. treatment and the i.v. treatment already reported in the present Section.

Sample collection

(a) Pre-treatment sera

Two days before homidium treatment, 50 ml blood samples were collected aseptically from each animal via the jugular vein into plain vacutainers. The procedures for the preparation of sera were as described in Chapter 2 (Materials and Methods). A large pool was prepared from these serum samples, aliquoted and stored at -20°C until required. Each of the aliquots prepared was enough to last one week at +4°C once thawed. The rest of the sera were stored as individual samples. The pooled serum samples were used as negative controls and in the preparation of homidium-spiked and quality control standards.

(b) Serum samples for drug assays

Ten ml blood samples were collected as described above immediately prior to treatment and at the following times after treatment: one and 8 hours, twice daily for the first week, daily for the second week, thrice during the third week, twice during the fourth week and once a week from the fifth week to the end of the observation period of 90 days. Procedures followed in the

preparation and storage of sera for drug analysis were as described in Chapter 2.

Data evaluation

Procedures followed for the evaluation of data from the drug assays were as described in Chapter 2. Statistical evaluation included determination of the standard deviation and coefficient of variation of sample replicates, and the inter- and intra-sample variation.

The student's t-test was used to test for significance at both 95 and 99% confidence limits in comparing means of various pharmacokinetic parameters obtained between the different groups of cattle treated with homidium.

Pharmacokinetic evaluation

Non-compartmental pharmacokinetic analysis was carried out on serum homidium concentration-versus-time data following i.m. drug administration following procedures described in Chapter 2. The parameters determined using included the AUC, the AUMC, MRT, F and MAT.

The F and the MAT were calculated using i.v. data reported above.

Results

Plasma homidium concentrations

The drug concentrations in serum showed an exponential decline. Semi-log plots of the serum homidium concentration-versus-time data for the five individual cattle are shown in Figs. 4.3(a) to 4.3(e). The plot showing the mean ± SD serum drug concentration-versus-time is given in Fig. 4.4. The first sample collection was one hour following treatment when a mean serum drug concentration of 72.5±2.2 ng ml^{-1} was attained. This declined to 9.8±1.8 ng ml^{-1} at 24 hours, 0.22±0.02 ng ml^{-1} at 60 days and 0.15±0.07 ng ml^{-1} at 90 days (limit of detection: 0.1 ng ml^{-1}). The values for C_{max} and t_{max} were not

Fig. 4.3(a) to 4.3(e)

Serum homidium concentration in five individual Friesian cattle following i.m. treatment with homidium bromide at 1 mg kg^{-1} b.w.

Fig. 4.3(a)
Animal No. 04

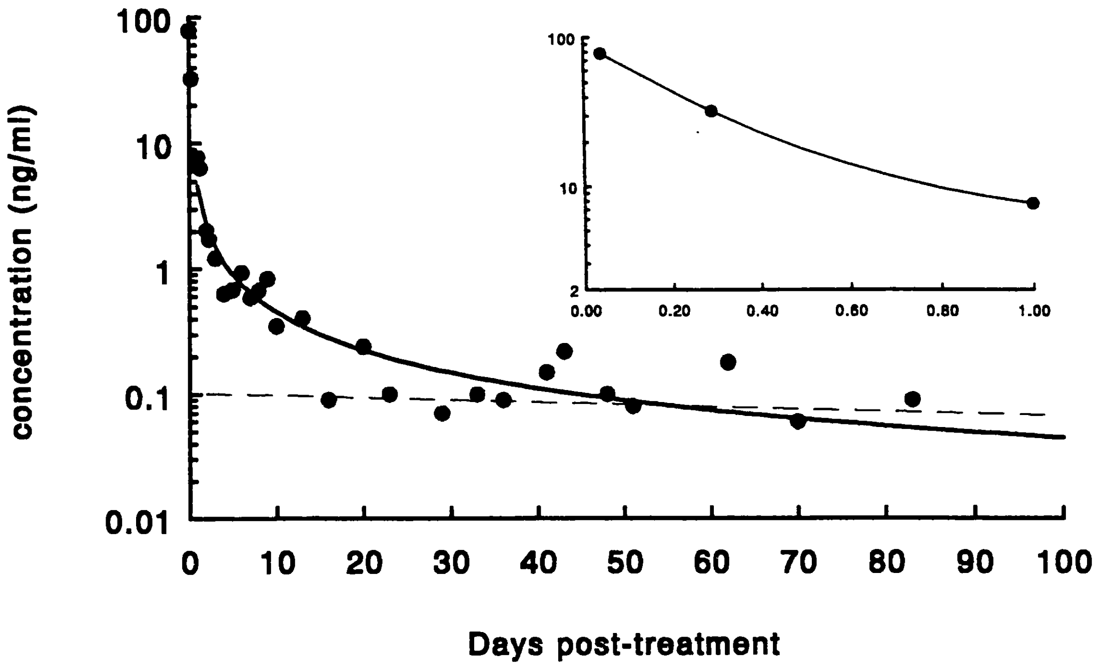


Fig. 4.3(b)
Animal No. 11

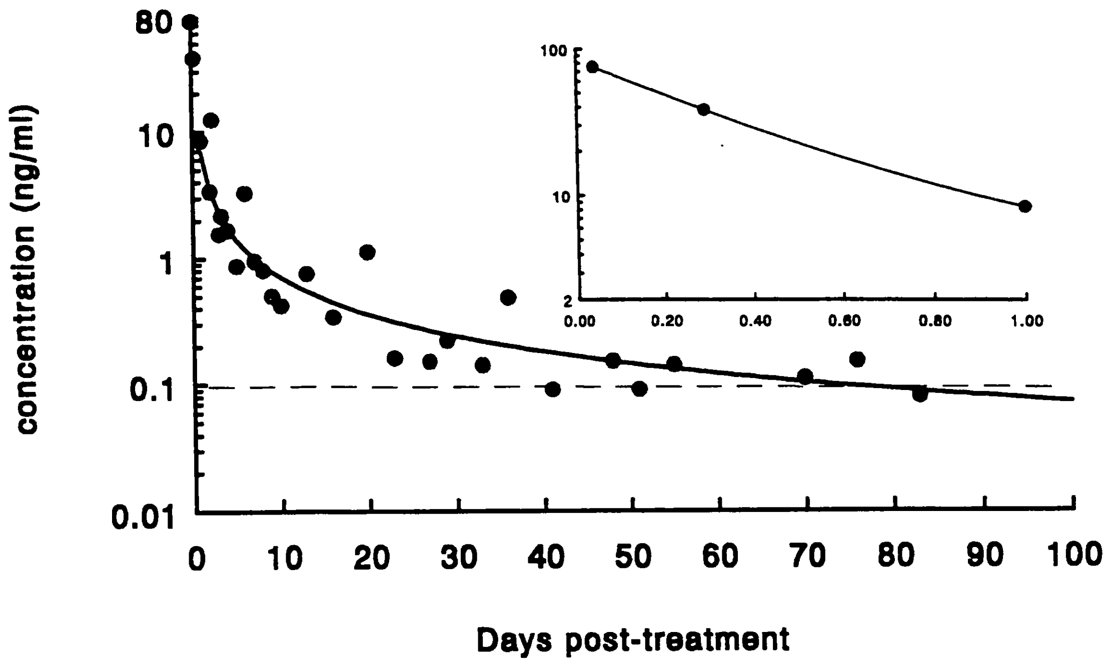


Fig. 4.3(c)
Animal No. 16

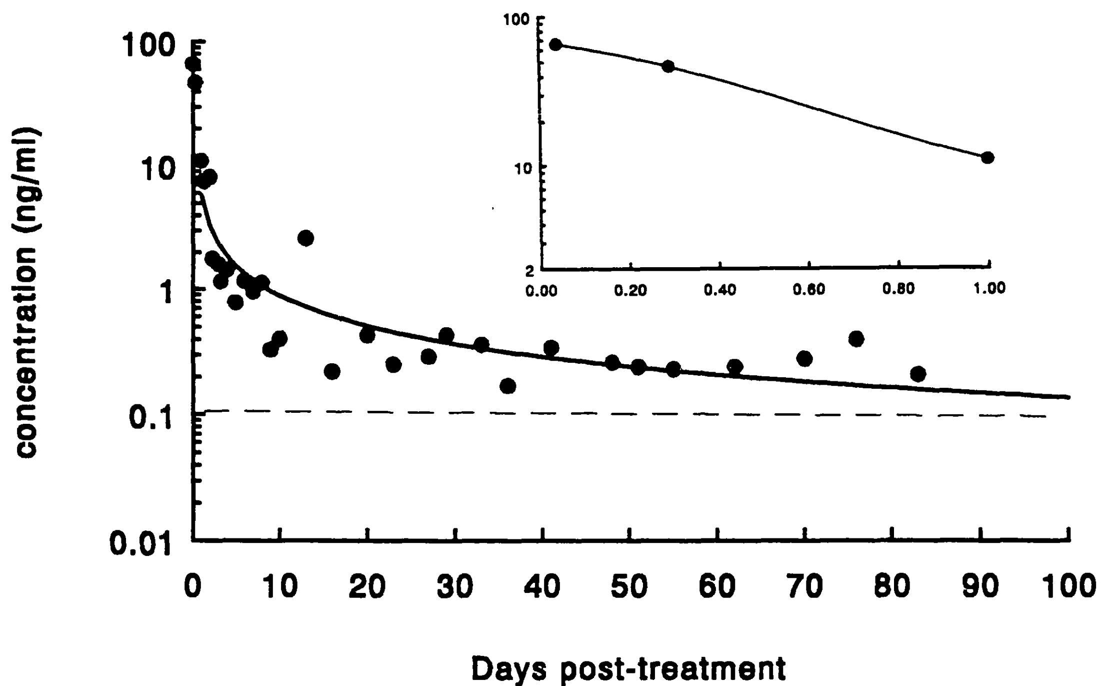


Fig. 4.3(d)
Animal No. 17

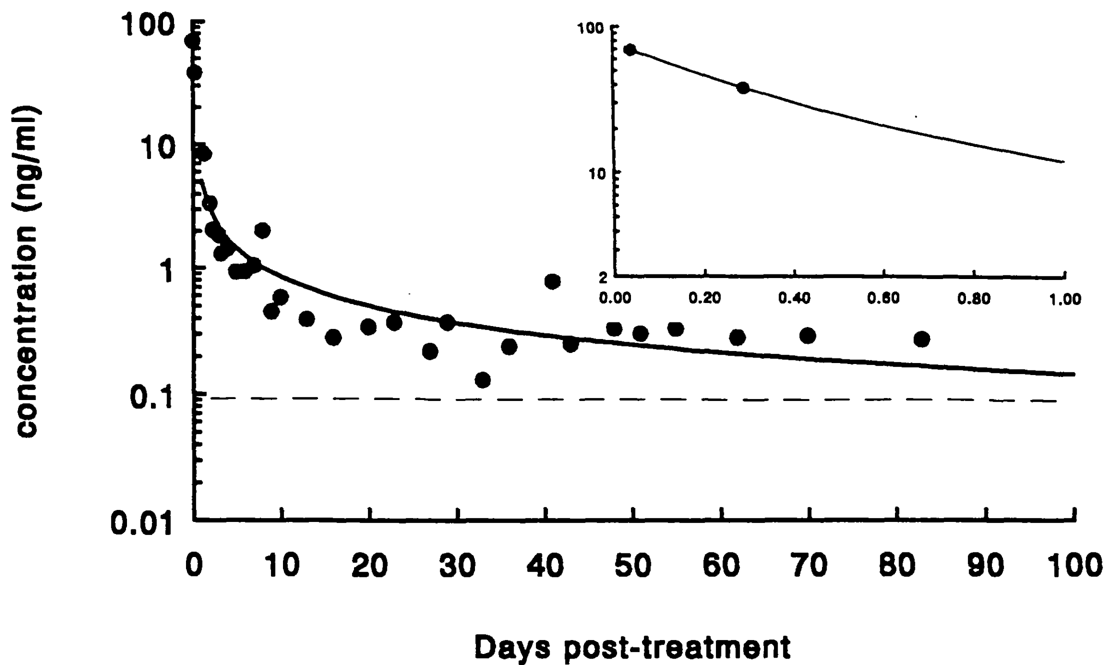


Fig. 4.3(e)
Animal No. 18

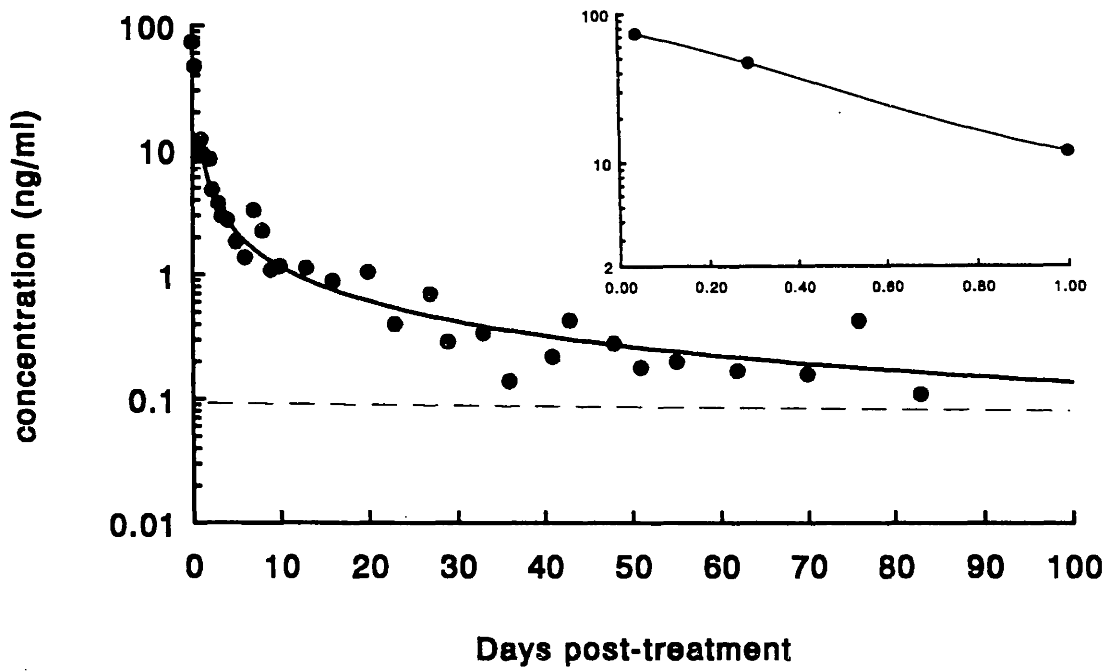
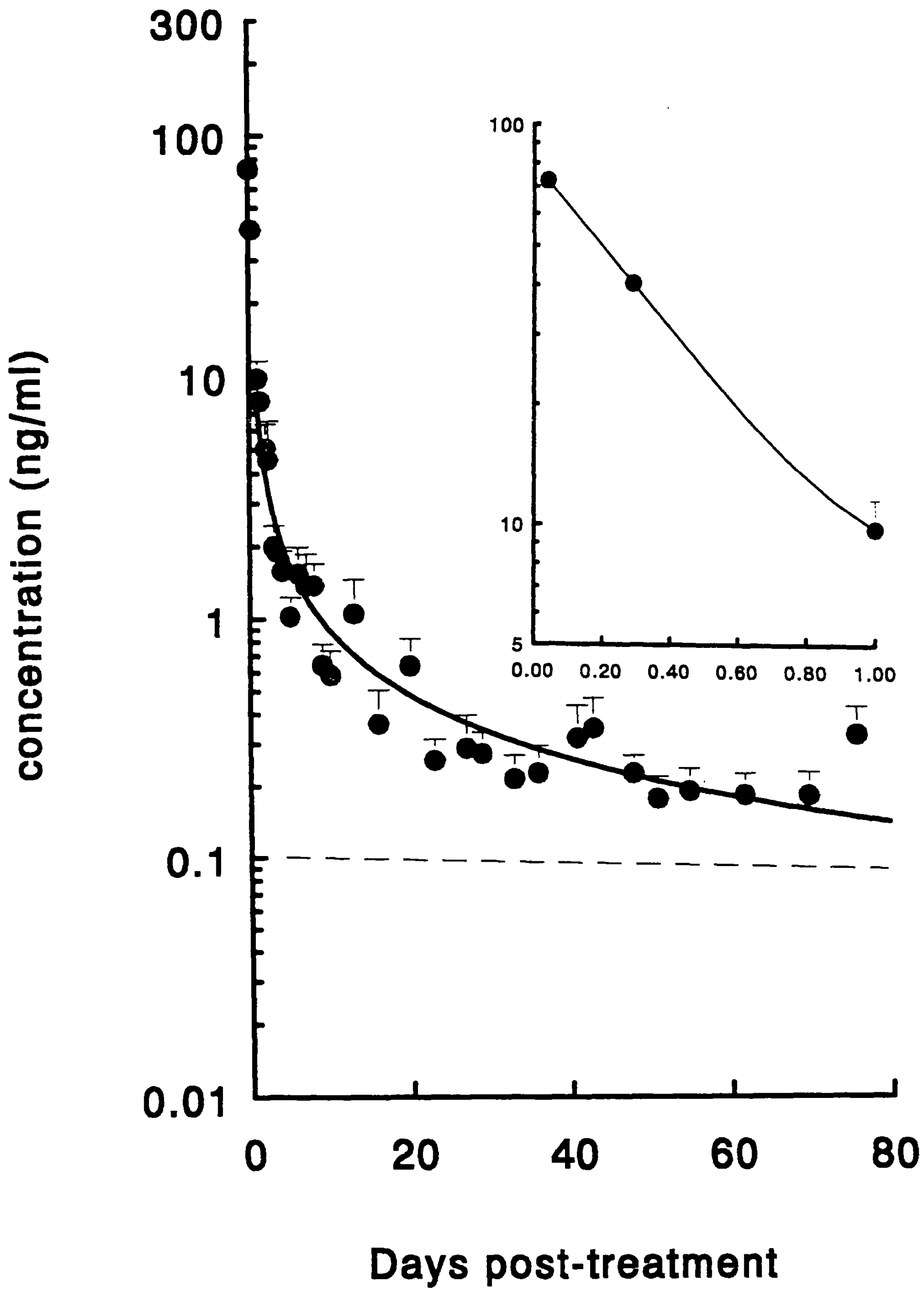


Fig. 4.4

The mean (±SD; n = 5) serum homidium concentration in Friesian cattle following i.m. treatment with homidium bromide at 1 mg kg^{-1} b.w.

Fig. 4.4



determined since the first sample was collected one hour following treatment at which time the drug was already in the distribution phase.

Non-compartmental pharmacokinetic analysis

The pharmacokinetic parameters are given in Table 4.3. The extrapolated observed mean AUC and AUMC values were 1935±397 ng.h ml^{-1} (range 1245 - 2345) and 890244±466474 $ng.h^2\ ml^{-1}$ (range 351600 - 1545188). The value for the $MRT_{0-\infty}$ observed was 439.7±185.1 hours (range 261.3 - 724.3).

A comparison of homidium pharmacokinetics in Friesian cattle following intravenous and intramuscular drug administration

Homidium serum concentration

A comparison of the mean (±SD) serum concentration versus time plot between i.v.-treated and i.m.-treated cattle is given in Fig. 4.5. A mean serum homidium concentration of 31.9±2.1 ng ml^{-1} obtained one hour following i.v. treatment was markedly lower than 72.5±2.2 ng ml^{-1} obtained one hour following i.m. treatment. Similar differences in drug concentrations were observed 24 hours following treatment; whilst a mean drug concentration of 3.9±0.4 ng ml^{-1} was obtained following i.v. treatment, the value was 9.8±1.8 ng ml^{-1} in the i.m.-treated cattle.

No drug was detectable after 17 days in four of the five cattle treated by the i.v. route whilst low levels remained in the circulation of five cattle for over 10 weeks following i.m. treatment.

Pharmacokinetic parameters

As shown in Table 4.4, a comparison in the non-compartmental pharmacokinetic parameters obtained from the i.v-treated cattle data and the

Table 4.3

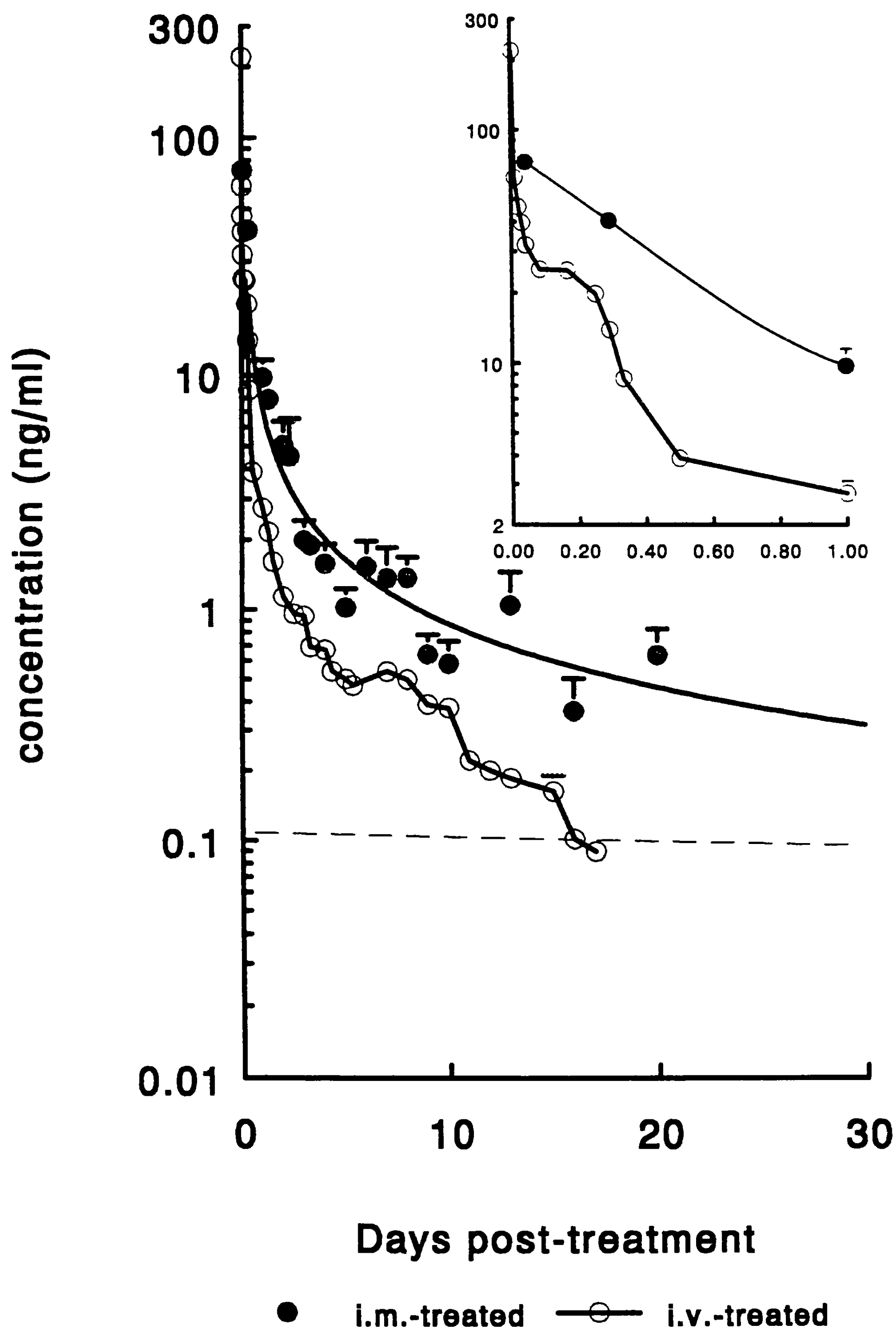
Pharmacokinetic parameters of homidium in normal Friesian cattle after intramuscular injection of homidium bromide at a dose rate of 1 mg kg^{-1} b.w. : Non-compartmental analysis

Parameter	Animal No.					Mean ± SD
	4	**11**	**16**	**17**	**18**	
β	0.002	0.002	0.001	0.001	0.002	0.002±0.001
t½β (h)	372.7	337.3	680.7	695.4	355.1	488.2±163.6
$AUC_{0\text{-last}}$ (ng.h ml^{-1})	1196	1712	1996	1862.6	2288	1811±361
$AUC_{0\text{-}\infty}$ observed (ng.h ml^{-1})	1245	1751	2202	2133	2345	1935±397
$AUC_{0\text{-}\infty}$ predicted (ng.h m^{-1})	1209	1725	2137	2019	231943	1882±388
$AUMC_{0\text{-last}}$ (ng.h^2 ml^{-1})	229198	360972	691565	733913	650890	533308±200646
$AUMC_{0\text{-}\infty}$ obs. (ng.h^2 ml^{-1})	351600	457477	1304932	1545188	792024	890244±466474
$AUMC_{0\text{-}\infty}$ pred. (ng.h^2 ml^{-1})	261310	394590	1109941	1203133	729285	739652±374143
$MRT_{0\text{-last}}$ (h)	191.6	210.9	346.5	394.0	284.5	285.5±77.3
$MRT_{0\text{-}\infty}$ observed (h)	282.5	261.3	592.5	724.3	337.8	439.7±185.1
$MRT_{0\text{-}\infty}$ predicted (h)	216.2	228.7	519.5	595.8	314.4	374.9±154.9
$MAT_{0\text{-}\infty}$ observed (h)	219.5	198.3	529.5	661.3	274.8	376.7±185.1
% F	47.0	33.4	26.6	27.5	25.0	31.9±8.8

Fig. 4.5

A comparison of the mean ± SD (n = 5) serum homidium concentration between i.v.-treated and i.m.-treated Friesian cattle following treatment with homidium bromide at 1 mg kg^{-1} b.w.

Fig. 4.5



i.m.-treated cattle data showed that the values from the i.v.-treated cattle were all highly significantly lower than those obtained in cattle treated by the intramuscular injection ($p < 0.05$; Table 4.4). Using the i.v. data, the bioavailability of the intramuscular dose was calculated as 31.9±8.1% and the MAT as 376.7±185.1 hours.

SECTION TWO

Homidium pharmacokinetics in Boran cattle following intramuscular drug administration and comparison with data obtained from Friesian cattle

Materials and Methods

Drug administration, blood sample collection, data handling and pharmacokinetic evaluation were as described in Section One of the present Chapter.

Experimental cattle

Five Galana Boran (*Bos indicus*) cattle used in this study were bred at the Kenya Trypanosomiasis Research Institute (KETRI), Kikuyu, Kenya, and had no previous exposure to tsetse and/or trypanocidal drugs. The cattle were aged between 16 and 20 months, and weighed between 180 and 200 kg. Their management before and during the experiments were as described in Chapter 2.

Experimental design

All five Boran cattle were treated with homidium bromide by deep i.m. injection at a dose rate of 1 mg kg^{-1} b.w. into the neck muscles. Serum samples were then collected for a period of 90 days following i.m. treatment. The sera

Table 4.4

A comparison of the mean ± SD (n = 5) of the pharmacokinetic parameters of homidium in normal Friesian cattle following either intravenous or intramuscular injection of homidium bromide at a dose rate of 1 mg kg^{-1} b.w.: Non-compartmental analysis

Parameter	intravenous	intramuscular	Significance
β	0.0122±0.0003	0.002±0.001	***
$t½\beta$ (h)	58.58±1.52	488.2±163.6	**
$AUC_{0\text{-last}}$ (ng.h ml^{-1})	582.5±69.4	1811±361	**
$AUC_{0\text{-}\infty}$ observed (ng.h ml^{-1})	585.6±67.9	1935±397	**
$AUC_{0\text{-}\infty}$ predicted (ng.h ml^{-1})	583.7±69.5	1882±388	**
$AUMC_{0\text{-last}}$ (ng.h^2 ml^{-1})	34877±5480	533308±200646	**
$AUMC_{0\text{-}\infty}$ observed (ng.h^2 ml^{-1})	36757±4754	890244±466474	*
$AUMC_{0\text{-}\infty}$ predicted (ng.h^2 ml^{-1})	35589±5575	739652±374143	*
$MRT_{0\text{-last}}$ (h)	59.87±5.63	285.5±77.3	**
$MRT_{0\text{-}\infty}$ observed (h)	62.98±5.91	439.7±185.1	*
$MRT_{0\text{-}\infty}$ predicted (h)	60.98±5.81	374.9±154.9	*

*** $p < 0.001$

** $p < 0.01$

* $p < 0.05$

were thereafter analysed using the second of the two homidium assays (Assay 2) described in Chapter 3. Non-compartmental pharmacokinetic analysis was carried out on the serum homidium concentration-versus-time data. The pharmacokinetic parameters obtained were compared to those obtained in similarly treated Friesian cattle reported in Section One of the present Chapter.

Results

Serum homidium concentrations

Following treatment of the Boran cattle, there was a rapid decline in the serum homidium concentrations within the first 24 hours. Plots of the serum homidium concentration-versus-time data for the five individual cattle are given in Figs. 4.6(a) to 4.6(e). The mean serum homidium concentration after one hour following drug administration was 112.1±40.3 ng ml^{-1}. This declined to 13.0±3.3 ng ml^{-1} in 24 hours. Sixty and 90 days following treatment, these values were 0.18±0.05 ng ml^{-1} and 0.17±0.09 ng ml^{-1}, respectively. The mean (±SD, n = 5) serum homidium concentration versus time plot following treatment of Boran cattle is shown in Fig. 4.7.

Non-compartmental pharmacokinetic analysis

The pharmacokinetic parameters obtained from non-compartmental analysis of the i.m. data are shown in Table 4.5. The AUC and the AUMC values were 2056±341 ng.h ml^{-1} (range 1585 - 2401) and 177351±37189 ng.h^2 ml^{-1} (133392 - 219725), respectively. The mean value for MRT calculated from extrapolated observed AUC and AUMC values was 92.3±21.1 hours (range 61.9 - 123.2).

Fig. 4.6(a) to 4.6(e)

Serum homidium concentration in five individual Boran cattle following i.m. treatment with homidium bromide at 1 mg kg^{-1} b.w.

Fig. 4.6(a)
Ainimal No. 295

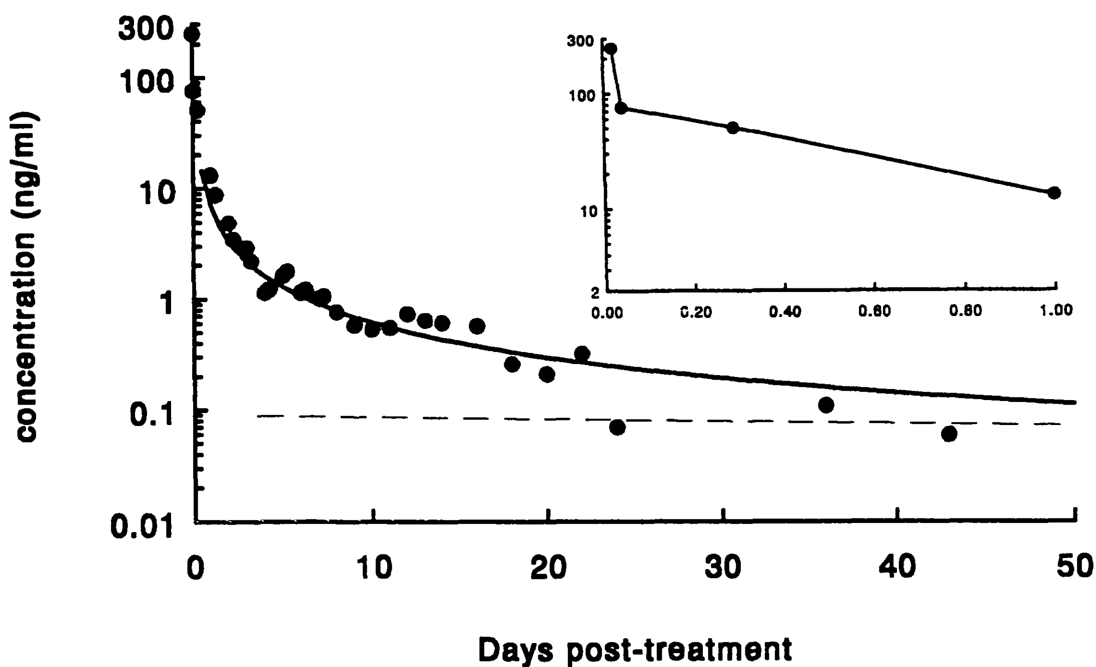


Fig. 4.6(b)
Animal No. 298

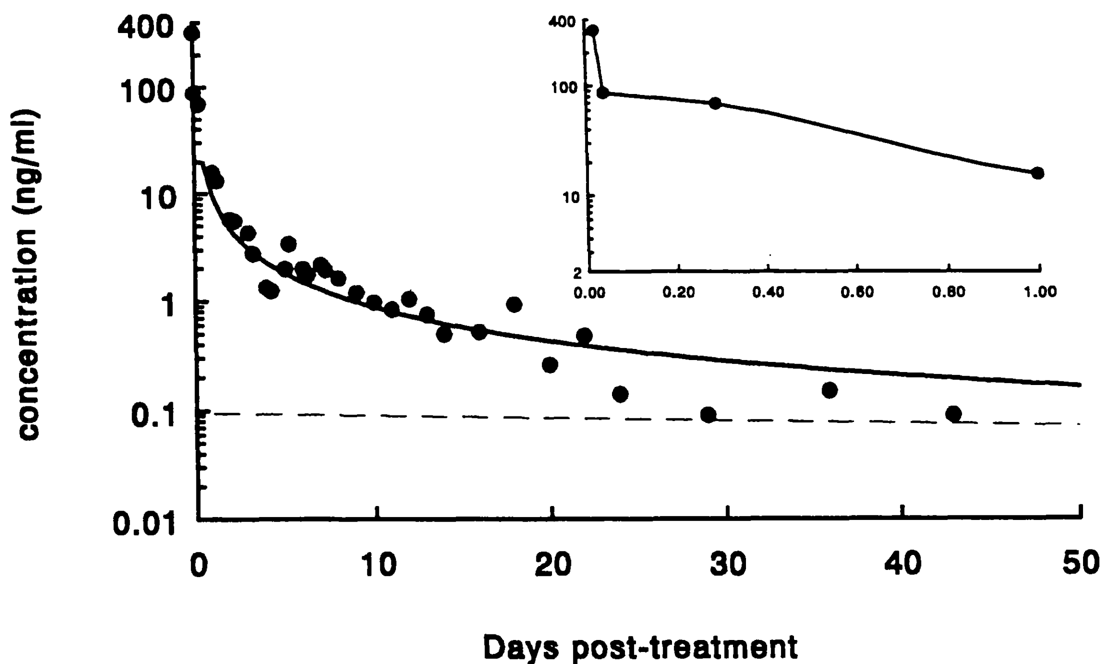


Fig. 4.6(c)
Animal No. 308

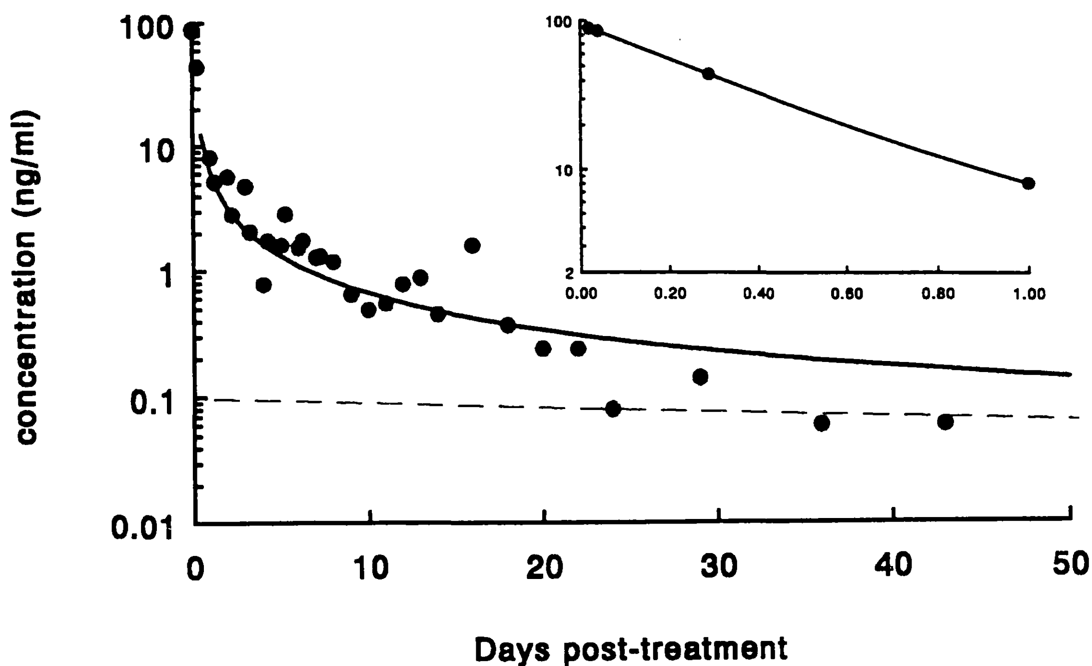


Fig. 4.6(d)
Animal No. 310

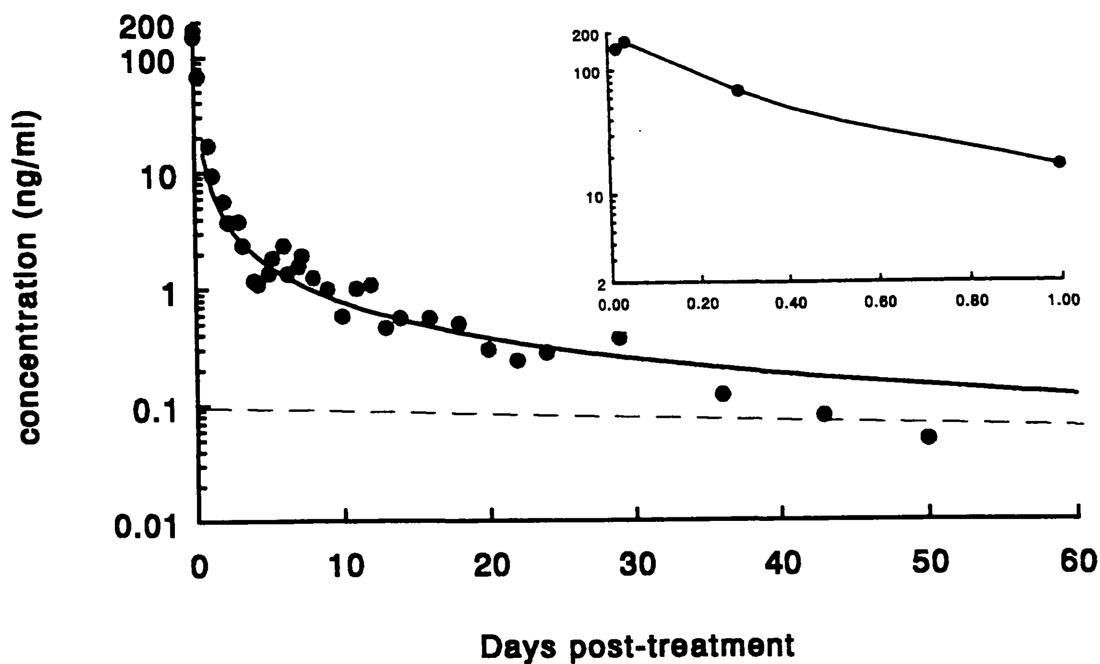


Fig. 4.6(e)
Animal No. 323

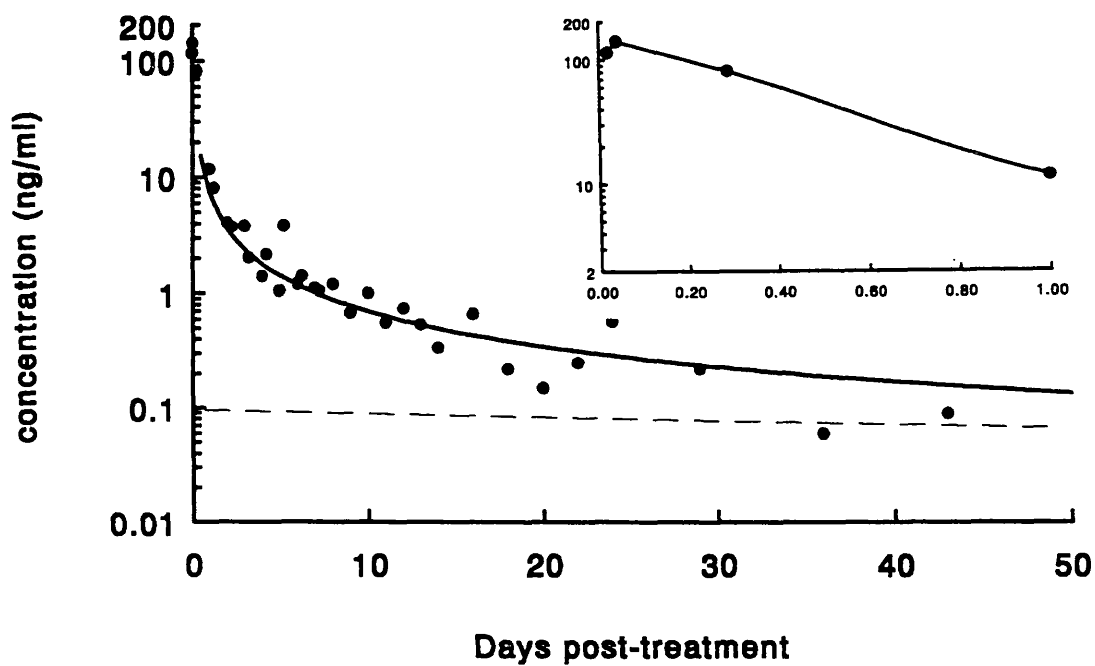


Fig. 4.7

The mean (±SD; n = 5) serum homidium concentrations in Boran cattle following i.m. treatment with homidium bromide at 1 mg kg^{-1} b.w.

Fig. 4.7

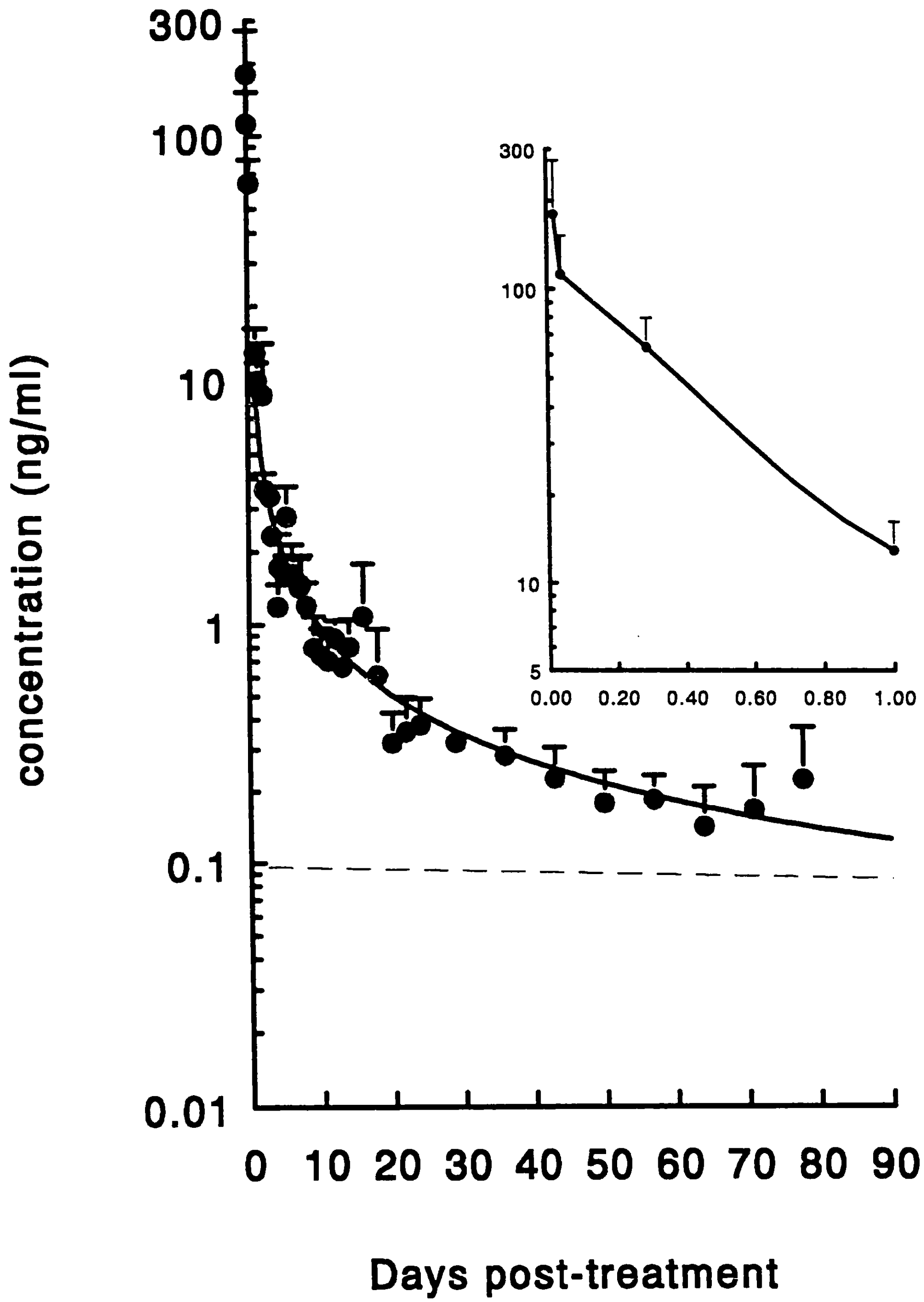


Table 4.5

Pharmacokinetic parameters of homidium in normal Boran cattle after intramuscular injection of homidium bromide at a dose rate of 1 mg kg^{-1} b.w. : Non-compartmental pharmacokinetic analysis

Parameter			Animal	Nos.		Mean ± SD
	295	298	308	310	323	Mean ± SD
β	0.005	0.004	0.003	0.004	0.005	0.004±0.001
t½β (h)	154.2	187.8	210.8	188.3	148.1	177.8±23.4
$AUC_{0\text{-last}}$ (ng.h ml^{-1})	1705	2337	1576	2398	2239	2051±341
$AUC_{0\text{-}\infty}$ observed (ng.h ml^{-1})	1705	2350	1585	2401	2236	2056±341
$AUC_{0\text{-}\infty}$ predicted (ng.h m^{-1})	1706	2339	1579	2400	2249	205453±342
$AUMC_{0\text{-last}}$ (ng.h^2 ml^{-1})	133392	219725	177005	218223	138408	177351±37189
$AUMC_{0\text{-}\infty}$ obs. (ng.h^2 ml^{-1})	133392	219725	177005	218223	138408	177351±37189
$AUMC_{0\text{-}\infty}$ pred. (ng.h^2 ml^{-1})	135859	223578	181956	221321	140859	180714±37651
$MRT_{0\text{-last}}$ (h)	78.2	94.0	112.3	91.0	61.8	87.5±16.8
$MRT_{0\text{-}\infty}$ observed (h)	78.2	104.9	123.2	93.1	61.9	92.3±21.1
$MRT_{0\text{-}\infty}$ predicted (h)	79.6	95.6	115.3	92.2	59.8	89.1±17.5

A comparison of serum homidium concentrations and pharmacokinetics between Friesian and Boran cattle following intramuscular treatment

Serum homidium concentrations

Fig. 4.8 shows a comparison of the mean ± SD (n = 5) serum homidium concentration-versus-time profiles between the Boran and Friesian cattle following i.m. treatment. The results show that the drug profiles were similar between the two breeds of cattle. However, the mean drug concentration of 112.1±40.3 ng ml^{-1} obtained one hour following treatment of Boran cattle was markedly higher than the value of 72.5±2.2 ng ml^{-1} obtained in the Friesian cattle. A wider variability in concentration one hour following treatment was observed between individual Boran cattle (66.0 - 169.6 ng ml^{-1}) than between individual Friesian cattle (65.9 - 78.4 ng ml^{-1}).

Pharmacokinetic parameters

Table 4.6 gives a comparison of the pharmacokinetic parameters for the Boran and Friesian cattle. The AUC values obtained were not significantly different from those obtained in Friesian. The first sampling time following treatment of the Boran cattle was 30 minutes whilst that of the Friesian was one hour. Therefore, the C_{max} and t_{max} were not determined since the drug was already in the distribution phase at both times. The mean residence time of 92.3±21.1 hours (range 61.9 - 123.2) obtained in Boran cattle was significantly lower than that obtained in the Friesian of 439.7±185.1 hours ($p < 0.01$).

Fig. 4.8

A comparison of the mean ± SD (n = 5) serum homidium concentration between Friesian and Boran cattle following i.m. treatment with homidium bromide at 1 mg kg^{-1} b.w.

Fig. 4.8

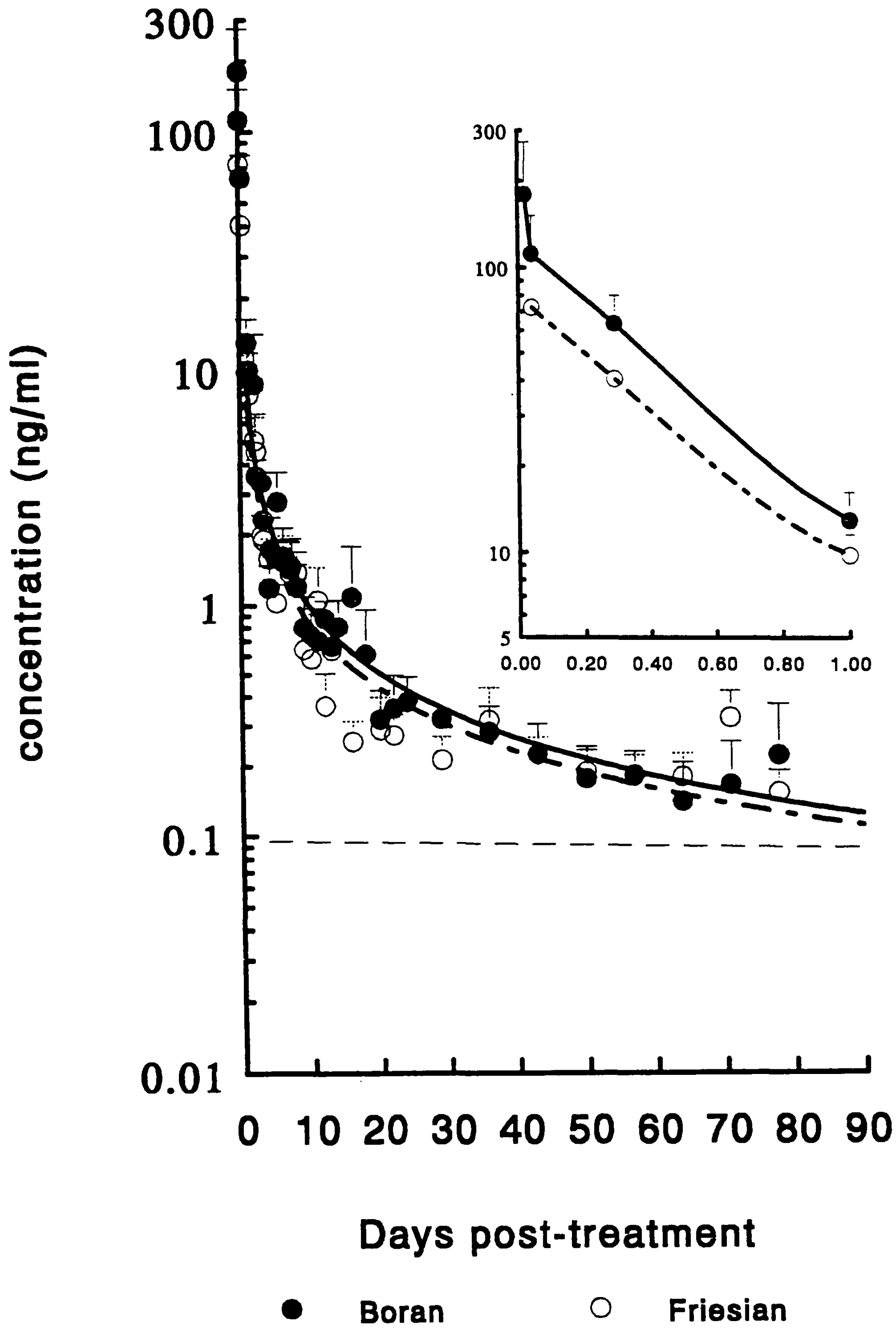


Table 4.6

Comparison of pharmacokinetic parameters of homidium between Friesian and Boran cattle following intramuscular injection of homidium bromide at a dose rate of 1 mg kg^{-1} b.w. : Assay 2, Non-compartmental analysis

Parameter	Friesian	Boran	Significance
tmax (h)	nd	nd	
Cmax (ng ml^{-1})	nd	nd	
β	0.002±0.001	0.004±0.001	***
$t½\beta$ (h)	488.2±163.6	177.8±23.4	***
$AUC_{0\text{-last}}$ (ng.h ml^{-1})	1811±361.2	2051±341.4	
$AUC_{0\text{-}\infty}$ observed (ng.h ml^{-1})	1935±397.3	2056±341.4	
$AUC_{0\text{-}\infty}$ predicted (ng.h ml^{-1})	1882±388	205453±342	
$AUMC_{0\text{-last}}$ (ng.h^2 ml^{-1})	533308±200646	177351±37189	
$AUMC_{0\text{-}\infty}$ observed (ng.h^2 ml^{-1})	890244±466474	177351±37189	*
$AUMC_{0\text{-}\infty}$ predicted (ng.h^2 ml^{-1})	739652±374143	180714±37651	*
$MRT_{0\text{-last}}$ (h)	285.5±77.3	87.5±16.8	**
$MRT_{0\text{-}\infty}$ observed (h)	439.7±185.1	92.3±21.1	*
$MRT_{0\text{-}\infty}$ predicted (h)	374.9±154.9	89.1±17.5	*

*** $p < 0.001$

** $p < 0.01$

* $p < 0.05$

nd = not determined

DISCUSSION

This Chapter describes investigations into the pharmacokinetics of homidium bromide in Friesian cattle following i.v. and i.m. treatment, and in Boran cattle following i.m. treatment. The studies were made possible following successful development and validation of the highly sensitive ELISA methods reported in Chapter 3, one of which was used. The results showed rapid elimination of the drug from circulation within approximately three weeks following i.v. treatment whilst the drug could be monitored successfully for periods over 10 weeks following i.m. treatment.

The results obtained in Friesian cattle following i.v. treatment showed a rapid exponential decline in the serum homidium concentrations (Fig. 4.2). Following i.v. treatment, the mean drug concentrations had declined from over 200 ng ml^{-1} at five minutes to approximately 30 ng ml^{-1} at one hour. Within 24 hours the value was about 4 ng ml^{-1}. No drug was detectable within 17 days of treatment in four of five cattle following i.v. treatment. However, following i.m. treatment of the Friesian cattle, the mean homidium levels after one hour and 24 hours were approximately 70 ng ml^{-1} and 10 ng ml^{-1} respectively showing the decline was more rapid in the i.v. treated cattle than in the i.m. treated cattle. Levels of about 0.2 ng ml^{-1} were detected within 90 days in the cattle treated by i.m. injection. These results suggest rapid elimination of the drug from the body following i.v. treatment and that the low levels in circulation approximately three weeks following i.m treatment could be attributed to the injection site drug depot.

In the pharmacokinetic evaluation, the results obtained by fitting an open compartmental model showed that the i.v. data was best described by tri-exponential equations. The half-lives obtained were 0.064±0.037 hours for $t½\alpha$, 7.17±1.87 hours for $t½\beta$ and 106.3±6.6 hours for $t½\gamma$. These results showed rapid distribution of the drug within minutes of treatment. The low AUC and

MRT values reflect the rapid disappearance of the drug from serum following i.v. treatment.

The differences observed in values for the rate constants between k_{12} and k_{21} and also between k_{13} and k_{31} showed that no equilibrium was established between the free drug concentrations in the blood and tissues and therefore, no steady state. Despite the non-attainment of a steady state in the drug distribution/elimination, the calculated volume of distribution in all the five animals was extremely large. The volume of the central compartment of 2.81±2.45 l kg^{-1} and that of apparent volume of distribution of 111.6± 18.0 l kg^{-1} were extremely large and far exceeded the normal plasma volume for cattle of approximately 35.9 ml kg^{-1} and the normal total blood volume of approximately 51.2 ml kg^{-1} (Ruckebusch et al, 1991). These large calculated volumes of distribution suggest extensive extravascular distribution of the drug. This observation also suggests that the serum drug concentration-versus-time data could have fitted models with more than three compartments. Determination of the rate constants k_{12}, k_{21}, k_{13} and k_{31} could possibly give an insight into the interactions between homidium and the host following treatment. Determination of these constants revealed that homidium movement into tissues was unsaturated at the recommended dose of 1 mg kg^{-1}.

Use of a non-compartmental model to fit the data from Friesian cattle treated by i.v. injection was necessary so that the extrapolated AUC and MRT values could be calculated. The results showed no marked differences in the AUC and the MRT values between the two pharmacokinetic models, compartmental and non-compartmental. In the determination of MAT in the i.m treated Friesian cattle, the mean MRT value from the non-compartmental model fitting of the i.v. data was therefore, used.

Following the establishment of pharmacokinetic parameters of homidium in Friesian after both i.v. or i.m drug administration, a study was carried out in Boran cattle, a breed indigenous to the tsetse infested areas of

sub-Saharan Africa following i.m. treatment. After i.m. treatment of Boran cattle, the mean serum homidium concentration obtained one hour following treatment was approximately 115 ng ml^{-1}. This level fell to about 15 ng ml^{-1} and 0.2 ng ml^{-1} after 24 hours and 90 days respectively, following drug administration. In the Friesian cattle these values were approximately 70 ng ml^{-1}, 10 ng ml^{-1} and 0.2 ng ml^{-1} after one hour, 24 hours and 90 days, respectively following i.m. treatment. Although the mean serum homidium concentrations obtained in Boran and Friesian cattle were not significantly different after 24 hours following treatment, those in the Boran showed a wider variability between individual animals than those in the Friesian. The mean (±SD) concentration versus time plots were however, not markedly different (Fig. 4.8).

AUC values for Friesian and Boran cattle were not significantly different. However, there were significant differences in the estimated AUMC values ($p < 0.05$) which were probably due to the differences in the initial sampling periods. Usually, the AUC is estimated from the drug concentration versus time plot but the AUMC is estimated from the plot of the product of drug concentration and time versus time. The estimated values for the two parameters would therefore be inherently different. The first sampling time following i.m. treatment was 30 minutes in Boran and one hour in Friesian cattle. Since, the estimation of the AUMC is based on the plot of the product of concentration and time versus time, the differences in the initial sampling times between the two breeds of cattle could have been responsible for the large differences observed in the AUMC values. This suggests that the time intervals at which samples are collected will clearly influence the estimation of the secondary pharmacokinetic parameters. A protocol that included as many data points as possible around the serum drug concentration peak would give more accurate estimates of the secondary pharmacokinetic parameters. The sampling protocol used in the present study had only three data points up to 24

hours following treatment. The estimated parameters were therefore high due to the uncertainties in the estimation of the AUMC following i.m. injection.

Previous studies on homidium pharmacokinetics were conducted using a radiometric technique (Gilbert and Newton, 1982). Details of these studies and comparisons with the results of the ELISA method are discussed in Chapter 8. In the present study high values for Vd and the large differences observed between the rate constants k_{12} and k_{21} and between k_{13} and k_{31} suggested extensive extravascular distribution of the drug and the demonstrated unsaturable pharmacokinetics. Similar observations were made in results reported in Chapter 8 using radioisotopes. Besides the use of radiolabelled drug, no other earlier techniques were able to detect and monitor the drug for sufficiently long periods of time to make estimates of pharmacokinetic parameters.

The results of the present study have demonstrated non-attainment of equilibrium between the free serum homidium concentrations in the blood and tissues following treatment at the recommended therapeutic dose suggesting that the transport of the drug into host cells could be unsaturated at pharmacological concentrations.

It has been demonstrated that tissue levels of homidium remain high for long periods following either i.v. or i.m. drug administration (Gilbert and Newton, 1982) which suggests covalent binding of the drug to tissue proteins may occur. Contribution of these residues to the circulating drug concentrations appears negligible as has been shown by the results of the present study in which it was demonstrated that levels fell below the detection limit within 17 and 22 days in animals treated by the i.v. route. In the i.m. treated cattle however, levels in circulation remained detectable for over 10 weeks, suggesting that the injection site depot was the only primary source of possibly the intact drug from two weeks following treatment onwards.

Although the nature of binding of homidium to tissue proteins is not

well understood, Newton (1976) showed that the drug intercalates with DNA and has a high selectivity for mitochondrial DNA; a property that has enabled it to be used extensively as a biochemical probe. Other studies have shown that the binding of homidium to DNA *in vivo* may involve more than simple intercalation. Mahler and Bastos (1974), studying the uptake of ^{3}H-homidium by *Saccharomyces cerevisiae* found that homidium associated with mitochondrial DNA accounted for approximately 2.3% of total homidium bound by DNA. There was some evidence that it might be a (metabolic) derivative of homidium formed within the mitochondrion which becomes covalently bound to mitochondrial DNA.

Our present knowledge of homidium metabolism is limited. Gilbert and Newton (1982) investigated the possibility of metabolites in sera from ^{14}C homidium-treated rabbits and cattle (some infected with *T. congolense*) using thin layer chromatography (TLC) and gel chromatography. No evidence of metabolites was obtained using TLC, a relatively insensitive semi-quantitative technique, but two non-homidium radiolabelled components were detected in the urine and bile. These accounted for 46% (with one bearing 38%) of the total radioactivity eluted.

Results of the present studies and those reported previously (Gilbert and Newton , 1982) suggest rapid elimination of much of the injected dose of homidium. The present study using the more sensitive ELISA technique, has however, demonstrated measurable concentrations of homidium for longer periods than was possible using the earlier, less sensitive technique. Homidium could be detected with ELISA for as long as 17 days following intravenous treatment of cattle. Following intramuscular treatment, the drug was measurable for up to 10 weeks, a duration in keeping with the 8 to 17 week period of prophylaxis reported for homidium use in the field (Dolan et al., 1990). This suggests that the ELISA could prove to be a useful tool in investigating the relationship between the circulating drug concentrations and

protection against trypanosome challenge in chemoprophylactic situations, and might on long term basis assist in the planning of appropriate prophylactic and therapeutic regimens for field use. The relationships between homidium concentrations and chemotherapy and chemoprophylaxis of *T. congolense* infections in Boran cattle are investigated further in the two following Chapters (Chapters 5 and 6).

CHAPTER FIVE

INVESTIGATION INTO THE EFFECTS OF *TRYPANOSOMA CONGOLENSE* INFECTIONS ON THE PHARMACOKINETICS OF HOMIDIUM IN BORAN CATTLE

INTRODUCTION

Control of trypanosomiasis in endemic areas has depended largely on the use of chemotherapeutic or chemoprophylactic agents. One such agent is homidium which was classified mainly as a chemotherapeutic agent but has been reported to offer some protection against infection in the field (Mwambu, 1971; Dolan et al., 1990). After its introduction, several studies were carried out to determine the minimum curative and maximum tolerated doses (Wilson and Fairclough, 1953; Wilde and Robson,1953; Unsworth, 1954a, b). These studies are covered in detail in Chapter 1, Section Two.

The normal use of homidium has been in the treatment of infections due to both *T. congolense* and *T. vivax* in cattle, sheep and goats at the recommended dose rate of 1 mg kg^{-1} bodyweight (b.w.) and several million doses are administered to African livestock each year. However, so far there has been no detailed pharmacokinetic evaluation of the drug in either non-infected or trypanosome-infected cattle following treatment. The only previous study by Gilbert and Newton (1982) using ^{14}C homidium bromide, lacked details on pharmacokinetics.

The results of the study by Gilbert and Newton (1982) carried out in non-infected and trypanosome-infected rabbits and calves showed that there were no differences in homidium concentrations in blood and tissue fluids in both non-infected and *T. congolense*-infected cattle. Blood and tissue fluid drug levels reached maximum levels of 120 - 170 ng ml^{-1} one hour following i.m. treatment of calves at 1 mg kg^{-1} b.w. The levels fell rapidly within 24 hours and then slowly to 15 ng ml^{-1} in 8 days. Separation of trypanosomes from other blood components in samples collected from an ear vein at 1, 6 and 12 hours after treatment of calves at 1 mg kg^{-1} demonstrated that approximately 80% of total radioactivity detected in the blood was bound to trypanosomes.

Following i.m. treatment of non-infected rabbits at 1 mg kg^{-1}, Gilbert and Newton (1982) showed that serum homidium concentrations reached maximum levels of 180 ng ml^{-1} in blood and 50 ng ml^{-1} in tissue fluid one hour following i.m. treatment. These levels fell to less than 10 ng ml^{-1} within 96 hours. Increase in the dose rate from 1 to 10 mg kg^{-1} b.w. increased the blood and tissue drug levels by only 2 - 3 fold. Intramuscular treatment of rabbits at 1 mg kg^{-1} or 10 mg kg^{-1} 8 days after *T. congolense* infection ($1x10^6$ trypanosomes) caused parasitaemia to become sub-patent within 48 hours. No cure was effected despite the fact that these trypanosomes were drug-sensitive. Infections initiated in mice from stabilates or by sub-inoculation from infected rabbit blood after homidium treatment were always cured by homidium at 1 mg kg^{-1} b.w. after the infection had become patent.

They also demonstrated that infection of previously non-infected and re-infection of infected rabbits 5 days after treatment with 1 mg kg^{-1} revealed an apparent prophylactic effect in the case of the infected animal. The pre-patent period lasted 8 days compared to 3 days observed in either non-infected rabbits or homidium-treated non-infected rabbits. Despite these observations in both non-infected and infected rabbits, the drug concentrations in the blood and tissue fluid were found to be similar.

Determination of anti-trypanosome antibody in the sera from *T. congolense* and *T. brucei*-treated rabbits revealed that the levels of antibody were unaffected by the homidium treatment. The trypanocidal activity in the serum samples, measured by the method of Goodwin and Tierney (1977) was compared with the levels of radioactivity in the same sera. Their results showed higher levels of trypanocidal activity following i.m. treatment of infected rabbits with homidium than in the non-infected rabbits. Following treatment of infected rabbits, Gilbert and Newton (1982) observed a smaller initial fall in activity during the first 3 days after treatment after which serum trypanocidal activity was sustained for a further 30 days.

Removal of IgG from serum taken 5 hours after homidium treatment of infected rabbits resulted in the reduction of the trypanocidal activity to 46% that of serum from which IgG had not been removed, taken at the same time. Removal of IgG from serum taken 10 and 19 days after homidium treatment caused a complete loss of *in vitro* trypanocidal activity. In both cases, all trypanocidal activity was present in the IgG containing fraction. Removal of the IgG from sera of homidium-treated non-infected rabbits did not affect the *in vitro* trypanocidal activity of these samples.

Despite the observations involving homidium as a chemotherapeutic drug, little information was previously available on the effects that trypanosome infections might have on the pharmacokinetics of the drug and subsequent prophylactic activity. The availability of the highly sensitive ELISA method (Chapter 3, Assay 2) for the detection and monitoring of homidium levels in cattle has made the present study possible.

STUDY OBJECTIVES

The objectives of this study were:

(a) To determine the pharmacokinetics of homidium in cattle, using ELISA, infected with one of two trypanosome populations of *T. congolense*; one drug-sensitive and the other drug-resistant.

(b) To compare the pharmacokinetics of homidium in cattle infected with *T. congolense* and in non-infected cattle (reported in Chapters 4 and 6).

MATERIALS AND METHODS

Experimental cattle

A total of 10 castrated male Boran calves obtained from Ol Maisor Farm, Laikipia District, Central Province, Kenya, were used. This area is free from endemic trypanosomiasis. The calves were six months old and weighed

between from 120 and 140 kg. The procedures followed in the management of the animals, before and during the experimental period, were as described in Chapter 2.

Pre-infection sera

Collection and storage of large pools of pre-infection sera were carried out as described for 'pre-treatment sera' in Chapter 2.

Trypanosomes

The trypanosome populations used and preparation of the infecting inoculum were as described in Chapter 2 (Materials and Methods).

Experimental design

On the day of infection the 10 animals were divided into two groups. One group of five calves was inoculated with $1x10^5$ homidium-sensitive trypanosomes (*T. congolense* IL 1180) whereas the other group of five calves was inoculated with $1x10^5$ homidium-resistant trypanosomes (*T. congolense* IL 3330). The inoculum was given by intravenous injection to all animals. All calves were treated with homidium bromide 7 days after trypanosomes were first demonstrated in the peripheral blood of all animals in each group.

Assessment of parasitaemia and PCV

Following infection, animals in both groups were monitored for parasitaemia and PCV. Ear vein blood samples were taken daily from the first day of infection in heparinised capillary tubes and the presence of trypanosomes and PCV determined as previously described in Chapter 2.

Drug treatment

Homidium bromide (Ethidium®, Camco, UK; Lot No. B4B3) was used to treat the animals following infection. A 2.5% (w/v) solution of the drug was prepared in sterile water immediately prior to treatment. The dose given was 1 mg kg^{-1} body weight (b.w.). The drug was given as a single bolus by deep intramuscular (i.m.) injection into the muscles of the neck.

Sample collection

Sampling for parasitaemia and PCV continued following treatment. In addition, blood samples were collected for drug assays at the following intervals after treatment: 5, 10, 15, 30 minutes, 1, 2, 4, 6, 8 and 12 hours, twice a day during the first week, daily during the second week, thrice during the third week, twice during the fourth week, and thereafter weekly to the end of the observation period of 90 days. Total serum protein and serum albumin levels were determined in all the samples.

Determination of total serum protein

Total serum protein was determined by using the Coomassie® Plus Protein Assay Reagent. The procedure followed is described in Chapter 2.

Determination of serum albumin

For the determination of serum albumin, a quantitative, colorimetric procedure (Procedure No. 631, Sigma Diagnostics) was used, as described elsewhere (Chapter 2).

Determination of homidium concentrations

Serum homidium concentrations were analysed using Assay 2 of the homidium-ELISA methods. This method is described in Chapter 3.

Handling of homidium ELISA data

The procedures followed in handling of ELISA are as described in Chapter 2.

Pharmacokinetic statistical evaluation

The procedures followed in the pharmacokinetic and statistical evaluation of the experimental data are as described in Chapter 4.

RESULTS

Boran cattle infected with a drug-susceptible population of *T.congolense* (IL 1180) and later treated with homidium bromide at 1 mg kg^{-1} b.w. by the intramuscular route

Parasitaemia

Fig. 5.1 shows the parasitaemia in cattle following trypanosome inoculation and the time of homidium treatment. Trypanosomes were first detected in the peripheral blood of four out of the five cattle in this group seven days following infection; the remaining animal was first detected parasitaemic on the eighth day. Individual animals exhibited different levels of parasitaemia, some animals being more heavily parasitised than others at the time of treatment, on day seven following demonstration of parasites in the peripheral blood of all the five animals (day 15 after trypanosome inoculation) (Table 5.1). On treatment with homidium at 1 mg kg^{-1} b.w., trypanosomes were cleared from the blood within 24 hours of drug administration in 4 out of the 5 cattle of the homidium-sensitive group, the remaining animal cleared within 48 hours (Table 5.1). This latter animal had the highest parasite density before treatment. All the animals remained negative to the end of the observation period, which was 90 days.

Fig. 5.1

A comparison of parasitaemia in Boran cattle treated (↓) with homidium at 1 mg kg^{-1} b.w. following infection with either a drug sensitive (*T. congolense* IL 1180) or a drug-resistant trypanosome population (*T. congolense* IL 3330)

Fig. 5.1

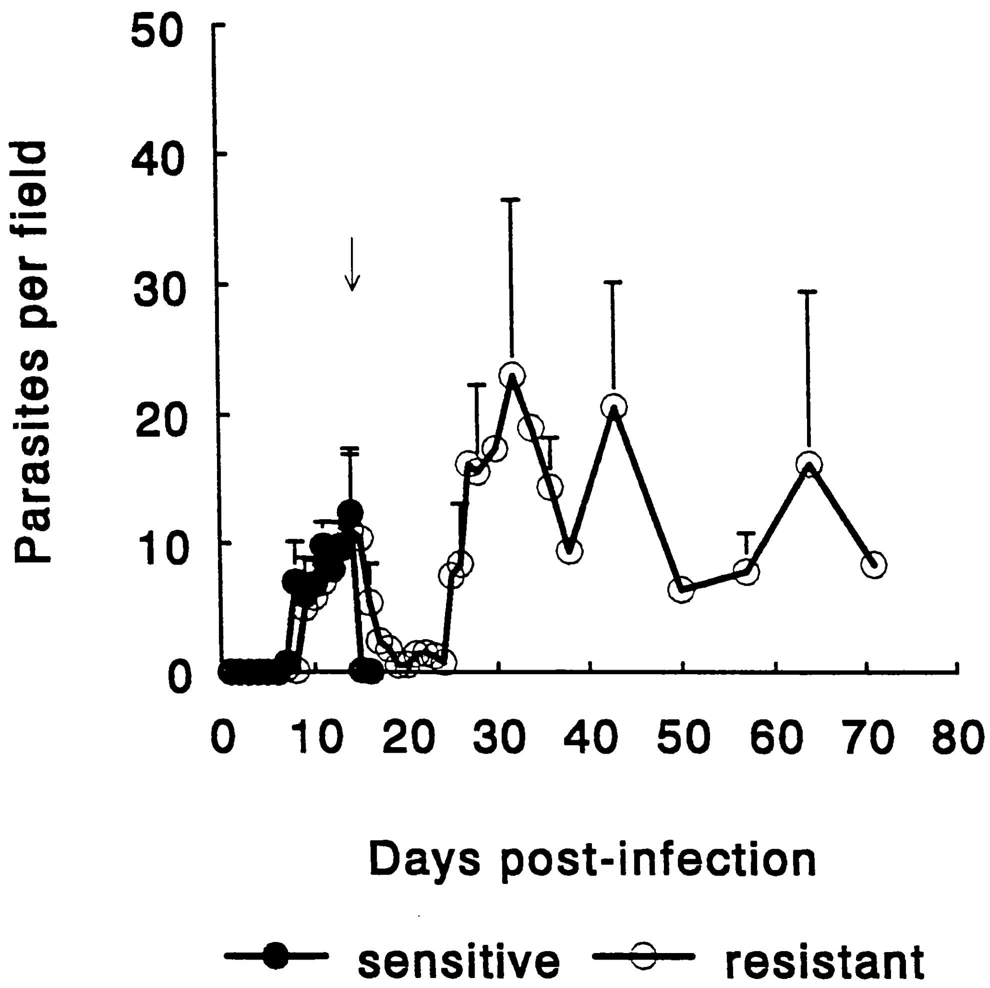


Table 5.1

No. of trypanosomes per field, estimated by the thick blood smear method (Mag. x 400), at the time of homidium treatment of cattle infected with either a drug-sensitive or a drug-resistant trypanosome population

Animal No.	No. of trypanosomes per field	Homidium conc. at 5 min. following treatment (ng ml^{-1})	Time taken to clear parasites following treatment
410†	16	34.1	24 hours
411†	12	57.9	24 hours
412†	20	74.1	48 hours
413†	8	40.9	24 hours
414†	8	174.7	24 hours
Mean ± SD	12.8±4.7	76.4±51.1	
415††	10	78.7	not cleared (resistant)
416††	20	104.3	not cleared (resistant)
417††	8	121.2	not cleared (resistant)
418††	14	173.1	not cleared (resistant)
419††	3	113.4	not cleared (resistant)
Mean ± SD	11.0±5.7	118.1±31.0	

† = cattle infected with the drug-sensitive trypanosome population

†† = cattle infected with the drug-resistant trypanosome population

Serum drug concentrations

Figs. 5.2(a) to 5.2(e) show the serum homidium concentration-versus-time plots of individual animals. The mean drug concentration 5 minutes following treatment was 76.4±51.1 ng ml^{-1} (range 34.1 - 174.7 ng ml^{-1}; Table 5.1). The mean ± SD peak serum concentration (C_{max}) was 180.4±34.7 ng ml^{-1} (range 112.0 - 204.3), and the mean time at which this peak occurred (t_{max}) was 0.226±0.029 hours (range 0.168 - 0.240). Only animal No. 413 had a t_{max} value of 0.17 hours. The value of 0.24 hours was obtained in all the remaining four animals of this group. By day 36 post-treatment, the concentration of homidium in serum ranged from 0.15 - 0.30 ng ml^{-1}.

Pharmacokinetics

An exponential decline in the serum concentration was observed and the non-compartmental pharmacokinetic model was used to describe the experimental data. Pharmacokinetic parameters are given in Table 5.2. The $AUC_{0-\infty}$ and the $AUMC_{0-\infty}$ observed values were 1667±233 ng.h ml^{-1} (range 1453 - 2117) and 480580±239402 ng.h^2 ml^{-1} (range 177106 - 806586), respectively. Using these values, the $MRT_{0-\infty}$ observed was calculated as 296.7 ±158.9 hours (range 121.9 - 504.6). The results showed wide variations in the MRT values between individual infected animals (Table 5.2).

Haematological indices

From the onset of parasitaemia, there was a rapid drop in PCV from a mean pre-infection value of 38.4±4.4% to 24.8±1.6% within 7 days of demonstration of parasites in the peripheral blood (Fig. 5.3). The PCV values returned to the pre-infection levels within one week of drug administration in all animals. The animals in this group fed well and generally gained weight throughout the experiment.

Figs. 5.2(a) to 5.2(e)

Serum homidium concentration in five individual Boran cattle treated with homidium at 1 mg kg^{-1} b.w. following infection with a homidium-sensitive *Trypanosoma congolense* population (IL 1180)

Fig. 5.2(a)
Animal No. 410

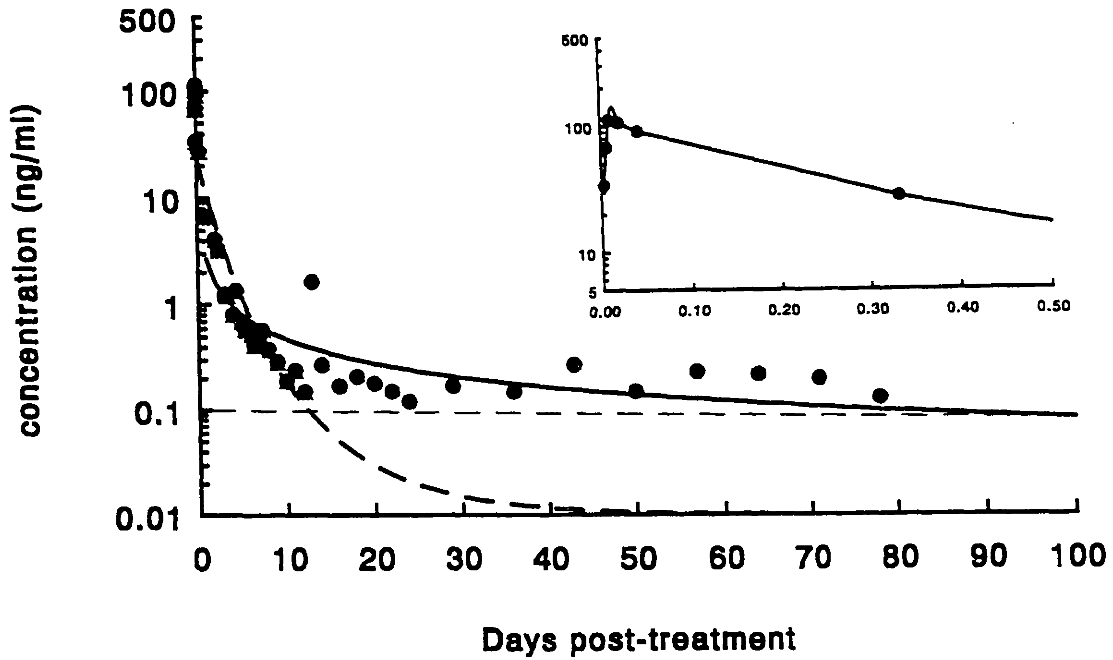


Fig. 5.2(b)
Animal No. 411

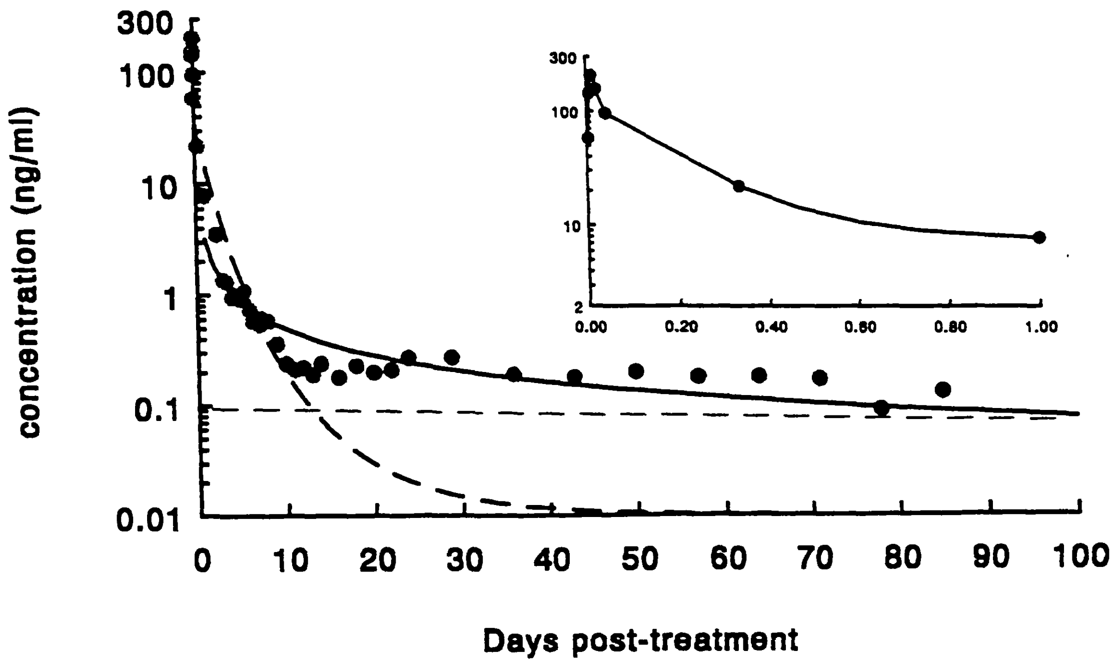


Fig. 5.2(c)
Animal No. 412

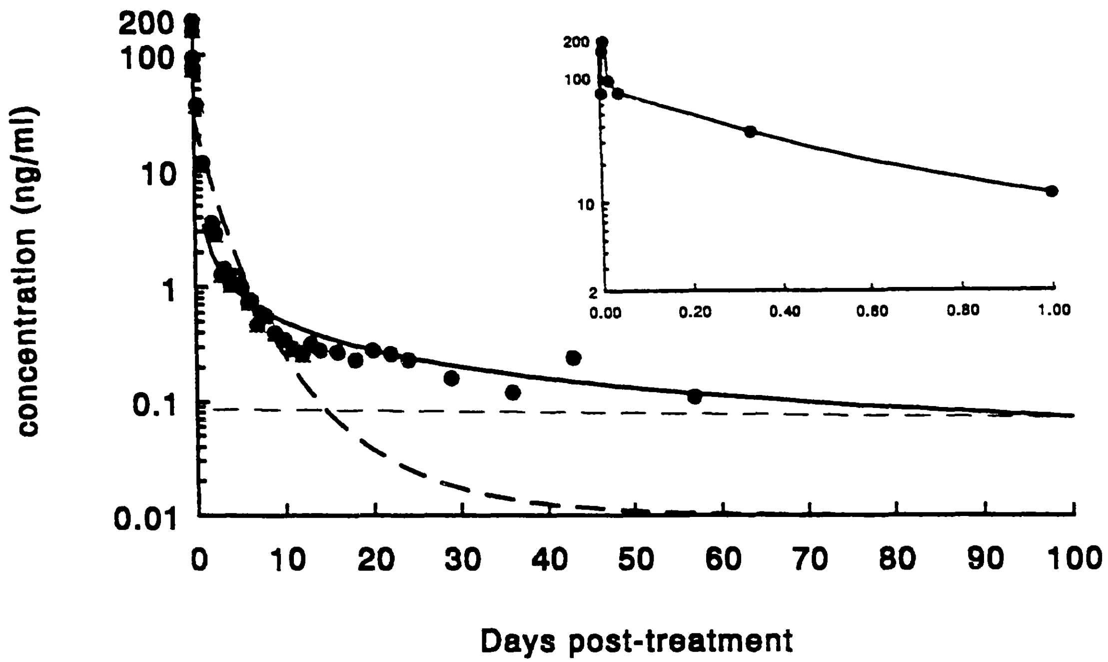


Fig. 5.2(d)
Animal No. 413

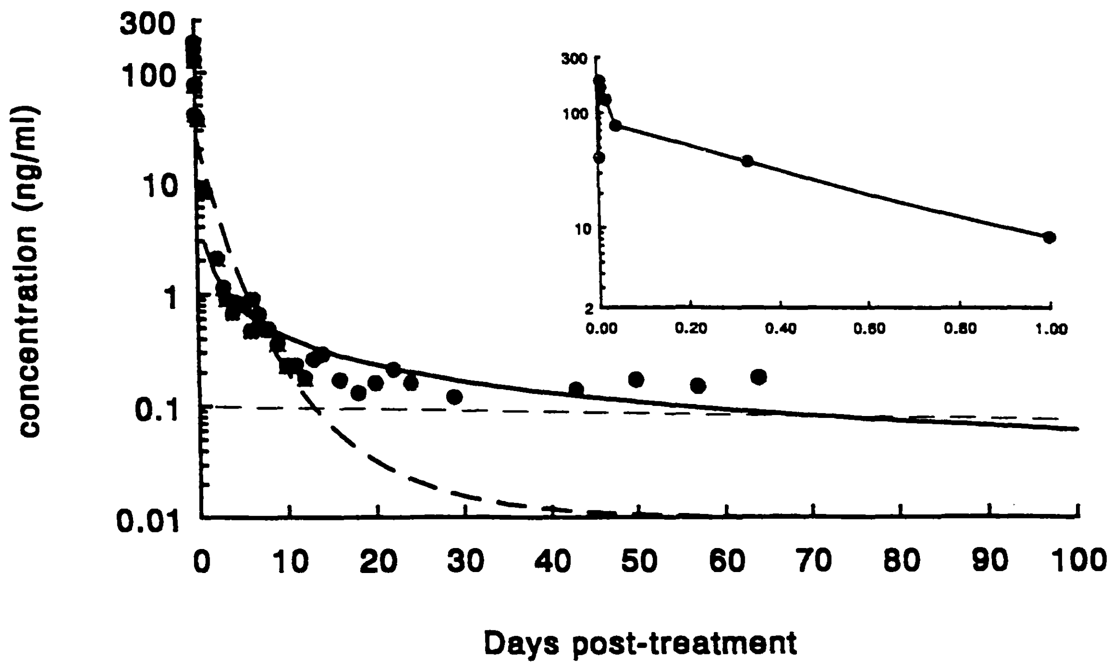


Fig. 5.2(e)
Animal No. 414

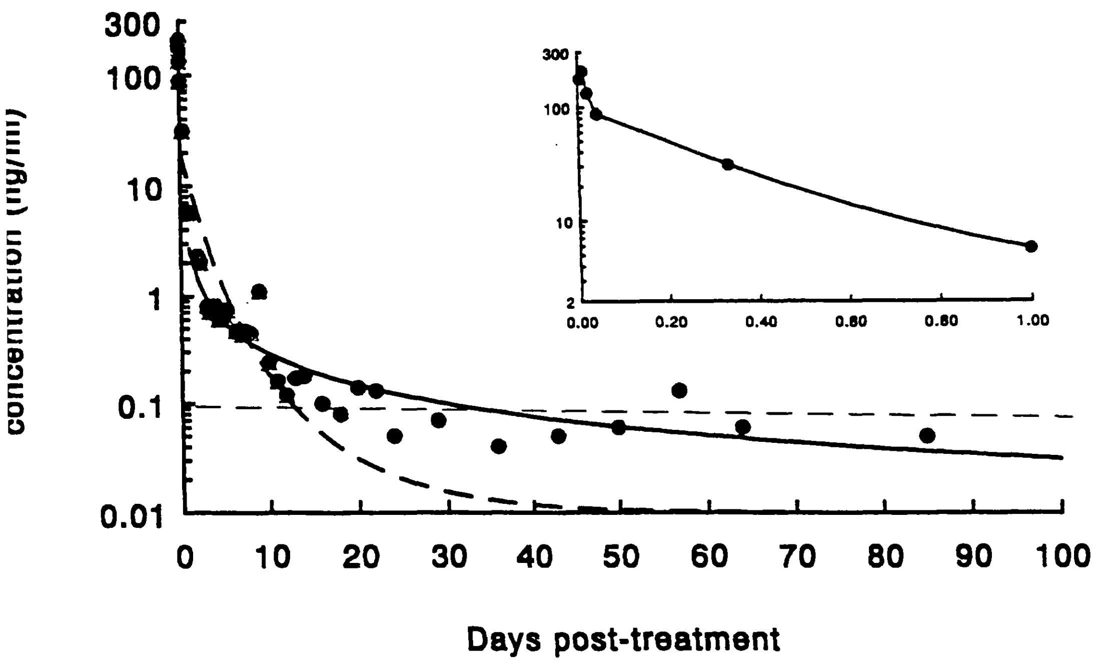


Table 5.2

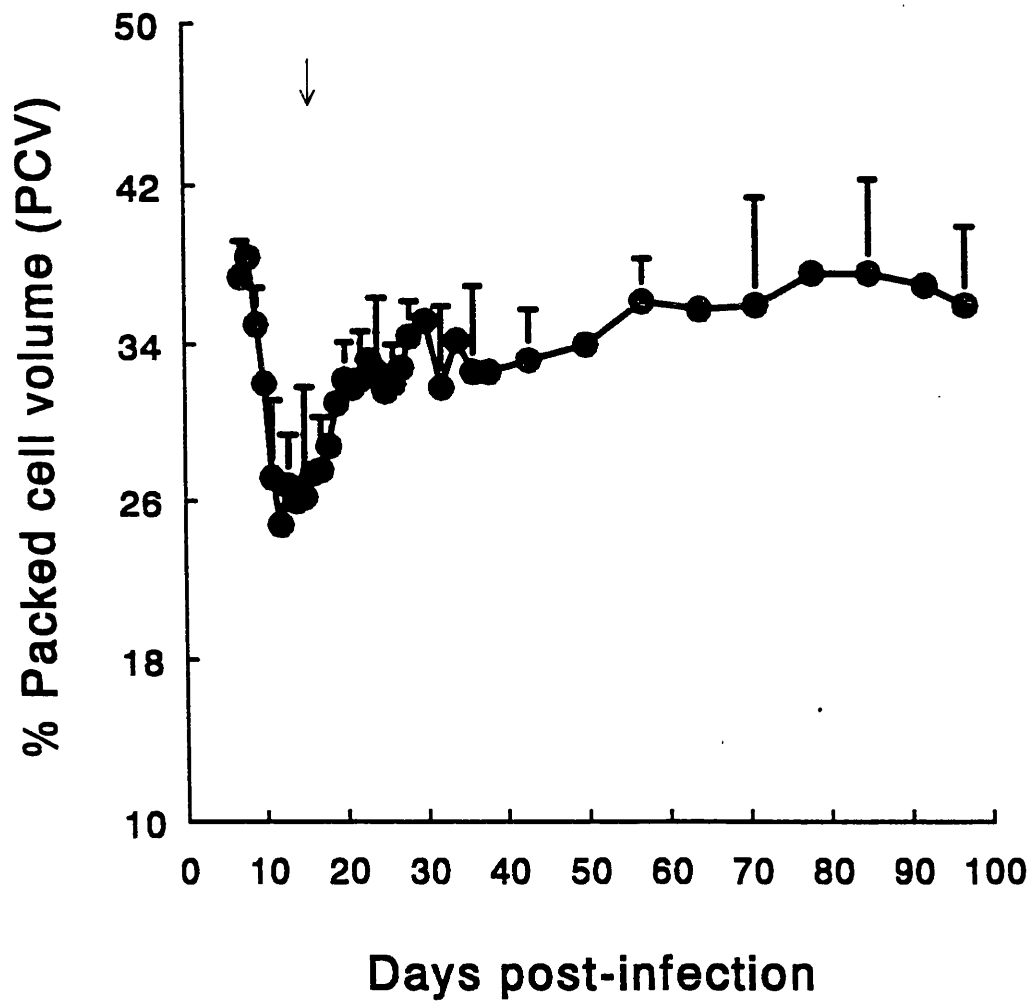
Pharmacokinetic parameters of homidium in Boran cattle infected with a sensitive population of *Trypanosoma congolense* (IL 1180) and later treated with homidium bromide at a dose rate of 1 mg kg^{-1} b.w.

Parameter	Animal No.					
	410	411	412	413	414	Mean ±SD
t_{max} (h)	0.24	0.24	0.24	0.17	0.24	0.22±0.03
C_{max} (ng ml^{-1})	112.0	204.3	194.4	187.8	203.7	180.4±34.7
β	0.0011	0.0013	0.0028	0.0019	0.0020	0.0018±0.0006
t½β (h)	655.6	519.0	245.3	358.8	339.6	423.7±145.6
$AUC_{0\text{-last}}$ (ng.h ml^{-1})	1475	1409	1453	1625	2117	1616±261
$AUC_{0\text{-}\infty}$ observed (ng.h ml^{-1})	1606	1536	1453	1625	2117	1667±233
$AUC_{0\text{-}\infty}$ predicted (ng.h ml^{-1})	1556	1465	1454	1642	2122	1648±247
$AUMC_{0\text{-last}}$ (ng.h^2 ml^{-1})	439434	368996	177106	425345	291257	340428±96872
$AUMC_{0\text{-}\infty}$ observed (ng.h^2 ml^{-1})	806586	702608	177106	425345	291257	480580±239402
$AUMC_{0\text{-}\infty}$ predicted (ng.h^2 ml^{-1})	680675	517926	181156	465474	305119	430070±172827
$MRT_{0\text{-last}}$ (h)	297.8	262.0	121.9	261.8	137.6	216.2±72.0
$MRT_{0\text{-}\infty}$ observed (h)	504.6	457.4	121.9	261.8	137.6	296.7±158.8
$MRT_{0\text{-}\infty}$ predicted (h)	437.4	353.4	124.6	283.5	143.8	268.5±120.2

Fig. 5.3

Mean ± SD (n = 5) packed cell volume (%) in Boran cattle treated (↓) with homidium at 1 mg kg^{-1} b.w. 15 days following inoculation with a homidium-sensitive trypanosome population (*T. congolense* IL 1180)

Fig. 5.3



Total serum proteins levels

The mean total serum protein of the five cattle obtained from pre-treatment sera was 46.05±2.25 mg ml^{-1}. Within 15 days of inoculation with the drug-sensitive *T. congolense* i.e., at the time of treatment, this value was 39.5±0.4 mg ml^{-1}, which was significantly different from the pre-infection value ($p < 0.05$). On treatment of the cattle the levels increased to the pre-treatment values within one week (Fig. 5.4) and continued to rise to an average value of approximately 50 mg ml^{-1} within 14 days of treatment, which was maintained to the end of the experimental period of 90 days.

Serum albumin levels

The mean pre-infection serum albumin value of the five cattle was 26.0±2.5 mg ml^{-1}. Fifteen days following trypanosome inoculation at the time of drug treatment, this value was 22.2±2.4 mg ml^{-1} (Fig 5.5). This value was not significantly different from that obtained before trypanosome inoculation. Pre-infection serum albumin levels were generally maintained to the end of the observation period of 90 days after treatment (Fig. 5.5).

Total serum globulin levels

Fig. 5.6 shows the total serum immunoglobulin levels in the five cattle infected with the homidium-sensitive trypanosome population. Total serum globulin levels were calculated from the difference between the total serum protein levels and the serum albumin levels. The results showed an initial drop in globulin levels during the first 14 days post-infection from pre-infection values of 20.4±1.5 mg ml^{-1} to approximately 16±1.9 mg ml^{-1}. Approximately 13 days following treatment, concentrations of 24.05±2.0 mg ml^{-1} were attained, peaking at approximately 30 days post-infection (approximately 15 days after treatment) (Fig. 5.6). After the peak, values of 24.9±1.8 mg ml^{-1}

Fig. 5.4

Mean ± SD (n = 5) total serum protein levels in Boran cattle treated (↓) with homidium at 1 mg kg^{-1} b.w. 15 days following inoculation with a homidium-sensitive trypanosome population (*T. congolense* IL 1180)

Fig. 5.4

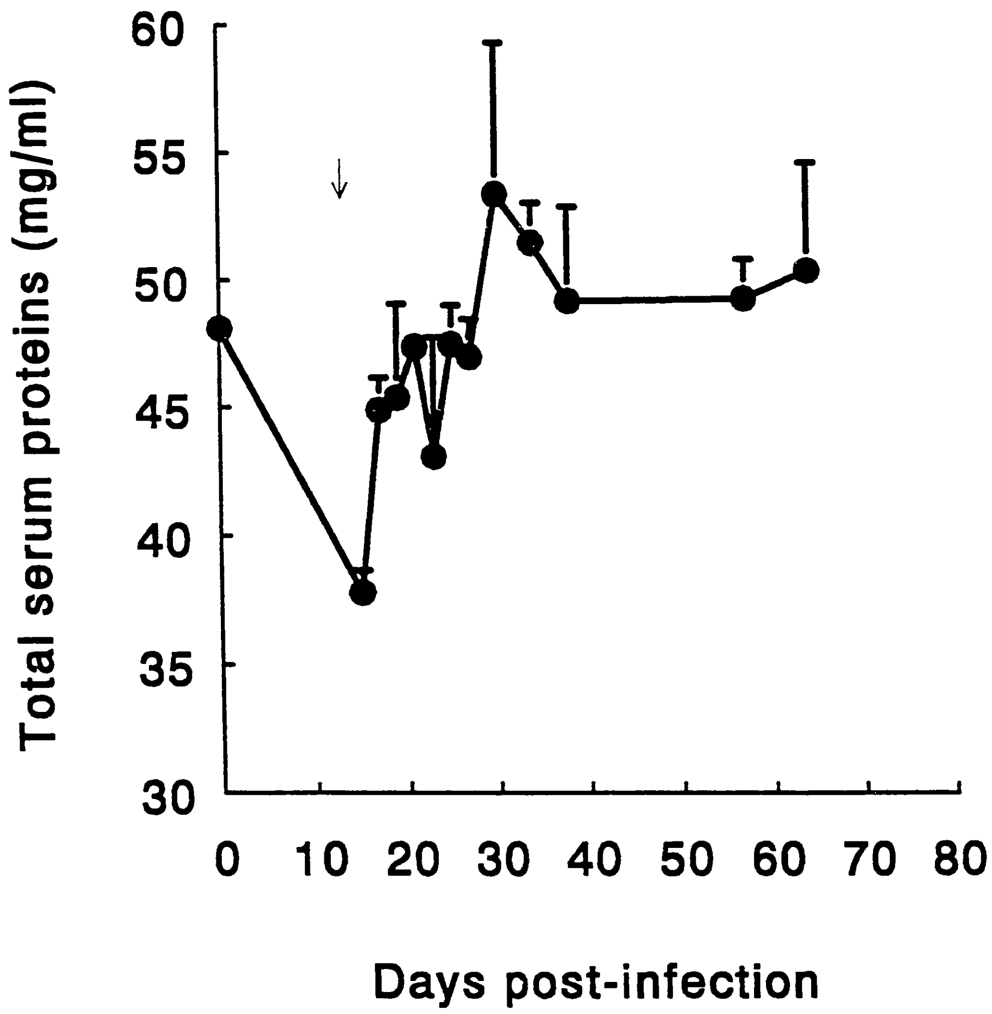


Fig. 5.5

Mean ± SD (n = 5) serum albumin levels in Boran cattle treated (↓) with homidium at 1 mg kg^{-1} b.w. 15 days following inoculation with a homidium-sensitive trypanosome population (*T. congolense* IL 1180)

Fig. 5.5

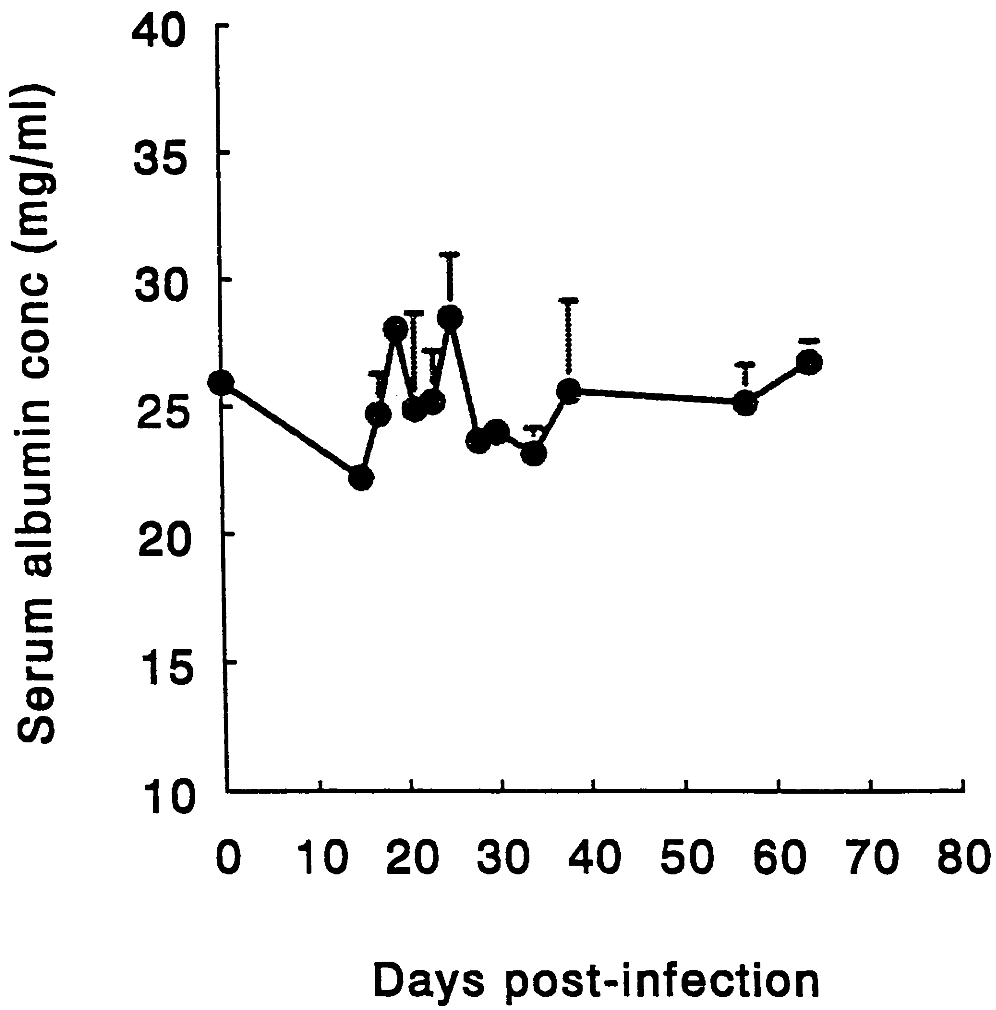
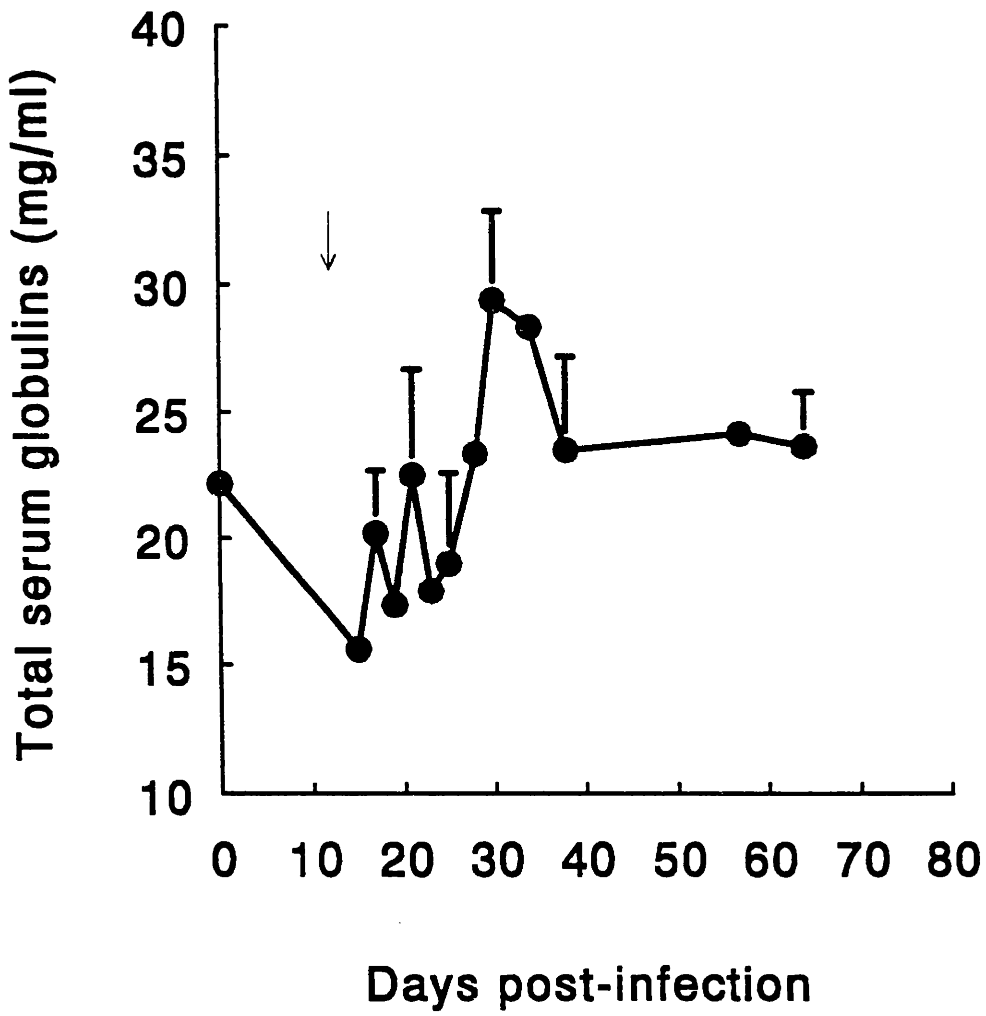


Fig. 5.6

Mean ± SD (n = 5) total serum globulin levels in Boran cattle treated (↓) with homidium at 1 mg kg^{-1} b.w. 15 days following inoculation with a homidium-sensitive trypanosome population (*T. congolense* IL 1180)

Fig. 5.6



were attained approximately 25 days following treatment. These levels were generally maintained to the end of the observation period of 90 days.

Boran cattle infected with a drug-resistant population of *T. congolense* (IL 3330) and later treated with homidium bromide at 1 mg kg^{-1} b.w. by the intramuscular route

Parasitaemia

Fig. 5.1 shows the parasitaemia in cattle following trypanosome inoculation and the time of homidium treatment. Trypanosomes were first demonstrated in the peripheral blood of all the five animals in this group seven days following infection. On the day of treatment, which was 7 days after all animals were first detected parasitaemic (14 days post-inoculation), individual animals exhibited different levels of parasitaemia (Table 5.1). After treatment, trypanosomes did not completely disappear from the blood. However, there was a decline in the parasitaemia in all animals. This drop in parasitaemia lasted approximately 10 days (Fig. 5.1), after which trypanosomes reappeared in the circulation and rose to a series of peaks.

Serum drug concentrations

The homidium concentration versus time plots for animal Nos. 415 to 419 are given in Figs. 5.7(a) to 5.7(e), respectively. Five minutes following treatment of the group of cattle infected with homidium-resistant trypanosome population, the circulating drug level in serum was 118.1±31.0 ng ml^{-1} (range 78.7 - 173.1 ng ml^{-1}) (Table 5.1). The mean peak serum drug concentration (C_{max}) was 179.6±29.3 ng ml^{-1} (range 131.8 - 220.2) and the time at which the maximum concentration occurred (t_{max}) was 10-15 minutes. As already mentioned, trypanosomes did not clear from the circulation following treatment. Ten days following treatment, no drug was detectable in serum of any of the five animals in this group. The results showed that in the presence

Figs. 5.7(a) to 5.7(e)

Serum homidium concentration in five individual Boran cattle treated with homidium at 1 mg kg^{-1} b.w. following infection with a homidium-resistant *Trypanosoma congolense* population (IL 3330)

Fig. 5.7(a)
Animal No. 415

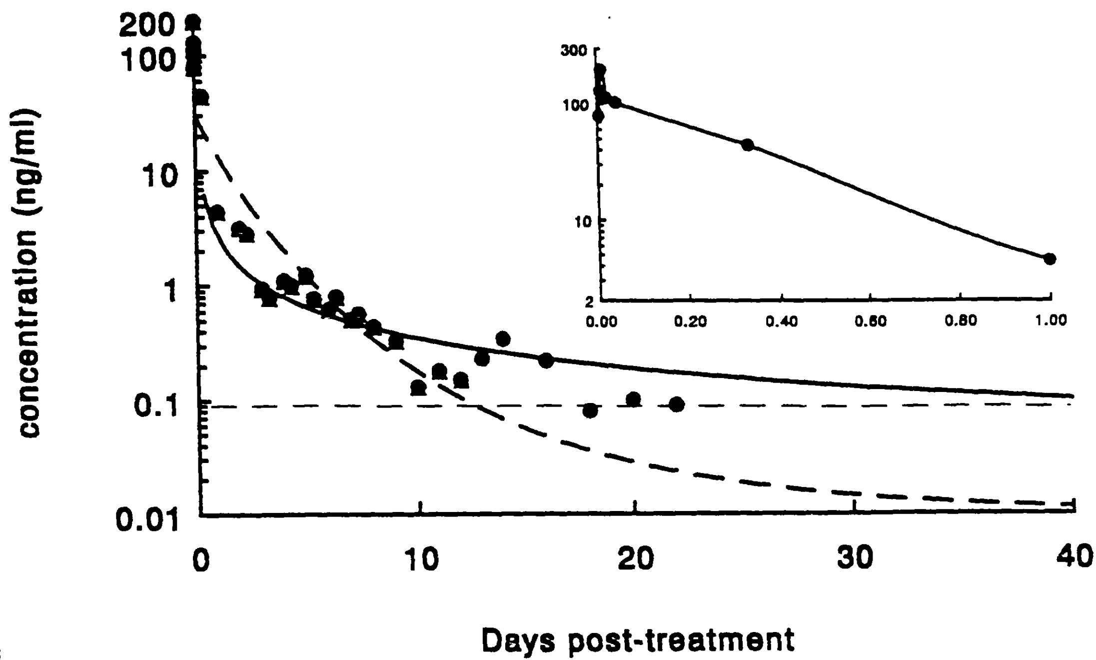


Fig. 5.7(b)
Animal No. 416

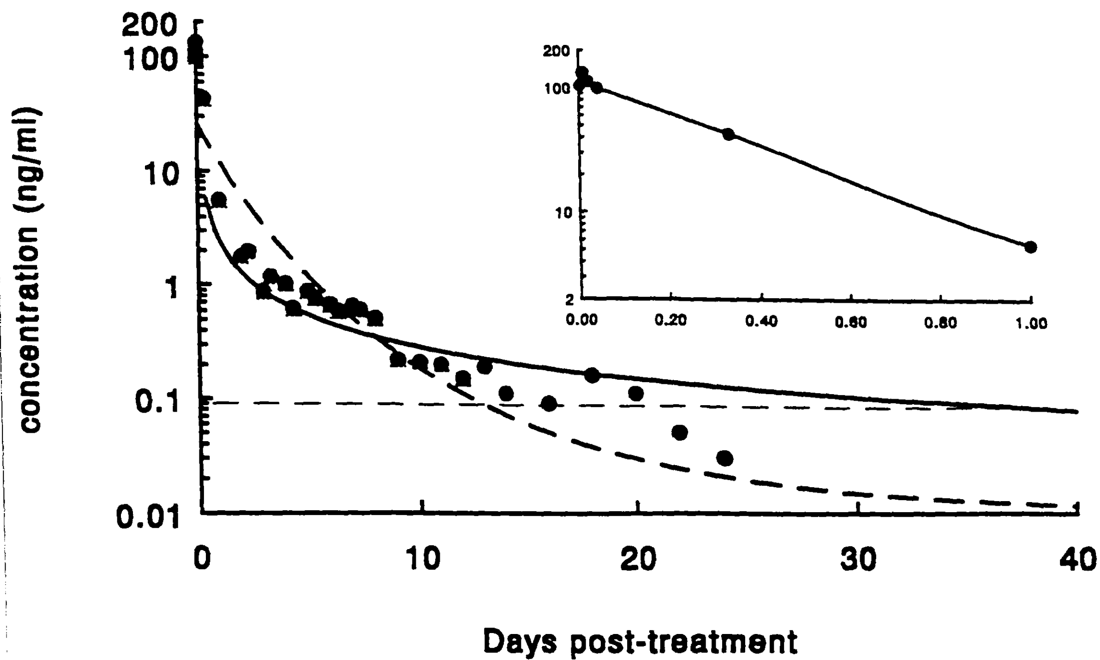


Fig. 5.7(c)
Animal No. 417

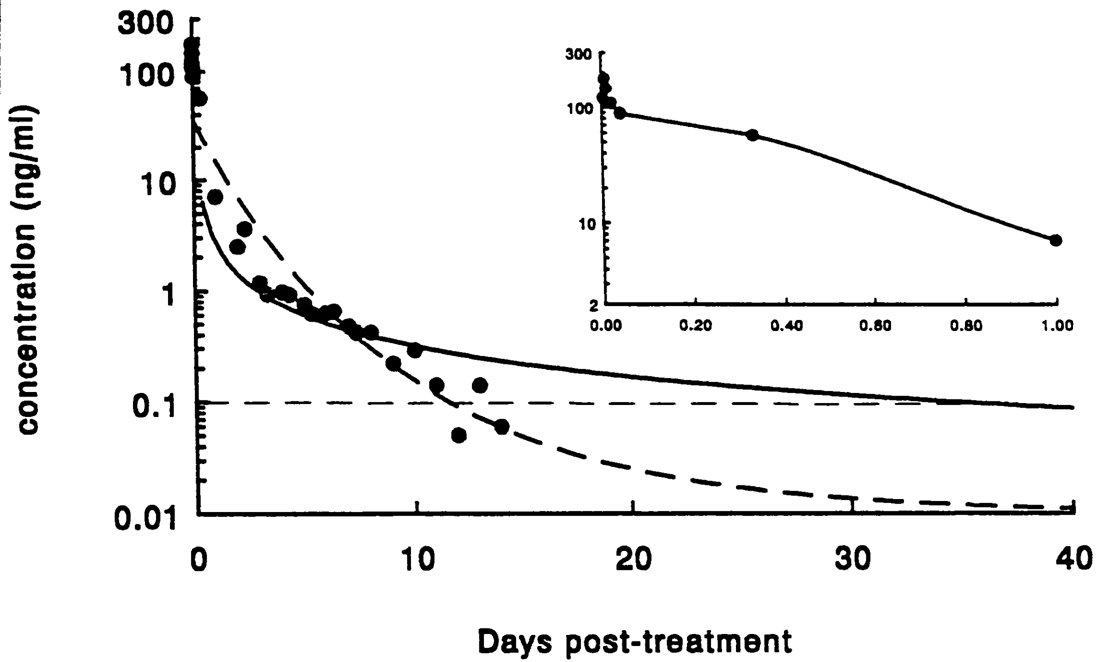


Fig. 5.7(d)
Animal No. 418

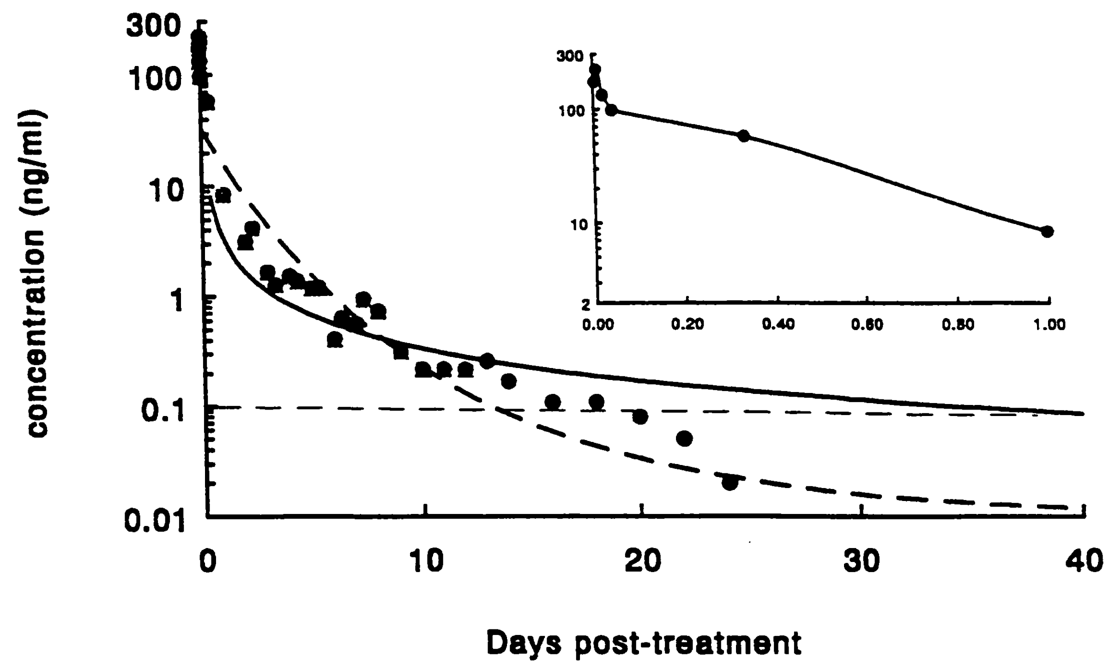
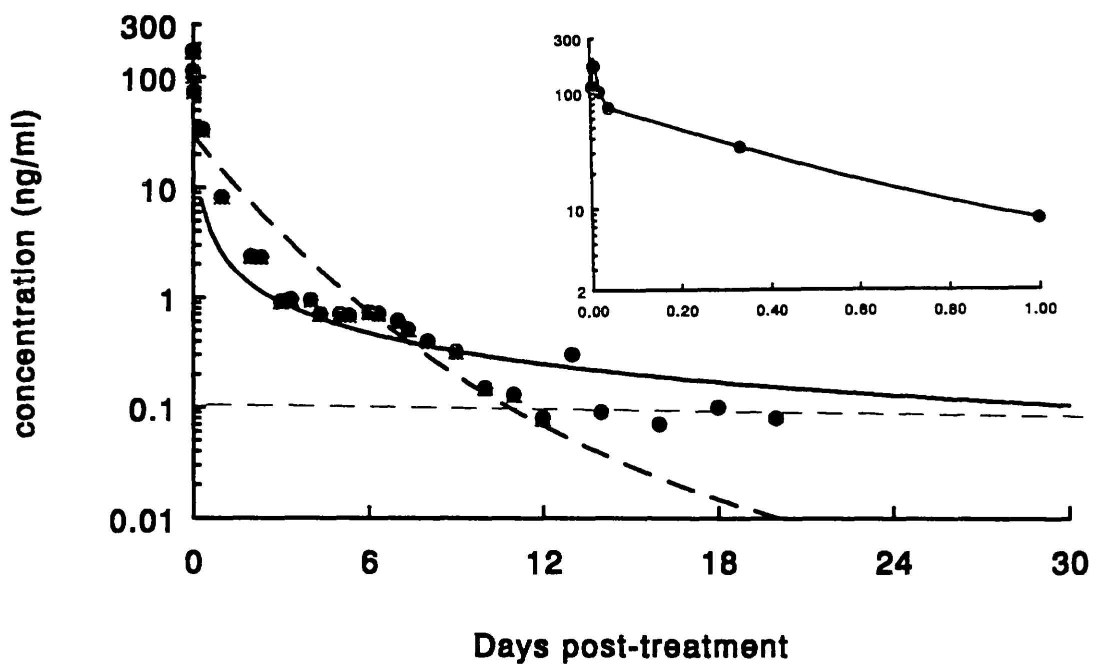


Fig. 5.7(e)
Animal No. 419



of a homidium-resistant trypanosome population, the rate of disappearance of the drug from serum was accelerated.

Pharmacokinetics

Decline in serum homidium concentration was exponential and the non-compartmental pharmacokinetic model was used to describe the experimental data. The results are given in Table 5.3. From the $AUC_{0-\infty}$ and $AUMC_{0-\infty}$ observed values of 1329±157 ng.h ml^{-1} (range 1110 - 1561) and 43513±7554 ng.h^2 ml^{-1}, the $MRT_{0-\infty}$ observed value was calculated as 32.8±4.5 hours (range 25.5 - 37.5). Large variations in the MRT values between individual infected animals were observed.

Haematological indices

The drop in PCV in the animals infected with the homidium-resistant trypanosomes was gradual following trypanosome inoculation from a mean pre-infection value of 38.4±2.5% to 31.0±3.1%. There was a slight elevation in PCV to approximately 35% following treatment, after which the drop was rapid, reaching levels of 19.0±1.2% at 70 days as the peaks of parasitaemia continued (Fig. 5.8). Over this period, there was loss of appetite, accompanied by deterioration in body condition and loss in body weight. The lowest recorded PCV value was 17%. Because the animals were in poor condition, they were removed from the experiment 77 days after treatment, when each animal was treated with three doses of diminazene aceturate; the first dose was 14 mg kg^{-1} b.w. followed by 7 mg kg^{-1} b.w. on the second day and 7 mg kg^{-1} b.w. on the third day. No parasites were found in the blood of any of the animals when examined for trypanosomes after this treatment. Up to a period of 60 days following diminazene treatment, no trypanosomes were detected in their peripheral blood. Three of the animals were still too weak even after the diminazene treatment and were humanely killed three days

Table 5.3

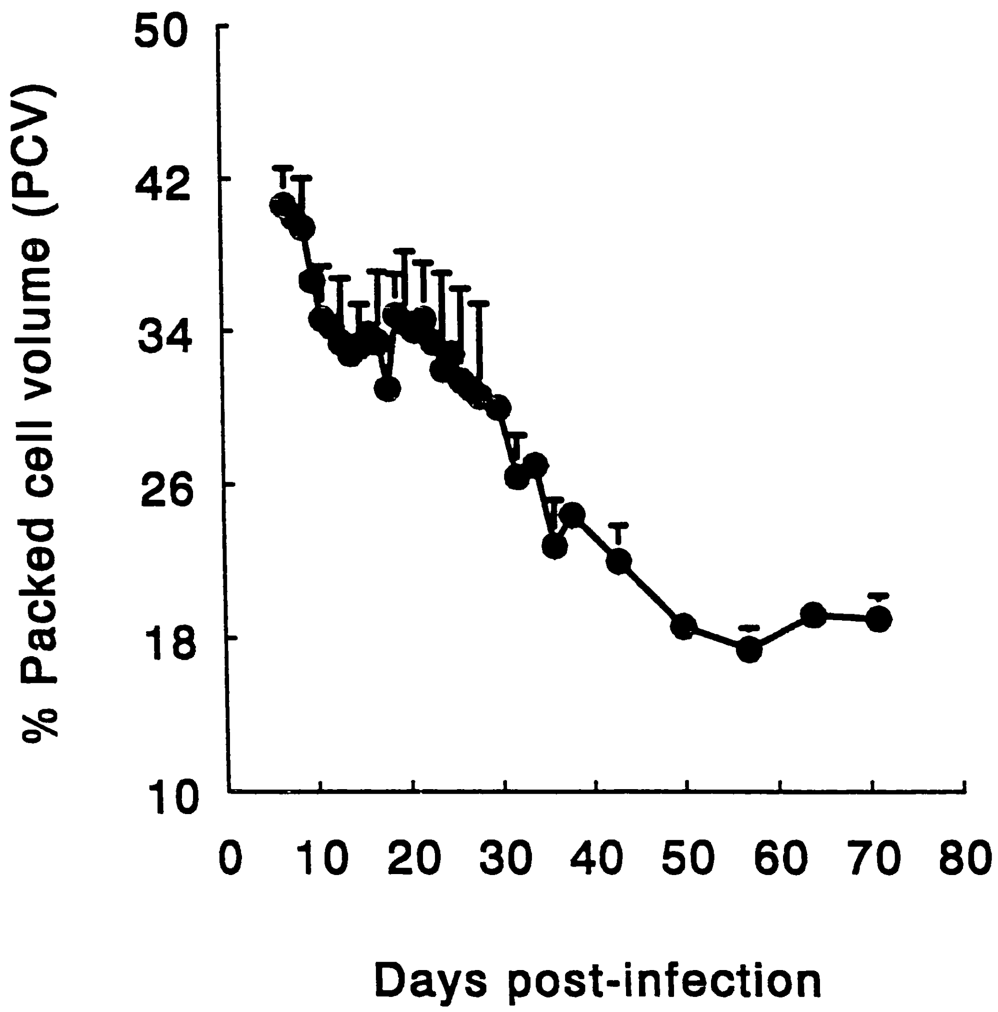
Pharmacokinetics of homidium in Boran cattle infected with a resistant population of *Trypanosoma congolense* (IL 3330) and later treated with homidium bromide at a dose rate of 1 mg kg^{-1} b.w.

Parameter	Animal Nos.					
	415	416	417	418	419	Mean ± SD
t_{max} (h)	0.240	0.168	0.168	0.0168	0.240	0.197±0.035
C_{max} (ng ml^{-1})	197.1	131.8	176.5	220.2	172.3	179.6±29.3
β	0.0091	0.0071	0.01198	0.0077	0.0125	0.0097±0.0022
$t½β$ (h)	76.3	98.2	57.9	89.5	55.4	75.5±16.9
$AUC_{0\text{-last}}$ (ng.h ml^{-1})	1309	1210	1424	15504	11044	1320±157
$AUC_{0\text{-}\infty}$ observed (ng.h ml^{-1})	1320	1226	1428	1561	1110	1329±156
$AUC_{0\text{-}\infty}$ predicted (ng.h ml^{-1})	1314	1219	1425	1558	1107	1325±157
$AUMC_{0\text{-last}}$ (ng.h^2 ml^{-1})	41681	36287	34058	47384	30731	38028±5881
$AUMC_{0\text{-}\infty}$ observed (ng.h^2 ml^{-1})	48174	45975	36411	53677	33327	43513±7554
$AUMC_{0\text{-}\infty}$ predicted (ng.h^2 ml^{-1})	44554	41574	34744	52022	32195	41018±7087
$MRT_{0\text{-last}}$ (h)	31.83	29.99	23.62	30.56	27.83	28.83±2.77
$MRT_{0\text{-}\infty}$ observed (h)	36.49	37.51	25.5	34.39	30.03	32.78±4.45
$MRT_{0\text{-}\infty}$ predicted (h)	33.90	34.12	24.38	33.39	29.08	30.97±3.79

Fig. 5.8

Mean ± SD (n = 5) packed cell volume (%) in Boran cattle treated (↓) with homidium at 1 mg kg^{-1} b.w. 14 days following inoculation with a homidium-resistant trypanosome population (*T. congolense* IL 3330)

Fig. 5.8



following diminazene treatment. However, two of the animals survived and gained weight.

Pathology

On post-mortem of the three cattle from the group which was infected with the drug-resistant trypanosome population, it was found that the animals had enlarged lymph nodes and splenomegaly. In addition, there was evidence of muscle wasting, depletion of the body fat and accumulation of oedematous fluid in the pericardium. The surfaces of the liver, kidneys and lungs showed focal areas of haemorrhage.

Total serum protein levels

The mean (±SD) pre-infection total serum protein level was 47.12±1.34 mg ml^{-1}. Fourteen days following infection, at the time of treatment, the mean total serum protein concentration was 42.05±4.63 mg ml^{-1}. The pre-infection value was slightly higher but not significantly different from the value at 15 days at 95% confidence limit. Following homidium treatment, concentrations rose to pre-infection values within one week, despite the fact that the animals were not cured. Thereafter, there was a gradual drop to 39.68±2.18 mg ml^{-1} on day 36 day following treatment (Fig 5.9).

Serum albumin levels

Pre-infection serum albumin levels were 25.7±1.4 ng ml^{-1}. A mean ± SD value of 24.5±2.5 mg ml^{-1}, obtained 14 days after trypanosome inoculation (Fig. 5.10) was lower but not significantly different from the pre-infection levels. Within 36 days of trypanosome inoculation, the serum albumin levels had dropped to only 21.5±0.37 mg ml^{-1}. This was found to be significantly lower from the pre-infection value ($p < 0.05$)

Fig. 5.9

Mean ± SD (n = 5) total serum protein levels in Boran cattle treated (↓) with homidium at 1 mg kg^{-1} b.w. 14 days following inoculation with a homidium-resistant trypanosome population (*T. congolense* IL 3330)

Fig. 5.9

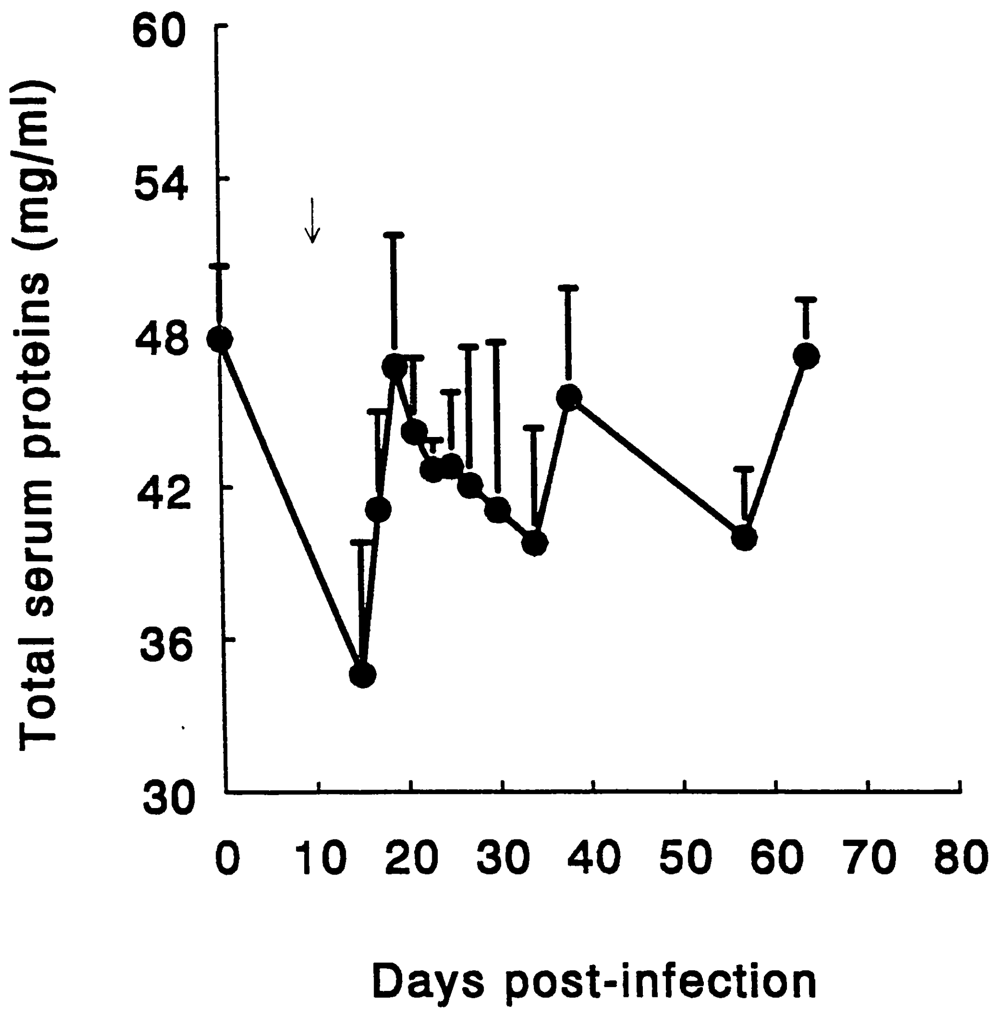
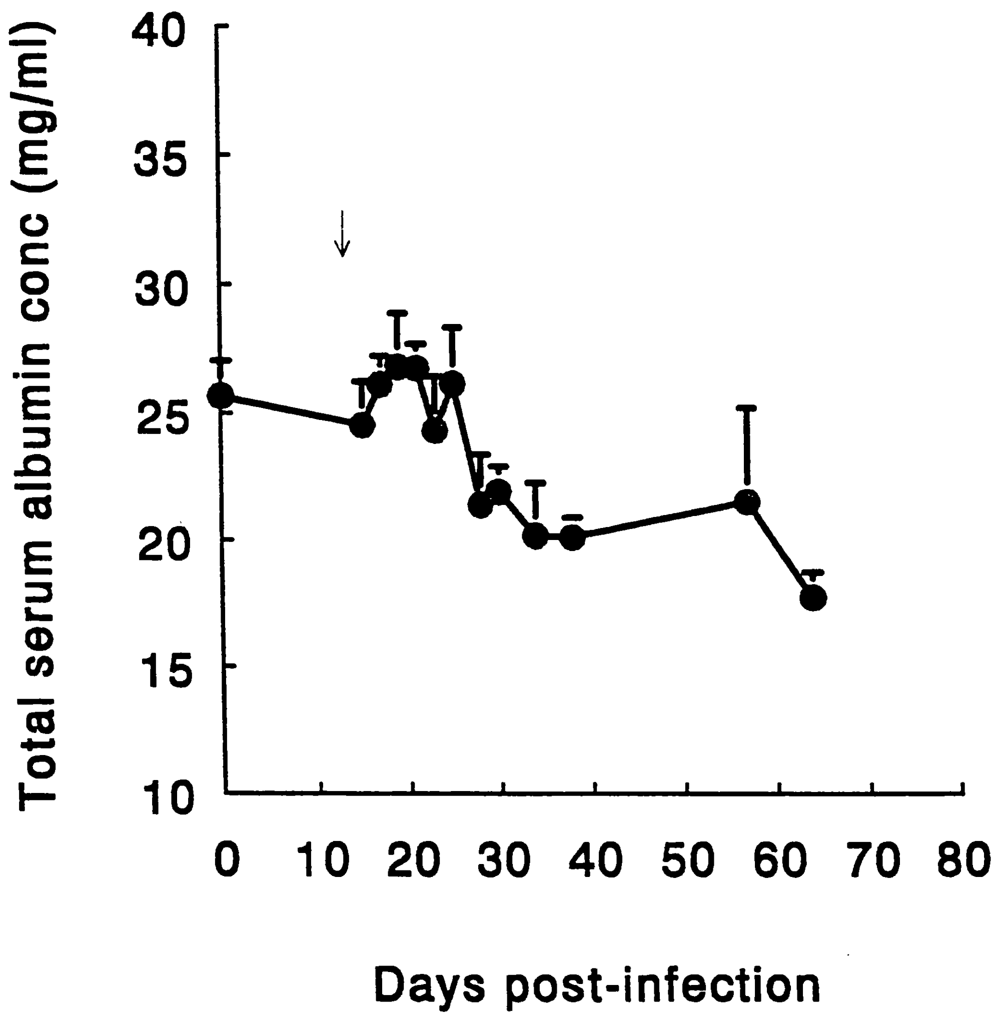


Fig. 5.10

Mean ± SD (n = 5) serum albumin levels in Boran cattle treated (↓) with homidium at 1 mg kg^{-1} b.w. 14 days following inoculation with a homidium-resistant trypanosome population (*T. congolense* IL 3330)

Fig. 5.10



Total serum globulin levels

Fourteen days following infection, there was a drop in the total serum globulin concentrations from a pre-infection value of 22.58±0.98 mg ml^{-1} to approximately 20.6±0.5 mg ml^{-1} (Fig. 5.11). Concentrations did not appear to change significantly over a two week period following treatment, after which two peaks were observed at 40 and 65 days post-infection which appeared to coincide with parasitaemia peaks. A mean value of 21.5±0.4 mg ml^{-1} was recorded 36 days following homidium treatment.

Comparison of homidium serum concentration and pharmacokinetics between trypanosome-infected and non- infected cattle reported in Chapters 4 and 6

Serum homidium concentrations

Fig. 5.12 and 5.13 show a comparison of the mean ± SD serum homidium concentrations between non-infected cattle and cattle infected with either a sensitive or a resistant population of *T. congolense*. The values for non-infected cattle were obtained from the experiments described in Chapter 4. The earliest sampling time for the non-infected cattle reported in Chapter 4 was 30 minutes. Data on serum homidium concentrations in these animals before 30 minutes post-treatment are therefore lacking. However, the serum homidium concentrations five minutes after the treatment of ten non-infected cattle are available from the experiment reported in Chapter 6 and are used in the comparison in Table 5.4.

Five minutes following homidium treatment, the mean drug concentration in cattle infected with a drug-sensitive trypanosome population (76.4±51.1 ng ml^{-1}) was significantly lower than that obtained in non-infected Boran cattle, (235.6±118.3 ng ml^{-1}; n= 10; Chapter 6) ($p < 0.05$). Similarly, the mean peak serum drug concentration of 180.4±34.7 ng ml^{-1} obtained in cattle

Fig. 5.11

Mean ± SD (n = 5) total serum globulin levels in Boran cattle treated (↓) with homidium at 1 mg kg^{-1} b.w. 14 days following inoculation with a homidium-resistant trypanosome population (*T. congolense* IL 3330)

Fig. 5.11

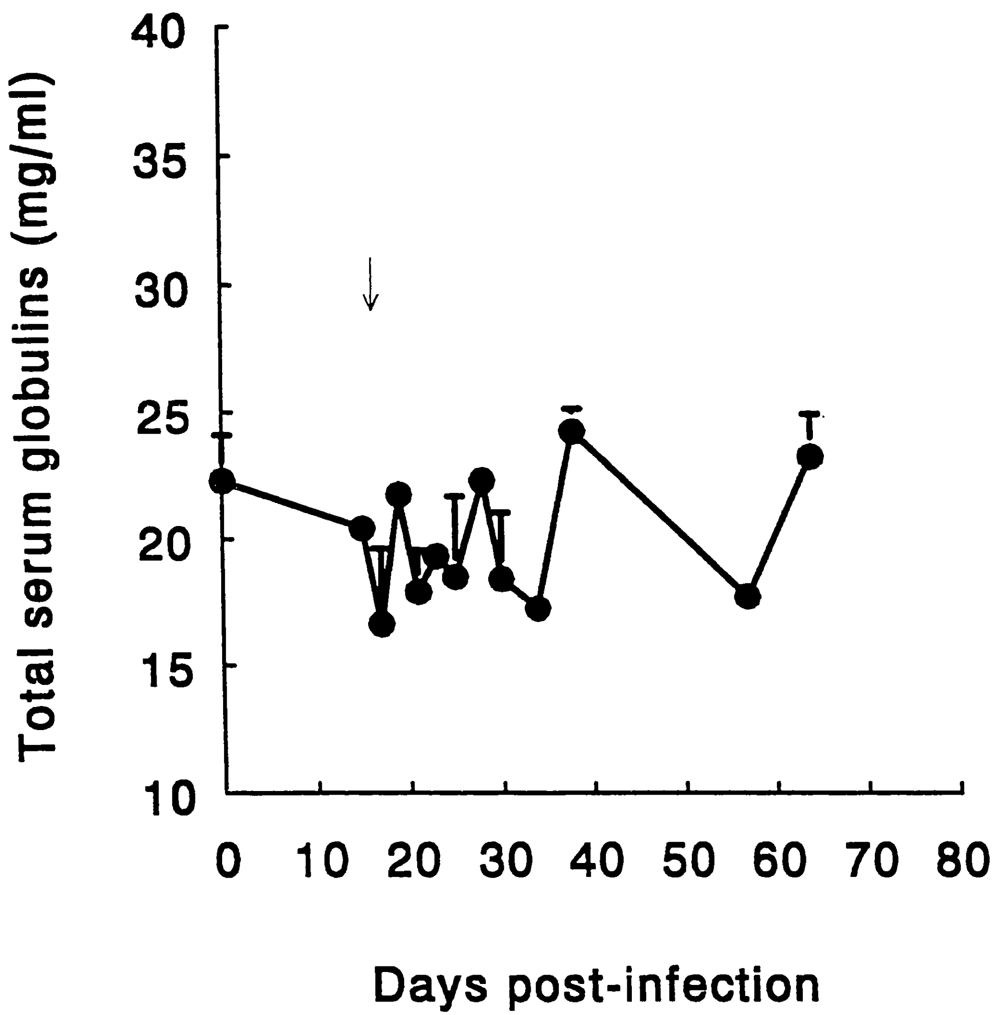


Fig. 5.12

Mean ± SD (n = 5) serum homidium concentrations in non-infected cattle and cattle infected with a homidium-sensitive trypanosome population (*T. congolense* IL 1180).

Fig. 5.12

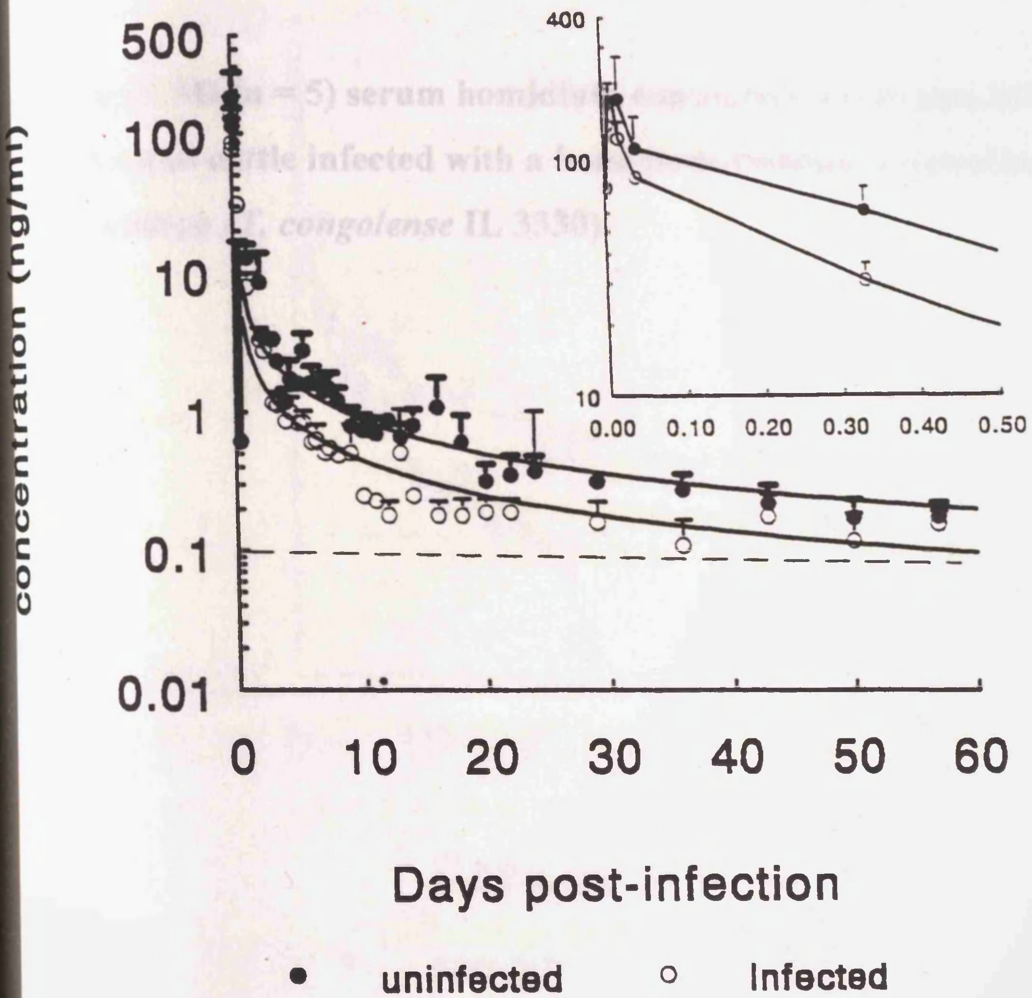
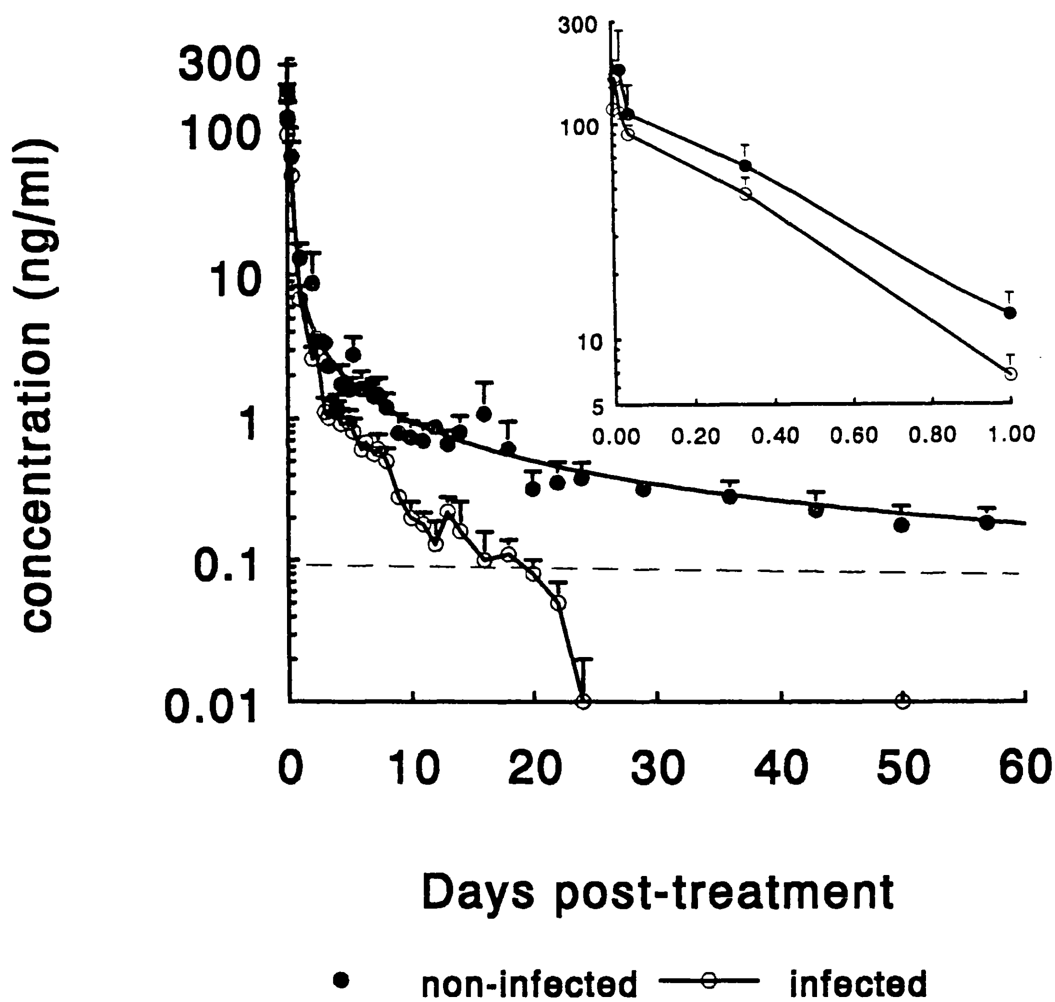


Fig. 5.13

Mean ± SD (n = 5) serum homidium concentrations in non-infected cattle and cattle infected with a homidium-resistant trypanosome population (*T. congolense* IL 3330).

Fig. 5.13



• non-infected —o— infected

Table 5.4

Serum homidium concentrations five minutes following treatment of non-infected and cattle infected with either a sensitive or a resistant population of *T. congolense*

(a) Non-infected cattle

Animal No.	concentration (ng ml^{-1})	Animal No.	Concentration (ng ml^{-1})
426	169.9	435	454.2
429	332.7	437	95.6
430	226.3	438	356.8
433	146.7	442	87.6
434	490.2	444	250.4
Mean ± SD (n = 10)	261.1±135.7		

(b) Sensitive trypanosome population

Animal No.	concentration (ng ml)
410	34.06
411	57.92
412	74.13
413	40.91
414	174.74
Mean ± SD	76.35±51.12* *

(c) Homidium-resistant trypanosome population

Animal No.	concentration (ng ml)
415	78.66
416	104.3
417	121.2
418	173.1
419	113.4
Mean ± SD	118.11±31.0*

Comparison with the mean value of uninfected cattle using the student's t-test

* * $p \leq 0.01$

* $p \leq 0.05$

infected with the sensitive trypanosome population was significantly lower than 276.2±83.4 ng ml^{-1} reported in non-infected cattle ($p < 0.05$). Following treatment of cattle infected with a drug-resistant trypanosome population, the mean serum drug concentration obtained at five minutes, 118.1±31.0 ng ml^{-1}, was significantly lower than that obtained in non-infected cattle ($p < 0.05$). Similarly, the peak concentration of 179.6±29.3 ng ml^{-1} was found to be significantly lower than that observed in non-infected cattle ($p < 0.05$) but not significantly different from the value obtained in the cattle infected with the drug-sensitive trypanosome population.

For the remaining comparison of non-infected and infected cattle, the values obtained in non-infected cattle reported in Chapter 4 rather than Chapter 6 were used because data up to 90 days post-treatment were available for the experiment described in Chapter 4. The serum homidium concentration-versus-time plot obtained following treatment of cattle infected with a drug-sensitive trypanosome population showed an accelerated rate of drug elimination within the first 10 days of treatment which reverted to normal following the disappearance of trypanosomes from the circulation (Fig. 5.12) and an elevation in PCV. However, the accelerated rate of drug elimination observed in cattle treated following infection with a drug-resistant trypanosome population continued until the drug was no longer detectable in the circulation (approximately 10 days following treatment) (Fig. 5.13).

Pharmacokinetics

A comparison of the pharmacokinetic parameters between non-infected cattle and cattle infected with either the sensitive or resistant populations of *T. congolense* is given in Table 5.5. The mean AUC value obtained following treatment of cattle infected with the sensitive trypanosome population was higher but not significantly different from the value in non-infected cattle. The

Table 5.5

Homidium pharmacokinetic parameters in non-infected Boran cattle and cattle infected with either a sensitive (IL 1180) or a resistant (IL 3330) population of *T. congolense* following treatment with homidium bromide at 1 mg kg^{-1} b.w.

Parameter	Non-infected Boran cattle	Boran cattle Infected with IL 1180 sensitive	Boran cattle Infected with IL 3330 resistant
t_{max} (h)	nd	0.2256±0.0288	0.1968±0.0353
C_{max} (ng ml^{-1})	nd	180.4±34.8	179.6±29.3
β	0.0040±0.0005	0.0018±0.0006	0.0097±0.0022
$t_{½β}$ (h)	177.8±23.4	423.7±145.6**	75.46±16.89**
$AUC_{0\text{-last}}$ (ng.h ml^{-1})	2051±341	1616±261	1320±157*
$AUC_{0\text{-}\infty}$ observed (ng.h ml^{-1})	2056±341	1667±233	1329±156*
$AUC_{0\text{-}\infty}$ predicted (ng.h ml^{-1})	205453±342	1648±247	1325±157
$AUMC_{0\text{-last}}$ (ng.h^2 ml^{-1})	177351±37189	340428±96872	38028±5881
$AUMC_{0\text{-}\infty}$ observed (ng.h^2 ml^{-1})	177351±37189	480580±239401	43513±7554
$AUMC_{0\text{-}\infty}$ predicted (ng.h^2 ml^{-1})	180714±37651	430070±172827	41018±7087
$MRT_{0\text{-last}}$ (h)	87.5±16.8	216.2±72.0*	28.82±2.78*
$MRT_{0\text{-}\infty}$ observed (h)	92.3±21.1	296.7±158.8*	32.78±4.46*
$MRT_{0\text{-}\infty}$ predicted (h)	89.1±17.5	269.5±120.2	30.97±3.78

nd = not determined.
Group comparisons of means between non-infected and infected cattle:
** $p < 0.01$
* $p < 0.05$

MRT value of 296.6±158.9 hours obtained in cattle infected with the drug-sensitive trypanosome population was, however, significantly higher than the value obtained in non-infected cattle of 92.27±21.14 hours ($p < 0.05$), and the value of 32.78±4.46 hours obtained in cattle infected with the drug-resistant trypanosome population ($p < 0.01$).

The AUC and MRT values obtained in cattle treated following infection with the drug-resistant trypanosome population were found to be significantly lower than the values obtained in non-infected cattle and in cattle infected with the sensitive trypanosome population ($p < 0.01$) (Table 5.3).

DISCUSSION

The present study has demonstrated that *T. congolense* infections can have pronounced effects on serum homidium's concentration profile and pharmacokinetics following treatment of infected cattle with homidium bromide at the recommended therapeutic dose rate (1 mg kg^{-1}). Evidence was obtained showing that drug elimination rate was accelerated in the presence of an infection. The mean residence time of the drug in cattle treated following infection with a drug-sensitive trypanosome population was significantly higher than that observed in non-infected cattle in cattle infected with a drug-resistant trypanosome population.

Following i.m treatment, trypanosomes were cleared within 24 hours from the peripheral blood of four out of five cattle infected with a homidium-susceptible trypanosome population. The remaining animal, which had the highest parasitaemia density became aparasitaemic within 48 hours. No trypanosomes were detected in the peripheral blood of any of these animals to the end of the observation period (90 days), suggesting complete cure. The parasitology results showed that with the homidium-sensitive parasites there was only a slight variability in the time taken for the trypanosomes to clear from blood following homidium treatment.

Five minutes following homidium treatment of cattle infected with the drug-sensitive trypanosome population, the mean ± SD serum drug concentration was 76.4±51.1 ng ml^{-1} (range 34.1 - 174.7) (Table 5.4). The drug profile results showed an acceleration in rate of decline in serum concentrations in the infected cattle within the first 10 days following treatment after the peak which was observed at 10 minutes. The rate reverted back to normal following the elimination of parasites from circulation (Fig. 5.12) and at a time when an elevation in PCV levels was observed (Fig. 5.3). The accelerated rate of the elimination of the drug from the circulation during the first 10 days could have been due to the changes in the host following infection, such as fever, PCV and parasitaemia. Other important changes could be changes in the protein levels and tissue damage caused by the infection, which are also mentioned in this discussion at a later stage.

A mean residence time of 296.6±158.9 hours (range 121.9 - 504.6) obtained in cattle infected with the drug-sensitive trypanosome population following treatment was quite long. The results suggest a longer mean residence time for the drug in cattle following treatment of cattle infected with the drug-sensitive trypanosome population than was observed in non-infected cattle suggesting that such cattle would be protected for a much longer period in case of repeated exposure to trypanosome infections than the non-infected cattle. This would in turn reduce the number of treatments required per animal per year, thereby reducing the cost of keeping the animals in a tsetse- infested area. This finding is supported by the observation made by Whiteside (1962) in which he demonstrated a reduction in the number of treatments from five to one in a year following repeated exposure to trypanosome infections and successful cure with diminazene. He attributed this to the combined effect of the immune response and drugs.

Studies by Gilbert and Newton (1982) showed that i.m. treatment of *T. congolense* infected and *T. brucei*-infected rabbits with ^{14}C homidium bromide

at 1 and 10 mg kg^{-1} b.w. did not result in cure. Only an increase in the pre-patent period from 3 days (homidium-treated non-infected rabbits) to 8 days (homidium-treated infected rabbits) was observed. The trypanosome populations used were not drug-resistant. Their observations showed similarities in homidium concentrations between infected and non-infected rabbits. The differences between these results and those obtained in the present study could be as a result of a species difference in drug absorption, distribution and elimination.

One interesting observation made by Gilbert and Newton (1982) was that despite the fact that the rabbits were not cured following treatment at the two dose rates, sub-inoculation of the blood from these rabbits into mice resulted in cure at a dose rate of 1 mg kg^{-1} b.w. Removal of IgG from serum taken five hours after homidium treatment of infected rabbits resulted in the reduction of the trypanocidal activity to 46% that of serum from infected homidium-treated rabbits taken at the same time. All trypanocidal activity in samples collected at 10 and 19 days following treatment of infected rabbits was associated with IgG and this was demonstrated by the complete loss of trypanocidal activity in serum *in vitro* when IgG was removed. These results of Gilbert and Newton (1982) support the above observations made by Whiteside (1962) on the contribution of the host's immune response to chemotherapy.

The high values for MRT obtained following treatment of cattle infected with a drug-sensitive trypanosome population could possibly be as a result of the presence of anti-trypanosome antibodies. This is also supported by the elevated total serum globulin levels observed.

Following i.m treatment of the cattle infected with the homidium-resistant trypanosomes, the parasites did not clear from the circulation although there was a drop in the trypanosome density for about 10 days (Fig. 5.1), after which the peaks of parasiteamia continued over the observation

period of 70 days. The homidium-resistant trypanosomes were therefore confirmed to be highly resistant to the recommended therapeutic dose of homidium of 1 mg kg^{-1} b.w. Five minutes following treatment, the mean serum drug concentration in these animals was 118.1±31.0 ng ml^{-1} (range 78.7 - 173.1) (Table 5.4). The value for C_{max} was 179.6±29.3 ng ml^{-1} (range 131.8 - 220.2) and this occurred between 10 minutes (animal Nos. 416, 417 and 418) and 15 minutes (animals 415 and 419) following treatment (Table 5.3). However, no drug could be detected 10 days post-treatment in these cattle, showing that the homidium-resistant trypanosome infection markedly altered the drug's concentration profile (Fig. 5.13).

The mean residence time of 32.8±4.5 hours (range 25.5 - 37.5) was obtained in the animals infected with the drug-resistant trypanosome population. All the animals in this group showed low MRT values. Low values for MRT obtained in these cattle suggest that either the drug was not held for long periods at the injection site due to physiological changes in the host, or that it was released as metabolites by the trypanosomes, undetected by the ELISA method used.

Comparisons of the mean ± SD serum drug concentrations between the two infected groups of cattle and non-infected cattle showed that five minutes following homidium treatment, there were significant differences in drug concentrations. For instance, the mean drug concentration at this time in the cattle infected with the drug-sensitive trypanosome population was significantly lower than that in non-infected cattle ($p < 0.01$; Table 5.4). During the same period (five minutes following treatment), the mean drug concentration in the cattle infected with the drug-resistant trypanosome population was also significantly lower than that in the non-infected cattle ($p < 0.05$). However, the C_{max} values between the two groups of infected cattle were not significantly different. This suggests a significant uptake of the drug by trypanosomes and is supported by the observation by Gilbert and Newton

(1982) using ^{14}C homidium in which approximately 80% of the drug was bound to trypanosomes separated from ear vein blood collected at 1, 6 and 12 hours post-treatment. Although blood samples obtained at 30 minutes following treatment were not examined, it appears that appreciable uptake of the drug takes place within minutes of treatment when the parasitaemia is high (Fig. 5.1).

It is of interest that homidium uptake by trypanosomatids *Crithidia fasculata* and *C. seymouri* has been demonstrated to be biphasic and that the characteristics of the two phases are consistent with the hypothesis that there is an initial specific external binding followed by a very slow process of transport into the cells (Coolbear and Midgely, 1986). It was also demonstrated in the same study that homidium uptake by the trypanosomatids could not be saturated over a large concentration range. The study of Sutherland et al. (1992) on isometamidium uptake by *T. congolense* demonstrated an energy dependent transport of the drug into trypanosomes.

The acceleration in the rate of decline in the serum drug levels observed in both groups of infected cattle during the first 10 days following treatment reverted back to normal only in the cattle infected with the drug-sensitive trypanosome population but not in the cattle infected with the drug-resistant trypanosome population (Figs. 5.12 and 5.13). Although the rate of drug elimination reverted to normal in the group of cattle infected with the drug-sensitive trypanosome population, the drug concentrations remained generally low compared to those observed in non-infected cattle (Fig. 5.12). This acceleration in the rate of drug elimination could have been as a result of several factors which include drug uptake by trypanosomes, changes in the host physiology or a combination of these factors. Whilst no drug was detectable in the peripheral blood of all animals infected with the drug-resistant trypanosome population within approximately 10 days of treatment, drug levels of between 0.1 and 0.3 ng ml^{-1} were still detectable 8 to 13 weeks

post-treatment in non-infected cattle and in cattle infected with the homidium-sensitive trypanosomes. The observation of elevated rate of drug elimination in trypanosome-infected cattle is similar to that of Eisler et al. (1994) who observed this phenomenon in cattle with isometamidium.

The pharmacokinetics of homidium in cattle infected with the drug-sensitive trypanosome population indicated that the MRT values were significantly higher than the values obtained in non-infected cattle ($p < 0.01$; Table 4.5). A mean residence time of 296.6±158.9 hours obtained in cattle infected with the drug-sensitive trypanosome population was significantly higher than 92.27±21.14 obtained in non-infected cattle. The same value obtained in cattle infected with the drug-resistant trypanosome population 32.78±4.46 hours, was even lower, showing that a persistent trypanosome infection could lead to drug failure by increasing the rate of disappearance following treatment.

Differences in homidium's pharmacokinetics were therefore observed between (i) the non-infected cattle and cattle infected with either homidium-sensitive or homidium resistant trypanosome populations and (ii) the two groups of infected animals. The results demonstrated that the mean residence time of the drug cattle in infected with drug-sensitive trypanosomes was longer than in non-infected animals and in animals in which the trypanosomes persisted following treatment.

The changes in pharmacokinetics of homidium in trypanosome-infected cattle may be linked to changes in host metabolism. Groothuis et al. (1978) studied the effect of experimental *Echerichia coli* endotoxaemia on ampicillin : amoxycillin blood levels after oral and parental administration of either ampicillin or amoxycillin to each of the calves (8 calves per group) at standard dose rates equivalent to 1 g amoxycillin and 1 g amplicillin. They demonstrated that there was a significant delay in absorption of the drugs following infection and treatment and that the drug concentrations were

markedly lower in the febrile than in the non-febrile controls. In further work, it was shown that effects on drug absorption reverted back to normal following the disappearance of fever.

In similar work, van Miert et al. (1976) carried out experiments to evaluate the effect of endotoxin-induced fever on the biotransformation and blood levels of sulphafurazole in adult goats, and observed that the blood drug levels were higher in four out of six goats during the febrile episode. During fever, the half-life of the non-metabolised sulphafurazole was shortened by 12% and was attributed to increased blood flow in the kidneys and liver. The pattern of urinary metabolites was also altered during the period of fever. Van Miert et al. (1976) also illustrated how changes in haemodynamics in the absence of fever could affect drug kinetics possibly due to reduced blood flow in the liver and kidneys. They suggested a modification of the dosage regimen of a drug or of the route of administration in such situations. However, sufficient data would be needed to permit such modifications.

Studies on blood sulphonamide levels in feedlot cattle with respiratory disease (Young 1973) has demonstrated that depressed rumen motility leads to lowered sulpha absorption rates and sub-optimal plasma sulphonamide concentrations of the drug administered orally.

In the field of animal trypanosomiasis, infections have been associated with peaks of fever, which usually coincide with the appearance of trypanosomes in the blood, and peaks of parasitaemia.

The occurrence of fever in various diseases, including trypanosomiasis, poses potential problems in drug treatment if fever *per se* alters the absorption, distribution, biotransformation and/or excretion of drugs. For homidium, it is difficult to predict how the drug's metabolism may be affected by febrile periods. The significant changes in drug concentrations observed five minutes following treatment would suggest that the initial rapid disappearance of the drug could possibly be due to trypanosomes metabolising the drug. However,

elevated temperatures and an associated increase in metabolic rate (Katunguka-Rwakishaya et al., 1993) could also play a part.

Since most drugs are bound to serum albumin, it is also possible that in the presence of decreased amounts of serum albumin during trypanosome infection, as shown in the present study, the movement of the drug to target cells could be affected.

Besides episodes of fever and enhancement in the rate of host metabolism and the low serum albumin levels associated with trypanosome infections, other host changes include changes in the PCV and damage to tissue. PCV has been used in trypanosomiasis to determine the severity of the disease. Katunguka-Rwakishaya et al. (1993) showed that the observed anaemia was due to accelerated extravascular destruction of red blood cells and heamodilution. Van Miert et al. (1976), in their work using amoxycillin in goats observed that haemodynamic changes may influence drug kinetics. Therefore, the haemodilution associated with trypanosome infections could have contributed to the changes in the drug pharmacokinetics observed in the present study possibly also due to reduced blood flow in the liver and kidneys.

The results of the present study showed the drop in PCV during the first seven days of infection was more gradual in the group infected with the homidium-resistant than the group infected with the drug-sensitive trypanosomes suggesting that the drug-sensitive trypanosome population was possibly more virulent than the drug-resistant trypanosome population. The results showed that PCV levels were approximately 24% in the cattle infected with the homidium-sensitive trypanosomes compared to approximately 34% in cattle infected with the homidium-resistant trypanosomes on the day of treatment (14 to 15 days following infection). Pre-infection levels were 38.4±4.6%. Whilst PCV levels attained 14 days following trypanosome infection with the drug-sensitive trypanosome population were elevated within one week following homidium treatment (Fig. 5.3), levels in the group

infected with the drug-resistant trypanosome population continued to decline, although the treatment helped maintain levels nearly constant for approximately 10 days, between day 14 and day 24 of infection.

In work on serum changes in *T. congolense*-infected cattle, Wellde et al. (1974) observed that total serum protein concentrations fell sharply during the first 5 weeks of infection, then increased gradually to normal levels. Serum albumin levels followed a similar pattern for the first 5 weeks, then remained at relatively low levels. Although gamma globulin levels also declined during the first 5 weeks their levels were gradually elevated, surpassing the pre-infection values.

In another study, Nielsen et al. (1978) investigated changes in serum immunoglobulins and complement components in infected animals. The results obtained after infecting calves with 5.5×10^8 parasites of *Trypanosoma congolense*, showed that some animals were more parasitised than others; parasitaemia levels ranged from heavy to medium to low. There was no change in the IgG_1 and IgG_2 levels. IgM increased early in infection and the amount of increase appeared related to the parasite burden. The amounts of IgA and IgE were both very much decreased and this appeared to be related to the numbers of parasites in the blood. They were able to demonstrate a correlation between immunoglobulin levels and parasiteamia by treating each animal as a single entity rather than use of average values.

The results reported in this Chapter which included variations in parasite density following infection (14 - 15 days post-infection) between individual animals, a drop in PCV following the establishment of infection and decreased levels of serum albumin are in agreement with the findings of Wellde et al. (1974) and Nielsen et al. (1978). However, the present study showed a general decrease in serum globulin levels in both groups of infected cattle following infection. The increase in the globulin levels in the cattle treated following infection with the homidium-sensitive population of

trypanosomes was quite evident. In contrast, the total globulin levels in the cattle infected with the drug-resistant trypanosome population were generally maintained at pre-infection values except for two peaks at approximately 40 and 65 days following infection which appeared to coincide with the parasitaemia peaks (Figs. 5.1 and 5.11).

Most tissues and organs are damaged during the course of trypanosome infection although some are more severely affected than others. One organ which undergoes severe damage is the heart. Initially, lesions occur beneath the epicardium and the endocardium. However, in advanced cases, the entire myocardium may be involved (Murray and Morrison, 1980) leading to degeneration of the myocardial fibres. The pericardium eventually fills with fluid probably due to increased vascular permeability which allows leakage from the blood of the protein-rich fluid. Results of this study showed that the animals which were humanely killed approximately 8 weeks following infection with drug-resistant trypanosome population (after drug failure) had an accumulation of fluid in the pericardium. Tissue damage which included the heart, the liver and the spleen as observed in the present study would therefore possibly lead to vigorous changes in micro-circulation, likely to have affected circulation of the drug following treatment.

Although the physiological changes occurring in the animals appeared to be much faster in the animals infected with the drug-sensitive trypanosome population as demonstrated by the rapid drop in PCV compared to the animals infected with the drug-sensitive trypanosome population, these changes were easily reversed following treatment.

Results of the present study indicate that even with homidium-sensitive trypanosomes, the parasites take at least 24 hours to clear from the circulation following treatment with homidium bromide at 1.0 mg kg^{-1} b.w. and even longer in more heavily parasitised animals. Unsworth (1954b) demonstrated that trypanosomes took as long as 84 hours to clear from the circulation

following treatment with homidium bromide at 0.1 mg kg^{-1} b.w. With dose rates of 0.3 and 0.9 mg kg^{-1} b.w., trypanosomes took 48 and 32 hours respectively to clear from the circulation.

In conclusion, the aforementioned observations made on the pharmacokinetics of homidium in infected cattle were made possible through the availability of the highly sensitive ELISA method. The changes observed in the serum drug concentration-versus-time profiles were similar to those made in cattle using isometamidium (Eisler et al., 1994). These could be due to the fact that the two compounds share structural similarities. The present results have, however, given an insight into one important factor that could contribute to drug failure following treatment, thereby reducing the efficacy of the drug; that is, drug resistance.

Holmes and Torr (1988) suggested several possible explanations for drug failure which included: underdosing, preparation, administration, fraud, not treated, cryptic foci, reinfection and drug resistance

In the field, these explanations may not only occur singly but in combination and/or sequentially. Administration of trypanocidal drugs in the field is often carried out by the farmer who may not have adequate knowledge on how to prepare and administer the drugs which are easily acquired from the drug retailers. Inaccessibility to diagnostic facilities could lead to treatment of animals based on clinical signs which may not be due to trypanosomiasis. Lack of weighing scales and economic hardships faced my the majority of small scale farmers would suggest that animals could be underdosed. Therefore, the ease at which trypanocidal drugs are acquired, lack of knowledge on drug preparation and administration combined with lack of facilities and economic hardships could contribute to the development of drug resistance in the field.

The results of the present study showed that presence of drug-resistant trypanosomes would lead to drug failure. Therefore, ways have to be found of

minimising the development of drug resistance in the field. This could be through stricter control in the application of the drugs which would involve qualified personnel and not left solely in the control of the farmer.

Treatment regimens such as that suggested by some workers (Whiteside, 1962; Mwambu, 1971) involving alternating homidium and diminazene every two years once drug-resistance has been identified could assist in the control of the spread of drug resistance. Identification of drug resistant trypanosomes with some degree of certainty would be the first important step in tackling the problem of resistance. Several methods have been tried which include studies in animals and use of tissue culture methods. The alternative approach suggested by Holmes and Torr (1988) was the development of laboratory tests to quantify drug concentrations in treated cattle. Presence of trypanosomes in cattle with drug levels which are known to be normally trypanocidal could be a valuable index of drug resistance. The highly sensitive ELISA method used in the present study and the work reported in Chapter 4 has demonstrated the value of this assay in studies towards this goal.

CHAPTER SIX

INVESTIGATION INTO HOMIDIUM AS A CHEMOPROPHYLACTIC AGENT

INTRODUCTION

In addition to therapeutic activity, homidium has also been reported to offer protection against trypanosomiasis ranging from a few weeks to several months. Leach et al. (1955) reported limited prophylactic activity of homidium at a dose rate of 1 mg kg^{-1} b.w. since it protected cattle against *T. congolense* infection for 4 weeks and *T. vivax* for 6 weeks. In contrast, Whiteside (1962), Mwambu (1971) and Dolan et al. (1990) have reported prophylactic activity of several months.

Mwambu (1971), in his studies observed the fact that sporadic treatment of suspected, or positively identified animals often excluded healthy carriers and designed a block treatment regimen using homidium bromide for cattle trypanosomiasis in an endemic area in Bukedi District, Uganda. The results showed that the incidence of infections was reduced and maintained at a low level for at least three months following homidium treatment compared to the control herd (in which diminazene aceturate was used to treat only positively diagnosed cases at 7.0 mg kg^{-1} b.w.). The results also showed that block treatment of herds of cattle with homidium maintained good condition longer than when individual cases were sporadically treated with diminazene aceturate. By reducing the number of treatments required in a year through block treatment, the control of the disease was more economical and efficient than when only positively diagnosed cases were treated with diminazene aceturate.

Dolan et al. (1990) carried out a study to investigate homidium prophylaxis in two breeds of cattle, the trypanotolerant Orma Boran and the improved Kenya Boran (Galana Boran) exposed to natural tsetse challenge on the Galana Ranch, Coastal Region Kenya. Each breed was divided into two groups; one treated with homidium bromide at 1 mg kg^{-1} b.w. and the other not treated but kept as control herd. Treatment of the homidium herd of each breed depended on the trypanosome infection rate in the control herds;

homidium treatment was given when 20% of the control herd were detected positive in a 29 day period. Subsequent homidium treatments were given if three breakthrough infections were detected in the homidium-treated herd. The study lasted 12 months in which all the animals were herded together. During the 12-month period, all animals detected positive in either the control or the homidium-treated herd of each breed were treated with diminazene aceturate at 7.0 mg kg^{-1} b.w.

No animals died due to the effects of trypanosomiasis in any of the groups during the 12 months. The number of infections in the Orma Boran remained low throughout the year, therefore, no homidium treatment was given. A total of 16 infections were recorded in the Orma control herd compared to 58 in the Galana control herd within 12 months. Only the Galana Boran received homidium treatment. The first treatment was given three months after the trial began, at the time when trypanosomes were detected in 20% of the control herd. No infections were recorded in these homidium-treated animals for 19 weeks following drug administration. The second homidium treatment was given to the Galana Boran 5 months after the first treatment. From the time of this second treatment to the 18th week when one animal was detected parasitaemic, no trypanosomes were detected in any of the animals in this herd; during the same 18-week period, 34 infections were recorded in the control group. Thirty infections out of the 58 detected during the 12-month study period were due to *T. congolense* and the remainder due to *T. vivax.*

In all the above experiments, drug levels were not determined due to lack of a sensitive and accurate method for the analysis of homidium. Therefore, it was not possible to relate the observed prophylactic periods to the circulating drug concentrations.

STUDY OBJECTIVES

1. To treat cattle with homidium, as would occur in the field when prophylactic cover is given to non-infected animals, and monitor the drug concentrations using ELISA.

2. To use the above data to determine whether homidium pharmacokinetics in individual animals before exposure to trypanosome challenge influence the length of the observed prophylaxis following trypanosome infection of the treated animals.

3. To challenge the animals at intervals of 30 days after homidium administration with either a drug-resistant or a drug-sensitive population of *Trypanosoma congolense* while continuing to monitor the serum drug concentrations until trypanosome infections were detected.

3. To attempt to correlate serum drug concentrations with the duration of prophylaxis following trypanosome challenge of treated cattle in an effort to determine the minimum protective serum homidium concentrations for the different trypanosome populations used for challenge.

MATERIALS AND METHODS

Experimental cattle

A total of 15 steers aged 9 months and weighing between 150 and 165 kg body weight were used. The steers were purchased from the Kapiti Farm, Machakos District, Eastern Province of Kenya; an area free from endemic trypanosomiasis. The management of the animals before and during the experiments were as described in Chapter 2.

Trypanosome populations

Two stabilates of *T. congolense* were used for the challenge infections (IL 1180 and IL 3330). The former was known to be relatively drug sensitive (CD_{50} in mice = 0.018 mg kg^{-1} b.w. with isometamidium chloride and 2.3 mg kg^{-1} b.w. with diminazene aceturate [Peregrine et al., 1991]) and the latter homidium resistant (CD_{50} in mice > 20 mg kg^{-1} b.w. for homidium chloride, 20 mg kg^{-1} b.w. for isometamidium chloride and > 30 mg kg^{-1} b.w. for diminazene aceturate [Codjia et al., 1993]). In cattle, *T. congolense* IL 1180 is sensitive to 0.001 mg kg^{-1} b.w. for isometamidium chloride (Sones et al., 1988) and IL 3330 is resistant to 7.0 mg kg^{-1} b.w. diminazene aceturate and 0.5 mg kg^{-1} b.w. isometamidium chloride (Codjia et al., 1993). Both *T. congolense* IL 1180 and *T. congolense* IL 3330 had also been tested *in vitro* using metacyclic forms. These trypanosomes were incubated at 35°C for 48 hours with concentrations ranging from 0.5 ng - 50 μg ml^{-1}. of either homidium bromide or homidium chloride. The trypanosomes were thereafter transferred to cultures containing bovine endothelial cell monolayers to assess their viability over the following five days. Sensitivity was expressed as the minimum effective drug concentration which killed 100% of trypanosomes in a given population within five days. The results were compared to controls incubated without drug. *T. congolense* IL 1180 was shown to be sensitive to 100 ng ml^{-1} of either homidium bromide or chloride with a minimum level of sensitivity of 50 ng ml^{-1}, whilst the IL 3330 population was resistant to 1000 ng ml^{-1} of either homidium salts (Gray and Peregrine, 1993).

Preparation of trypanosome inoculum and infection of cattle

Groups of five sub-lethally irradiated male Swiss white mice were inoculated with the either *T. congolense* IL 1180 or *T. congolense* IL 3330 stabilates. Trypanosome inoculations for the infection of cattle were prepared in mice as described in Chapter 2. The cattle Nos. 426, 429, 430, 433 and 434

were inoculated with IL 1180 whilst Nos. 435, 437, 438, 442 and 444 were inoculated with IL 3330. The number of trypanosomes given was 1 x 10^5 by subcutaneous (s.c.) injection. The s.c. route was chosen since it was the closest possible to the inoculation of an animal with trypanosomes during a tsetse bite.

Drug treatment

The drug compound used was homidium bromide (Ethidium®, Laprovet, France; Batch No. 52072/N [NP30L]). A 2.5% (w/v) solution was prepared in sterile water immediately prior to use. Administration of the drug was by a deep intramuscular injection at 1 mg kg^{-1} into the neck muscles.

Experimental design

Cattle were divided into three groups of five animals; those challenged with the homidium-sensitive population (Group HS), those challenged with the homidium-resistant population (Group HR), and the challenge controls (Group CC). The HS and HR groups were first treated with homidium bromide, then challenged at intervals of 30 days with either a homidium-sensitive (Group HS) or a homidium-resistant trypanosome (Group HR) population. The remaining five animals (Group CC), which did not receive homidium treatment were used as untreated controls. The challenge of homidium-treated cattle was repeated every 30 days until infections were detected. At each 30 day challenge of either Group HS or HR, one control from group was challenged in order to test the viability of the trypanosomes used.

Homidium concentrations were determined in all the treated animals for as long as it was detectable after trypanosome breakthrough infections had been detected. Following the development of trypanosome infections in the drug-treated cattle they were removed from the experiment and treated with

diminazene aceturate at 7.0 mg kg^{-1} b.w. for the homidium-sensitive trypanosome population and at 14 mg kg^{-1} b.w. for the homidium-resistant population when they attained a PCV of 15%. A dose rate of 7.0 mg kg^{-1} was chosen for the drug-sensitive trypanosome population since this is the dose rate usually employed in the field to treat breakthrough infections in cattle under a prophylactic regime. For the drug-resistant trypanosome population, a dose rate of 14 mg kg^{-1} b.w. was chosen since the population has been reported to be resistant to a dose rate of 7.0 mg kg^{-1} b.w. in cattle (Codjia et al., 1993).

Detection of anti-trypanosome antibodies

The main objective of the present study was to investigate the relationship between serum homidium concentrations and the observed period of prophylaxis following trypanosome challenge of homidium-treated cattle. It was also important to find out whether anti-trypanosome antibodies developed during the challenge periods and whether these contributed to the observed period of prophylaxis. The serum samples examined for anti-trypanosome antibodies were collected immediately before each trypanosome challenge at 30, 60 and 90 days post-treatment in Groups HS and HR and at 0 and 30 days post-infection in Group CC. The method used is described in Chapter 2.

Collection of samples

Pre-treatment sera

Two days before homidium treatment, fifty millilitres of blood samples were collected aseptically into plain vacutainer tubes by venipuncture from each animal. The procedures followed for the subsequent preparation and storage of sera were as described in Chapter 2. These serum samples were used as negative control sera in the preparation of spiked homidium standards and the quality control standards.

Pre-infection sera

On each day of the trypanosome challenge, ten millilitres of blood were collected from each animal immediately before trypanosome challenge, sera were prepared and stored as described in Chapter 2 for pre-treatment sera. This sera was used to determine individual animal serum homidium concentration at the time of trypanosome inoculation and also the presence of anti-trypanosome antibodies. The sera collected at 30 days when the first challenge was given were used to determine the pre-infection values for total serum proteins, serum albumin and the total serum globulins.

Blood samples for packed cell volume (PCV) and parasitological examination

Blood samples from cattle for PCV and parasitological examination were collected in heparinised capillary tubes before and after trypanosome inoculation following the sampling protocol described in Chapter 2. The blood-filled capillary tubes were centrifuged in a haematocrit centrifuge at 12000 g for 10 minutes after which they were read with a micro-haematocrit reader. After determination of the PCV, the buffy coat was examined for trypanosomes using the phase contrast/buffy coat method of Murray et al. (1977).

Determination of serum homidium levels

Ten ml blood samples were collected immediately prior to treatment and at the following intervals after treatment; 5, 15, 30, 45 minutes, 1, 2, 4, 8, 12 hours, twice daily during the first week, daily during the second week, thrice during the third week, twice during the fourth week and thereafter once a week to the end of the observation period (determined by the detection of trypanosomes in all animals). Sera were prepared and stored as described in Chapter 2. Homidium concentrations were determined using Assay 2 of the

enzyme-linked immunosorbent assays (ELISA) for homidium described in Chapter 3.

Data handling

The procedures for data handling were as described in Section 2.8.

Pre-challenge pharmacokinetic evaluation

Pharmacokinetic evaluation of the serum concentration-versus-time data was carried out using data collected up to 30 days after homidium treatment (i.e., before the first trypanosome challenge). The procedures used were as described in Chapter 2.

RESULTS

Cattle treated with homidium bromide and challenged at monthly intervals with a homidium-sensitive trypanosome population

Pre-challenge pharmacokinetics of homidium

The pre-challenge pharmacokinetic parameters obtained from a non-compartmental analysis of the serum drug concentration data are shown in Table 6.1. The mean $AUC_{0-\infty}$ observed value was 1681±177 ng.h ml^{-1} (range 1540 - 1960). The $MRT_{0-\infty}$ observed was 82.2±16.7 hours (range 64.7 - 110.0). The results showed wide variation in the observed MRT values between individual animals. MAT values could not be determined since data of serum drug concentration following i.v. injection of non-infected Boran cattle was not available.

Serum homidium concentrations

The individual animal serum homidium concentration-versus-time plots are given in Figs. 6.1(a) to 6.1(e). The mean serum homidium concentration-

Table 6.1

Pre-challenge pharmacokinetic parameters of homidium in individual Boran cattle of Group HS treated with homidium bromide at 1 mg kg^{-1} b.w.

Parameter	Animal No.					Mean±SD
	426	429	430	433	434	
t_{max} (h)	0.24	0.07	0.07	0.24	0.24	0.17±0.08
C_{max} (ng ml^{-1})	240.8	332.7	226.3	231.9	316.5	269.6±45.4
β	0.0039	0.0065	0.0069	0.0063	0.0051	0.0057±0.0011
t½β (h)	177.5	107.5	100.1	109.3	135.2	125.9±28.4
$AUC_{0\text{-last}}$ (ng.h ml^{-1})	2002	1525	1698	1521	1511	1636±161
$AUC_{0\text{-}\infty}$ observed (ng.h ml^{-1})	1960	1559	1743	1559	1540	1681±177
$AUC_{0\text{-}\infty}$ predicted (ng.h ml^{-1})	1968	1539	1712	1529	1523	1654±172
$AUMC_{0\text{-last}}$ (ng.h^2 ml^{-1})	1452175	75931	92634	111073	84331	101837±24615
$AUMC_{0\text{-}\infty}$ observed (ng.h^2 ml^{-1})	220214	100873	124873	144284	111103	140269±42537
$AUMC_{0\text{-}\infty}$ predicted (ng.h^2 ml^{-1})	186557	86530	102565	118523	95677	117970±35853
$MRT_{0\text{-last}}$ (h)	75.43	49.80	54.55	73.03	55.83	61.73±10.43
$MRT_{0\text{-}\infty}$ observed (h)	109.99	64.71	71.64	92.56	72.15	82.21±16.72
$MRT_{0\text{-}\infty}$ predicted (h)	94.81	56.21	59.91	77.49	62.82	70.25±14.25

Figs. 6.1(a) to 6.1(e)

Serum homidium concentrations in individual Boran cattle after treatment with homidium bromide at 1 mg kg^{-1} and challenged at 30 day intervals (↑)with a drug-sensitive population of *T. congolense* (IL 1180)

Fig. 6.1(a)
Animal No. 426

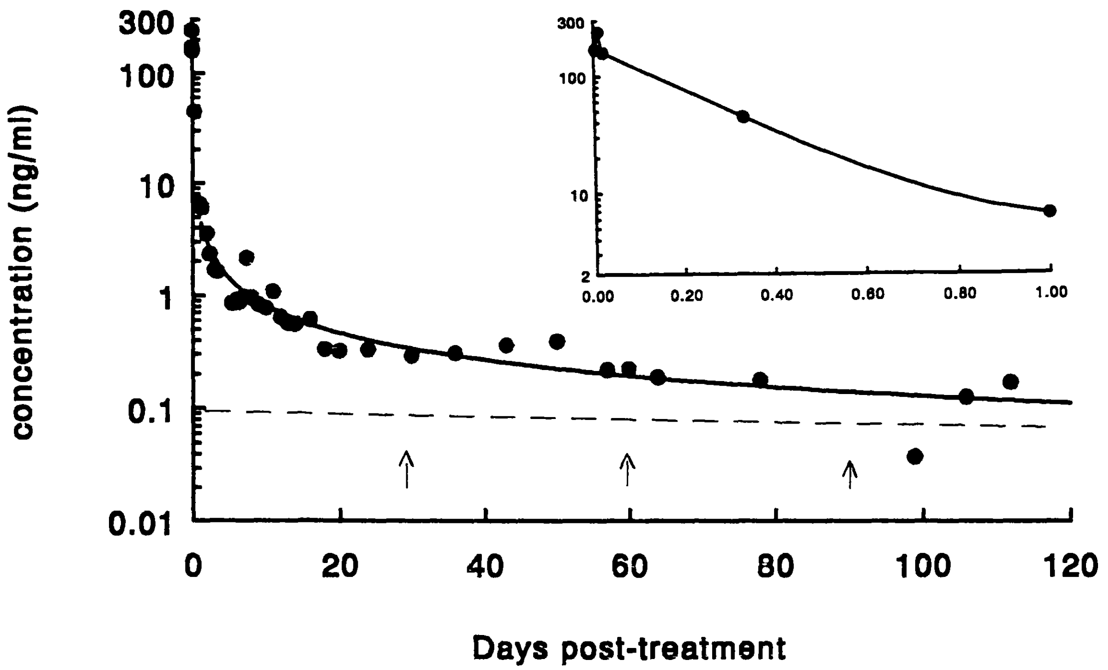


Fig. 6.1(b)
Animal No. 429

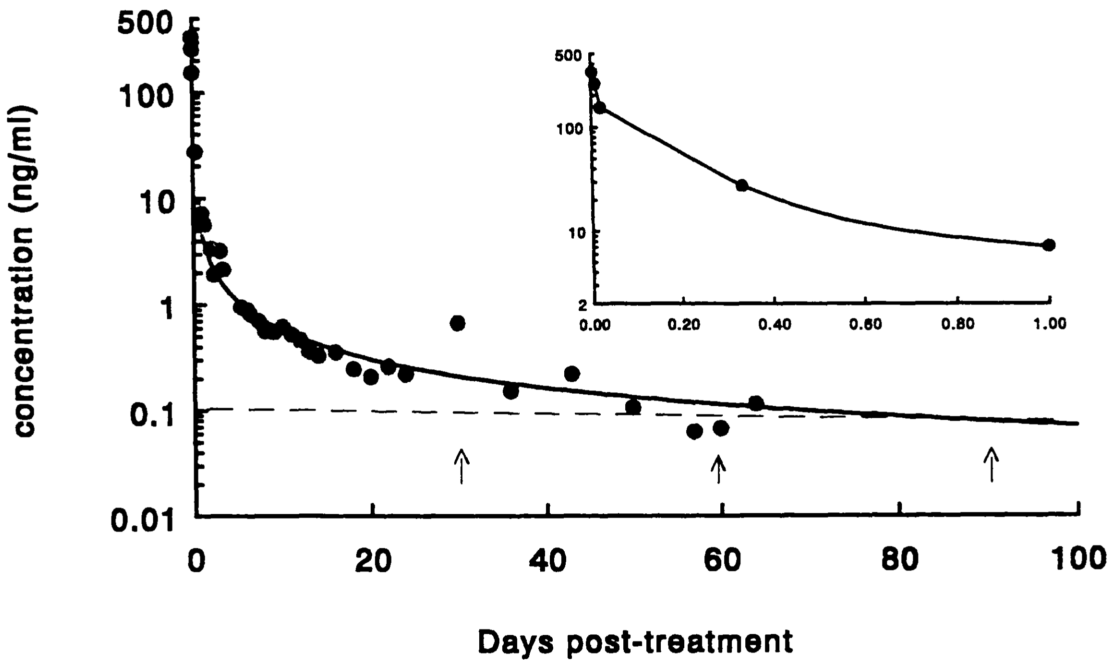


Fig. 6.1(c)
Animal No. 430

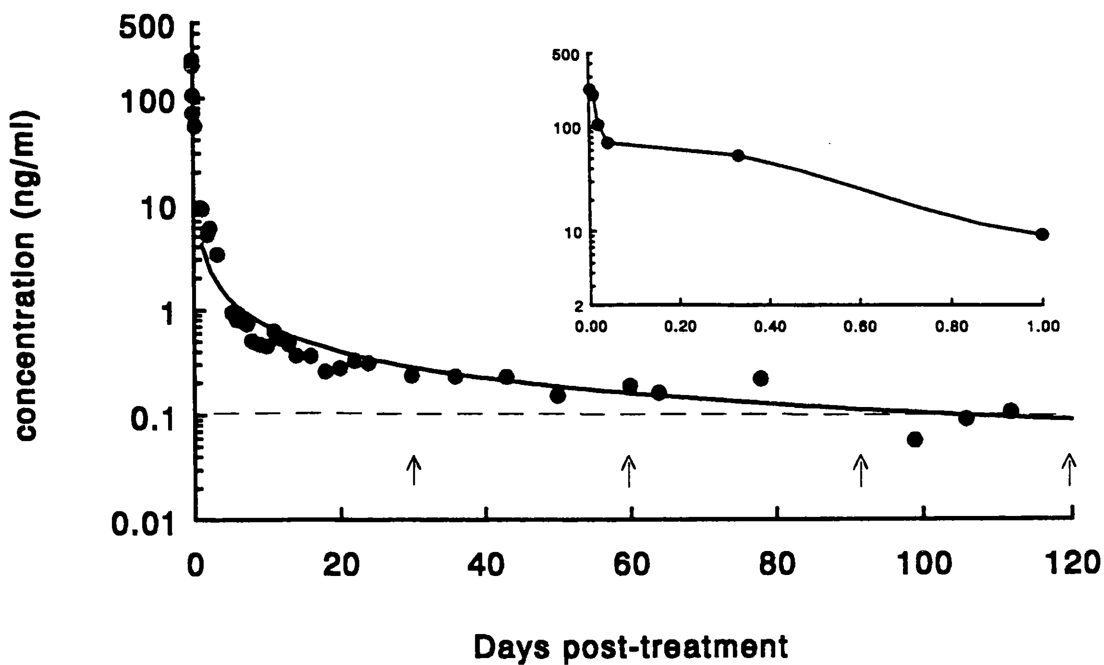


Fig. 6.1(d)
Animal No. 433

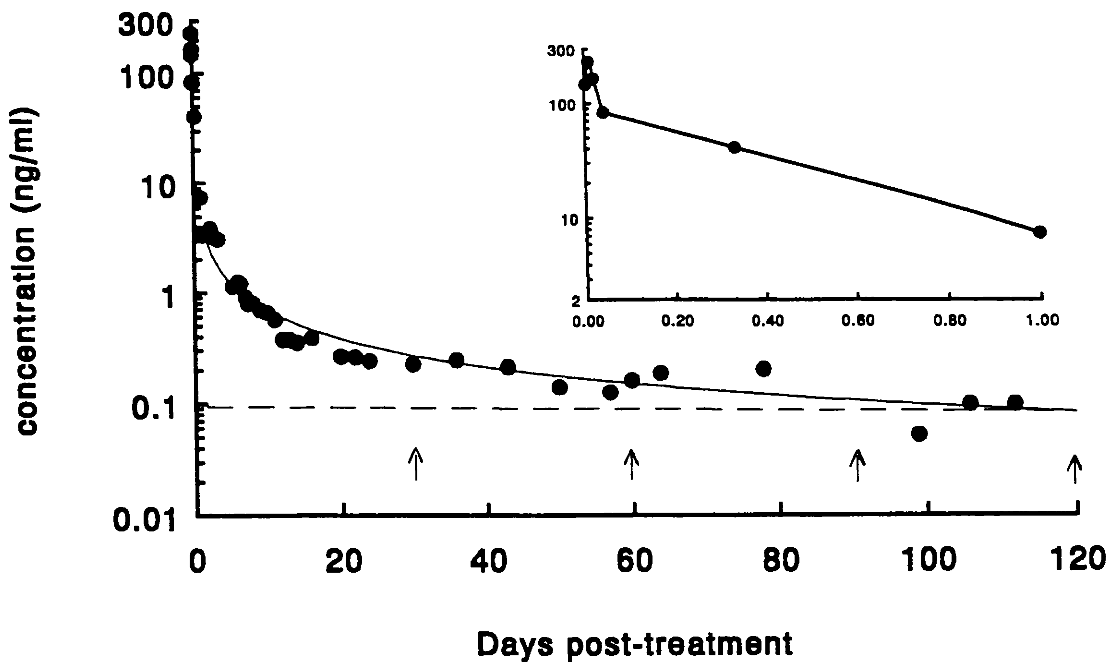
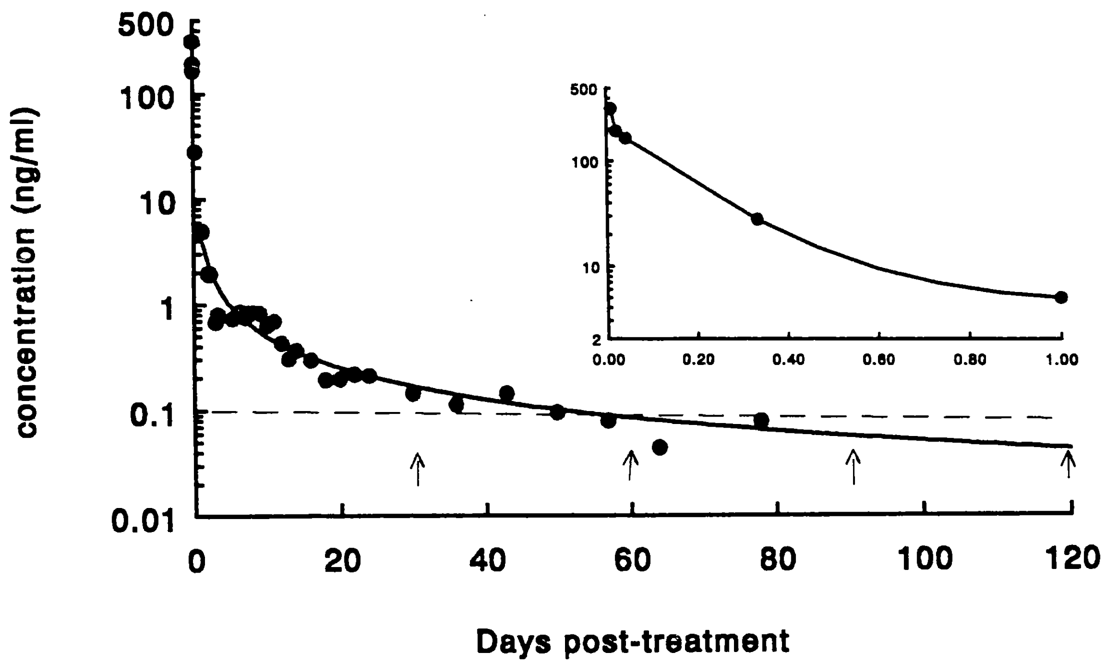


Fig. 6.1(e)
Animal No. 434



versus-time plot is shown in Fig. 6.2. The results showed a rapid decline in the concentrations during the first 24 hours of drug administration followed by an extremely slow terminal phase. Five minutes following treatment, the mean serum drug concentration was 218.9± 71.8 ng ml^{-1} The mean peak serum homidium concentration (C_{max}) was 269.6±45.4 ng ml^{-1} attained between 5 and 15 minutes after drug administration. Twenty four hours later, the mean circulating drug concentration was 7.1±1.4 ng ml^{-1}. Thereafter, the decline was extremely slow, reaching levels of 0.22±0.05 and 0.18±0.06 ng ml^{-1} at 30 and 60 days, respectively. Ninety days following treatment, three of the animals had concentrations between 0.13 and 0.2 ng ml^{-1} in circulation whereas two others had concentrations below the assay detection limit (0.1 ng ml^{-1}). The decline in the serum concentrations was similar to that observed in non-infected Boran cattle reported in Chapter 4 following i.m. injection.

Trypanosome infections

Group CC

Following the 30-day trypanosome challenge of homidium-treated cattle, the challenge control animal at each challenge developed an infection 8 days following trypanosome inoculation.

Group HS

Following the trypanosome challenge at 30 days after homidium treatment with a drug-sensitive stabilate of *T. congolense*, no trypanosomes were detected in any of the treated animals whilst trypanosomes were demonstrated in the peripheral blood of the challenged untreated control animal. The five homidium-treated Group HS cattle were therefore considered protected. These Group HS cattle were later challenged at 60, 90 and 120 days following treatment. Table 6.2 shows the individual as well as the group mean serum concentrations at the time of trypanosome challenge and the number of

Fig. 6.2

Mean ± SD serum homidium concentrations in Boran cattle (n = 5) following challenge at 30 day intervals with a drug-sensitive population of *T. congolense* (IL 1180) after treatment with homidium at 1 mg kg^{-1} b.w.

Fig. 6.2

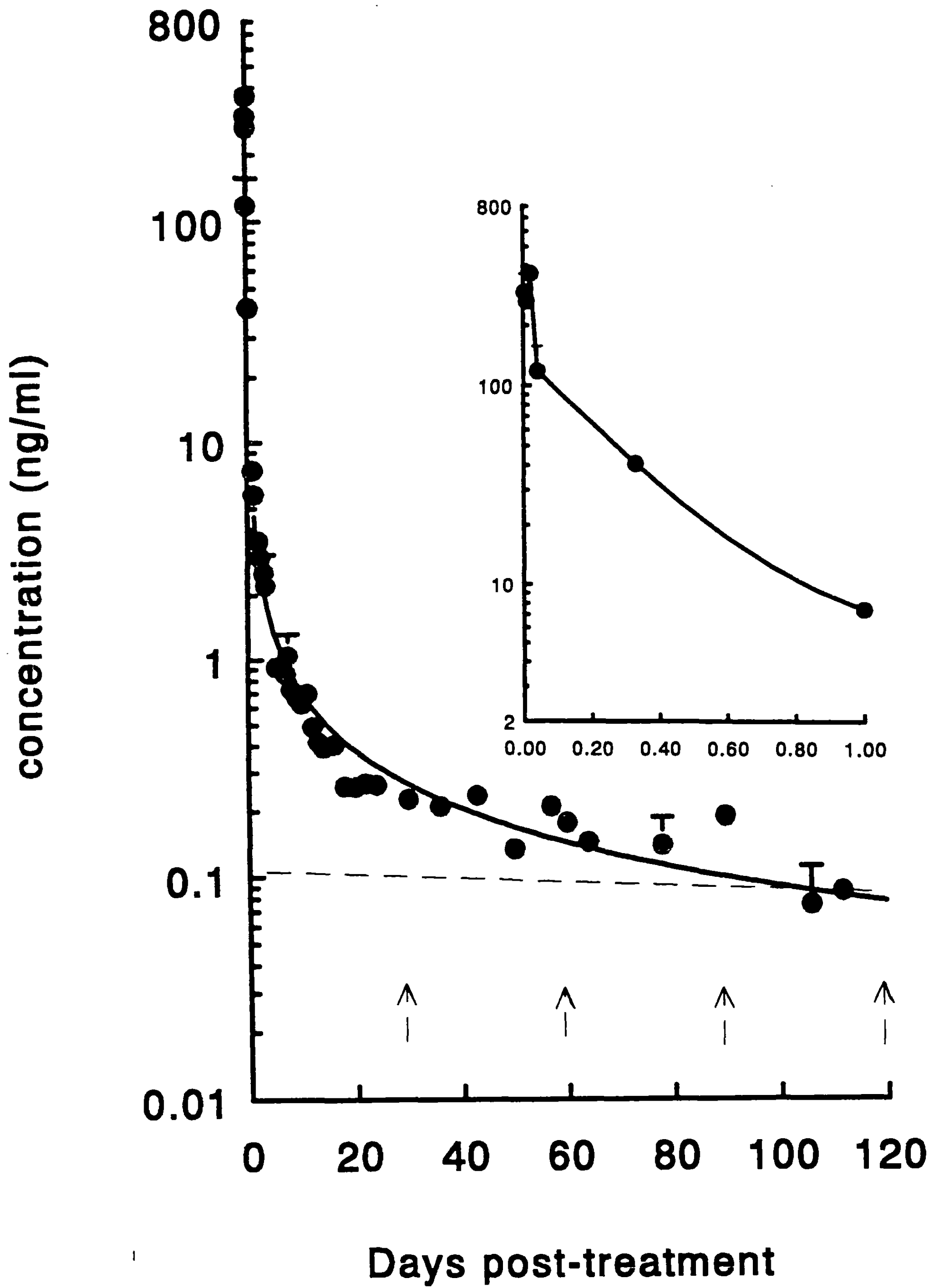


Table 6.2

Serum homidium concentrations (ng ml^{-1}) and response to challenge at 30 day intervals with a homidium-sensitive population of *Trypanosoma congolense* (IL 1180), in individual Boran cattle after administration of homidium bromide at 1 mg kg^{-1} b.w. (Group HS)

Time post-treatment (days)	Animal No. 426	429	430	433	434	Mean ± SD n = 5	protected /treated
30	0.30^{+}	0.22^{+}	0.24^{+}	0.22^{+}	0.15^{+}	0.22±0.05	5/5
60	0.19^{+}	0.12^{+}	0.16^{+}	0.19^{+}	$<0.1^{+}$	0.18±0.06	5/5
90	0.18^{+}	$<0.1^{*}$	0.22^{+}	0.20^{+}	$<0.1^{+}$	0.12±0.09	4/5
120	0.10^{*}	$<0.1^{*}$	$<0.1^{*}$	$<0.1^{*}$	$<0.1^{*}$	0.09±0.05	0/5

Assay limit of detection = 0.1 ng ml^{-1}

+ = animal protected

* = animal not protected

animals protected in Group HS cattle. Trypanosomes were first detected in peripheral blood of one animal (No. 429) 30 days after the third challenge on day 90; the same day the fourth challenge was due to be given. No trypanosomes were detected in the remaining four cattle at day 90 and they were therefore, considered protected. Trypanosomes were first detected in the peripheral blood of these four animals in Group HS 14, 17, 23 and 24 days after challenge at 120 days following drug administration. These four animals were therefore, not protected at 120 days following homidium treatment. Pre-patent periods determined from the time of trypanosome inoculation to the time when the trypanosomes were first detected in the blood of all five Group HS cattle ranged from 14 to 30 days. Table 6.3 shows a comparison in pre-patent periods between Group CC and Group HS animals. The periods between treatment and the first detection of trypanosomes in the peripheral blood of the five animals ranged from 120 to 144 days (Table 6.3).

For animal No. 429 which became infected following trypanosome challenge on day 90 post-treatment, the serum drug concentration at this time was already below the detection limit (0.1 ng ml^{-1} [Table 6.2]). This same animal had the lowest MRT value in the group. Animal No. 434 in which trypanosomes were first detected on day 144 following homidium treatment did not become infected following trypanosome challenges at 60 and 90 days post-treatment when homidium serum concentrations were below the limit of detection at both times (Table 6.2). The individual pre-challenge pharmacokinetics of the two animals (Nos. 429 and 434) which showed the shortest and the longest periods between treatment and demonstration of trypanosomes in the blood are given in Table 6.1. The two animals had similar C_{max} and AUC values but animal 429 showed markedly lower values for t_{max}, and MRT.

Table 6.3

A comparison in the pre-patent periods following trypanosome inoculation at 90 and 120 days post-treatment (p.t.) between Groups HS and CC

Group HS **Animal No.**	**Limit of protection (days)**	**Pre-patent period Group HS (days)**
426	120	14
429	90	30
430	120	23
433	120	17
434	120	24

Group CC Animal No.	**Date when challenge was given**	**Pre-patent period (days)**
436	90	8
431	120	8

Pathogenesis

All the untreated challenge control animals developed clinical signs that were similar to those reported in Chapter 5 following trypanosome infection. These signs included loss of appetite, a drop in PCV levels and loss in weight. In contrast, all the homidium-treated cattle remained healthy until after breakthrough trypanosome infections were detected; no significant changes were observed in the PCV from the pre-infection values of approximately 40% until trypanosome breakthrough infections were established. After establishment of infection, the changes observed were similar to those reported in Chapter 5. Following treatment of infected Group HS cattle with diminazene when they had attained a PCV of approximately 15%, trypanosomes were cleared from the peripheral blood within 48 hours. Thereafter, all the above clinical signs were reversed with animals feeding well and an elevation of the PCV to pre-infection values within one week. The animals then remained aparasitaemic during a follow-up period of two months.

The pre-infection mean total serum protein of Group HS animals was 44.9 ± 4.0 mg ml^{-1} serum. The values obtained after 60 and 90 days were 46.1 ± 3.0 and 42.8 ± 2.6 mg ml^{-1}, respectively. There were no significant differences between these values and those obtained before infection (Fig. 6.3). Similarly, no significant differences were observed between pre-treatment serum albumin and total globulin levels and the levels obtained over the same period of 90 days (Figs. 6.4 and 6.5).

Anti-trypanosome antibodies

No anti-trypanosome antibodies were detected by IFAT in the samples collected at 30, 60, 90 days following treatment of Group HS (collected immediately prior to trypanosome challenge). However, antibodies were detected in the serum samples collected from the untreated challenge controls at 30 and 60 days following trypanosome inoculation.

Fig. 6.3

Mean ± SD total serum protein levels in Boran cattle (n = 5) following challenge at 30 day intervals with a drug-sensitive population of *T. congolense* (IL 1180) after treatment with homidium at 1 mg kg^{-1} b.w.

Fig. 6.3

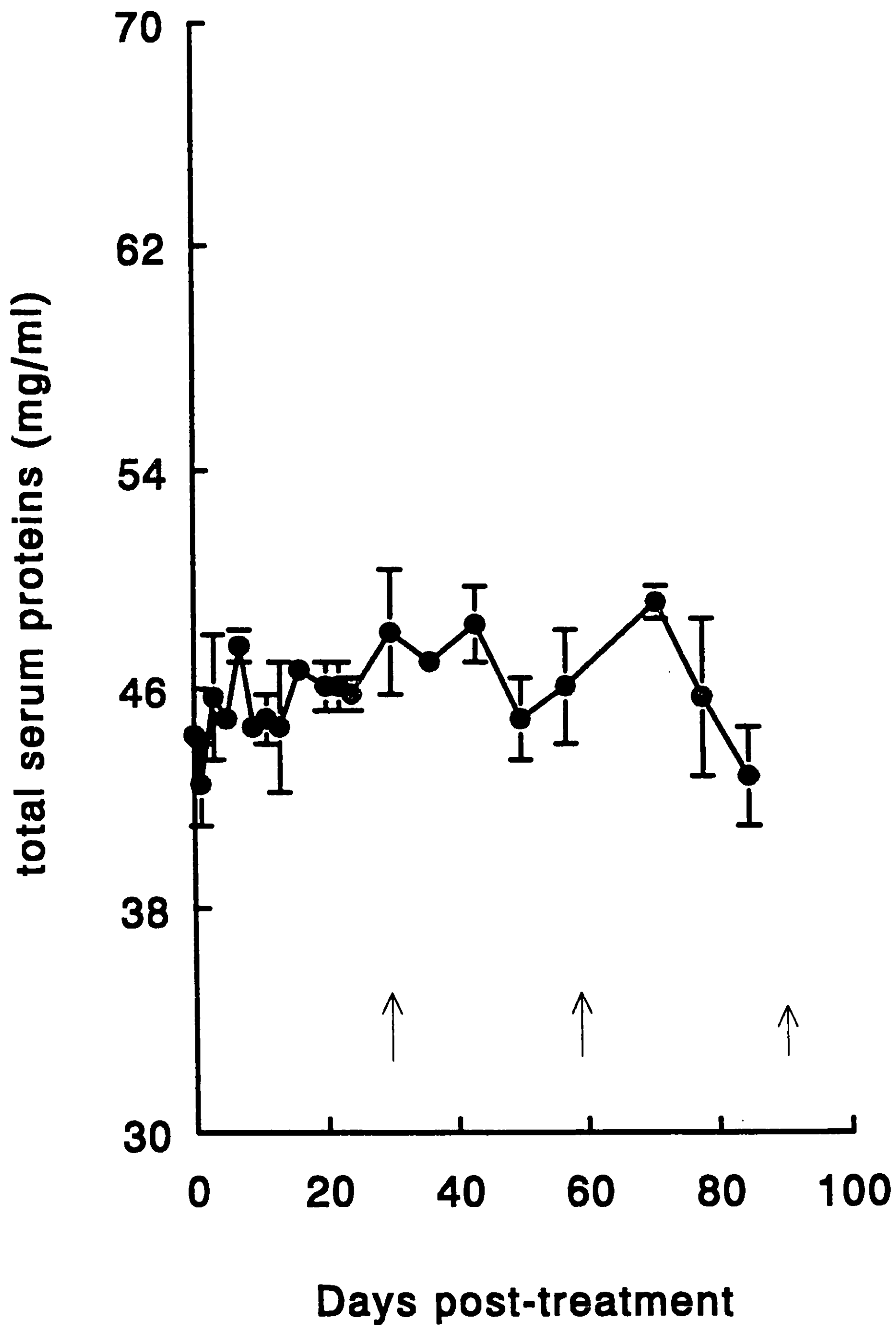


Fig. 6.4

Mean ± SD serum albumin levels in Boran cattle (n = 5) following challenge at 30 day intervals with a drug-sensitive population of *T. congolense* (IL1180) after treatment with homidium at 1 mg kg^{-1} b.w.

Fig. 6.4

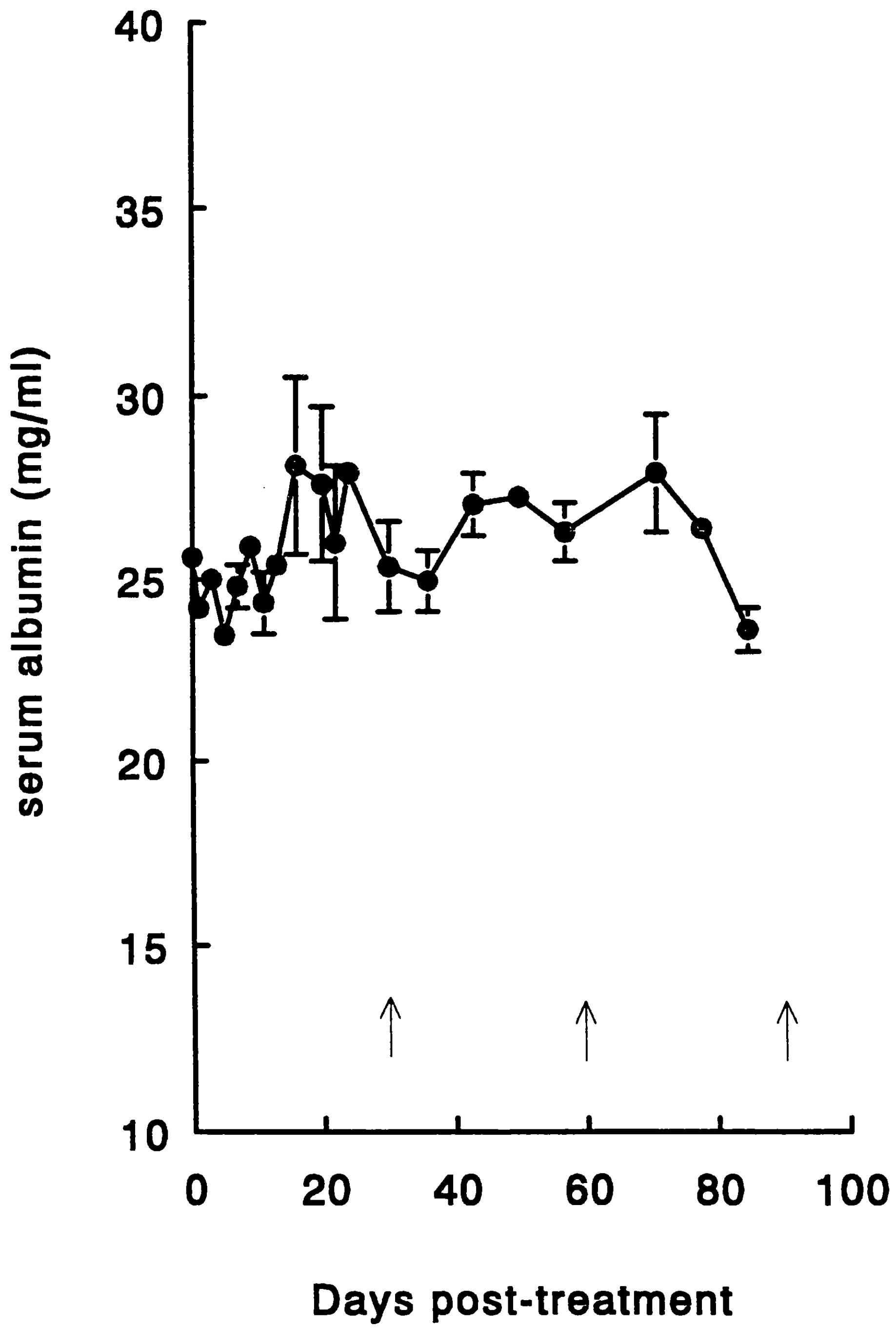
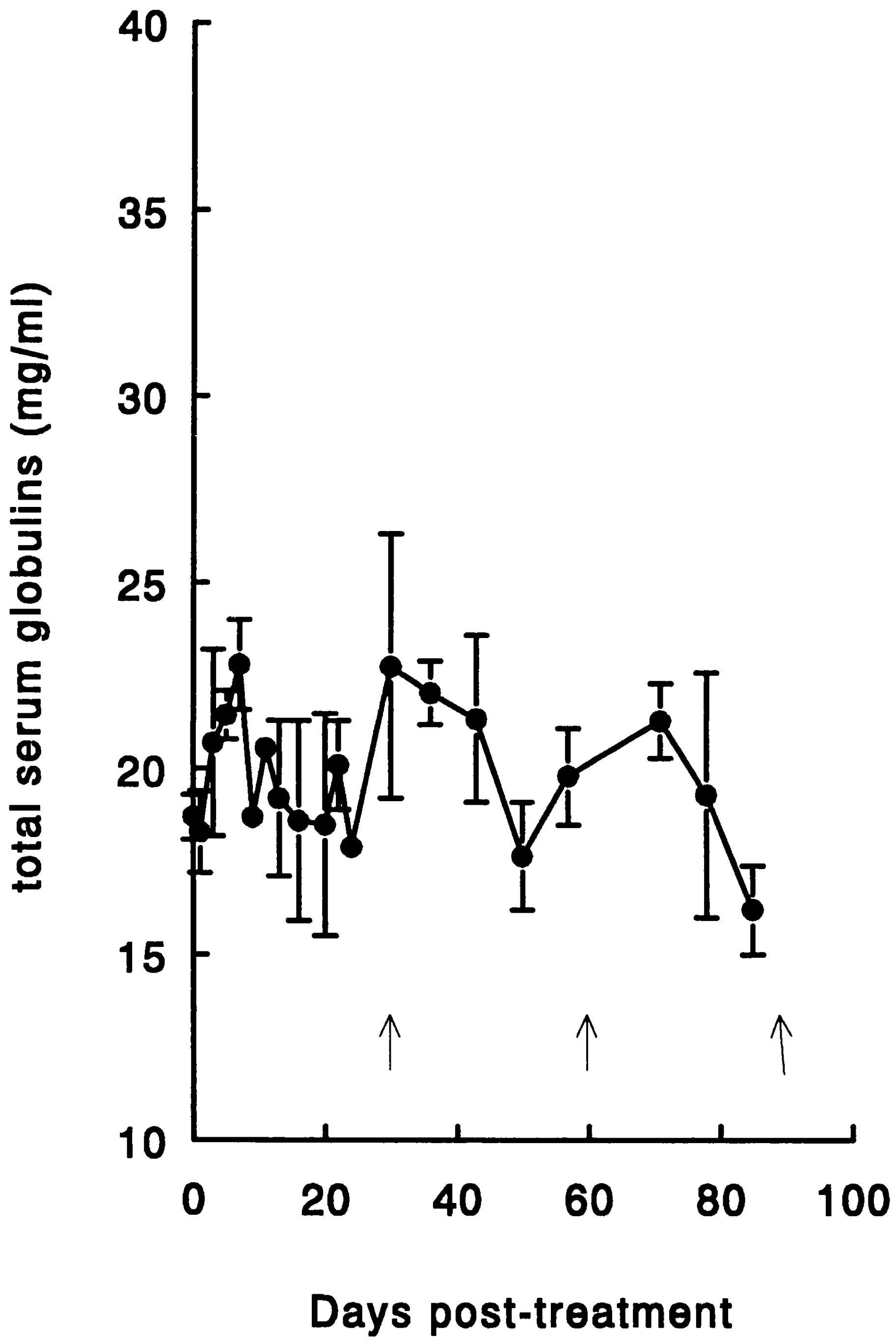


Fig. 6.5

Mean ± SD total serum globulin levels in Boran cattle (n = 5) following challenge at 30 day intervals with a drug-sensitive population of *T. congolense* (IL1180) after treatment with homidium at 1 mg kg^{-1} b.w.

Fig. 6.5



Cattle treated with homidium bromide and challenged at monthly intervals with a homidium-resistant trypanosome population

Pre-challenge pharmacokinetics of homidium

The pre-challenge pharmacokinetics obtained from a non-compartmental analysis are shown in Table 6.4. The mean $AUC_{0-\infty}$ observed value was 1556±173 ng.h ml^{-1}. The $MRT_{0-\infty}$ observed values was 72.3±13.4 hours. These pre-challenge pharmacokinetic parameters were found to be similar to the pre-challenge pharmacokinetic parameters obtained in Group HS cattle and in Boran cattle reported in Chapter 4 following i.m. treatment.

Serum homidium concentrations

Serum homidium concentration-versus-time plots for the individual animals in Group HR are given in Figs. 6.6(a) to 6.6(e). The mean peak serum concentration was 282.8±108.5 ng ml^{-1} attained between 5 and 15 minutes following treatment. The serum homidium concentration-versus-time profile obtained during the first 30 days following treatment of Group HR cattle was similar to that observed in Group HS cattle during the same period. At the time of the first trypanosome challenge at 30 days post-treatment, the mean homidium concentration in serum was 0.20±0.05 ng ml^{-1} (range 0.15 - 0.30). Fig. 6.7 shows the mean (±SD) serum homidium concentration-versus-time profile following challenge of homidium-treated Group HR cattle

Following the first trypanosome challenge at 30 days after homidium treatment of the Group HR cattle, the challenge control was detected parasitaemic 8 days later. Trypanosomes were detected in four of five Group HR cattle after 9 days and in the remaining one animal 10 days following trypanosome inoculation. Thus, the pre-patent periods were only one or two days longer than the Group CC animal. The results showed that the serum homidium concentrations at the time of trypanosome inoculation were not protective against establishment of infection.

Table 6.4

Pre-challenge pharmacokinetic parameters for homidium in individual Boran cattle of Group HR treated with homidium bromide at 1 mg kg^{-1} b.w.

Parameter	Animal No.					Mean±SD n = 5
	435	**437**	**438**	**442**	**444**	
t_{max} (h)	0.072	0.24	0.07	0.48	0.07	0.19±0.16
C_{max} (ng ml^{-1})	454.2	187.1	356.8	165.5	250.4	282.8±108.5
β	0.0066	0.0085	0.0071	0.0044	0.0080	0.0069±0.0014
$t_{½β}$ (h)	105.6	81.3	97.9	157.6	86.7	105.7±27.3
$AUC_{0\text{-last}}$ (ng.h ml^{-1})	1477	1531	1361	1846	1398	1523±172
$AUC_{0\text{-}\infty}$ observed(ng.h ml^{-1})	1513	1556	1401	1883	1429	1556±173
$AUC_{0\text{-}\infty}$ predicted(ng.h ml^{-1})	1484	1538	1375	1864	1406	1533±175
$AUMC_{0\text{-last}}$ (ng.h^2 ml^{-1})	105149	248265	91147	89823	58991	118675±66526
$AUMC_{0\text{-}\infty}$ observed (ng.h^2 ml^{-1})	1370284	99454	119515	124281	79261	111908±20312
$AUMC_{0\text{-}\infty}$ predicted (ng.h^2 ml^{-1})	111530	87034	101222	106698	64119	94121±17100
$MRT_{0\text{-last}}$ (h)	71.30	53.80	66.96	48.65	42.20	56.56±10.94
$MRT_{0\text{-}\infty}$ observed (h)	90.55	63.93	85.32	66.01	55.47	72.25±13.38
$MRT_{0\text{-}\infty}$ predicted (h)	75.15	56.60	73.60	57.23	45.61	61.64±11.20

Figs. 6.6(a) to 6.6(e)

Serum homidium concentrations in individual Boran cattle after treatment with homidium bromide at 1 mg kg^{-1} and challenged at 30 day intervals (↑) with a drug-resistant population of *T. congolense* (IL 3330)

Fig. 6.6(a)
Animal No. 435

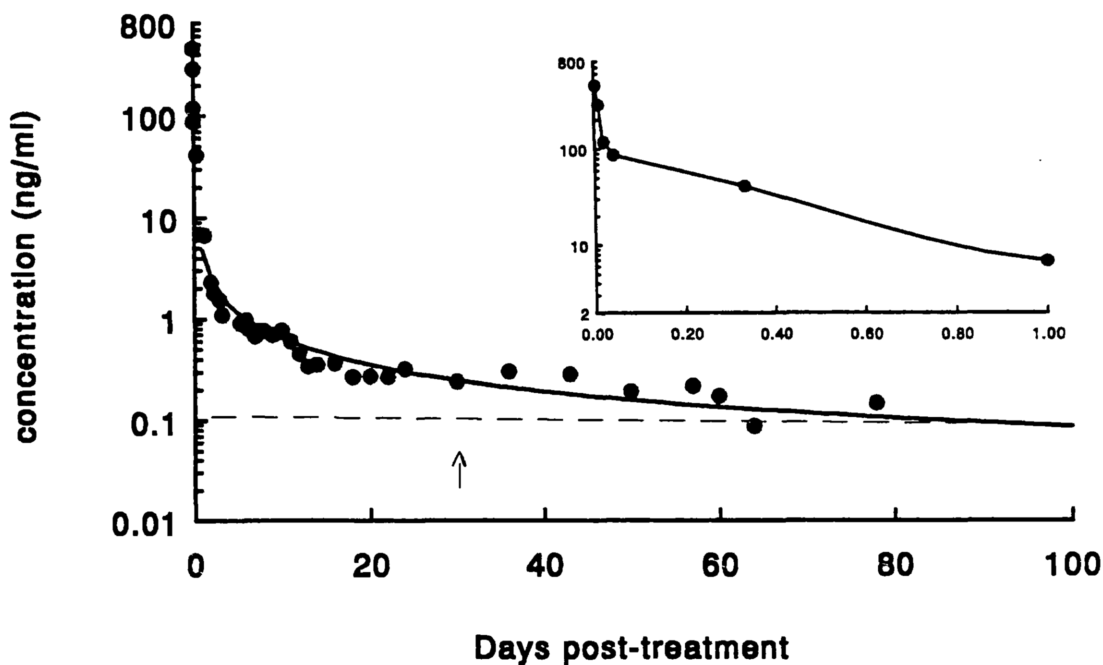


Fig. 6.6(b)
Animal No. 437

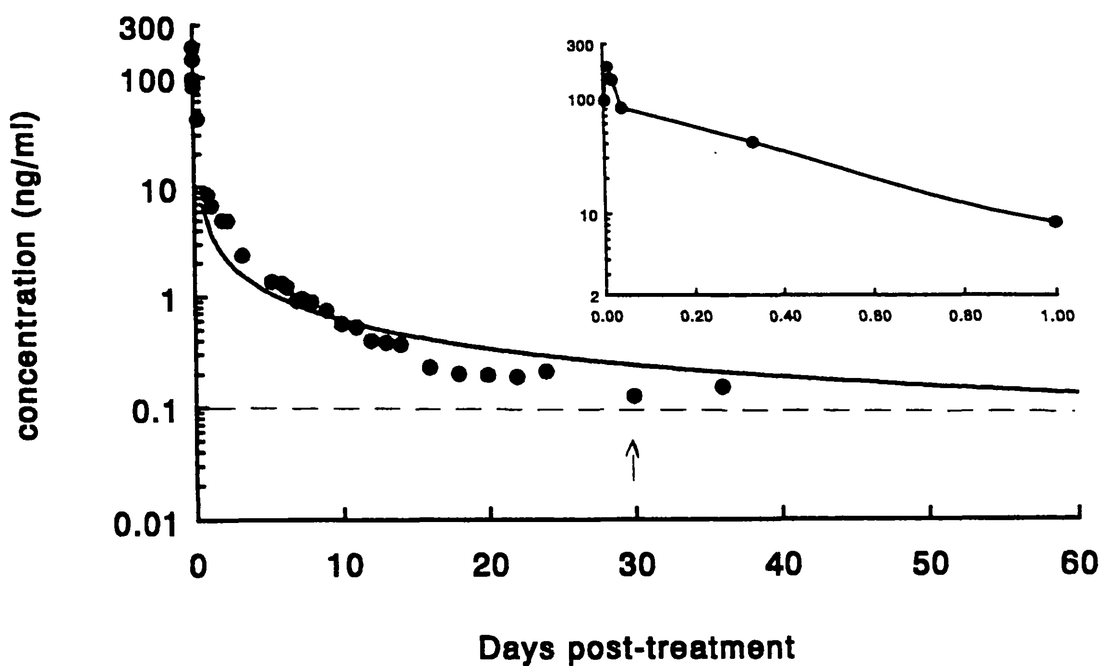


Fig. 6.6(c)
Animal No. 438

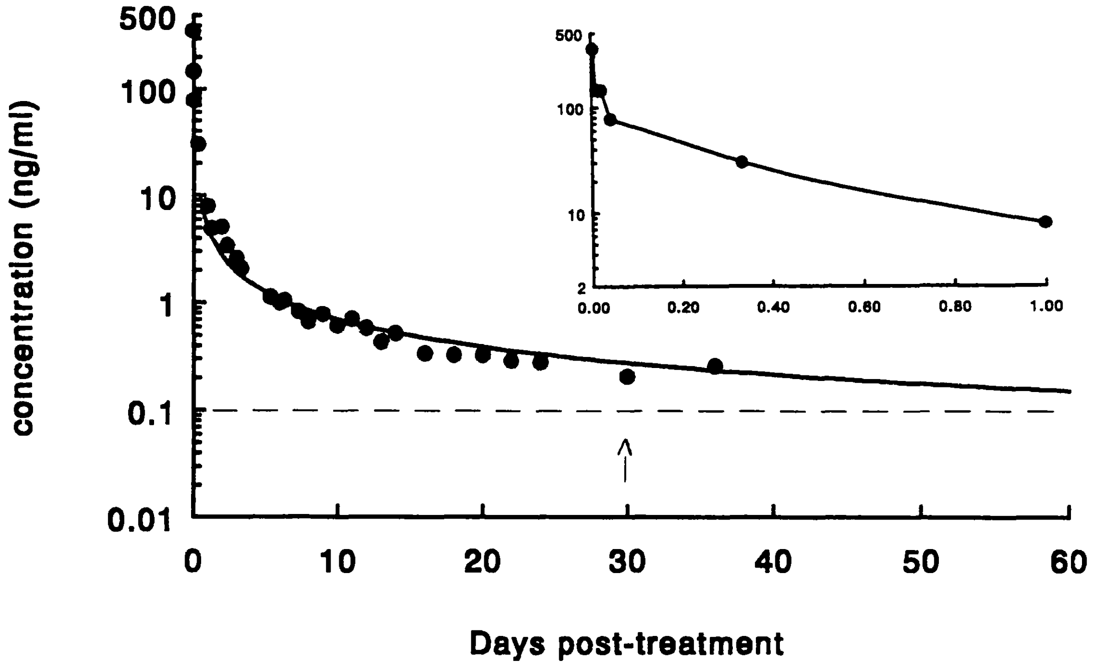


Fig. 6.6(d)
Animal No. 442

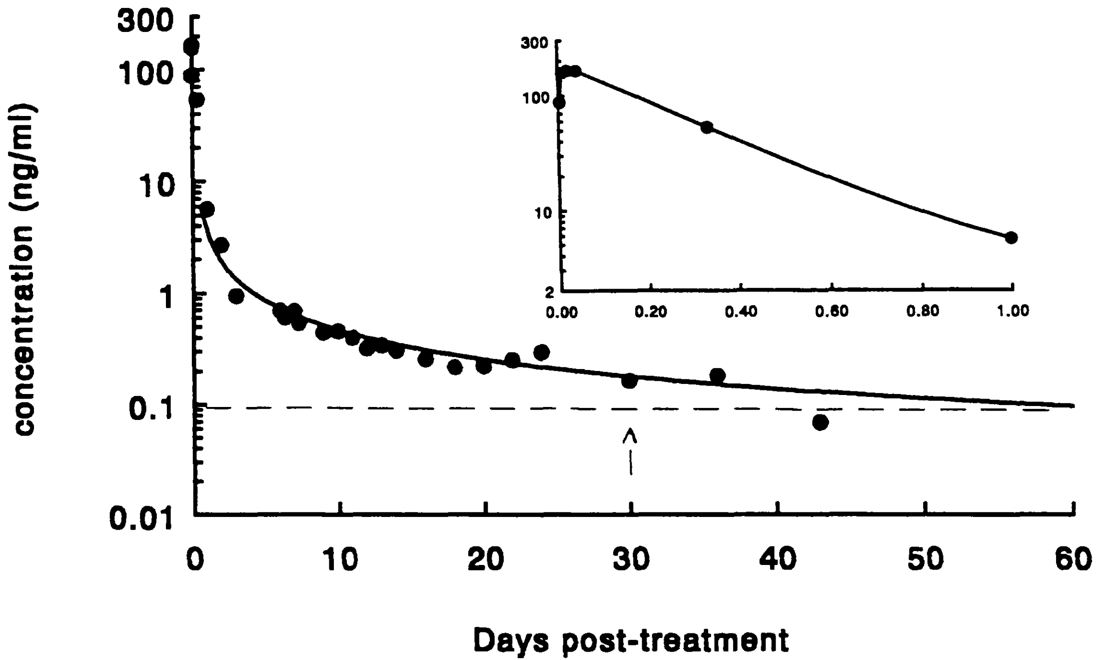


Fig. 6.6(e)
Animal No. 444

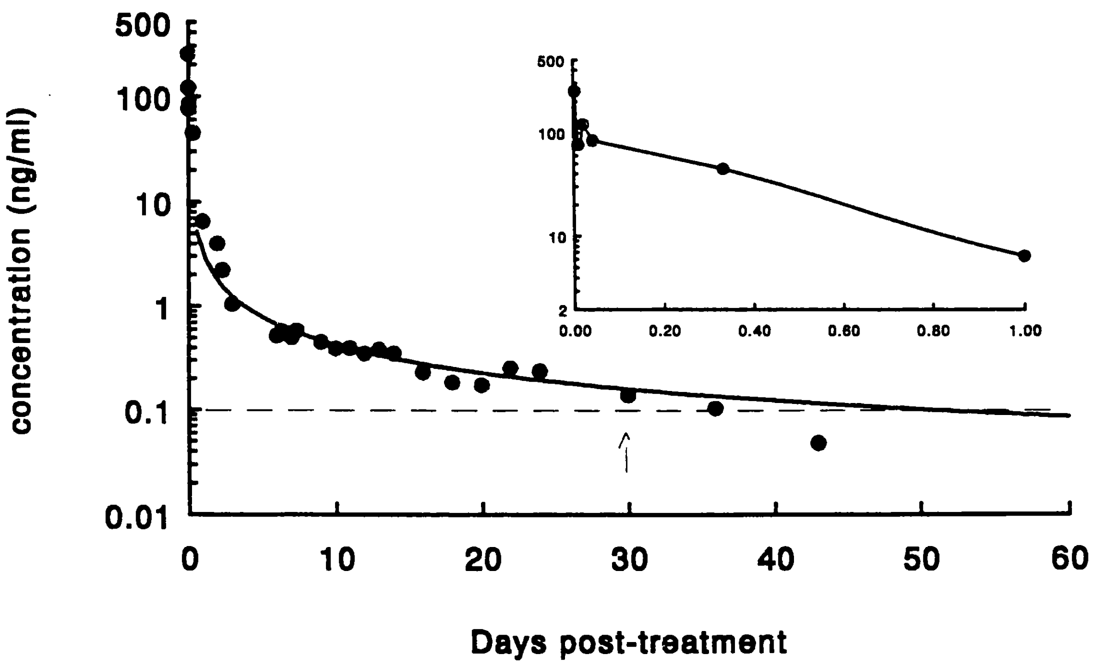
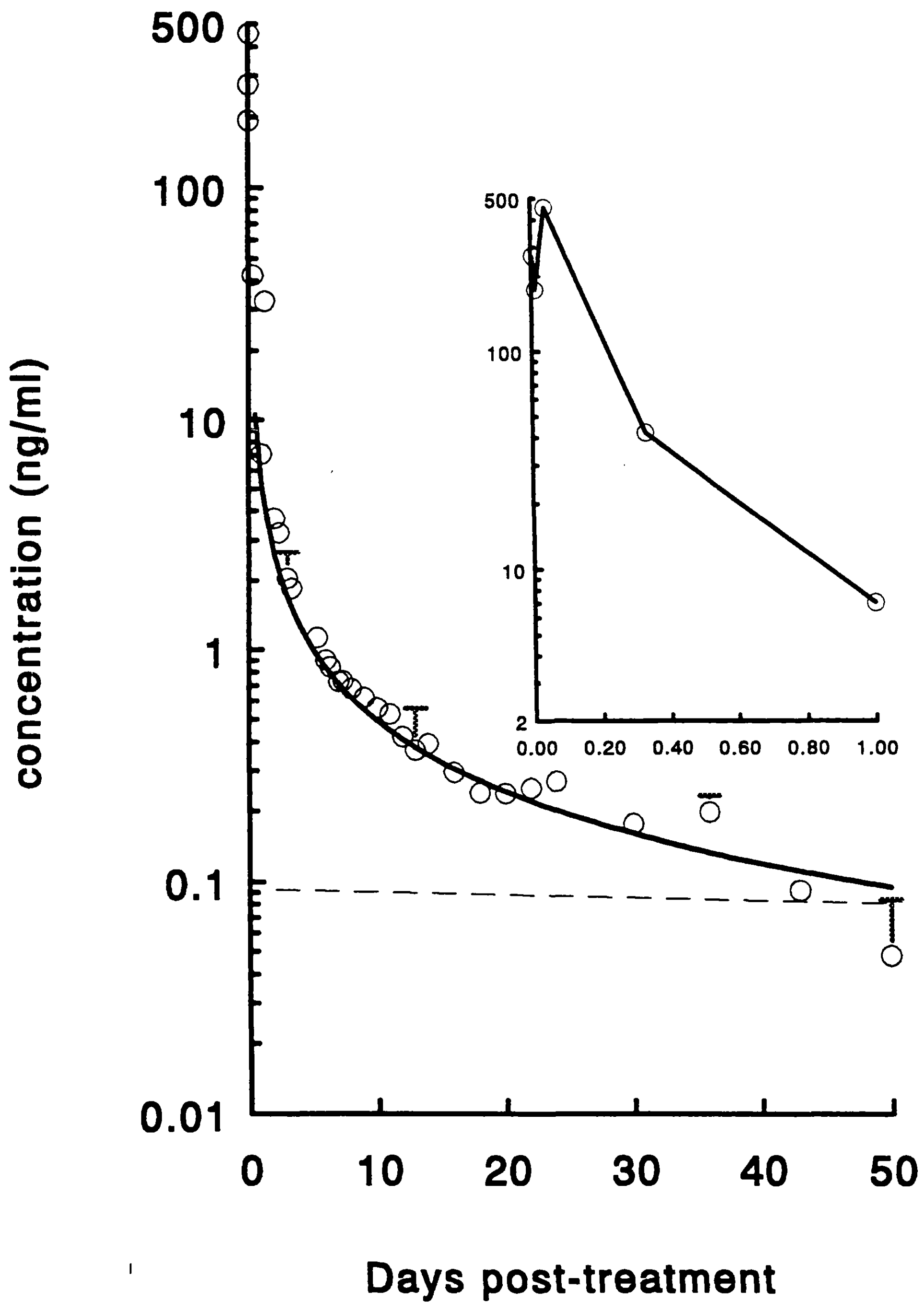


Fig. 6.7

Mean ± SD serum homidium concentrations in Boran cattle (n = 5) following challenge at 30 day intervals with a drug-resistant population of *T. congolense* (IL 3330) after treatment with homidium at 1 mg kg^{-1} b.w.

Fig. 6.7



No drug was detected in the circulation of all Group HR cattle 13 days following the first trypanosome challenge given at 30 days (approximately 43 days following treatment; 6 days after trypanosomes were first detected in the peripheral blood). An acceleration in the drug disappearance rate in serum was observed during the 13 days (Fig. 6.7). The decline in serum homidium concentrations following establishment of infection was more rapid than that observed in cattle which did not become infected until the third or fourth trypanosome challenges. This accelerated rate of decline in drug concentration was, however, similar to that obtained in cattle infected with the resistant trypanosome population, reported in Chapter 5. Fig. 6.8 shows a comparison of the mean ± SD serum homidium concentrations for cattle in Groups HS and HR following trypanosome challenge after homidium treatment.

Pathology and pathogenesis

As in the cattle challenged with the homidium-sensitive trypanosomes, there were no significant changes in the packed cell volume from the time of homidium treatment until the time of the first trypanosome challenge which was carried out 30 days later. But after the establishment of parasitaemia, there was a drop in the PCV from 35% to 20% within two weeks of challenge.

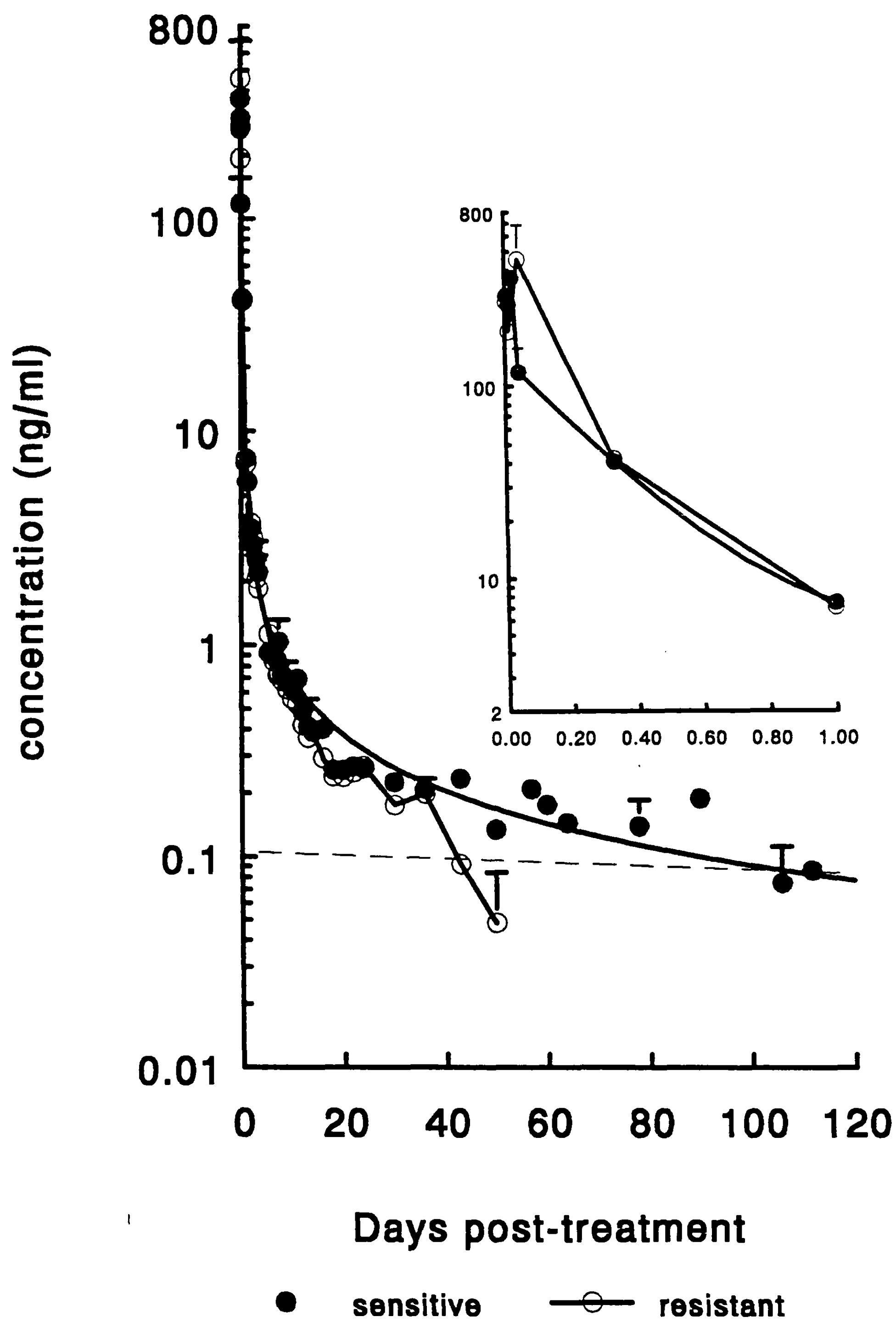
Out of the six animals challenged with the homidium-resistant trypanosome population (one control and five homidium treated), one died on day 85 following homidium treatment when the PCV was 16%, three were treated with diminazene at a dose rate of 14 mg kg^{-1} b.w. on day 86 following homidium treatment (56 days following trypanosome challenge) on the day they attained a PCV of 15%. The remaining two animals died on days 88 and 92 respectively, one day after both attained a PCV of 15% and before diminazene could be given.

In the diminazene-treated animals, trypanosomes were cleared from peripheral blood of all the three animals within 24 hours. Thereafter, two

Fig. 6.8

Mean ± SD serum homidium concentrations in Boran cattle (n = 5) following challenge at 30 day intervals (↑) with either a drug-sensitive (IL 1180) or a drug-resistant (IL 3330) population of *T. congolense* after treatment with homidium bromide at 1 mg kg^{-1} b.w.

Fig. 6.8



remained negative during a two-month follow-up period whereas one relapsed 39 days after diminazene treatment.

The three animals which died from trypanosomiasis in Group HR were still in reasonable physical body condition although they had lost up to 21% of body weight. On post-mortem, petechial haemorrhages of the kidneys, oesophagus, abomasum, small intestine, colon and the vascular system were observed. Lymph nodes were enlarged and haemorrhagic. The larynx, trachea, bronchi and lungs appeared frothy. The pericardium and peritoneal cavity were filled with straw-coloured fluid and the heart appeared flabby. Enlargement of the liver and the spleen were also observed.

Determination of the total protein levels in Group HR cattle showed that from a mean pre-infection value of 44.9±4.0 mg ml^{-1}, the values were 37.0±5.9 and 39.7±8.2 mg ml^{-1} after 60 and 90 days, respectively. These values were not significantly different from pre-infection values (Fig. 6.9). Sixty and 90 days post-treatment, the serum albumin levels were 21.3±0.3 and 19.3±0.5 mg ml^{-1}, respectively, a significant drop from the mean infection value of 27.8±0.3 mg ml^{-1} (Fig. 6.10). There was however, a slight elevation in the total immunoglobulin levels from the pre-infection value of 17.1±1.5 mg ml^{-1} to 20.4±1.8 mg ml^{-1} within 90 days of treatment (Fig. 6.11).

DISCUSSION

Having demonstrated in the previous studies reported in this thesis (Chapters 4 and 5) that serum homidium concentrations of between 0.1 and 0.3 ng ml^{-1} are detectable in circulation of non-infected cattle for periods of over 10 weeks, it was decided that studies be carried out to determine whether these low drug concentrations are protective against trypanosome infections. The results of the present study in which cattle were challenged with either a drug-sensitive or a drug-resistant trypanosome population following homidium prophylactic treatment showed that the low serum homidium concentrations

Fig. 6.9

Mean ± SD total serum protein levels in Boran cattle (n = 5) following challenge at 30 day intervals with a drug-resistant population of *T. congolense* (IL 3330) after treatment with homidium at 1 mg kg^{-1} b.w.

Fig. 6.9

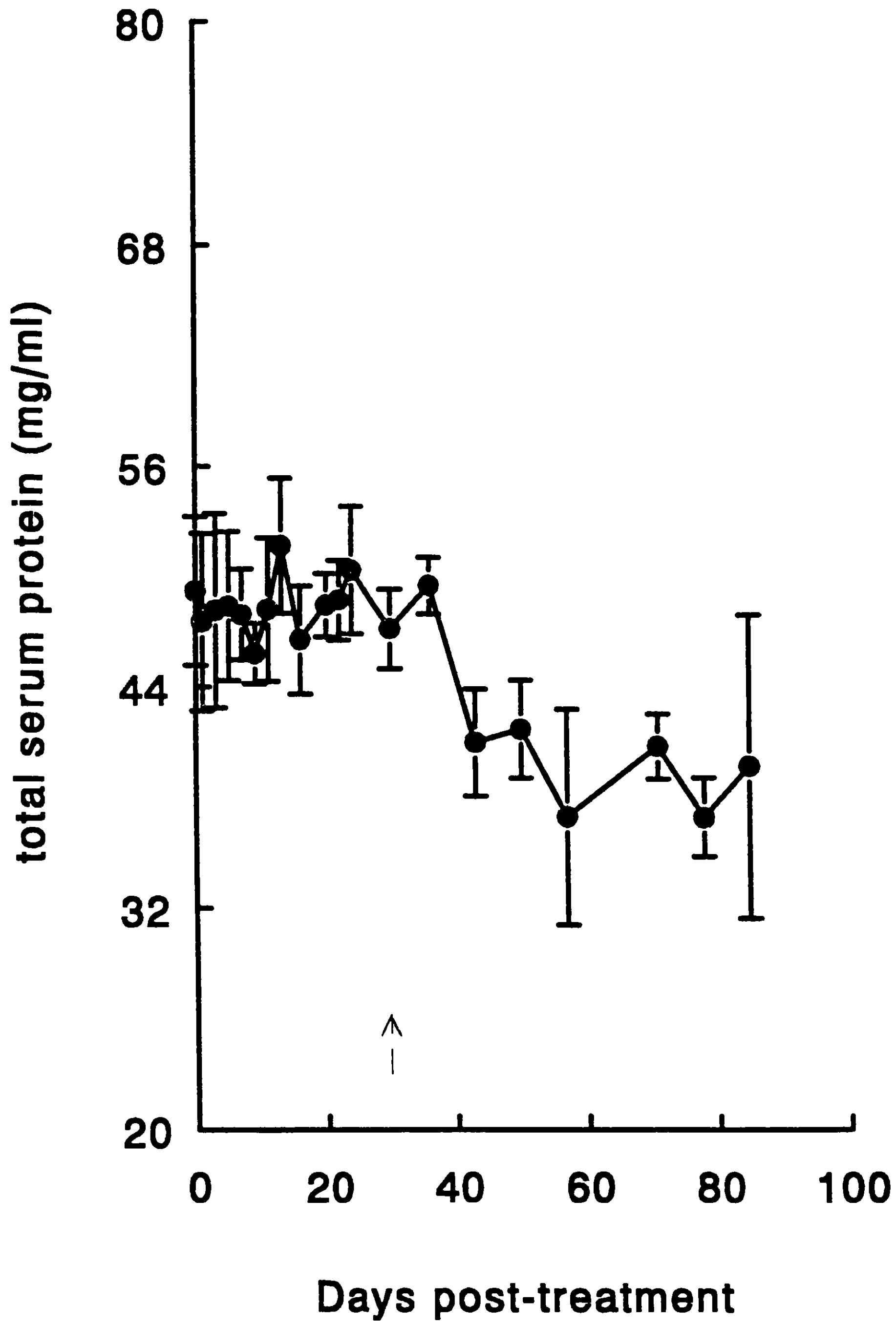


Fig. 6.10

Mean ± SD serum albumin levels in Boran cattle (n = 5) following challenge at 30 day intervals with a drug-resistant population of *T. congolense* (IL3330) after treatment with homidium at 1 mg kg^{-1} b.w.

Fig. 6.10

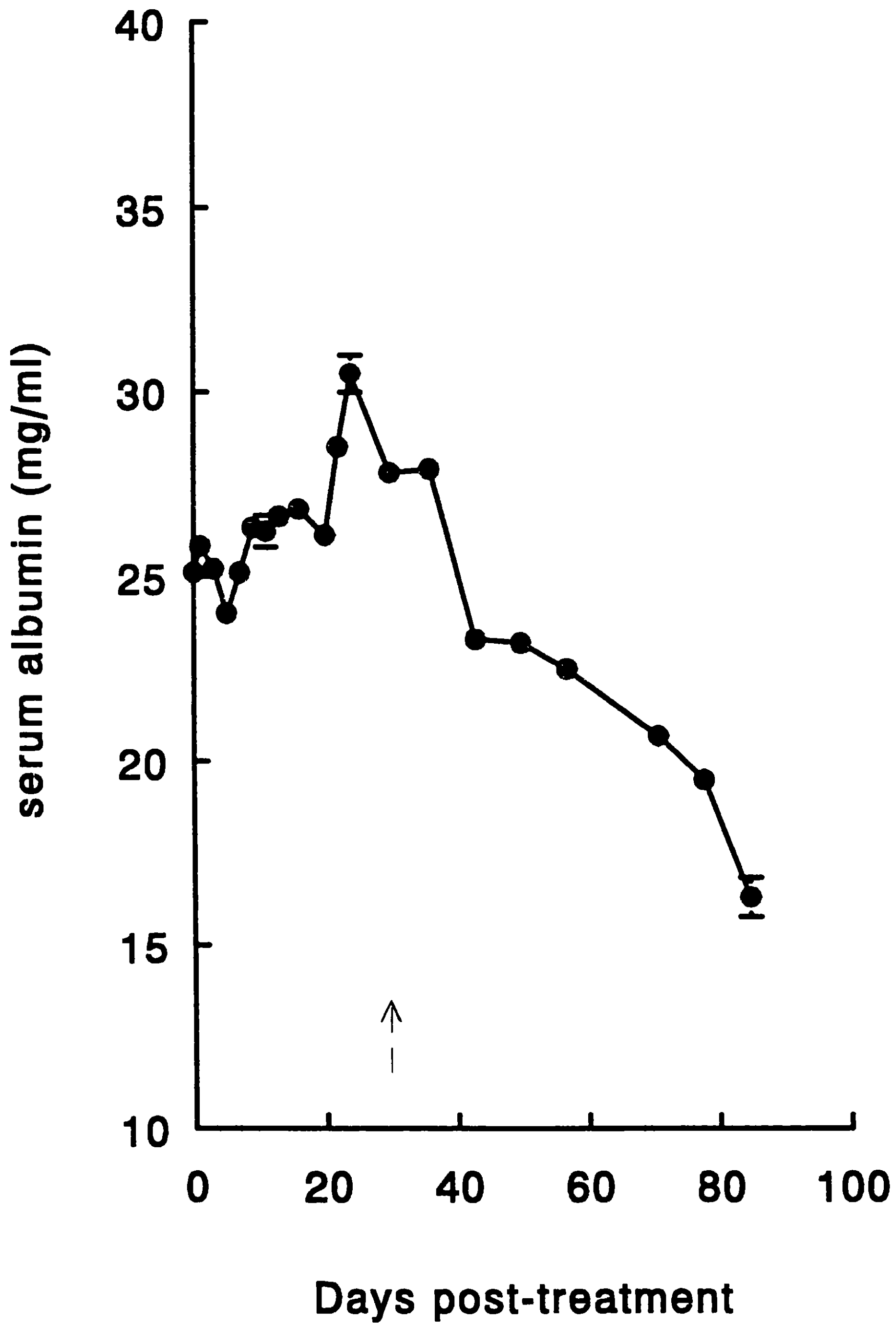
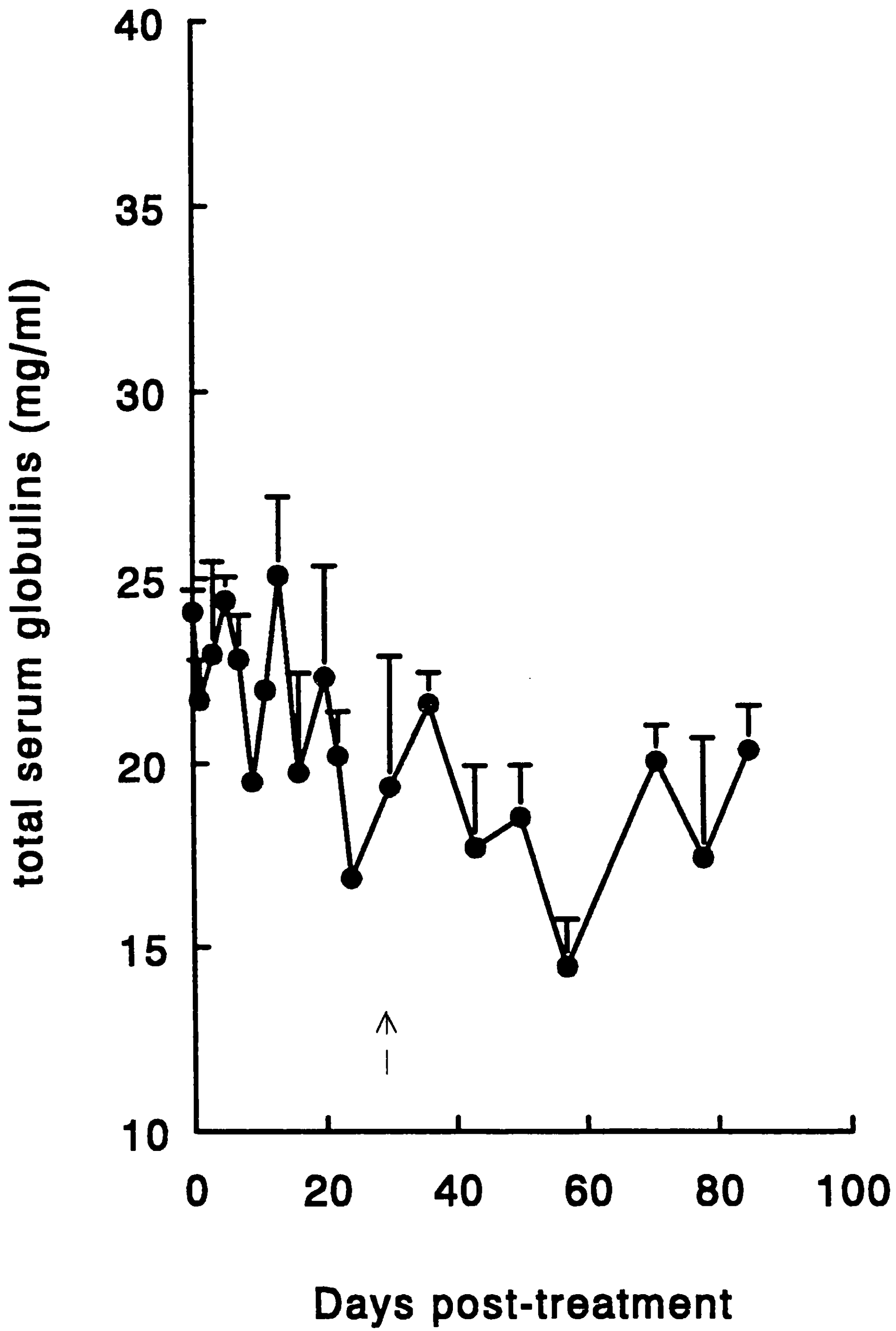


Fig. 6.11

Mean ± SD total serum globulin levels in Boran cattle (n = 5) following challenge at 30 day intervals with a drug-resistant population of *T. congolense* (IL3330) after treatment with homidium at 1 mg kg^{-1} b.w.

Fig. 6.11



were protective against challenge with a drug-sensitive trypanosome population for periods of over 90 days in four of five cattle in the group. Presence of anti-trypanosome antibodies was not demonstrated. However, prophylaxis against challenge by a drug-resistant trypanosome population was not achieved at 30 days post-treatment.

Following i.m. treatment of Group HS cattle with homidium, the drug decline was exponential with an initial rapid fall during the first 24 hours, followed by a slow phase of elimination of the drug. Pre-challenge pharmacokinetics were similar to those reported in Chapter 4 of this thesis for non-infected Boran cattle. However, the results showed wide variations in MRT values between individual animals suggesting that the difference in the mean residence time of the drug in a group of animals given similar treatment could vary as much as two-fold.

At each trypanosome challenge of Group HS cattle carried out at 30 day intervals after treatment, each of the challenged untreated controls were detected parasitaemic on day 8 following trypanosome inoculation showing that the trypanosomes were infective.

Following the 90 day trypanosome challenge of Group HS cattle, trypanosomes were first detected in animal No. 429, 120 days after homidium treatment and on the day it was due for the fourth challenge. The pre-patent period was 30 days. The remaining four animals in this group became parasitaemic following the fourth challenge carried out on day 120 after homidium treatment. The exact periods from homidium treatment to first detection of trypanosomes in the four animals were 134, 137, 143 and 144 days with pre-patent periods of 14, 17, 23 and 24 days. These periods were similar to those reported in the field using homidium bromide at dose rates of 1.0 mg kg^{-1} b.w. (Unsworth, 1954b; Mwambu, 1971; Dolan et al, 1990).

The results of the present study showed that homidium's pre-challenge pharmacokinetics appeared to contribute to the length of the observed period

between treatment and demonstration of parasites in the circulation in the Group HS cattle. Animal No. 429 in which trypanosomes were first detected on day 120 following treatment had an extrapolated MRT value of approximately 65 hours which was the lowest recorded in the group (Table 6.1). This low MRT value would suggest that the drug did not reside in the body for long periods of time and hence reduced period of protection compared to the other four animals in the group.

At the first trypanosome challenge of Group HS cattle carried out at 30 days following homidium treatment, the group mean serum drug concentration in the animals was 0.22±0.05 ng ml^{-1} which was effective against establishment of trypanosomes following challenge. Similarly, a mean concentration of 0.18±0.06 ng ml^{-1} at the second challenge carried out at 60 days following treatment, was significant to protect cattle against challenge by a drug-sensitive population of *T. congolense*. However, at this time, one animal (No. 434) had a drug concentration below the limit of detection of the assay but did not develop a trypanosome infection. At 90 days post-treatment, two animals (Nos. 429 and 434) showed drug concentrations below the limit of detection (0.1 ng ml^{-1}), but when challenged one animal (No. 429) developed a trypanosome infection whereas the other (No. 434) did not. Interestingly, animal No. 434, in which homidium was undetectable at 60 days remained negative even after the third challenge at 90 days post treatment. These two animals showed marked variations in t_{max} and MRT values. Whilst the peak serum homidium concentration in animal No. 429 appeared at 0.07 hours, that of animal No. 434 occurred at 0.24 hours. The MRT values were approximately 65 and 72 hours in animal Nos. 429 and 434, respectively (Table 1) demonstrating differences in the mean average time drug molecules resided in the body. Although the drug was undetectable in the serum of animal No. 434 at 60 and at 90 days post-treatment, the drug levels appeared to be protective.

From the present study, it has been established that the periods between homidium treatment and first detection of parasites in peripheral blood ranged between 120 and 144 days with pre-patent periods ranging from 14 to 30 days. However, at the time parasite infections were detected in Group HS cattle, serum homidium concentrations in all five animals were undetectable and it was not possible to determine the minimum effective serum drug concentration. Animal No. 429 was fully protected from infection by the serum homidium concentration at day 60 challenge but not at day 90 challenge, at which time the homidium concentration was below 0.1 ng ml^{-1}.

Akol and Murray (1982) demonstrated that appearance of trypanosomes in the peripheral blood and lymph nodes occur simultaneously. The high drug levels in the superficial lymph nodes as demonstrated in studies reported in Chapter 9 using ^{14}C homidium could play some role in protecting the animals from infection especially in cases where circulating drug levels could not be demonstrated as in the present study.

No anti-trypanosome antibodies were detected in any of the serum samples collected from Group HS cattle immediately before challenge at 30, 60 and 90 days, suggesting that the prophylactic period observed was solely attributed to the presence of the drug and not the presence of anti-trypanosome antibodies. A similar observation was made by Whitelaw et al. (1986) in studies on isometamidium prophylaxis against *T. congolense* challenge in Boran cattle in which drug residues were demonstrated to effectively limit trypanosome multiplication; immune responses were not detectable in any of the animals under chemoprophylaxis. Peregrine et al. (1988) demonstrated that antibodies to metacyclic trypanosomes that were used to challenge the cattle did not appear in any of the animals under isometamidium prophylactic cover except in the animals challenged with $5x10^5$ metacyclics. In the present study, low antigen levels not capable of producing a measurable antibody response could possibly have been due to the low inoculum used ($1x10^5$ trypanosomes)

and the presence of the drug which did not allow establishment and multiplication of trypanosomes.

Pre-challenge pharmacokinetics obtained in Group HR cattle were similar to those reported in the Group HS cattle (Table 6.1 and 6.5). Subsequent to the 30-day trypanosome challenge of Group HR cattle (given homidium and challenged with the resistant trypanosome population), no protection was observed as all animals became parasitaemic. The parasitaemia pre-patent periods were one or two days longer than that observed in the untreated challenge control animal which was detected parasitaemic 8 days following trypanosome inoculation. It therefore appears that homidium concentrations of between 0.15 and 0.3 ng ml^{-1} at the time of trypanosome inoculation could not inhibit trypanosomes from establishing and producing disease symptoms. These findings were not unexpected since this particular stabilate of trypanosomes has been demonstrated to be resistant to homidium bromide at the recommended therapeutic dose rate of 1 mg kg^{-1} b.w. as discussed in Chapter 5.

The results of the present study have demonstrated that the development of parasitaemia following challenge of homidium-treated animals with homidium-resistant trypanosomes markedly alters homidium's pharmacokinetics in cattle by enhancing elimination of the drug following establishment of the infection. Gilbert and Newton (1982) in their studies on homidium pharmacokinetics demonstrated that approximately 80% of the drug in the circulation at 1, 8 and 12 hours following treatment of calves with ^{14}C homidium was bound to trypanosomes suggesting substantial drug uptake of homidium by trypanosomes immediately following treatment. This could partly explain the increased rate of drug disappearance from circulation in the presence of trypanosomes.

As discussed in Chapter 5 of this thesis, trypanosome infections in cattle and sheep have been associated with fever, accelerated rate of

metabolism, fall in packed cell volume, haemodilution and decrease in serum albumin, among others (Katunguka-Rwakishaya et al., 1993). These changes may also have contributed to the accelerated rate of drug elimination in cattle with patent infections (Chapter 5).

Although several factors could be attributed to the variation in length of prophylactic period observed in the field, such as fly density (intensity of challenge), infection rates in flies and host susceptibility, the results obtained in this study have shown that serum homidium concentrations in individual animals and individual animal pre-challenge pharmacokinetics as well as the drug sensitivity of challenging trypanosome infections play an important role.

As already discussed in Chapter 5, in natural trypanosome populations there is considerable variation in drug sensitivity between individual trypanosome populations (Unsworth, 1954a; Peregrine, 1991). In the presence of drug-sensitive trypanosome population, the results of the present study suggest that animals would remain protected for long periods of time especially in the presence of low tsetse challenge. Protection of the animals was associated with the circulating homidium concentrations ranging between 0.1 and 0.3 ng ml^{-1}. Four out of the five Group HS cattle became parasitaemic when homidium concentrations had fallen below the assay limit of detection (0.1 ng ml^{-1}). However, these drug levels do not protect homidium-treated cattle against challenge by drug-resistant trypanosome populations.

Following the establishment of drug resistant trypanosome populations, the rate of drug disappearance was enhanced in a similar manner to the observations made in Chapter 5. This emphasises the urgent need for development of methods that can be used in identifying drug resistant trypanosomes, factors that contribute to development of drug resistance and ways of minimising its occurrence and spread in the field. Results of a field study investigating the use of homidium as a chemoprophylactic are reported in Chapter 7.

CHAPTER SEVEN

INVESTIGATION INTO THE EFFICACY OF HOMIDIUM BROMIDE IN THE CONTROL OF BOVINE TRYPANOSOMIASIS AT GALANA RANCH, COAST PROVINCE, KENYA

INTRODUCTION

Studies on homidium prophylaxis reported in Chapter Six demonstrated that homidium bromide administered at a dose rate of 1 mg kg^{-1} b.w. can offer protection to cattle against trypanosome challenge with homidium-sensitive trypanosomes for periods ranging from 120 to 144 days. After establishing this long period of protection in controlled studies in the laboratory, it was decided that a field study should be carried out to determine the efficacy of this drug under field conditions. Dolan et al. (1990) reported that homidium conferred periods of prophylaxis of over 18 weeks following treatment of cattle with homidium bromide at a dose rate of 1 mg kg^{-1} in work carried out on Galana ranch, Coastal Region, Kenya during 1989. However, later work also at Galana (Dolan et al., 1992) carried out in 1990 suggested resistance to phenathridine derivatives (isometamidium and homidium) and this work did not include monitoring of homidium concentrations. As a result, it was not possible to relate the periods of protection observed to the circulating drug levels. The development of homidium-detection ELISAs has now permitted such a study to be undertaken at Galana Ranch to investigate this relationship between the duration of protection and circulating drug levels in homidium-treated cattle.

STUDY OBJECTIVES

1. To determine the incidence of trypanosomiasis in the Dakabuku area of Galana Ranch, Coast Province, Kenya, and the effectiveness of homidium in controlling infections.

2. To measure and determine individual variations in serum homidium concentrations in cattle under prophylactic cover in the field.

3. To investigate the relationship between homidium concentrations and the length of prophylactic cover in cattle exposed to natural tsetse challenge.

4. To look for evidence of drug resistance

MATERIALS AND METHODS

Study area

The experiments were carried out at the Dakabuku area of Galana Ranch, Coastal Region, Kenya; an area of endemic trypanosomiasis. Tsetse challenge in this area has previously been reduced by the use of a 'pour-on' insecticide for two years before the present study.

Experimental animals

A herd of 160 Boran cattle were selected for use in this study from a group of 350 animals which had been ear-tagged, branded, weighed and kept in an area of low tsetse challenge for nearly two months. A few days prior to treatment, the cattle were moved to Dakabuku but kept close to the dip tank, where the tsetse challenge was low, until after treatment.

Experimental design

The cattle were divided into two groups. Eighty were treated with homidium bromide on the day before being moved to an area of high natural tsetse challenge, whilst the remaining group of 80, which did not receive homidium, served as controls and grazed along with the homidium-treated animals. The experiment was planned to coincide with the beginning of the rainy season when trypanosome challenge was expected to be high. The experiment was therefore carried out over the months of December/March 1994/95 which included one rainy season (mid-December/mid-January). Any cattle in either the control herd or the homidium-treated herd found to be

infected with trypanosomes were treated with diminazene aceturate at 7.0 mg kg^{-1} b.w.

Collection of pre-treatment blood samples

On the day before treatment, venous blood was collected into plain vacutainer tubes from all 160 cattle. These samples were allowed to clot, centrifuged to separate the sera, which was then frozen immediately and transported to the Kenya Trypanosomiasis Research Institute (KETRI) Laboratories for use as negative control sera in drug analysis.

Prophylactic drug treatment

A 2.5% (w/v) solution of homidium bromide (Ethidium®, Laprovet, France, Batch No. B18V2), prepared as already described in Chapter 2 (General Materials and Methods) was given by deep intramuscular injection into the middle one third of the neck at a dose rate of 1 mg kg^{-1} b.w. This prophylactic treatment was administered only once, at the start of the experiment.

Collection of blood samples for drug analysis

Ten millilitre blood samples were collected weekly from animals in the homidium-treated group, following trypanocidal treatment and every four weeks in the control group, and sera prepared as described above for pre-treatment sera. These samples were frozen and transported to KETRI laboratories for drug analysis.

Exposure of cattle to natural tsetse challenge

From the day following prophylactic treatment, all the 160 cattle (80 homidium-treated and 80 controls) were driven daily into the tsetse infested bush around the Dakabuku dip tank.

Parasitological examination

All animals were monitored for trypanosomes on the day before prophylactic treatment and at weekly intervals to the end of the observation period which lasted 14 weeks. Procedures followed in the detection of trypanosomes were as described in Chapter 2.

The incidence of trypanosome infections was determined weekly. In the control herd, the calculation was based on the number of animals exposed, excluding those that had been treated with diminazene the previous week. This was based on the assumption that the animals were still protected by the diminazene following treatment (diminazene offers protection against infection for a period of two weeks following treatment). And, if infected on day 14 the infection will not be detected by the third week. In the homidium-treated herd, all animals previously detected positive were excluded from the calculation of incidence of infection.

All the animals were weighed every month during the study period of 14 weeks.

RESULTS

Control herd

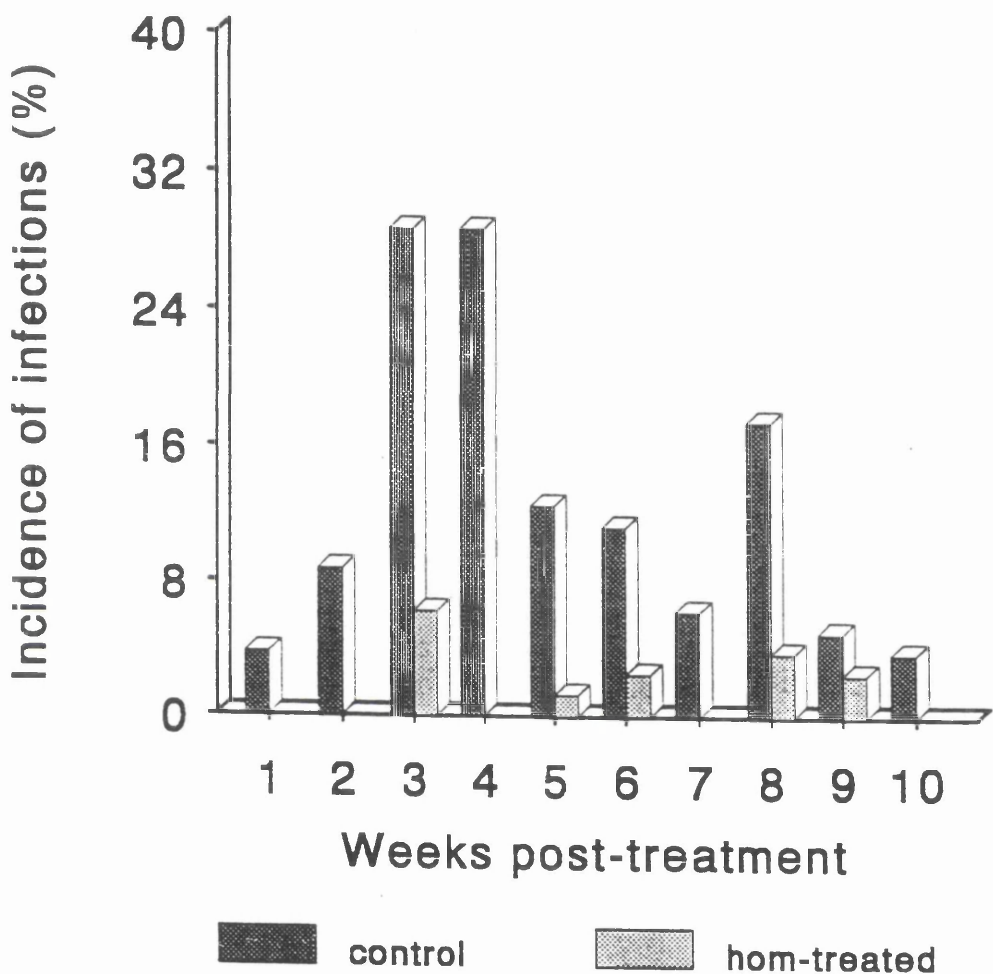
Incidence of infection

Fig. 7.1 shows the weekly incidence of trypanosome infections in the control herd which did not receive homidium treatment. During the 14 weeks in which the animals were examined, trypanosomes were detected in all the animals in the control herd. Most of the infections were recorded in the third and fourth weeks, which coincided with the period when the heavy rains were recorded. Any infections detected during this period were treated with diminazene aceturate. Within six weeks of treatment, 75 infections were

Fig. 7.1

Weekly incidence of trypanosome infections in the control herd and the homidium-treated herd following exposure to natural tsetse challenge

Fig. 7.1



detected in the 80 animals. Within ten weeks a total of 101 infections were detected and all the animals in the group had been detected infected at least once. Out of the 101 infections detected, 55 were due to *T. vivax* and the remaining due to *T. congolense*. One of the *T. vivax* infections was of the 'haemorrhagic type'; this animal died in spite of the diminazene treatment.

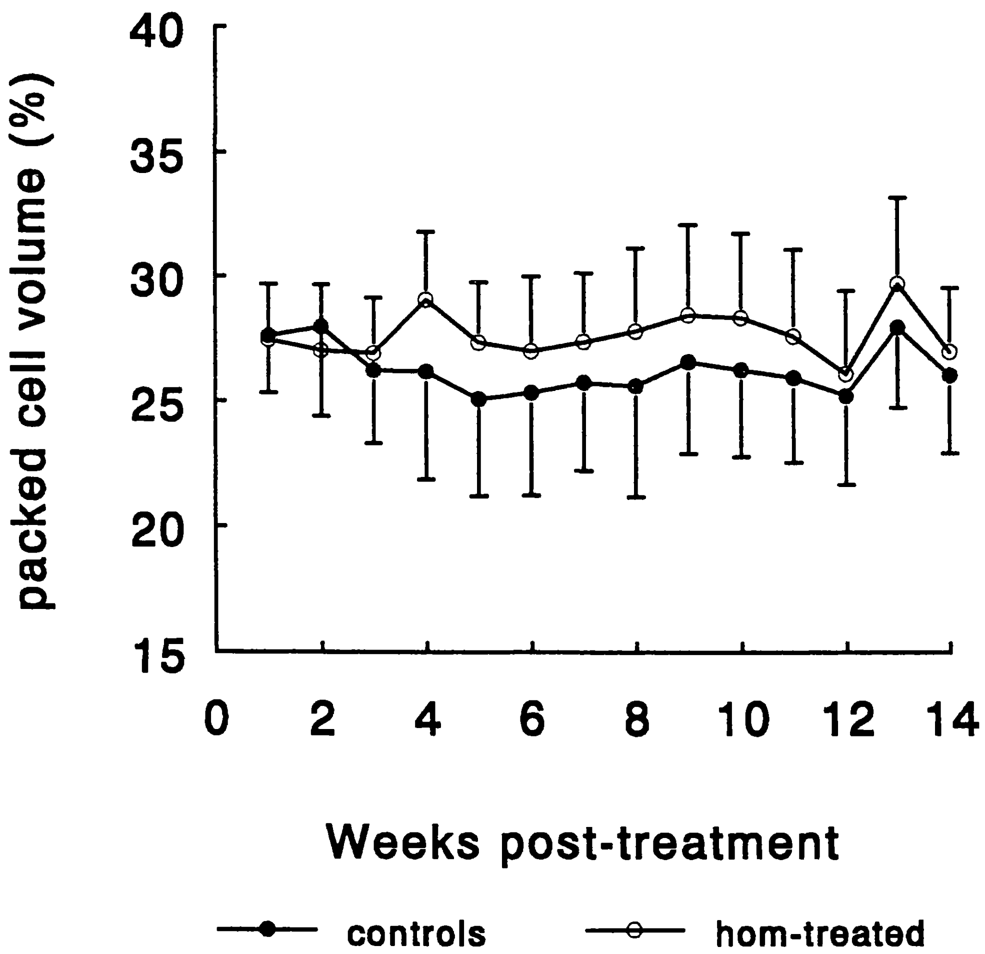
Packed cell volume

Fig. 7.2 shows the mean (±SD; n = 79) PCV values obtained over a period of 14 weeks. Individual animal values are shown in Appendix C. All animals which were detected positive with trypanosome infections were treated on the same day of trypanosome detection. The weekly PCV values calculated were based on the total number of animals in the group irrespective of whether they were infected or not. During the first week of the study period, a mean (±SD) PCV value of 27.6±2.3% was recorded. A slight increase in the mean PCV value was observed over the second and third weeks (Fig. 7.2). However, a significant drop was recorded during the fourth week ($p \leq 0.01$). Similarly, the mean PCV values recorded during the 8th and 12th weeks were also significantly lower than those obtained during the first week of the study ($p < 0.001$). PCV values as low as 14% were observed in some animals. Some animals showed consistently low PCV values over several weeks despite diminazene treatment. It was also observed that not all low PCV values were associated with detectable trypanosome infection and the contribution of concurrent infections to the low PCVs was suspected. Approximately half of the PCVs of 20% and below were associated with detectable trypanosome infections. The animal which died, possibly from the suspected haemorrhagic *T. vivax* had a PCV drop from 24 to 12 % within one week and died two days after trypanosomes were first detected in peripheral blood.

Fig. 7.2

Mean (±SD; n = 79) weekly packed cell volume (%) in the control herd and the homidium-treated herd following exposure to natural tsetse challenge

Fig. 7.2



Body weight gain

Fig. 7.3 gives the four-weekly values for live weight (kg) in the untreated control herd. At the beginning of the experiment, the mean (± SD ; n = 80) body weight of the untreated control animals was 179±15 kg. During the first four weeks, the mean live weight gain per animal was 12.6±6.9 kg. During this same period, weight loss was recorded in two animals whilst four animals maintained their original weights. The highest weight gain was 25 kg in five animals and 19 animals (including the five with weight gain of 25 kg) showed a weight gain of 20 kg and above.

During the second four-week period, the animals gained on average 10.4 kg per animal. However, two animals showed a loss in weight; these were not the same animals which lost weight during the first four weeks. Five animals maintained the weight they had attained during the first four-week period. At the end of the second four-week period, this latter animal did not show any gain in weight from pre-treatment values and showed persistently low PCV values throughout the 14 weeks of observation. During this same period, weight gain of 20 kg and above per animal was observed in eight animals including one animal which showed a weight gain of 25 kg.

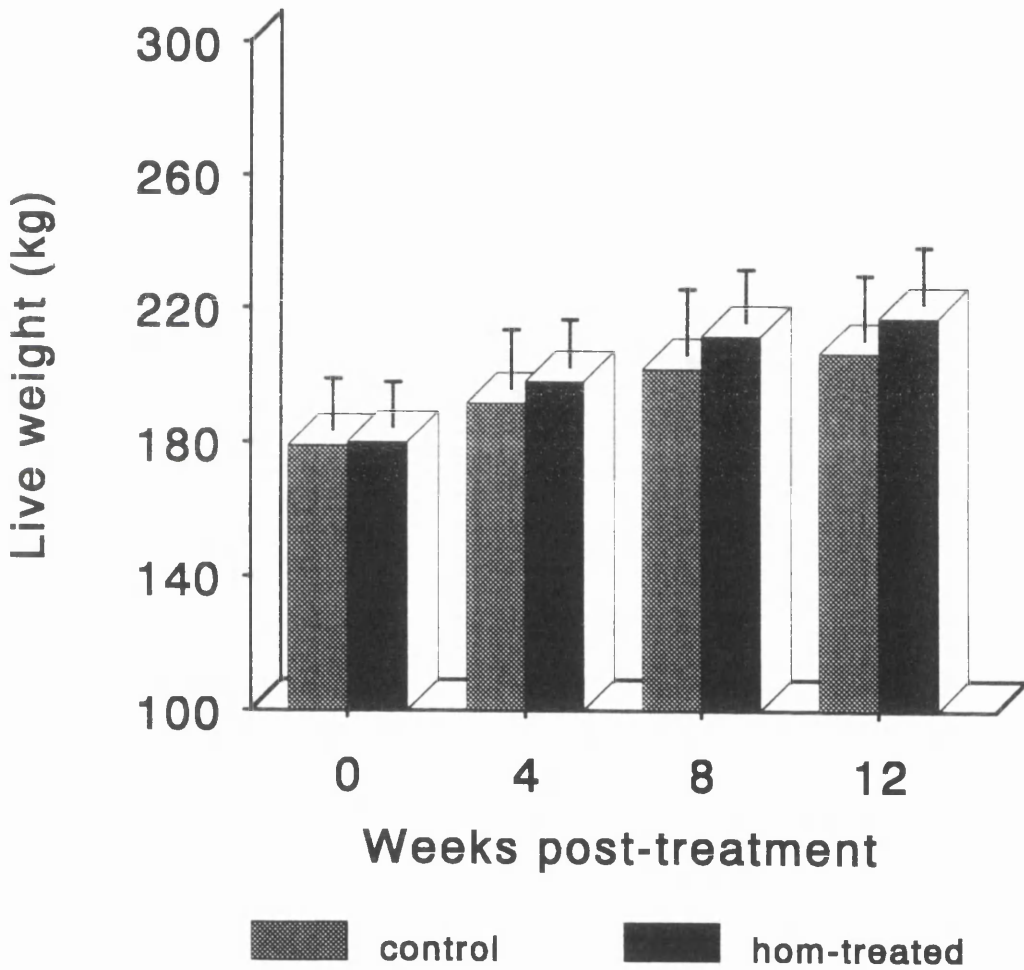
During the third four-week period following treatment (between weeks eight and twelve), the animals gained on average 4.6 kg. Nine animals showed a loss in weight whilst 14 maintained the weight gained during the second four-week period. The highest weight gain during this period was 15 kg per animal in three animals.

Overall, the mean (±SD; n = 79) body weight in the control animals at the end of the twelfth week was 207±19 kg compared with 179±15 kg recorded at the start of the experiment. The results showed that low weight gains were associated with low PCVs. However, low PCVs were not always associated with trypanosome infections.

Fig. 7.3

Mean (±SD; n = 79) four-weekly live weight (kg) in the control herd and the homidium-treated herd following exposure to natural tsetse challenge

Fig. 7.3



Homidium-treated herd

Incidence of infection

The weekly trypanosome incidence is shown in Fig. 7.1. The earliest trypanosome infections were detected three weeks following homidium treatment, when five animals were detected positive. Infections were also detected after five weeks (one animal), six weeks (one animal), eight weeks (three animals) and nine weeks (two animals). A total of 12 infections were therefore detected in the 14-week period. All infections, except one, were due to *T. vivax*. The only *T. congolense* infection detected was during the sixth week after drug administration.

The number of cattle protected in the homidium-treated group is shown in Table 7.1. A total of 75 out of the 80 animals were fully protected during the four weeks following treatment and which included the period of high trypanosome challenge which occurred during the third week. The results showed that the weekly percentage of animals remaining uninfected out of the total exposed (excluding the previous breakthrough infections) ranged between 93.8 - 100%.

Packed cell volume

Fig. 7.2 shows the mean (±SD) PCV (%) observed during the 14 weeks of observation following treatment. There was an increase in the mean PCV value of 27.5±2.2% to 29.1±2.8% within the first four weeks of treatment. By the fifth week, a mean value of 27.3±2.4% was observed; thereafter the PCV remained close to this value until the twelfth week after which an increase was recorded (Fig. 7.2).

Almost all the low PCV ≤ 20% were associated with trypanosome infections. The PCVs of all but one of the twelve animals in which infections were detected were elevated after treatment with diminazene aceturate (Table 7.2). However, four of the 12 animals in which trypanosome infections were

Table 7.1

Number of cattle in which new trypanosome infections were detected at Galana Ranch each week in animals given homidium bromide at 1 mg kg^{-1} b.w. and in animals not given homidium.

Time post-treatment (weeks)	controls +ve	controls infected/exposed	homidium herd +ve	homidium herd infected/exposed
1	3	3/80 (3.75%)	0	0/80 (0%)
2	7	7/77 (9.09%)	0	0/80 (0%)
3	23	23/73 (31.51%)	5	5/80 (6.25%)
4	23	23/57 (40.35%)	0	0/75 (0%)
5	10	10/57 (17.54%)	1	1/75 (1.33%)
6	9	9/70 (12.85%)	1	1/74 (1.35%)
7	5	5/71 (7.04%)	0	0/73 (0%)
8	14	14/75 (18.67%)	3	3/73 (4.11%)
9	4	4/66 (6.06%)	2	2/70 (2.86%)
10	3	3/76 (3.95%)	0	0/68 (0%)
11	0	0/77 (0%)	0	0/68 (0%)
12	0	0/79 (0%)	0	0/68 (0%)
13	0	0/79 (0%)	0	0/68 (0%)
14	0	0/79 (%)	0	0/68 (0%)

Table 7.2

Individual PCV (%) values of cattle in which breakthrough infections were detected following exposure to natural tsetse challenge after i.m. prophylactic treatment with homidium bromide at 1 mg kg^{-1} b.w.

Time post-treatment (weeks)	Animal No.												
	176	**179**	**182**	**186**	**187**	**218**	**220**	**234**	**241**	**242**	**249**	**259**	**Mean±SD**
1	30	28	27	30	26	29	29	24	25	25	23	29	27.45±2.24
2	31	25	28	26	24	27	28	23	25	25	19	31	27.03±2.64
3	24*	28	29	27	28	28	26*	20*	25*	27	20	30*	26.91±2.22
4	25	27	27	30	29	30	28	23	27	30	22	27	29.05±2.78
5	25	25*	30	26	29	25	30	26	27	28	21	29	27.34±2.43
6	25	28	26	27	21*	26	29	23	23	27	22	28	29.99±3.00
7	27	21	30	26	16	26	30	27	27	28	22	26	27.39±2.76
8	29	21	25	29	22	10**	29	27	25	21*	24*	31	27.81±3.36
9	29	22	14*	28*	23		30	26	23	25	16	31	28.46±3.65
10	28	21	17	29	22		26	25	24	29	16	30	28.35±3.40
11	26	26	19	30	22		27	25	25	30	19	27	27.61±3.52
12	26	26	19	23	16		26	24	24	25	15	30	26.10±3.33
13	31	28	20	25	24		30	24	29	25	20	31	29.73±3.46
14	29	26	21	25	22		27	25	29	27	20	27	27.00±2.57

* Breakthrough trypanosome infections (These animals were treated with diminazene aceturate when parasites were detected).
** Suspected haemorrhagic *T. vivax*; animal died one day after detection of trypanosomes.

detected (Table 2, Animal Nos. 182, 186, 187 and 249) maintained low PCV values even after diminazene treatment. One of these three animals had persistently low PCVs of 23% or below (Animal No. 249) before trypanosome infections were detected in the peripheral blood. Table 7.2 shows individual PCV values in homidium-treated cattle in which infections were detected.

Weight gain

Fig. 7.3 shows the mean (±SD) body weight (kg) of homidium-treated cattle measured at four-weekly intervals over the 12 weeks following homidium treatment. The mean (±SD;) live weight (kg) of the homidium-treated cattle at the beginning of the experiment was 180±13 kg. During the first four-week period, the average live weight gain was 18.18 kg per animal. One animal showed a loss in weight. A trypanosome breakthrough infection was detected in this animal during the fourth week. The highest weight gains recorded were 30 kg in two animals. The number of animals with a weight gain of 20 kg and above was 48.

During the second four-week period, the animals gained on average 13.61 kg. Two animals showed a loss in live weight. Breakthrough trypanosome infections were detected in both these animals; one in the sixth, the other in the ninth week following treatment. Two animals maintained weights measured after the first four weeks. These two animals developed breakthrough trypanosome infections during the fifth and eighth weeks following treatment. The highest weight gains observed were 20 kg, in 16 animals.

Between the eighth and the 12th weeks, the cattle gained on average 5.51 kg. Three animals showed a weight loss and 11 maintained the weight attained at the end of the second four week period following treatment. The highest weight gains recorded were 15 kg, in two animals.

Overall, the mean (±SD; n = 79) live weight of the homidium-treated cattle at the end of week 12 was 217±17 kg, an increase of 37 kg over the mean pre-treatment value.

Comparisons between homidium-treated and untreated control groups

Incidence of infection

Fig. 7.1 shows the weekly incidence of infection in the homidium-treated and the control and herds. Similarly, Table 7.1 shows the number of trypanosome infections detected in the homidium-treated and in the control herds over a period of 14 weeks. During the first six weeks, when 75 infections were recorded in the control herd, only seven infections were recorded in the homidium-treated animals. A total of 12 infections were detected in the homidium-treated animals in the 14-week period compared to 101 detected in the control herd. Whilst no new trypanosome infections were recorded after 10 weeks until the end of the observation period of 14 weeks in the homidium-treated group, only one infection was detected in the untreated control herd.

In the control herd the infections recorded were due equally to both *T. congolense* and *T. vivax*, but in the homidium-treated herd only one of the 12 breakthrough infections was due to *T. congolense* (46) and all the remainder were due to *T vivax* (55). In each group of cattle, one *T. vivax* infection resulted in a haemorrhagic syndrome condition.

Packed cell volume

Fig. 7.2 shows the mean (±SD) weekly PCV (%) of the homidium-treated and the untreated control groups following exposure to natural tsetse challenge. From the fourth week of the study period, the PCVs of the homidium-treated cattle were significantly higher than those of the untreated controls ($p < 0.001$). In the cattle under homidium prophylactic cover, PCV values of 20% and below were nearly always associated with trypanosome

infections. These low PCV values increased in all except four animals following treatment with diminazene aceturate. However, in the untreated control herd, animals with PCV of 20% and below were not always trypanosome infected. In the two cases of haemorrhagic *T. vivax*, the PCV drop was marked irrespective of whether the animal was under prophylactic treatment or not. These animals died within a day or two after detection of trypanosomes in circulation despite therapeutic intervention with diminazene aceturate.

Weight gain

Fig. 7.3 shows a comparison of the mean (±SD) four-weekly live weight between the untreated controls and the homidium-treated cattle. There were no significant differences in the mean (±SD; n = 80) live weight between the controls and the homidium-treated cattle at the start of the experiment. Thereafter, homidium-treated cattle showed a significant increase in live weight over the 12-week period (Table 7.3). The animals in the control herd required 101 diminazene doses over a 14-week period whilst the homidium herd received only one homidium treatment and 12 doses of diminazene over the same period. Although both groups showed an increase in live weight which was associated with the rainy period, the increase recorded in the homidium-treated group was significantly higher than that of the controls over the same period.

Serum homidium concentrations

Determination of serum homidium concentrations in individual animal pre-treatment sera showed that six out of 80 animals showed homidium concentrations above 0.1 ng ml^{-1} (assay detection limit) whilst the remaining animals were below this value. Fig. 7.4 shows the mean ± SD (n = 79) serum homidium concentration-versus-time plot.

Table 7.3

A comparison of the mean (±SD) four-weekly live weights (kg) between the untreated controls and homidium-treated cattle following exposure to natural tsetse challenge

Time post-treatment (weeks)	Untreated controls	Homidium-treated	Significance
0	179±15	180±13	
4**	192±18	198±14	*
8***	202±19	212±15	**
12***	207±19	217±17	**

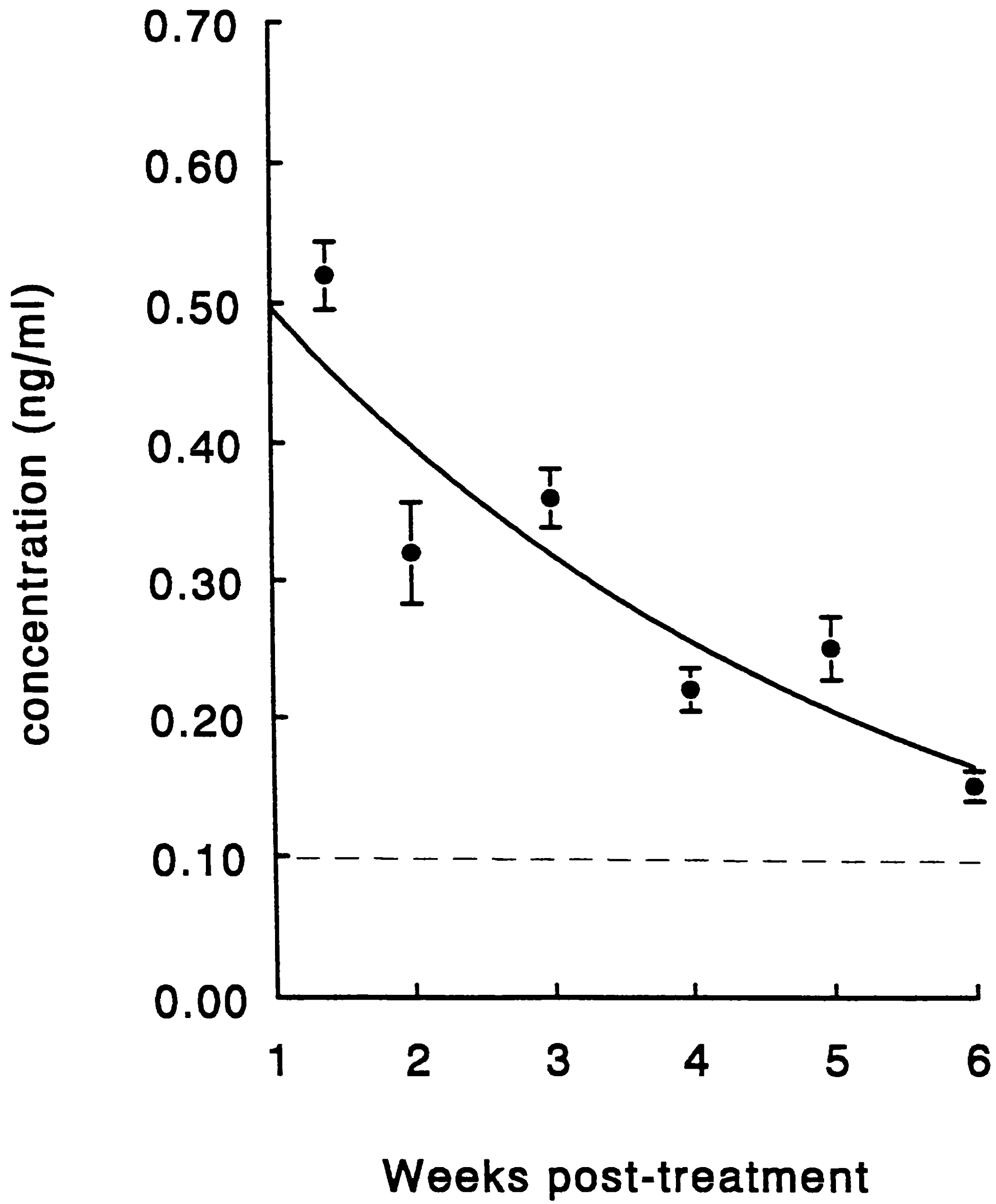
** p = 0.01

*** p < 0.001

Fig. 7.4

Mean ± SD (n = 79) serum homidium concentrations in cattle treated with homidium bromide at 1 mg kg^{-1} b.w. and exposed to natural trypanosomiasis challenge

Fig. 7.4



Ten days following homidium treatment, the mean serum homidium concentration was 0.52±0.02 ng ml^{-1}. At 10 days following treatment, only one animal had a concentration below the limit of detection. This particular animal did not become infected at all during the observation period of 14 weeks. Three weeks following homidium treatment when the highest number of breakthrough infections was recorded, the mean serum homidium concentration was 0.36±0.02 ng ml^{-1} (Table 7.4). The animals in which trypanosomes were detected had serum homidium levels of 0.12, 0.14, 0.27, 0.35, 0.40 and 0.57 ng ml^{-1} at the time of parasite detection. One animal which was found to be infected at week 5 after homidium treatment had a serum drug concentration of 0.12 ng ml^{-1}. By week 6 following treatment, the group mean (n = 80) serum drug level was 0.25±0.02 ng ml^{-1} but 15 animals had drug levels below the assay detection limit. Ten weeks after homidium treatment two more animals became infected; these were among the 15 in which the drug could no longer be detected by week 6.

DISCUSSION

The results of the present study carried out on Galana Ranch, Coastal Region, Kenya, demonstrated that homidium treatment conferred significant prophylactic cover against trypanosome infections in an area of high tsetse challenge. During a 14-week period, 101 trypanosome infections were detected in 80 cattle which did not receive homidium whilst in a similar number of cattle which received homidium at 1 mg kg^{-1} b.w. only 12 trypanosome infections were detected over the same period. Homidium prophylaxis was also associated with higher weight gains and haematocrit values.

The present study also demonstrated that serum homidium concentrations could be monitored successfully in blood samples of individual ranch cattle for at least six weeks following treatment using ELISA.

Table 7.4

Time at which breakthrough trypanosome infections were detected and the serum concentrations of individual cattle following prophylactic treatment with homidium bromide at a dose rate of 1 mg kg^{-1} b.w. and exposed to natural tsetse challenge at Galana Ranch

Animal No.	Time post-treatment (weeks)*	Homidium concentration (ng ml^{-1})
176	3	0.14**
220	3	0.57
234	3	0.40
241	3	0.27
259	3	0.35
179	5	0.12***

* Time (weeks) after treatment when trypanosomes were detected in individual cattle

** The mean (±SD) serum homidium concentration in the treated cattle at three weeks post-treatment was 0.36±0.02 ng ml^{-1}

*** The mean (±SD) serum homidium concentration in the treated cattle at five weeks post-treatment was 0.25±0.02 ng ml^{-1}

The weekly incidence of trypanosome infections in the homidium-treated and the untreated control cattle showed that infections were lower in the homidium-treated herd over the study period of 14 weeks. By six weeks following homidium prophylactic treatment, a total of seven infections were detected in the homidium-treated cattle compared to 75 detected in the control herd. This showed that although the challenge was high, 73 out of 80 homidium-treated animals did not develop trypanosome infections during that period.

Similar numbers of infections due to both *T. vivax* (55) and *T. congolense* (46) were recorded in the control group. However, only one animal in the homidium-treated group developed a *T. congolense* infection and the remaining 11 infections were with *T. vivax*. This finding suggests that the sensitivity of the *T. congolense* populations to homidium was higher than that of the *T. vivax* populations. Similar observations were made by Dolan et al. (1992) who showed that most of the infections detected in the homidium-treated herd on Galana Ranch were *T. vivax* where the prevalence of both *T. vivax* and *T. congolense* were similar.

The mean weekly PCV values obtained in the homidium-treated cattle were significantly higher ($p < 0.001$) than those obtained in the control herd from week four onwards. The low PCV values observed in individual homidium-treated animals were nearly always associated with trypanosome infections. In contrast, the low PCV values of 20% and below in the control herd were not always associated with detectable trypanosome infections. The persistently low PCV values in cattle in the control herd in the absence of detectable trypanosome infection could have been as a result of undiagnosed trypanosome or concurrent infections. In the two cases of haemorrhagic *T. vivax* (one in each group of cattle) a dramatic drop in PCV was observed and this was not prevented by diminazene treatment.

Both groups of animals showed an increase in body weight over the 12-week experimental period. The highest live weight gain was recorded during the fourth week in both groups; this was during the rainy season and at the time when the incidence of infection was highest. The lowest live weight gain was recorded between the 8th and the 12th weeks in both groups; this was at the end of the rainy season at a time when the incidence of infection was very low. The increase in body weight recorded in the homidium-treated herd was significantly higher than that recorded in the controls ($p \leq 0.01$; Table 7.3). This was despite diminazene treatments given to all positive cases.

Homidium concentrations in serum could be monitored in individual animals for at least six weeks following treatment. However, it was not easy to determine the minimum protective concentration from the present study. The results appear to suggest that even in the presence of extremely low concentrations in serum (below 0.1 ng ml^{-1}) protection was achieved in some cattle. The observation that six weeks following treatment fifteen of the treated animals had serum homidium concentrations below the detection limit and yet did not become infected throughout the rest of the 14-week observation period is consistent with the above suggestion that serum drug levels below 0.1 ng ml^{-1} may still be protective. A similar observation was made in Chapter 6 in which one animal had drug levels below 0.1 ng ml^{-1} at 60 days following treatment but did not become infected when challenged at either day 60 or day 90 following drug administration.

In the present study however, infections developed in some animals which had drug concentrations between 0.12 and 0.57 ng ml^{-1} serum on the day they were detected positive. The trypanosome populations of *T.vivax* causing these breakthrough infections were therefore apparently less sensitive to homidium than the *T congolense* population.

Some factors identified from the present study which may contribute to the length of the prophylactic period were (a) rainy period associated with

high incidence of infection possibly due to increase in tsetse challenge (information on tsetse populations was however, not included) (b) the nature of the trypanosome populations and their sensitivity to homidium; infections recorded in the control herd were equally due to both *T. vivax* and *T. congolense* but breakthrough infections in the homidium-treated herd were mostly *T. vivax* showing variations in the drug sensitivity (c) individual animal pharmacokinetics.

The use of homidium as a chemoprophylactic has had its problems, one of which has been the development of drug resistance, observed as early as 1966 (Jones-Davies and Folkers, 1966). In Kenya, failure of chemoprophylaxis has been reported with both homidium bromide and isometamidium chloride (Dolan et al., 1992; Munstermann et al., 1992; Maloo, 1993; Stevenson et al., 1995). In a similar study to that described by Dolan et al. (1990), Dolan et al. (1992) reported failure of prophylaxis on the Galana Ranch, Kenya, associated with both homidium and isometamidium administered at 1 mg kg^{-1} b.w. The study lasted 12 months, during which the trypanosome challenge was found to be exceptionally high; a total of 178 infections were detected in 90 animals in the control herd, 81% of which were *T. vivax* and the remainder *T. congolense*. Eight prophylactic treatments were given to each of the homidium and isometamidium herds over a period of 6-7 months.

The period of protection obtained in the present study ranged from three weeks to over 14 weeks in the same region on the Kenyan Coast where the studies reported by Dolan et al. (1990, 1992) were carried out. Findings from the present study that the animals which had breakthrough infections had different serum homidium concentrations would suggest the presence of trypanosome populations which varied in their sensitivity to homidium on the Galana Ranch. Failure of chemoprophylaxis in some animals could have been to be due to infection with drug-resistant trypanosomes.

The observation made by Dolan et al. (1992) on the Galana ranch that breakthrough trypanosome infections were mainly due to *T. vivax* was similar to the findings in the present study and occurred despite the similar incidence of *T. vivax* and *T. congolense* in the control herd. This would support the suggestion that the level of sensitivity to homidium in *T. congolense* populations at Galana is higher than that in *T. vivax* and higher levels of the drug may be needed to protect animals in this area against *T. vivax.*

The study of Stevenson et al. (1995) at Nguruman, Kenya, covering the period February 1990 to February 1991 which included a period of high incidence of infections (March to July) showed 215 infections (59% *T. vivax*, 27% *T. congolense*, 4% *T. brucei* and 10% mixed species) in the control herd of 30 animals. During the year, 25% of the animals in the control herd either died or were removed from the experiment because they were too weak suggesting failure of diminazene to provide effective control at 7 mg kg^{-1} b.w.

However, in the homidium-treated herd in the Nguruman studies, eleven homidium treatments (1 mg kg^{-1} b.w.) were required for a similar number of animals over the same period. Thirty eight new infections were detected in the homidium-treated herd over the year of which 60% were *T. congolense*. There was evidence of drug resistance in the *T. congolense* populations at Nguruman. These results were in contrast to the findings at the Galana Ranch both in the study by Dolan et al. (1992) and in the present study.

In the Dakabuku area of Galana Ranch, it could be concluded that homidium is an effective chemoprophylactic drug, a three to four monthly cover being appropriate during a period of low trypanosome challenge since significantly higher PCVs and weight gains were recorded compared to the controls. The low incidence of infections observed in the homidium-treated herd compared to the untreated controls suggested that most trypanosome

populations were drug-sensitive. However, detection of 12 infections in the homidium-treated cattle showing serum homidium concentrations that would have been effective in the presence homidium-sensitive trypanosomes suggests the presence of drug resistance. This drug resistance was however, not widespread.

The results of the present study showed that the ELISA method can be used effectively to determine serum homidium concentrations in individual ranch cattle. Although the homidium concentrations were determined for a period of only six weeks in the present study, the drug profile obtained was similar to that reported in Chapter 6 following challenge of homidium-treated cattle with a drug-sensitive trypanosome population. The results also showed that some of the animals were still protected even when the drug levels were below the limit of detection. Breakthrough trypanosome infections occurred in animals which still had detectable levels of homidium at concentrations which would be protective against drug-sensitive trypanosome populations (Chapter 6) and suggests the presence of drug resistance. Anti-trypanosomal antibodies were not determined in the present study. However, in the study reported in Chapter 6 it was demonstrated that immunological responses were not responsible for the observed variations in the prophylactic period. Further studies are therefore needed to investigate the contribution, if any, of anti-trypanosome antibodies to the observed period of prophylaxis in the field. Similarly, further studies are also needed to investigate the development and spread of drug resistance and the relationships between serum drug concentrations, drug resistance and immunity to trypanosome infections in field situations. The homidium ELISA makes these studies possible.

CHAPTER EIGHT

PHARMACOKINETICS AND TISSUE RESIDUES OF ^{14}C HOMIDIUM IN NON-INFECTED AND TRYPANOSOMA CONGOLENSE-INFECTED BORAN CATTLE

INTRODUCTION

Since its introduction into the field in 1952, homidium, a phenanthridinium compound has been widely used in the treatment of infections due to *Trypanosoma vivax* and *Trypanosoma congolense* in cattle, sheep and goats (Watkins and Woolfe, 1952). Mass treatment which followed its introduction (Wilson, 1960) was associated with the development of resistance in some countries.

Despite its continued widespread use and increasing reports of drug resistance, there is deficiency in the knowledge of the pharmacokinetic behaviour of the drug. The first report on homidium pharmacokinetics was by Gilbert and Newton (1982) who carried out studies using ^{14}C homidium in rabbits and calves given the drug by intramuscular injection. Details of the pharmacokinetic behaviour of homidium were, however, not given. Elsewhere, it was found that 50-55% of the drug given intraperitoneally at a dose rate of 15 mg kg^{-1} to mice was recovered in bile 16-18 hours later, most of it being monoacetyl-amino conjugates (MacGregor and Clarkson, 1971).

The present study examines in more detail the absorption, distribution and elimination characteristics of homidium in non-infected cattle treated either intramuscularly (i.m.) or intravenously (i.v.) with ^{14}C homidium chloride at a dose rate of 1 mg kg^{-1}. Results obtained using both the radiometric and ELISA methods in two of the animals have been included.

STUDY OBJECTIVES

1. To treat non-infected cattle with ^{14}C homidium by either intravenous (i.v.) or intramuscular (i.m.) injection and to determine the levels of radioactive drug in plasma and in tissues.

2. To treat *T. congolense* infected cattle with ^{14}C homidium by intramuscular (i.m.) injection and determine the levels of radioactive

drug in plasma/serum and in tissues at the end of the study period

3. To determine ^{14}C homidium pharmacokinetics and tissues distribution.

4. To compare pharmacokinetics between i.v.-treated and i.m.-treated cattle, inorder to determine absorption characteristics.

5. To compare serum homidium concentrations and pharmacokinetics between non-infected and infected cattle following i.m. treatment

6. To compare serum homidium concentrations and pharmacokinetic parameters with those obtained using the enzyme-linked immunosorbent assay (ELISA) in non-infected cattle reported in Chapters 4 following i.v. treatment.

7. To compare serum homidium concentrations and pharmacokinetic parameters obtained through the use of ^{14}C-homidium with those obtained using ELISA in non-infected and infected cattle reported in Chapters 4 and 5 following i.m. treatment.

MATERIALS AND METHODS

Experimental cattle

Eight healthy male Boran castrates weighing between 150 and 200 kg were obtained from a trypanosome free area and housed in a fly proof barn for three weeks to acclimatise to laboratory conditions before the start of experiments. Their management before and during the experiments was as described in Chapter 2. After the three weeks of acclimatisation, the animals were placed individually in metabolic cages.

Infection of cattle

A drug-sensitive population of *Trypanosoma congolense* IL 1180 (Sones et al., 1988; Peregrine et al., 1991) was used. An inoculum of $1x10^6$ trypanosomes prepared as described in Chapter 2 from a heavily infected mouse was given by intravenous injection into the jugular vein.

Haematological examination

The procedures followed for the determination of packed cell volume (PCV) were as described in Chapter 2.

Parasitological examination

Ear-vein blood was examined for the presence of trypanosomes following infection of the animals using the procedures described in Chapter 2.

Radiolabelled homidium, dosage and administration

The drug compound used for treatment was 6-^{14}C homidium chloride ethanolate of specific activity 69.13 μCi mg^{-1}. A 2%w/v solution was freshly prepared in sterile water immediately prior to use. The drug was administered as a single bolus at 1 mg kg^{-1} body weight by either the intravenous (i.v.) or intramuscular (i.m.) route deep into the neck muscles. Infected cattle were treated when they attained a PCV value of between 20 and 25%. The radio labelled drug was supplied by May and Baker, Dagenham, U.K through the International Atomic Energy Agency.

Experimental design

The cattle were divided into three groups. One group of three uninfected cattle were treated with ^{14}C homidium by i.v. injection and another group of three by i.m. injection. The remaining two animals were treated by

intramuscular injection after trypanosome infection. The infected cattle were treated on the day after attaining a PCV value of 25%. Following drug administration, levels of radioactivity were measured in plasma, serum, urine, faeces and tissues obtained at the end of the experimental period which ranged between 14 and 36 days.

Sample collection

Pre-treatment plasma/serum samples

Immediately prior to treatment, 10 ml samples of blood were collected by venipuncture into EDTA-coated vacutainers (Becton, Dickinson, Coventry, UK) from all the animals. Plasma was separated by centrifuging the blood at 1200 g for 30 minutes and stored at -20°C until required. In animals in which serum and blood samples were required besides the plasma, 15 ml blood samples were collected instead of 10 ml; 5 ml of the blood was stored in EDTA-coated vacutainer tubes, 5 ml was used in the preparation of serum using the procedures described in Chapter 3 and the remaining 5 ml were used to prepare plasma.

Plasma/serum samples for radioactive drug analysis

Ten millilitre blood samples were collected as described above from all the three animals following i.v. treatment. The blood samples were collected at the following intervals: 5, 10, 15, 30, and 45 minutes, 1, 2, 4, 6, 8, 12 and 24 hours, thereafter daily for 14 days and three times a week to the end of the experimental period which varied between 14 and 36 days. Similarly, blood samples were collected from cattle following i.m. drug administration but omitting the 5 and 10 minute sample collections. Plasma/serum was prepared and stored at -20°C until required.

Serum samples for ELISA

Ten millilitre blood samples were collected as described above from animal No. 368 following i.v-treatment and animal No. 369 following i.m. treatment at the following intervals: 5, 10, 15, 30, and 45 minutes, 1, 2, 4, 6, 8, 12 and 24 hours, thereafter daily for 14 days and three times a week to the end of the experimental period of 30 days. Serum was prepared as described in Chapter 2, aliquoted and stored at -20°C until required.

Faecal and urine samples for drug analysis

Twenty-four hour urine samples were collected in a receiver via a funnel fixed to the preputium of the animal. The volume was measured, samples aliquoted. One set of aliqouted samples was stored at -20°C until required whilst the radioactivity was measured within 24 hours of collection in the remaining aliquots. Similarly, 24-hour faecal samples were collected onto a rubber mat placed on the floor of the metabolic cage. Samples were thoroughly mixed through a sieve of 10 mm wire mesh and aliquoted, some of which was stored at -20°C whilst radioactivity was determined in the remainder within 24 hours of sample collection.

Tissue samples for drug analysis

Tissue samples were collected after the animals were killed at the end of the observation periods which were 14 and 21 days for the animals treated by the i.v. route and 14 and 28 days for the animals treated by the i.m. route. The tissue samples for immediate analysis were homogenised using an Ultra Turrax Homogeniser and aliquoted. The remaining un-homogenised samples were aliquoted (200 g aliquots) and stored at -20°C

Sample preparation and determination of radioactivity

Radioactivity was measured in a beta-counter (Tri-carb® 4000 Series

Liquid Scintillation Systems, Model 4530, Packard Instruments Co., Inc., U.S.A.). To one millilitre plasma/serum or urine was added 10 ml scintillation cocktail (Insta-Gel®, Packard Instruments Co., Inc., Meriden, U.S.A.) and radioactivity determined. Highly coloured samples were decolourised using hydrogen peroxide as described below. Blood samples were solubilised using the procedure described below for faecal samples. To 200 mg accurately weighed mixed faeces or tissue were added 1 ml Soluene®-350 (Packard Instruments Co., Inc., Meriden, U.S.A.), 0.5 ml isopropanol and the resulting mixture was incubated at 56°C overnight or until solubilisation was achieved. This was followed by the addition of 0.5 ml hydrogen peroxide for decolorisation. After the addition of the peroxide, samples were incubated for a further 20 minutes with the scintillation vial cap removed to expel all residual peroxide. Due to the high alkalinity of the Soluene®, one millilitre of 2M hydrochloric acid was then added to the mixture to neutralise the sample followed by 10 ml of the scintillation cocktail. Alternately, 200 mg sample of the mixed faeces were weighed into combustion boats, dried at 56°C for 2 hours and then combusted in a biological oxidiser (Biological Material Oxidiser OX-400, R.J. Instrument Corporation). Labelled carbon dioxide was collected in 15 ml ^{14}Carbon cocktail (OX-161, R.J. Harvey Instrument Corporation) and activity determined. Blank determinations were carried out using plasma/serum obtained prior to homidium treatment. Quench corrections were by the external standard source in-built in the counter. The radioactivity measured was expressed as disintegrations per minute (dpm).

ELISA method

Homidium ELISA method (Assay 2) described in Chapter 3 was used to determine homidium concentrations in the serum samples of two animals collected following either i.v. (animal No. 368) or i.m. (animal No. 369) injection.

Data analysis

During radioactivity determination, samples with low radioactivity were counted for at least 30 minutes until low coefficients of variation (CVs) as computed by the counter between the counts were attained. The cut-off point for acceptable CVs was 5%. The limit of detection was taken as four times the background count. Homidium concentration was calculated using the expression:

dpm sample/dpm std x dilution factor

obtained by repeatedly analysing diluted ^{14}C homidium (labelled:unlabelled) containing 1 ng undiluted ^{14}C homidium (≡ 170 dpm)

All concentrations have been expressed in nanograms per ml or per gram sample (Mean ± SD) calculated from the radioactivity measured. This did not take into account the metabolic state of the drug

Handling of the ELISA data was as described in Chapters 3 and 4.

Statistical evaluation

Comparisons of pharmacokinetic parameters between the various treatment groups and the various methods was carried out using the student's t-test at both 95 and 99% confidence limits.

Pharmacokinetic evaluation

Compartmental pharmacokinetic analysis was carried out on experimental data from the i.v.-treated cattle. The plasma/serum concentration versus time data following i.v. drug administration were fitted by monoexponential, biexponential and triexponential disposition functions by using a curve stripping procedure followed by a weighted non-linear least squares regression. Akaike's Information Criterion (AIC) described by Yamaoka et al. (1978) was used to compare the adequacy of monoexponential

versus multiexponential models. Both the i.v. and the i.m. experimental data were similarly analysed using a non-compartmental model from which the total area extrapolated to infinity was estimated. The area under the curve (AUC) and the area under the moment curve (AUMC) were determined using the trapezoidal rule from the concentration versus time plots. The formulae for the various pharmacokinetic parameters were as described by Gibaldi and Perrier (1982). These have been more fully covered in Chapter 4.

RESULTS

Non-infected Boran cattle treated with ^{14}C homidium by the intravenous route

Serum homidium concentrations

Plots of the plasma/serum homidium concentration versus time are shown in Figs. 8.1(a) to 8.1(c). The results show a rapid exponential decline in the plasma drug concentrations within the first 24 hours followed by slow elimination phase. A mean (± SD) plasma homidium concentration versus time plot is shown in Fig 8.2. Five minutes following treatment, the mean plasma concentration was 499.6±97.9 ng ml^{-1}. Concentrations of 226.4±22.3, 100.3±8.0 and 23.5±1.4 ng ml^{-1}, were obtained within 15 minutes, one hour and one day, respectively. The drug profiles obtained in animal No. 368 on homidium concentrations in plasma, serum and blood are shown in Fig. 8.3. Concentrations of approximately 5 ng ml^{-1} were detectable three weeks following treatment.

Compartmental, pharmacokinetic analysis

The plasma/serum concentration versus time profile best fitted a three-compartment open model. The pharmacokinetic parameters are shown in Table 8.1. Half-lives of 0.06±0.01, 4.14±0.49 and 177.1±5.3 hours for the distribution, post-distribution and elimination phases, respectively were

Fig. 8.1(a) to 8.1(c)

Serum homidium concentrations in individual Boran cattle following intravenous treatment with ^{14}C homidium at a dose rate of 1 mg kg^{-1} b.w.

Fig. 8.1(a)
Animal No. 214

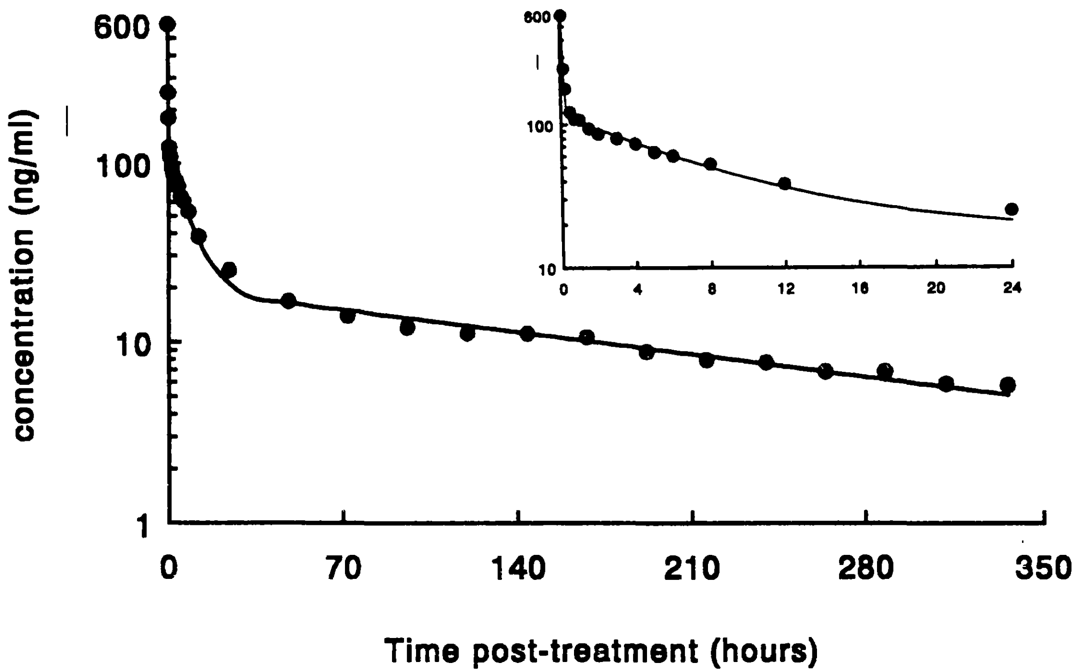


Fig. 8.1(b)
Animal No. 215

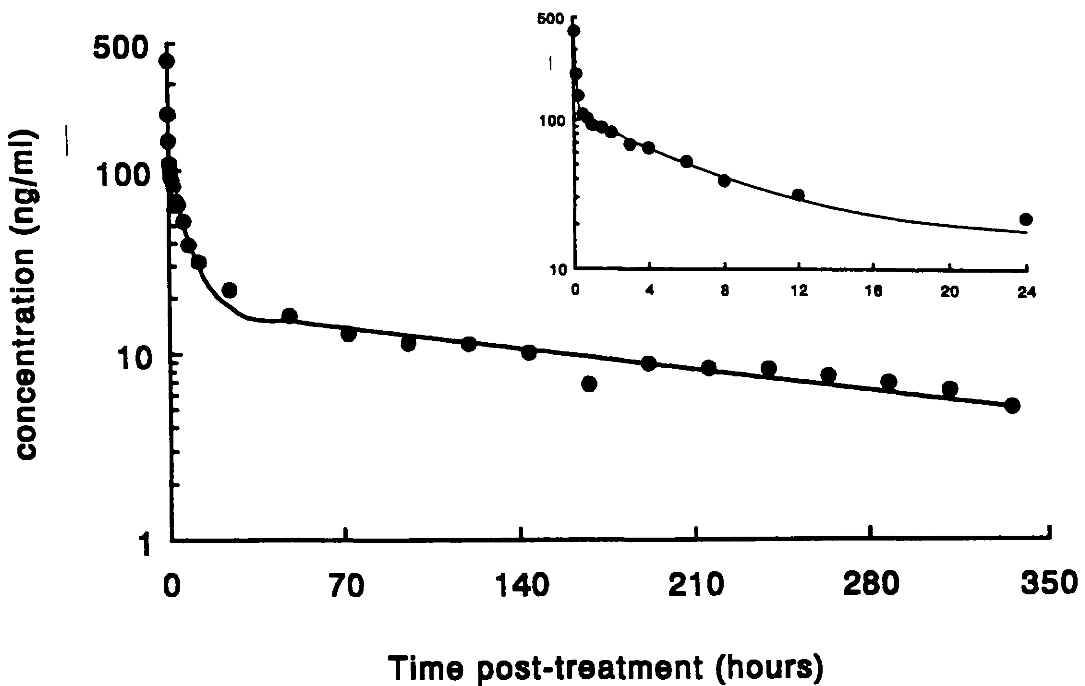


Fig. 8.1(c)
Animal No. 368

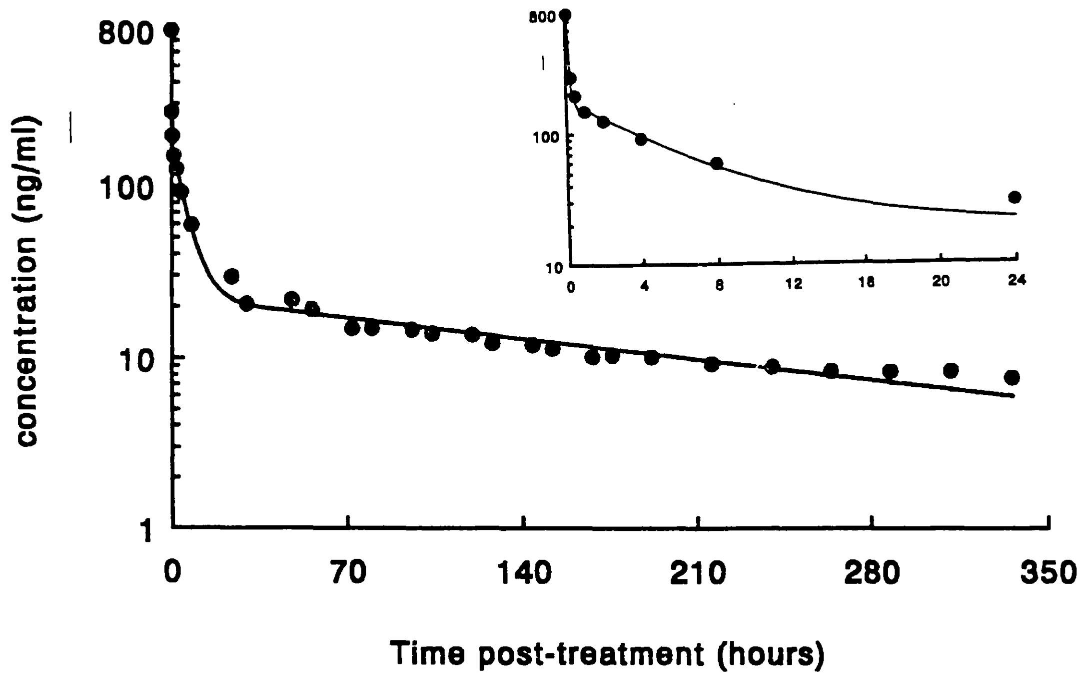


Fig. 8.2

Mean ± SD (n = 3) serum homidium concentrations in non-infected Boran cattle following intravenous treatment with ^{14}C homidium at a dose rate of 1 mg kg^{-1} b.w.

Fig. 8.2

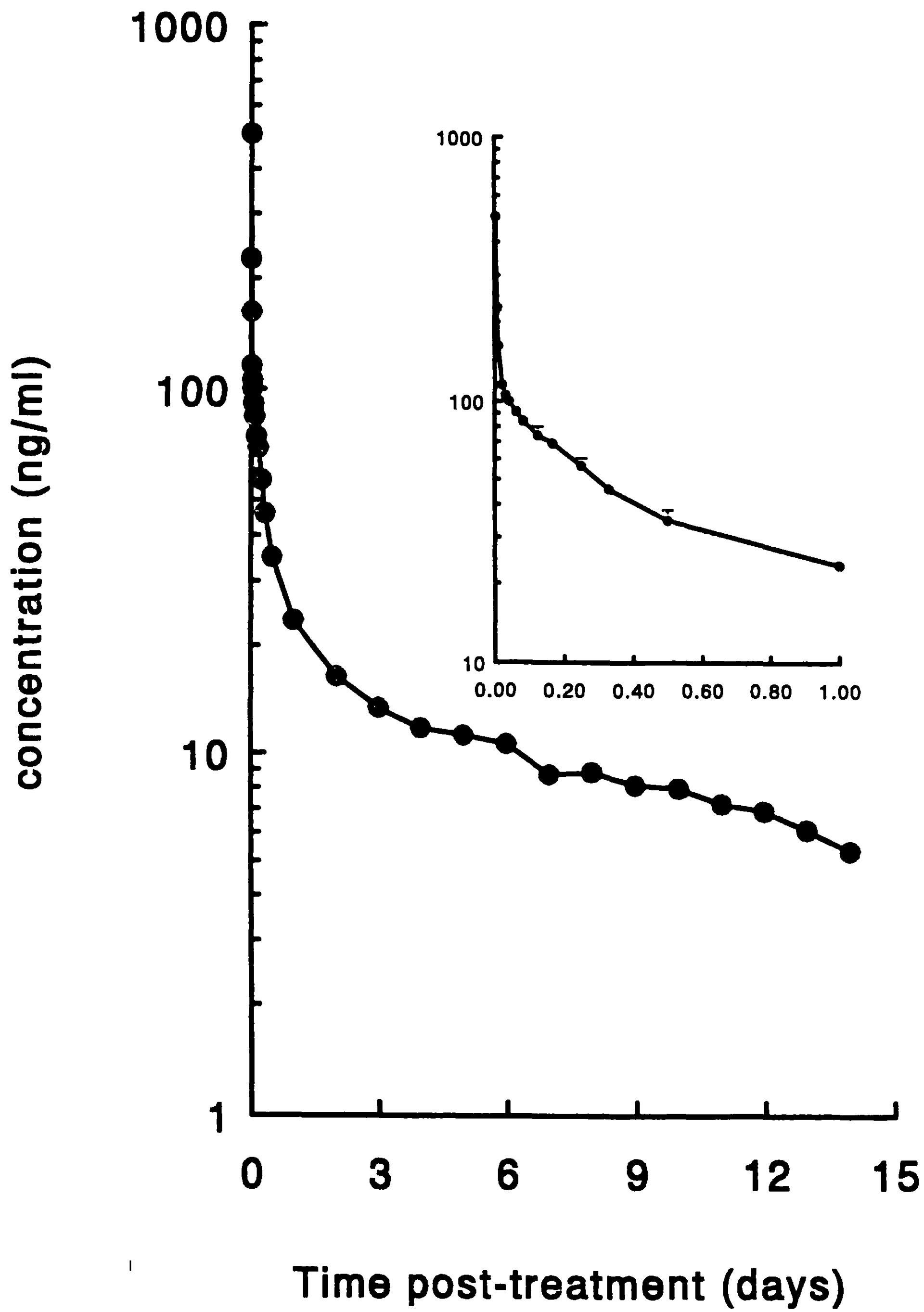


Fig. 8.3

Homidium concentrations in serum, plasma and blood following intravenous treatment of animal No. 368 with ^{14}C homidium at a dose rate of 1 mg kg^{-1} b.w.

Fig. 8.3
Animal No. 368

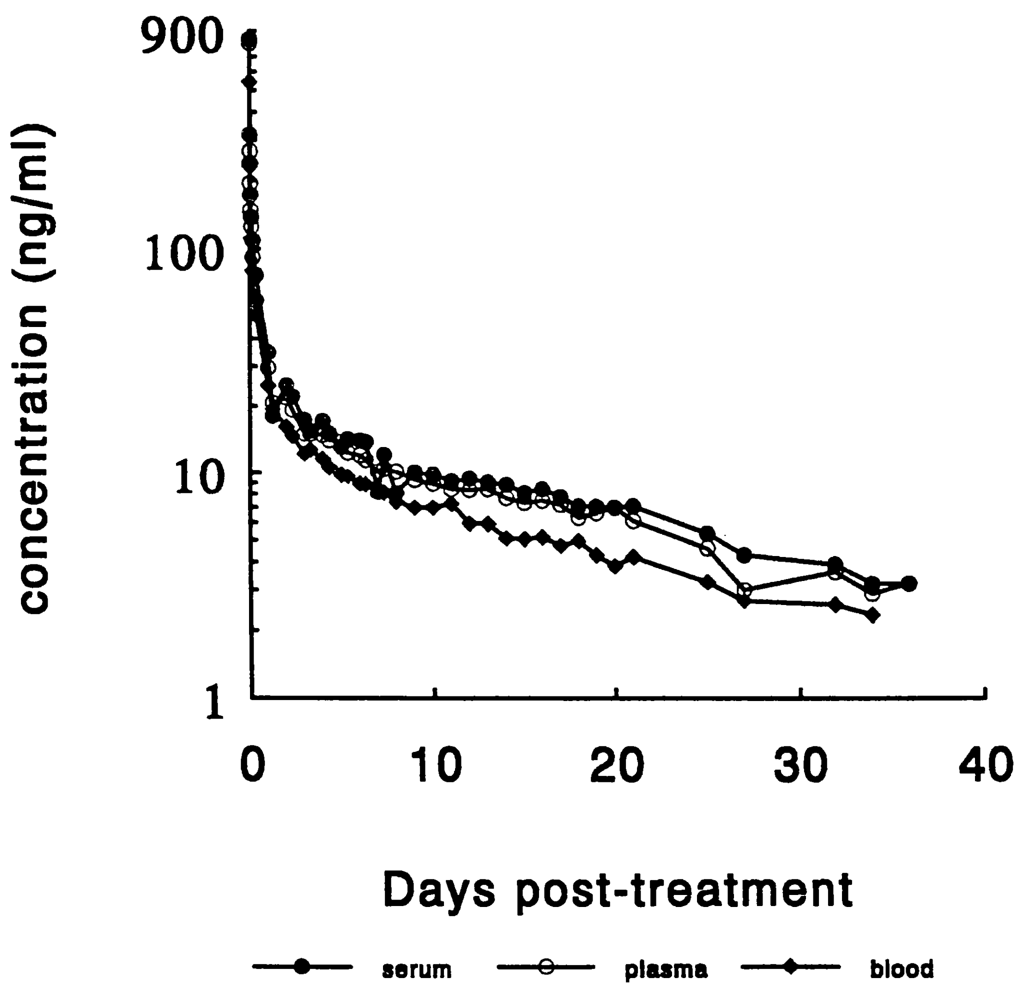


Table 8.1

Pharmacokinetic parameters of homidium in non-infected Boran cattle after intravenous drug administration of ^{14}C homidium chloride at 1 mg kg^{-1} body weight. Compartmental analysis

Parameter	Animal No. 214	Animal No. 215	Animal No. 368	Mean ± SD
A (ng ml^{-1})	1496	832	1545	1291±325
B (ng ml^{-1})	98.5	91.8	160.5	116.9±30.9
C (ng ml^{-1})	20.09	18.11	22.63	20.28±1.85
α	13.86	12.64	11.05	12.52±1.15
β	0.15	0.17	0.19	0.17±0.02
γ	0.004	0.004	0.004	0.004±0
$t½\alpha$ (h)	0.05	0.05	0.06	0.06±0.01
$t½\beta$(h)	4.76	4.10	3.57	4.14±0.49
$t½\gamma$(h)	171.5	184.1	175.7	177.1±5.3
Cp(0) (ng ml^{-1})	1617	942	1728	1426±348
Vc (l kg^{-1})	0.62	1.06	0.58	0.75±0.22
$AUC_{0\text{-last}}$ (μg.h ml^{-1})	5.75	5.42	6.70	5.96±0.54
$AUMC_{0\text{-last}}$ μg.h ml^{-1})	1232.1	1281.1	1458.5	1323.9±97.2
Vd_{area} (l kg^{-1})	37.3	43.6	32.5	37.8±4.6
Cl_b (ml h^{-1} kg^{-1}	173.9	184.5	147.9	168.8±15.4
k_{12} (h^{-1})	11.32	10.03	8.44	9.93±1.18
k_{21} (h^{-1})	1.13	1.60	1.33	1.35±0.19
k_{31} (h^{-1})	0.026	0.029	0.025	0.026±0.002
k_{13} (h^{-1})	1.26	0.98	1.21	1.15±0.12
MRT (h)	214.4	236.4	217.6	222.8±9.7

obtained. The plasma/serum concentration at time 0 (CpO) was 1426±348 ng ml^{-1} and the volume of the central compartment (Vc) 0.75±0.22 l kg^{-1}. The calculated rate constants k_{12} and k_{21} were 9.93±1.18 and 1.35±0.19 per hour, respectively. The k_{13} and the k_{31} values were 1.15±0.12 and 0.026±0.002 per hour, respectively. The mean value for the volume of distribution was 37.8±4.6 l kg^{-1}. The value obtained for the observed area under the curve AUC after i.v. treatment was 5.96±0.54 µg.h ml^{-1}. The value for the mean residence time was 222.8±9.7 hours.

Non-compartmental pharmacokinetic analysis following i.v. treatment

Table 8.2 gives the pharmacokinetic parameters of the non-compartmental model fitting of the i.v data. The mean value obtained for $AUC_{0-\infty}$ observed was 5.39±0.71 µg.h ml^{-1} and the $AUMC_{0-\infty}$ was 859.66±145.13 µg.h^2 ml^{-1}. Using these values, the mean residence time was calculated as 158.9±6.7 hours. The AUC values obtained using both compartmental and non-compartmental pharmacokinetic models were similar but marked variations were observed in the MRT values.

Non-infected cattle treated with ^{14}C homidium by the intramuscular route

Serum homidium concentrations

The serum homidium concentration versus time plots are given in Figs. 8.4(a) to 8.4(c). The maximum plasma drug concentration (C_{max}) obtained in non-infected cattle after i.m. treatment was 159.7±77.9 ng ml^{-1} plasma and the time at which it occurred (t_{max}) was 15 to 30 minutes. These plasma concentrations declined to 103.0±3.0 ng ml^{-1} and 10.8±1.4 within one and 24 hours, respectively. Four weeks later, the plasma concentrations of between 2 and 3 ng ml^{-1} were attained. The mean (± SD) plasma homidium concentration

Table 8.2

Pharmacokinetic parameters of homidium in non-infected Boran cattle after intravenous administration of ^{14}C homidium chloride at 1 mg kg^{-1} b.w. Non-compartmental analysis

Parameter	Animal No.660	Animal No. 663	Animal No.369	Mean ± SD
β	0.009	0.008	0.007	0.008±0.001
$t\frac{1}{2}\beta$(h)	77.55	82.28	93.25	84.36±6.57
$AUC_{0\text{-last}}$ (ng.h ml^{-1})	4458	4104	5333	4632±516
$AUC_{0\text{-}\infty}$ obs (µg.h ml^{-1})	5.08	4.71	6.37	5.39±0.71
$AUC_{0\text{-}\infty}$ pre (µg.h ml^{-1})	4.81	4.49	5.87	5.06±0.59
$AUMC_{0\text{-last}}$(µg.h^2 ml^{-1})	487.08	471.84	577.26	512.06±46.52
$AUMC_{0\text{-}\infty}$ obs(µg.h^2 ml^{-1})	766.17	748.18	1064.64	859.66±145.13
$AUMC_{0\text{-}\infty}$ pre(µg.h^2 ml^{-1})	644.62	646.37	830.80	707.27±87.36
$MRT_{0\text{-last}}$(h)	109.3	115.0	108.3	110.8±3.0
$MRT_{0\text{-}\infty}$ observed (h)	150.8	158.8	167.2	158.9±6.7
$MRT_{0\text{-}\infty}$ predicted (h)	134.0	144.0	141.5	139.8±4.3

Fig. 8.4(a) to 8.4(c)

Serum homidium concentrations in individual Boran cattle following intramuscular treatment with ^{14}C homidium at a dose rate of 1 mg kg^{-1} b.w.

Fig. 8.4(a)
Animal No. 369

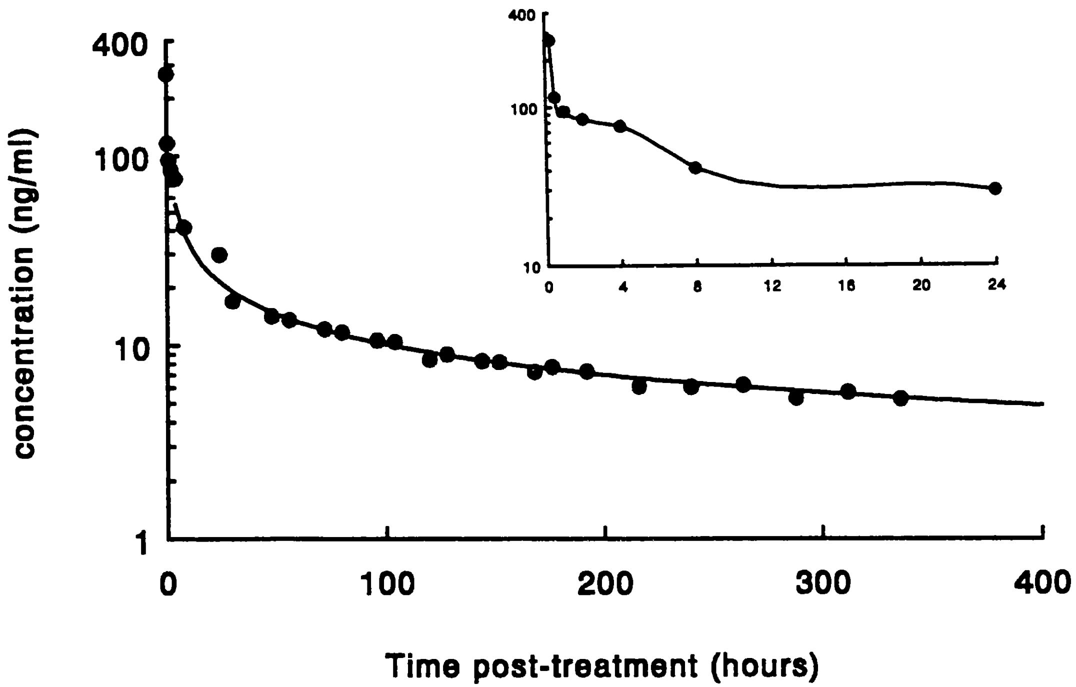


Fig. 8.4(b)
Animal No. 660

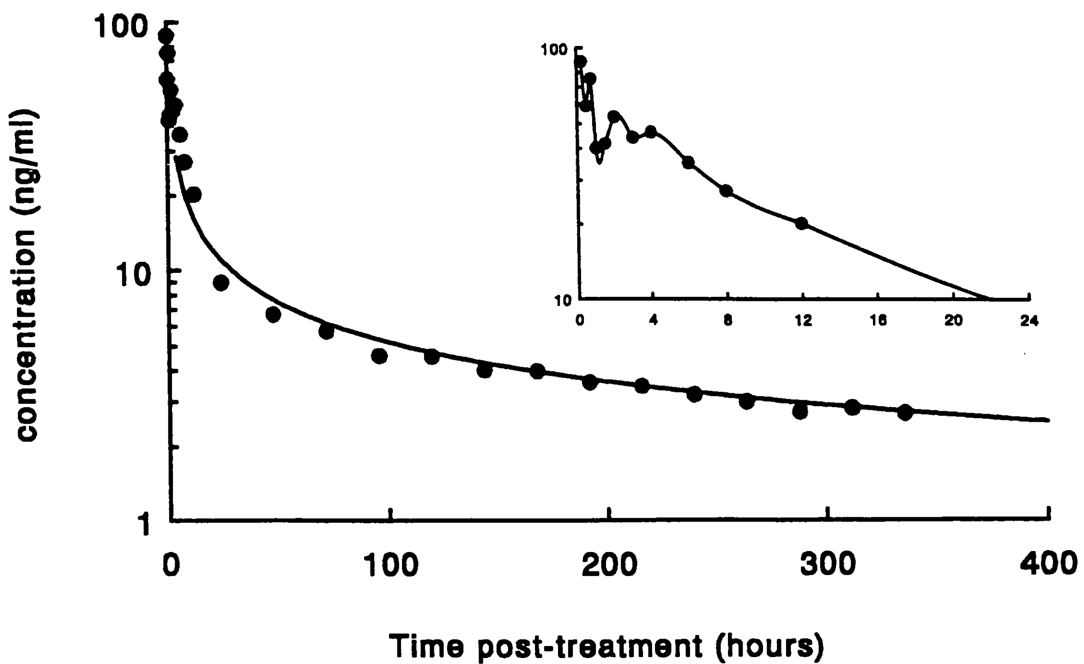
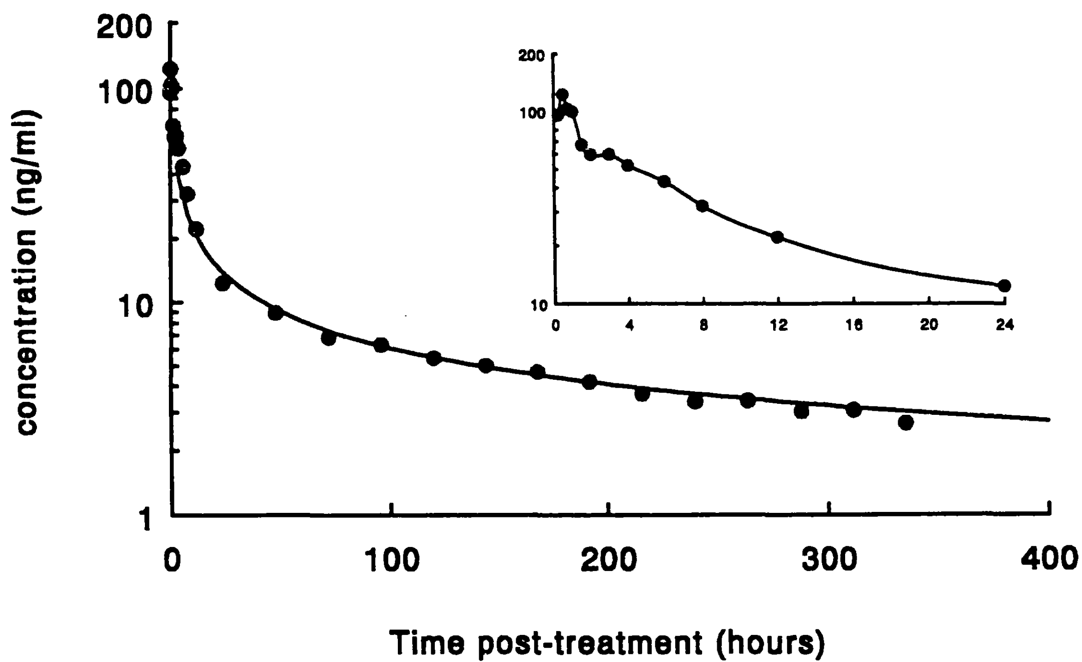


Fig. 8.4(c)
Animal No. 663



versus time plot is given in Fig. 8.5. The plasma, serum and blood homidium concentrations versus time plots of animal No. 369 are shown in Fig. 8.6.

Non-compartmental pharmacokinetic analysis

The mean $AUC_{0-\infty}$ (observed) value for i.m.-treated cattle was 3.37±0.87 $\mu g.h\ ml^{-1}$ Using the $AUC_{0-\infty}$ values obtained, mean value for bioavailability of the i.m. dose of homidium was 56.7±14.7%. The mean residence time was 200.10±62.48 hours giving a mean absorption time of 41.8±62.5 hours. The rest of the pharmacokinetic parameters are shown in Table 8.3.

A comparison in pharmacokinetic parameters between intravenous and intramuscular ^{14}C homidium-treatment

Compartmental and non-compartmental analysis after intravenous treatment

Following i.v. treatment, the observed total (extrapolated) AUC and MRT values using the non-compartmental pharmacokinetic analysis were 5.39±0.71 $\mu g.h\ ml^{-1}$ and 158.9±6.7 hours respectively. Using the compartmental open model, these values obtained were 5.96±0.54 $\mu g.h\ ml^{-1}$ and 222.8±9.7 hours. The variations in the AUC values were, therefore insignificant. Significant variations were however, observed in the MRT values ($p < 0.05$).

Non-compartmental analysis: after intravenous and intramuscular treatment

A comparison of the non-compartmental pharmacokinetic parameters between the i.v.-treated cattle and the i.m.-treated cattle is shown in Table 8.4. The results for AUC (extrapolated) and MRT following i.m. treatment were 3.37±0.87 $\mu g.h\ ml^{-1}$ and 200.10±62.48 hours respectively. The mean extrapolated AUC obtained following i.m. treatment was significantly lower

Fig. 8.5

Mean ± SD (n = 3) serum homidium concentrations in non-infected Boran cattle following intramuscular treatment with ^{14}C homidium at a dose rate of 1 mg kg^{-1} b.w.

Fig. 8.5

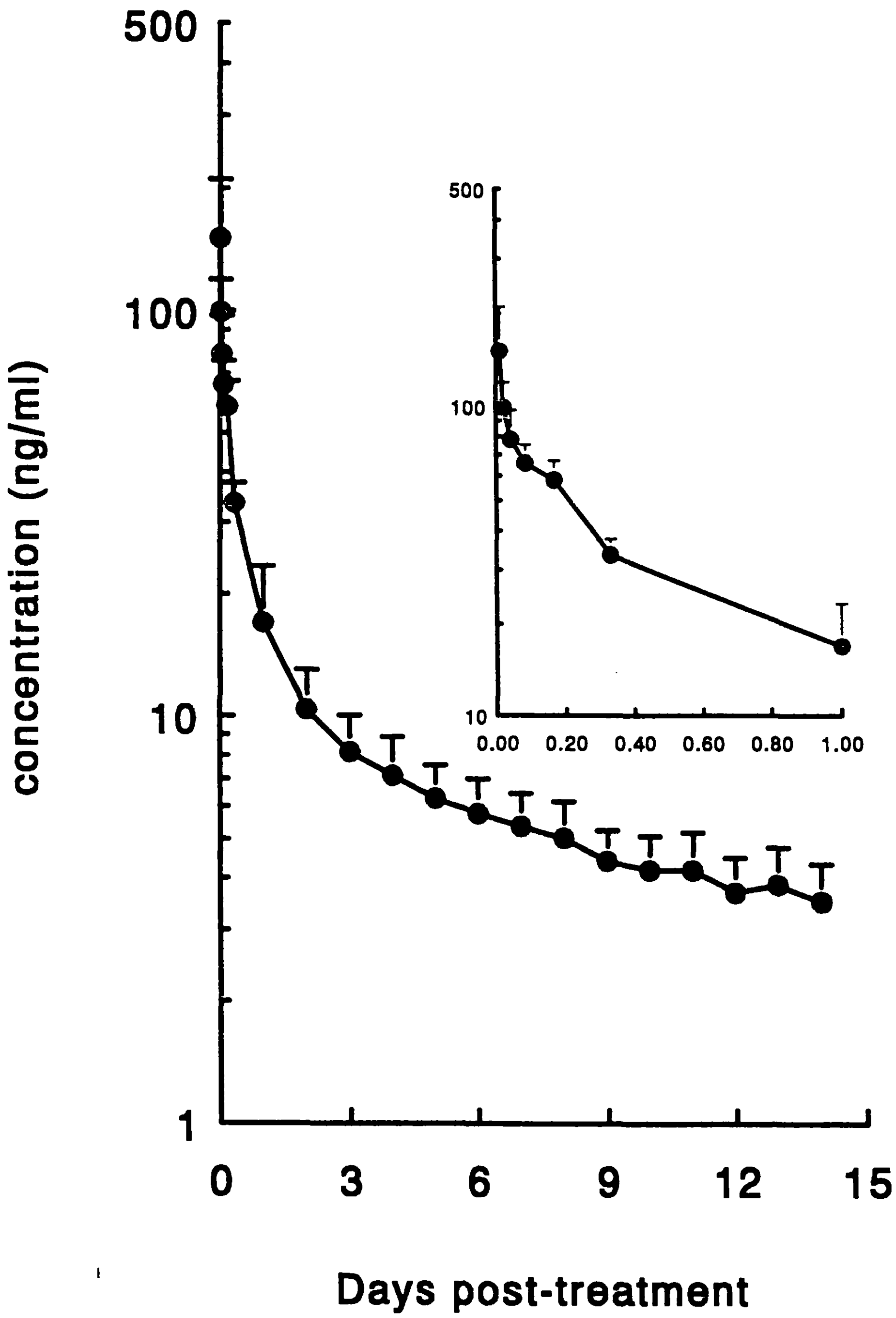


Fig. 8.6

Homidium concentrations in serum, plasma and blood following intramuscular treatment of animal No. 369 with ^{14}C homidium at a dose rate of 1 mg kg^{-1} b.w.

Fig. 8.6
Animal No. 369

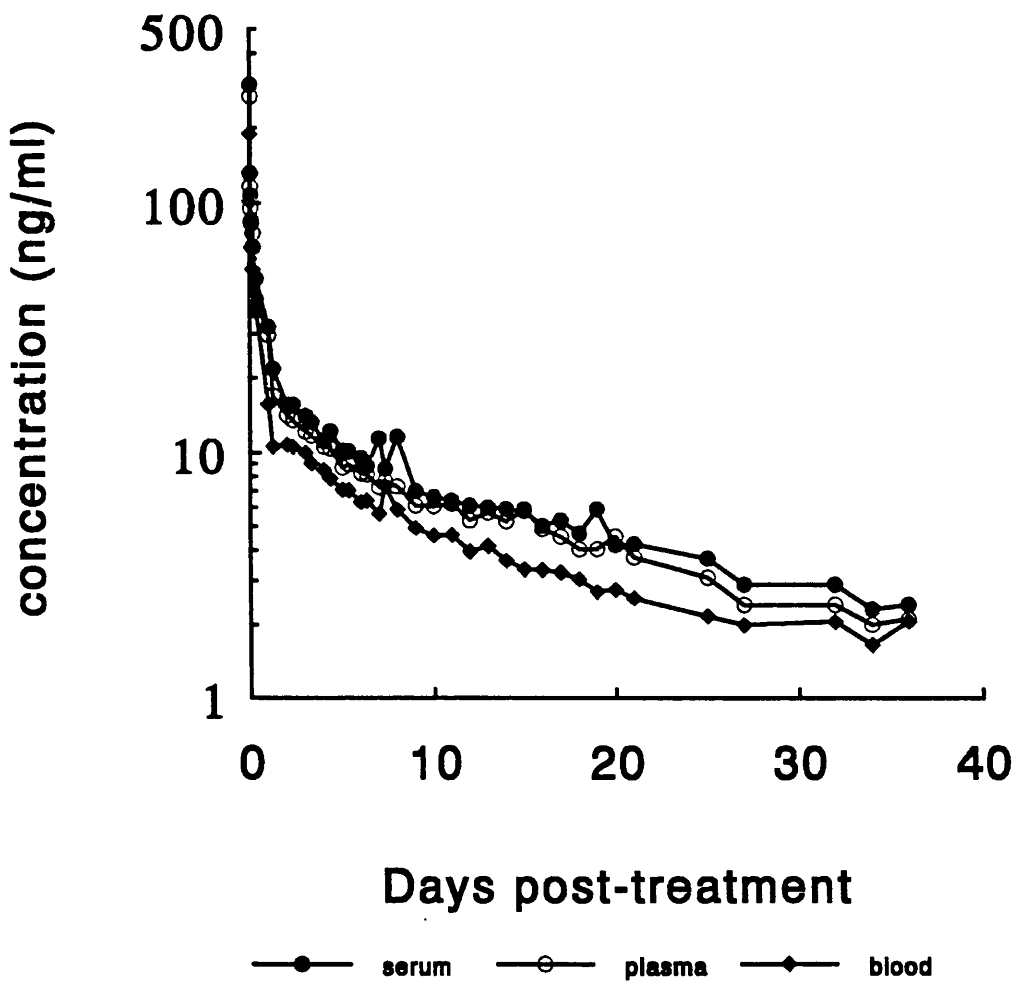


Table 8.3

Pharmacokinetic parameters of homidium in non-infected Boran cattle after intramuscular administration of ^{14}C homidium chloride at 1 mg kg^{-1} b.w. Non-compartmental analysis

Parameter	Animal No. 660	Animal No. 663	Animal No.369	Mean ± SD
t_{max} (h)	0.25	0.5	0.25	0.33±0.12
C_{max} (ng ml^{-1})	88.2	122.8	268.0	159.7±77.9
β	0.003	0.007	0.007	0.006±0.002
t½β(h)	233.9	94.0	97.7	141.9±65.1
$AUC_{0\text{-}last}$ (μg.h ml^{-1})	1.90	2.34	3.87	2.70±0.84
$AUC_{0\text{-}\infty}$ obs (μg.h ml^{-1})	2.81	2.70	4.6	3.37±0.87
$AUC_{0\text{-}\infty}$ pred (μg.h ml^{-1})	2.73	2.58	4.28	3.19±.077
$AUMC_{0\text{-}last}$(μg.h^2 ml^{-1})	200.89	232.02	403.88	278.93±89.26
$AUMC_{0\text{-}\infty}$ obs (μg.h^2 ml^{-1})	809.74	402.80	756.75	656.43±180.64
$AUMC_{0\text{-}\infty}$ pred(μg.h^2 ml^{-1})	759.12	344.75	600.06	567.98±170.68
$MRT_{0\text{-}last}$ (h)	105.65	99.21	104.51	103.12±2.81
$MRT_{0\text{-}\infty}$ observed (h)	288.62	149.14	164.35	200.10±62.48
$MRT_{0\text{-}\infty}$ predicted (h)	278.03	133.74	140.33	184.03±66.52
MAT (h)(approx.)	129.71	-9.77	5.44	41.8±62.5
% F	47.1	45.3	77.3	56.7±14.7

Table 8.4

A comparison of mean (±SD)pharmacokinetic parameters in normal cattle between the intravenous and the intramuscular routes following administration of ^{14}C homidium at 1 mg kg^{-1} b.w. Non-compartmental analysis

Parameter	Intravenous (n=3)	intramuscular (n=3)	Significance
β	0.008±0.001	0.006±0.002	
t½β(h)	84.36±6.57	141.85±65.07	
$AUC_{0\text{-last}}$ (µg.h ml^{-1})	4.63±0.52	2.70±0.84	
$AUC_{0\text{-}\infty}$ obs (µg.h ml^{-1})	5.39±0.71	3.37±0.87	*
$AUC_{0\text{-}\infty}$ pre (µg.h ml^{-1})	5.06±0.59	3.19±0.77	
$AUMC_{0\text{-last}}$ (µg.h^2 ml^{-1})	512.06±46.52	278.93±89.26	
$AUMC_{0\text{-}\infty}$ obs(µg.h^2 ml^{-1})	859.66±145.13	656.43±180.64	
$AUMC_{0\text{-}\infty}$ pre(µg.h^2 ml^{-1})	707.27±87.36	567.98±170.68	
$MRT_{0\text{-last}}$ (h)	110.82±2.96	103.12±2.81	
$MRT_{0\text{-}\infty}$ observed (h)	158.91±6.70	200.10±62.48	
$MRT_{0\text{-}\infty}$ predicted (h)	139.84±4.25	184.03±66.52	

* $p \geq 0.05$

Differences in the means between the rest of the parameters were insignificant at both 95 and 99% using the student's t test

than the values obtained following i.v. treatment ($p < 0.05$). Although the MRT values obtained in cattle following i.m. treatment appeared higher, they were not significantly different from those obtained following i.v. treatment.

T. congolense-infected cattle treated with ^{14}C homidium by the intramuscular route

Trypanosomes were detected in peripheral blood six days following infection and the animals were treated seven days later. From the time the parasites were demonstrated in blood to the time of treatment, a rapid drop was observed in the PCV from pre-infection values of approximately 40% to 25%. This was within a period of two weeks of following infection. On treatment, trypanosomes cleared from circulation within 48 hours and there was an elevation in the PCV to pre-infection values within a period of approximately one week.

Serum homidium concentrations

The serum homidium concentration versus time plots for animal Nos. 254 and 256 are given in Fig. 8.7(a) and 8.7(b). The decline in drug concentrations was exponential and similar to the observations made in non-infected cattle. However, there was an initial accelerated elimination of the drug from plasma during the first week of treatment after which the rate of decline reverted to normal (Fig. 8.8). This was more pronounced in animal No. 256 [Fig. 8.7(b)]. A mean ($n = 2$) peak serum drug level of 90.83 ng ml^{-1} was attained 15 minutes following treatment. Mean plasma drug concentrations of 64.9, 8.9 and 3 ng ml^{-1} were obtained within one hour, 24 hours and 28 days respectively.

Non-compartmental pharmacokinetics

The pharmacokinetic parameters obtained using a non-compartmental

Figs. 8.7(a) and 8.7(b)

Serum homidium concentrations in *T. congolense*-infected Boran cattle following intramuscular treatment with ^{14}C homidium at a dose rate of 1 mg kg^{-1} b.w.

Fig. 8.7(a)
Animal No. 254

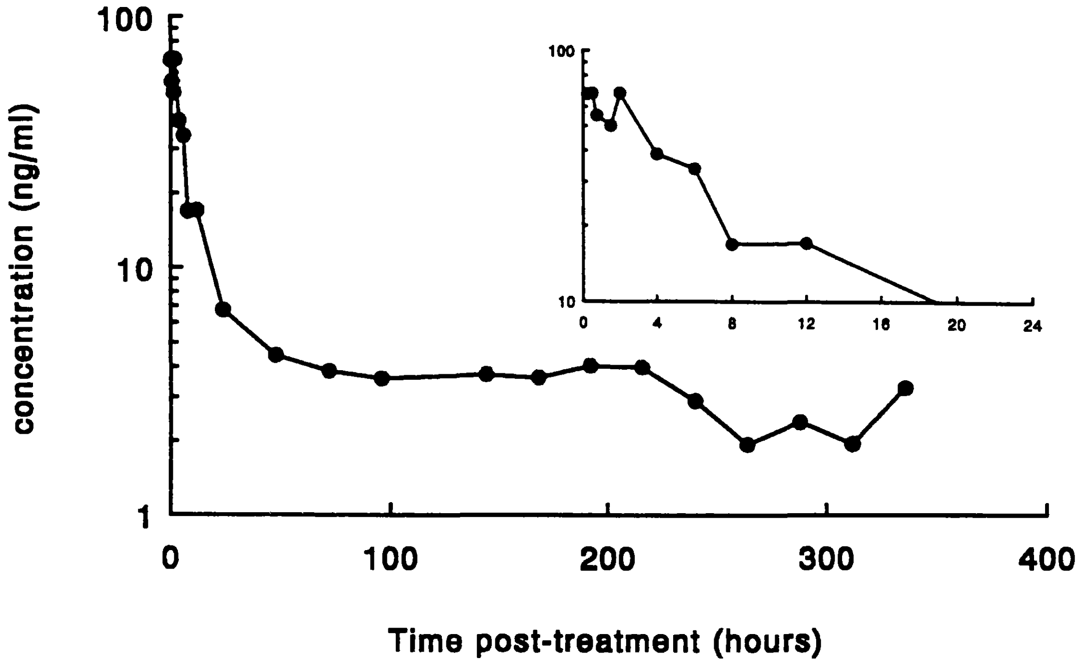


Fig. 8.7(b)
Animal No. 256

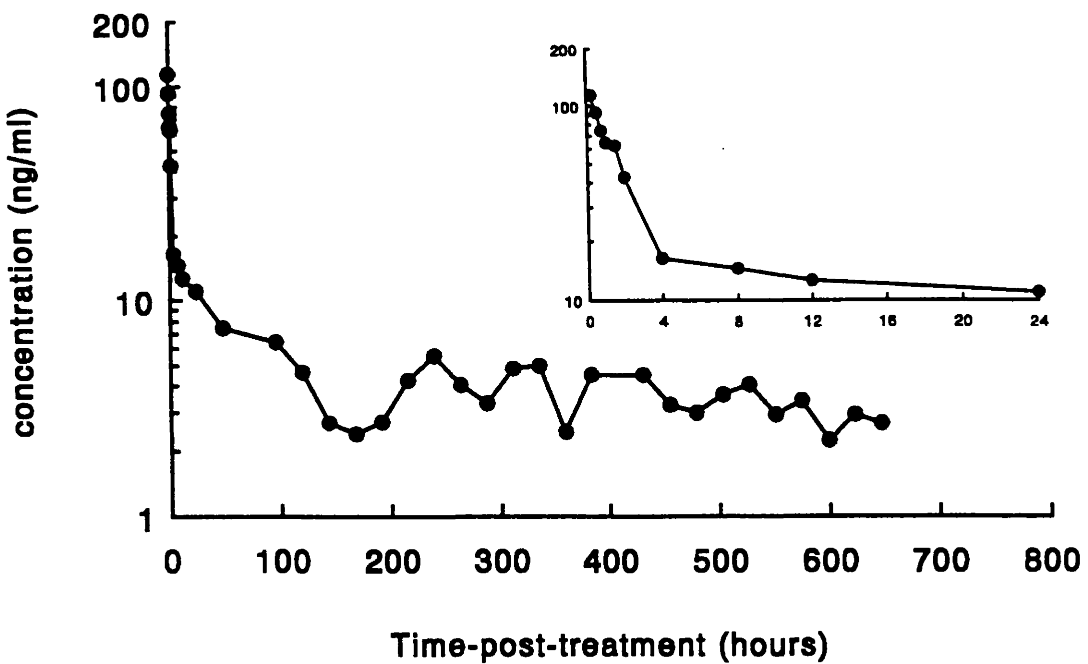
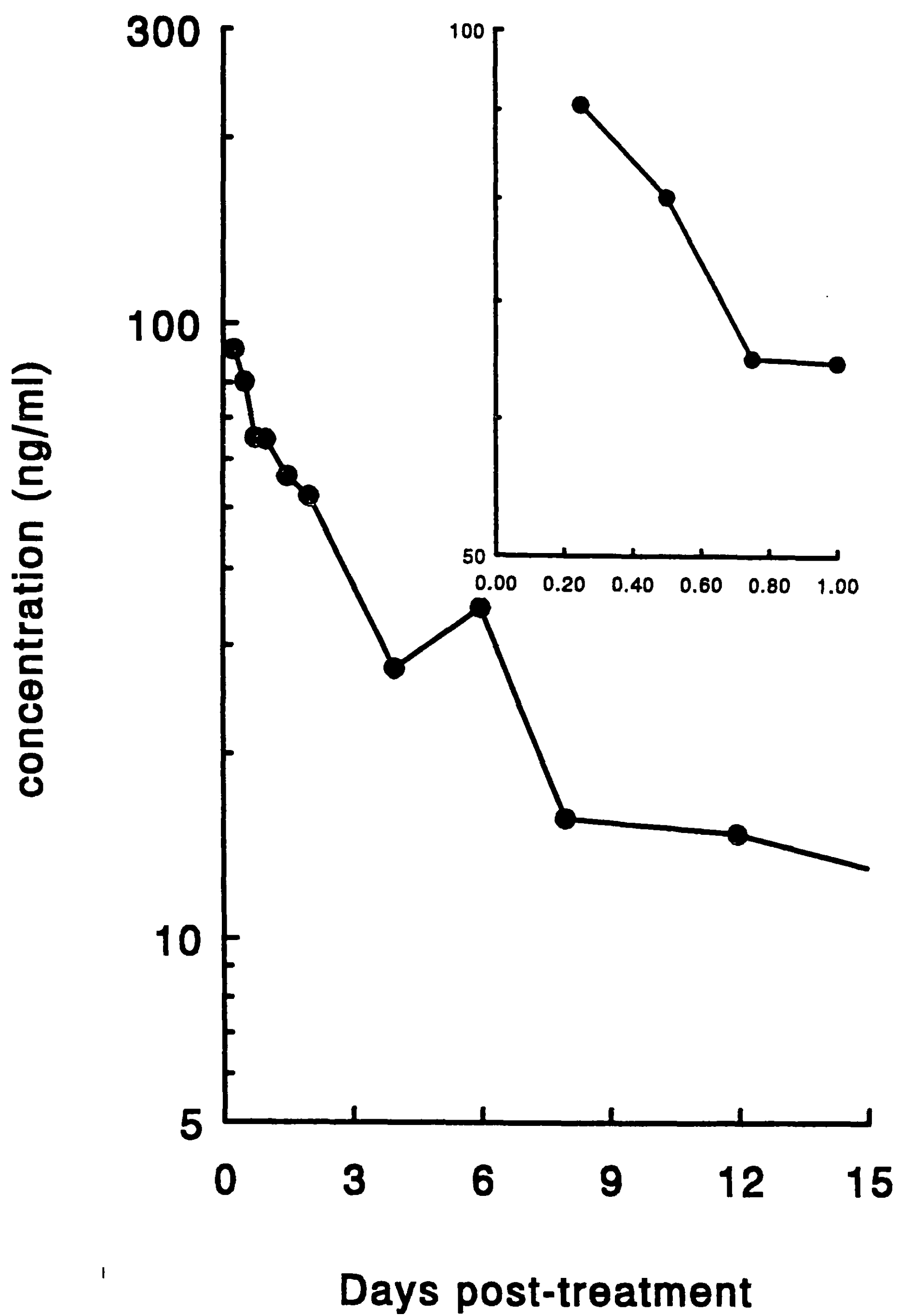


Fig. 8.8

Mean (n = 2) serum homidium concentrations in *T. congolense*-infected Boran cattle following intramuscular treatment with ^{14}C homidium at a dose rate of 1 mg kg^{-1} b.w.

Fig. 8.8



pharmacokinetic model are given in Table 8.5. The mean (n = 2) value obtained for AUC was 3.11 μg.h ml^{-1}. The mean value for MRT was 352.33 hours. Variations in the AUC values between individual animals were very small. Large variations were however, observed between individual animals in the MRT values.

A comparison of plasma homidium concentrations and pharmacokinetics between non-infected and *T. congolense*-infected cattle following i.m treatment with ^{14}C homidium

Following i.m. treatment of non-infected cattle with ^{14}C homidium, the mean (± SD, n = 3) peak serum concentration (Cmax) was 159.7±77.9 ng ml^{-1}. The plasma concentrations declined exponentially to 103.0±3.0 and 10.8±1.4 ng ml^{-1} within one and 24 hours respectively. Following i.m. treatment of *T. congolense*-infected cattle, the mean (n = 2) Cmax was 90.83 ng ml^{-1} and occurred 15 minutes following drug administration. Serum concentrations of 64.9 and 8.9 ng ml^{-1} were obtained within one and 24 hours respectively. Although the concentrations appeared higher in non-infected cattle, the variations between the two animals were not markedly different. Because of the small number of animals used, meaningful statistical analysis could not be carried out.

A comparison of the pharmacokinetic parameters between non-infected and infected cattle in given in Table 8.6. Following ^{14}C homidium treatment of non-infected cattle the mean value obtained for the AUC was 3.37±0.87 μg.h ml^{-1}. The values for the other pharmacokinetic parameters were 200.10±62.48 hours for the MRT, 41.79±62.5 hours for the MAT and 56.7±14.7% for F. Values obtained following similar treatment of infected cattle were 3.11 μg.h ml^{-1} for the extrapolated AUC and 352.33 hours for MRT. No values for MAT and F were determined due to lack of i.v. data in infected cattle. Marked variations were observed between non-infected and infected animals in the

Table 8.5

Pharmacokinetic parameters of homidium in *Trypanosoma congolense*-infected Boran cattle after intramuscular administration of ^{14}C homidium chloride at 1 mg kg^{-1} b.w. Non-compartmental analysis

Parameter	Animal No. 7	Animal No. 8	Mean
C_{max} (ng ml^{-1})	0.50	0.25	0.375
t_{max} (h)	67.78	113.88	90.83
β	0.002	0.005	0.004
t½β(h)	333.8	129.6	231.7
AUC_{0-last} (ng.h ml^{-1})	1.57	2.15	1.86
$AUC_{0-\infty}$ obs (ng.h ml^{-1})	3.15	3.08	3.11
$AUC_{0-\infty}$ pred (ng.h ml^{-1})	2.67	2.61	2.64
$AUMC_{0-last}$ (ng.h^2 ml^{-1})	170.10	255.73	212.91
$AUMC_{0-\infty}$ obs (ng.h^2 ml^{-1})	1465.27	738.51	1101.89
$AUMC_{0-\infty}$ pred (ng.h^2 ml^{-1})	1072.70	494.17	783.44
MRT_{0-last} (h)	108.33	118.84	113.58
$MRT_{0-\infty}$ observed (h)	464.51	240.15	352.33
$MRT_{0-\infty}$ predicted (h)	401.12	189.49	295.30

Table 8.6

Mean (±SD) pharmacokinetic parameters in non-infected and *T. congolense*-infected cattle following i.m. administration of ^{14}C homidium at 1 mg kg^{-1} b.w. Non-compartmental analysis.

Parameter	Normal (n=3) Mean ± SD	Infected (n=2) Mean
C_{max} (ng ml^{-1})	0.33±0.12	0.375
t_{max} (h)	159.7±77.9	90.83
β	0.006±0.002	0.004
t½β(h)	141.85±65.07	231.66
$AUC_{0\text{-last}}$ (μg.h ml^{-1})	2.70±0.84	1.86
$AUC_{0\text{-}\infty}$ obs (μg.h ml^{-1})	3.37±0.87	3.11
$AUC_{0\text{-}\infty}$ pred (μg.h ml^{-1})	3.19±0.77	2.64
$AUMC_{0\text{-last}}$ (μg.h^2 ml^{-1})	278.93±89.26	212.91
$AUMC_{0\text{-}\infty}$ obs (μg.h^2 ml^{-1})	656.43±180.64	1101.89
$AUMC_{0\text{-}\infty}$ pred (μg.h^2 ml^{-1})	567.98±170.68	783.44
$MRT_{0\text{-last}}$ (h)	103.12±2.81	113.58
$MRT_{0\text{-}\infty}$ observed (h)	200.10±62.48	352.33
$MRT_{0\text{-}\infty}$ predicted (h)	184.03±66.52	295.30

MRT values whilst the variations in the AUC values were not marked. As observed above, no meaningful statistical evaluation was possible due to the small number of animals used.

A comparison between the radiometric and ELISA methods

Serum homidium concentrations

Intravenous treatment

Using the radiometric method, the mean (±SD) serum homidium concentrations attained within 5, 15 minutes, one and 24 hours were 499.6± 97.9, 226.4±22.3, 100.3±8.0 and 23.5±1.4 ng ml^{-1} respectively. Using the ELISA method (Chapter 4), these values were 220.1±81.3, 62.1±14.4, 31.9± 4.3 and 3.9±0.9 ng ml^{-1} respectively. Concentrations of approximately 5 ng ml^{-1} were detected in circulation within three weeks of treatment using the radiometric method whilst concentrations were undetectable within 17 days of treatment using the ELISA method.

Following ^{14}C homidium treatment of animal No. 368, it was shown that the concentrations between plasma and serum were markedly similar (Fig. 8.3). Using the ELISA method to analyse serum samples from this ^{14}C homidium-treated animal, it was demonstrated that the serum drug levels fell below the limit of detection of the method (0.1 ng ml^{-1}) within approximately 20 days following i.v. treatment (Fig. 8.9). These findings support the observations reported in Chapter 4 in which drug levels were undetectable within 17 days of i.v. treatment with unlabelled homidium. However, variations in serum drug concentrations were observed as early as five minutes following i.v. treatment between the radiometric and the ELISA methods (Fig. 8.9). Using the radiometric method high homidium levels were demonstrated in circulation for over three weeks following i.v treatment whilst using the ELISA method drug concentrations were undetectable within approximately three weeks.

Fig. 8.9

Serum homidium concentrations obtained using both the ELISA and the radiometric methods following intravenous treatment of animal No. 368 with ^{14}C homidium at a dose rate of 1 mg kg^{-1} b.w.

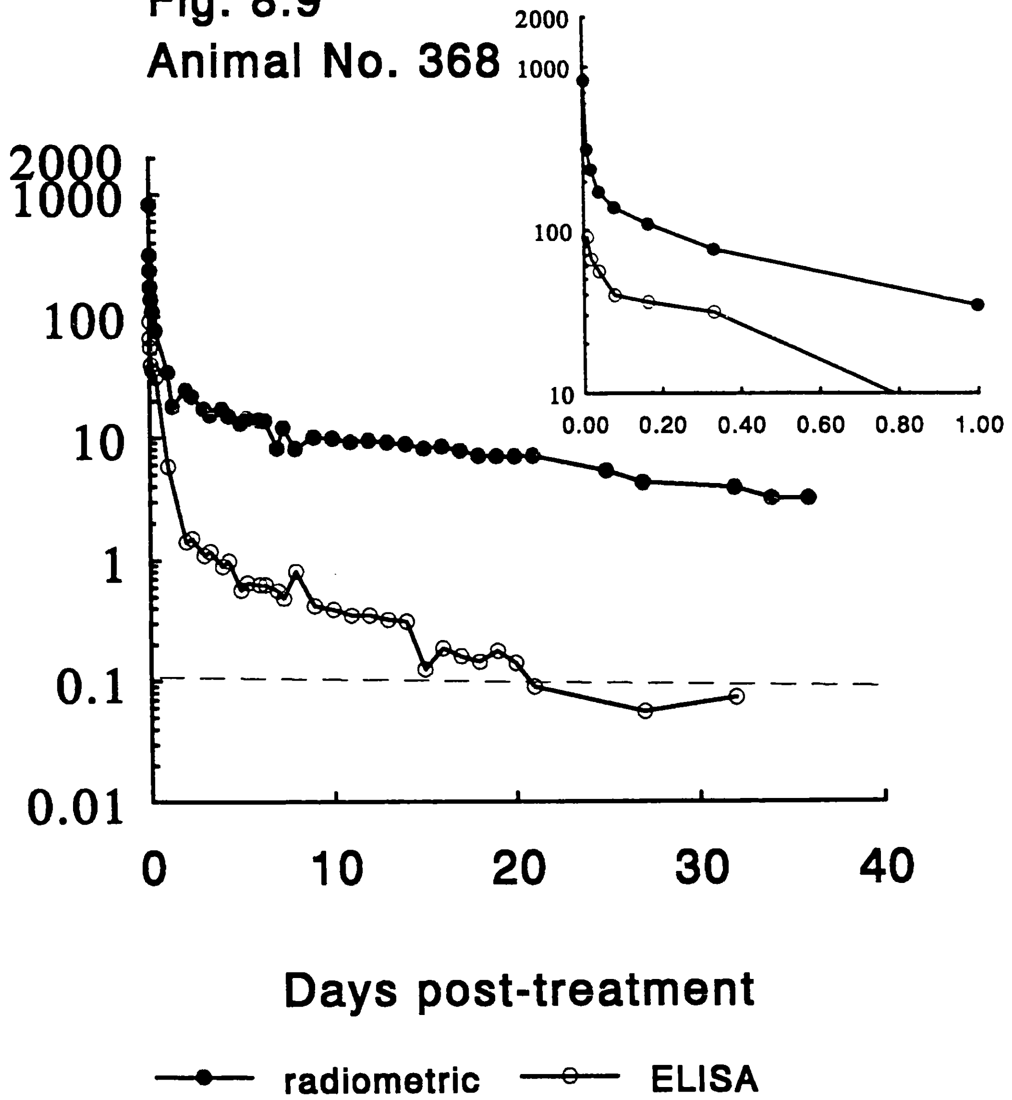


Fig. 8.9
Animal No. 368
concentration (ng/ml)
2000
1000
100
10
1
0.1
0.01
0
10
20
30
40
Days post-treatment
radiometric
ELISA
2000
1000
100
10
0.00
0.20
0.40
0.60
0.80
1.00

Intramuscular treatment

(a) Non-infected cattle

Following i.m ^{14}C homidium treatment, plasma drug concentrations remained generally lower than following i.v. treatment. Using the radiometric method, the mean (n = 3) peak serum homidium concentration was 159.7±77.9 ng ml^{-1}. One and 24 hours following treatment, drug concentrations of 103±3.0 and 10.8±1.4 ng ml^{-1} were attained. Using ELISA (Chapter 4), concentrations were 193.24±81.63 ng ml^{-1} for Cmax. Concentrations of 112.05±40.27 and 12.96±3.29 ng ml^{-1} attained after one and 24 hours respectively. Variations in homidium concentrations were insignificant up to 24 hours post-treatment. Thereafter, marked differences in concentrations were observed. Fig. 8.10 shows a comparison in homidium serum concentrations in animal No. 369 between radiometric and ELISA following i.m. treatment with ^{14}C homidium. The concentration versus time profiles were quite similar but with an approximately 10-fold difference in concentration between them.

(b) T. congolense-infected cattle

Using the radiometric method, the mean (n = 2) peak serum homidium concentration was 90.83 ng ml^{-1}. Concentrations after one and 24 hours were 64.6 and 8.9 ng ml^{-1}. Drug concentrations of approximately 3 ng ml^{-1} were still detectable within 28 days of treatment. Using ELISA (Chapter 5), the mean (n = 5) peak serum homidium concentration was 180.4±34.7 ng ml^{-1}. One and 24 hours following treatment, these values were 84.85±8.15 and 8.04±2.03 ng ml^{-1}. Low concentrations of between 0.2 and 0.3 ng ml^{-1} were detected 28 days following treatment. Using the radiometric method it was observed that the peak concentrations were lower that those obtained using the ELISA method. The initial acceleration in the rate of drug elimination within the first week of treatment after which the rate of decline reverted back to normal was observed using both the radiometric and the ELISA methods.

Fig. 8.10

Serum homidium concentrations obtained using both the ELISA and the radiometric methods following intramuscular treatment of animal No. 369 with ^{14}C homidium at a dose rate of 1 mg kg^{-1} b.w.

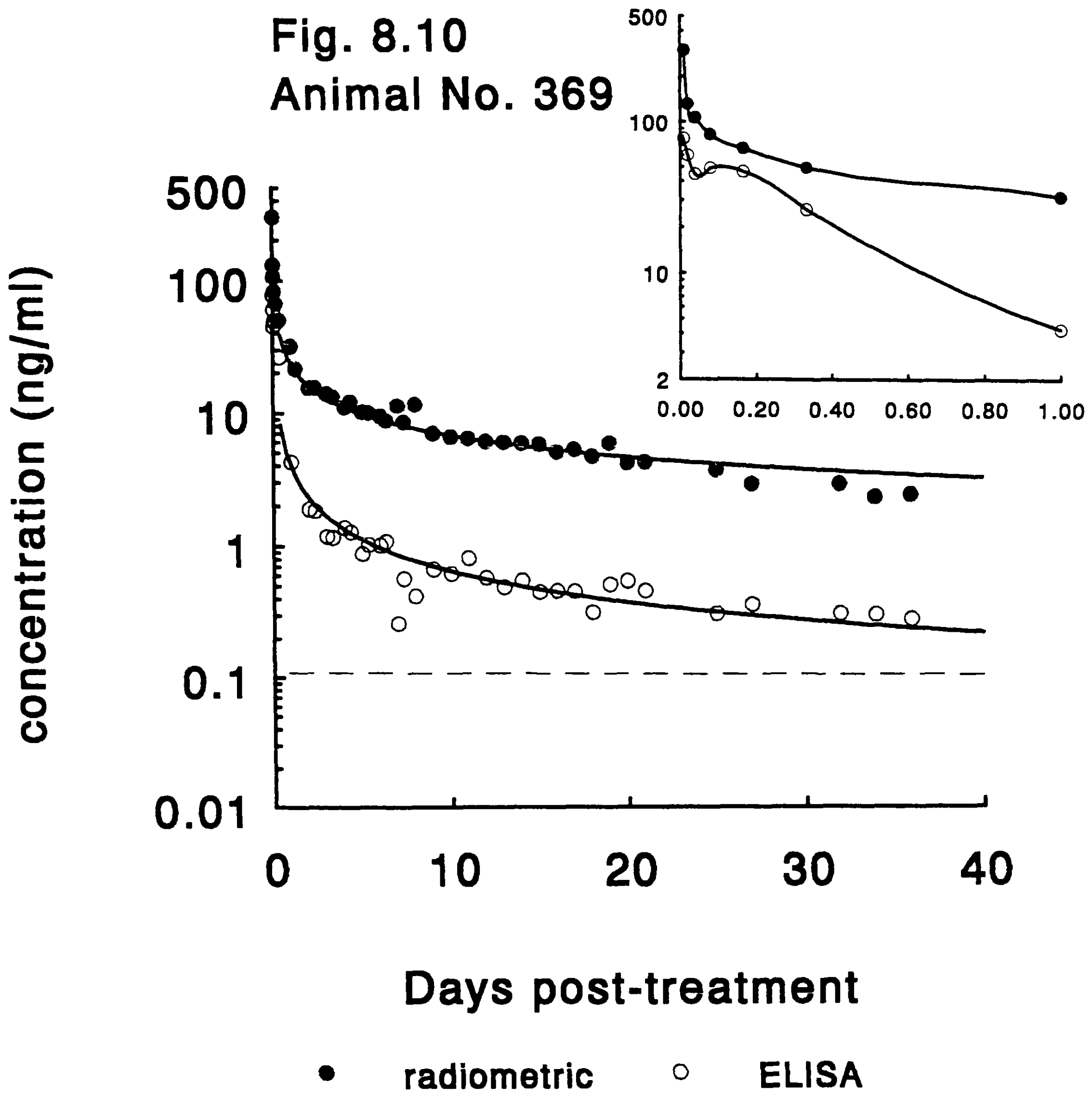


Fig. 8.10
Animal No. 369

Similar to the observations made above, the small numbers of animals used could not permit a meaningful statistical evaluation of the data. However, marked variations were observed in plasma/serum concentrations between the radiometric and ELISA methods reaching as high as 10-fold in 28 days following i.m. treatment of infected cattle.

Pharmacokinetic parameters

Intravenous treatment

(a) Compartmental analysis

Table 8.7 gives a comparison of the mean pharmacokinetic parameters using the compartmental, open model in non-infected cattle using the radiometric (n = 3) and the ELISA (n = 5, Chapter 4)) methods. Similarities were observed in some of the pharmacokinetic parameters which included the large volumes of distribution and the wide variations in the rate constants k_{12}, k_{21} and k_{13}, k_{31}. The mean value for the volume of distribution, although high using both techniques, was three-fold higher using ELISA than using the radiometric method. Some parameters such as the AUC and the MRT were markedly higher using the radiometric method. The approximately 10-fold difference in the serum drug concentrations have been reflected in the calculated AUC values (Table 8.7).

(b) Non-compartmental analysis

Table 8.8 shows a comparison of mean (±SD) pharmacokinetic parameters in non-infected cattle using the radiometric (n = 3) and the ELISA (n = 5, Chapter 4) following i.v. treatment. The observed extrapolated AUC values were 5.39±0.71 and 0.59±0.07 ng.h ml^{-1} using the radiometric and the ELISA methods, respectively. The MRT values calculated from the extrapolated AUC values were 158.9±6.7 hours using the radiometric method and 62.98±5.91 hours using the ELISA method. All the parameters obtained

Table 8.7

Mean (±SD) pharmacokinetic parameters in non-infected cattle obtained using both the radiometric and the ELISA methods following i.v administration of homidium at 1 mg kg^{-1}. Compartmental analysis

Parameter	Mean ± SD (n=3) Radiometric	Mean ± SD (n=5) ELISA	Significance
A (ng ml^{-1})	1291±325	545±293	
B (ng ml^{-1})	116.95±30.93	30.42±1.76	
C (ng ml^{-1})	20.28±1.85	1.38±0.17	
α	12.52±1.15	11.89±4.39	
β	0.17±0.02	0.10±0.02	*
γ	0.004±0.00	0.0065±0.0003	**
$t_{1/2}\alpha$ (h)	0.056±0.005	0.064±0.037	
$t_{1/2}\beta$ (h)	4.14±0.49	7.17±1.87	*
$t_{1/2}\gamma$ (h)	177.1±5.3	106.3±6.6	**
Cp(0) (ng ml^{-1})	1426±348	576.8±292.9	*
Vc (l kg^{-1})	0.75±0.22	2.81±2.45	
$AUC_{0\text{-}last}$ (µg.h ml^{-1})	5.96±0.54	0.58±0.08	**
$AUMC_{0\text{-}last}$ µg.h ml^{-1})	1323.91±97.24	36.68±7.33	**
Vd_{area} (l kg^{-1})	37.79±4.56	111.6±18.0	**
Cl_b (ml h^{-1} kg^{-1}	168.8±15.4	29.39±3.96	**
k_{12} (h^{-1})	9.93±1.18	9.56±3.76	
k_{21} (h^{-1})	1.35±0.19	0.85±0.25	*
k_{13} (h^{-1})	1.15±0.12	0.55±0.31	*
k_{31} (h^{-1})	0.026±0.002	0.0100±0.0006	**
MRT (h)	222.8±9.7	63.39±7.51	**

** $p < 0.01$
* $p < 0.05$

Table 8.8

Mean (±SD) pharmacokinetic parameters of homidium in non-infected Boran cattle obtained using both the radiometric and the ELISA methods following intravenous administration of homidium at 1 mg kg^{-1} b.w. Non-compartmental analysis

Parameter	Radiometric (n=3)	ELISA (n=5)	Significance
β	0.008±0.002	0.012±0.003	
$t^{1/2}\beta$ (h)	84.36±6.57	58.58±1.52	*
$AUC_{0\text{-last}}$ (μg.h ml^{-1})	4.63±0.52	0.58±.0.07	
$AUC_{0\text{-}\infty}$ obs (μg.h ml^{-1})	5.39±0.71	0.58±0.07	*
$AUC_{0\text{-}\infty}$ pre (μg.h ml^{-1})	5.06±0.59	0.58±0.07	
$AUMC_{0\text{-last}}$ (μg.h^2 ml^{-1})	512.06±46.52	34.88±5.48	
$AUMC_{0\text{-}\infty}$ obs(μg.h^2 ml^{-1})	859.66±145.13	36.76±4.75	*
$AUMC_{0\text{-}\infty}$ pre (μg.h^2 ml^{-1})	707.27±87.36	35.59±5.58	
$MRT_{0\text{-last}}$ (h)	110.82±2.96	59.87±5.63	
$MRT_{0\text{-}\infty}$ observed (h)	158.91±6.70	62.98±5.91	*
$MRT_{0\text{-}\infty}$ predicted (h)	139.84±4.25	60.98±5.81	

* $p < 0.01$

using the radiometric method were markedly higher than those obtained using ELISA. The 10-fold difference observed in the AUC between the two methods was similar to the results obtained using the compartmental analysis.

Intramuscular treatment

(a) Non-infected cattle

Table 8.9 shows a comparison of mean (±SD) pharmacokinetic parameters in non-infected cattle between the radiometric method (n = 3) and the ELISA (n = 5, Chapter 4) following i.m. treatment. Using the radiometric method, the values obtained for AUC and MRT were 3.37±0.087 μg.h ml^{-1} and 200.10± 62.49 hours respectively. The values for the same parameters using ELISA were 2.06±0.34 μg.h ml^{-1} and 92.27±21.14 hours, respectively. The pharmacokinetic parameters obtained using the radiometric method, though higher, the variations were only slightly significant ($p \geq 0.05$).

(b) T. congolense-infected cattle

Table 8.10 shows the comparison in the pharmacokinetic parameters in *T. congolense*-infected cattle between the radiometric and the ELISA (Chapter 5) methods. Using the radiometric method (n = 2) the values obtained for the AUC and MRT were 3.11 μg.h ml^{-1} and 352.33 hours, respectively. The values obtained using ELISA (n = 5, Chapter 5) were 1.68±0.23 μg.h ml^{-1} for the extrapolated AUC and 296.67±158.84 hours for MRT calculated from the extrapolated AUC values. No marked differences in the MRT values were observed between the two methods despite the variations in the AUC values.

Excretion and tissue residues following i.v. treatment

The amount of drug excreted in urine and faeces during the two weeks following treatment was approximately 76% of the total dose administered (Table 8.11). Tissue drug levels obtained fourteen days following treatment

Table 8.9

Mean (±SD) pharmacokinetic parameters of homidium in non-infected Boran cattle obtained using the radiometric ELISA methods after intramuscular administration of homidium at 1 mg kg^{-1} b.w. Non-compartmental analysis

Parameter	Radiometric (n=3) Mean ±SD	ELISA (n=5) Mean ±SD	Significance
C_{max} (ng ml^{-1})	0.33±0.12	nd	
t_{max} (h)	159.66±77.9	nd	
β	0.006±0.002	0.0040±0.0005	
t½β(h)	141.85±65.07	177.84±23.42	
AUC_{0-last} (μg.h ml^{-1})	2.70±0.84	2.05±0.34	
$AUC_{0-\infty}$ obs (μg.h ml^{-1})	3.37±0.87	2.06±0.34	*
$AUC_{0-\infty}$ pred (μg.h ml^{-1})	3.19±0.77	2.05±0.34	
$AUMC_{0-last}$ (μg.h^2 ml^{-1})	278.93±89.26	177.35±37.19	
$AUMC_{0-\infty}$ obs (μg.h^2 ml^{-1})	656.43±180.64	177.35±37.19	**
$AUMC_{0-\infty}$ pred (μg.h^2 ml^{-1})	567.98±170.68	180.71±37.65	
MRT_{0-last} (h)	103.12±2.81	87.47±16.8	
$MRT_{0-\infty}$ observed (h)	200.10±62.48	92.27±21.14	*
$MRT_{0-\infty}$ predicted (h)	184.03±66.52	89.07±17.47	
MAT (h) (approx.)	41.79±62.50	28.88±21.14	*
% F	56.7±14.7	32.0±5.7	*

nd = not determined, first interval of sample collection following treatment was 30 minutes

* $p \geq 0.05$

** < 0.05

Mean (±SD) pharmacokinetic parameters of homidium in non-infected and *T. congolense*-infected Boran cattle obtained using both the radiometric and the ELISA methods after intramuscular administration of homidium at 1 mg kg^{-1} b.w. Non-compartmental analysis

Parameter	Radiometric-Normal cattle Mean ±SD (n=3)	Radiometric-Infected cattle Mean (n=2)	ELISA - Normal cattle Mean ±SD (n=5)	ELISA - Infected cattle Mean ±SD (n=5)
t_{max} (h)	0.33±0.12	0.375	nd	0.226±0.03
C_{max} (ng ml^{-1})	159.66±77.9	90.83	nd	180.42±34.74
β	0.006±0.002	0.0037	0.0040±0.0005	0.0018±0.0006
$t½\beta$(h)	141.85±65.07	231.66	177.84±23.42	423.67±145.59
$AUC_{0\text{-last}}$ (µg.h ml^{-1})	2.70±0.84	1.86	2.05±0.34	1.62±0.26
$AUC_{0\text{-}\infty}$ obs (µg.h ml^{-1})	3.37±0.87	3.11	2.06±0.34	1.67±0.23
$AUC_{0\text{-}\infty}$ pred (µg.h ml^{-1})	3.19±0.77	2.64	2.05±0.34	1.65±0.25
$AUMC_{0\text{-last}}$ (µg.h^2 ml^{-1})	278.93±89.26	212.91	177.35±37.19	340.43±96.87
$AUMC_{0\text{-}\infty}$ obs (µg.h^2 ml^{-1})	656.43±180.64	1101.89	177.35±37.19	480.58±239.40
$AUMC_{0\text{-}\infty}$ pred (µg.h^2 ml^{-1})	567.98±170.68	783.44	180.71±37.65	430.07±172.83
$MRT_{0\text{-last}}$ (h)	103.12±2.81	113.58	87.47±16.8	216.21±71.97
$MRT_{0\text{-}\infty}$ observed (h)	200.10±62.48	352.33	92.27±21.14	296.67±158.84
$MRT_{0\text{-}\infty}$ predicted (h)	184.03±66.52	295.30	89.07±17.47	268.53±120.2

nd = not determined

are shown in Table 8.12. Tissue drug levels accounted for approximately 20% of the total dose given. High drug levels were observed in the major excretory organs persisting for longer periods in the liver which had values of 2195 and 2454 ng g^{-1} after 14 and 21 days respectively after treatment.

Excretion and tissue residues following i.m. treatment

Tables 8.11 and 8.12 show the excretion and tissue drug levels following either i.v. or i.m. drug administration using the radiometric method. Approximately 90% of the total dose given was excreted through urine and faeces within two weeks of drug administration. Twenty eight days following treatment, tissue residues accounted for approximately 13% of the total dose administered. Similar to the observations made following i.m treatment, the drug levels remained high in the major excretory organs.

DISCUSSION

The use of the radiometric method permitted homidium plasma/serum concentrations to be measured for up to 28 days post-treatment in both i.v and i.m.-treated cattle and pharmacokinetic parameters to be determined. Persistence of drug levels in tissues following treatment was demonstrated.

A comparison of data on plasma/serum homidium concentrations and pharmacokinetics in non-infected and *T. congolense*-infected cattle obtained in the present study using radiolabelled drug and those reported in Chapters 4 and 5 using ELISA showed some very interesting observations which are discussed.

Following i.v. ^{14}C homidium treatment and using the radiometric method to determine the homidium concentrations, a rapid decline in plasma/serum drug levels was observed. Drug levels declined exponentially from a mean (±SD) of 499.6±97.9 ng ml^{-1} obtained five minutes following treatment to 23.53±1.36 ng ml^{-1} in 24 hours. The drug concentrations generally

Table 8.11

Homidium excretion (cumulative percentage) in urine and faeces two weeks after administration ^{14}C homidium chloride at 1 mg kg^{-1} body weight to non-infected and trypanosome-infected Boran cattle

Treatment type	Drug in urine % (Mean±S.D)	Drug in faeces % Mean±S.D)	Drug excreted % (total)
Non-infected, i.v.-treated (n=3)	22.6±2.8	53.4±2.7	76.0
Non-infected, i.m.-treated (n=3)	17.8±0.6	72.0±4.1	89.8
Infected, i.m.-treated (n=2)	13.3	75.9	89.2

Table 8.12

Homidium tissue residues (ng g^{-1}) after *i.v. and i.m. administration of ^{14}C homidium chloride at 1 mg kg^{-1} body weight to Boran cattle

	Time post-treatment (days)					
	intravenous		intramuscular			
			normal			infected
Tissue Type	14 n=1	21 n=1	14 n=1	28 n=2	14 n=1	28 n=1
Liver	2195	2454	1411	1199	nd	1333
Kidneys	1354	904.2	648.8	448	485.7	161.1
Heart	48.5	51.1	32.2	16.4	42.9	19.5
Spleen	134.3	100.8	64.3	46.3	53.8	nd
Limb skeletal muscle	504.3	352.3	309.7	156.8	137.1	94.5
Lung	296.0	146.2	66.3	37.3	78.6	61.4
Skin	22.1	18.1	22.6	22.3	26.9	22.4
Small intestine wall	74.7	39.4	25.3	27.4	29.9	38.5
Large intestine wall	48.8	25.2	16.5	16.6	48.8	34.5
Abomasum	59.1	33.4	30.5	28.1	48	90.2
Omasum	31.5	18.4	24	9.4	18.7	54.4
Rumen	17.1	30.7	nd	8.3	29.8	nd
Reticulum	6.9	26.1	19.4	14.2	22.4	7.1
Tongue	273.3	233.0	342.9	160.2	199.4	57
Hump	188.9	220.6	nd	219.4	180.9	125.9
Superficial lymph nodes	549.0	nd	139.6	55.6	nd	nd
Pancreas	408.6	120.7	175.6	47.5	169.8	28.7
Bile	109.8	82.2	35.6	35.4	58.6	nd
Injection Site	nd	nd	nd	nd	41449	nd
Brain	16.3	12.6	nd	12.2	10	10
Urinary Bladder	26.5	24.1	26.2	21.7	28.5	10.7
Gall Bladder	19.7	14.8	26.4	21.8	13.7	21.8

nd = not determined

* tissue samples from only two of the animals analysed

remained higher in the animals treated by i.v. than those treated by i.m. injection. It was demonstrated that there were no marked differences in homidium concentrations between plasma and serum. However, the slightly lower values obtained in the blood could have been due to quenching of counts during determination of radioactivity or due to the insignificant binding to the red blood cells.

Compartmental pharmacokinetic evaluation of the i.v. data obtained using radiometric method showed high values for the volume of the central compartment and the apparent volume of distribution. The mean volume of distribution (Vdarea; 37.8±4.6 l kg^{-1}) was higher than normal plasma volume of approximately 35.9 ml kg^{-1} and normal total blood volume of approximately 51.2 ml kg^{-1} (Ruckebusch et al., 1991) and indicate that the drug was distributed extensively extravascularly following treatment. Large volumes of distribution inevitably indicate that the fraction of the drug in plasma is very small. This suggests that once in the tissues, the rate of movement of the drug back into the circulation was extremely slow. This is consistent with the observation that residues remained high for long periods of time. Further evidence in support of this is in the large differences in the rate constants k_{12} and k_{21} and also between k_{13} and k_{31}.

This persistence of the drug in the tissues suggests possibly covalent binding of the drug to tissue components, e.g. proteins. This situation suggests non-attainment of equilibrium and steady state between the plasma/serum drug concentrations and the tissues. The low drug concentrations in circulation following i.m. treatment were attributed mainly to the injection site depot following the observation that in the i.v. treated cattle, the drug was undetectable within 17 days of treatment using ELISA.

In the present study, the AUC values obtained using both compartmental and non-compartmental pharmacokinetic models following i.v treatment were similar but there was a marked variation in the MRT values.

Whereas the three-compartmental open model gave a mean value of 222.8±9.7 hours (range 214.36 - 236.36), the non-compartmental model gave mean values of 158.91±6.7 hours (range 150.77 - 167.18) based on the total AUC and AUMC values (extrapolated to infinity).

Following i.m. drug administration, disappearance of the drug from plasmaserum was rapid and, indeed, it was observed that following the i.m. dose, the drug concentrations fell from 159.7±77.9 ng ml^{-1} 15 minutes following treatment to 10.76±1.39 ng ml^{-1} within 24 hours and to levels between 2 and 3 ng ml^{-1} within 4 weeks; after the initial rapid fall in the plasma/serum concentrations, low concentrations remained in circulation for long periods of time. The mean residence time of value of 200.1±62.5 hours shows that the mean time drug molecules reside in the body is long. The bioavailability of the i.m dose was 56.6% showing that almost half of the i.m. dose given did not reach the systemic circulation.

Following i.m. treatment of infected cattle, the drug levels declined exponentially with an accelerated rate of elimination within the first week of treatment after which the rate of decline reverted back to normal following disappearance of trypanosomes from circulation. Similar to the non-infected cattle, low levels of the drug remained in circulation for long periods of time following treatment. The mean residence time of 352.52 hours was quite long.

In comparing the homidium concentrations and pharmacokinetics between the non-infected and infected cattle, it was shown that peak concentrations were markedly higher in non-infected cattle. The acceleration in the rate of decline of the drug during the first one week following i.m. treatment of infected cattle was not observed in uninfected cattle. Thereafter, variations in the concentrations between the non-infected and infected cattle were not markedly different. The low peak concentrations observed in the *T. congolense*-infected cattle could possibly have been as a result of either drug uptake by trypanosomes or the enhancement of elimination of the drug due to

changes in the host metabolism as a result of the infection as observed in Chapter 5. The mean extrapolated AUC value obtained was 3.11 μg.h ml^{-1} in infected cattle.

In comparing the radiometric and the ELISA methods, the plasma/serum drug concentrations obtained using radiometric method following i.v. treatment were found to be markedly higher than those obtained using ELISA (Chapter 4) from as early as five minutes following treatment. Whilst drug levels of approximately 5 ng were detected in circulation by the third week following i.v. treatment using the radiometric method, the drug levels were below the detection limit within three weeks of treatment using the ELISA method. This has been demonstrated in the results of the present study in animal No. 368 and in the study reported in Chapter 4.

The variations in the drug concentrations reflect the differences in the analytical methods used to determine homidium. The radiometric method detected the total radioactive species in solution all of which may not be attributed to intact drug. The disappearance of the drug from circulation within three weeks following i.v. treatment using the ELISA method and the detection of drug concentrations of approximately 5 ng ml^{-1} in similarly treated cattle using the radiometric method would suggest the presence of metabolites from as early as five minutes following treatment. These metabolites could possibly have been undetected by the ELISA method used.

Following i.m.-treatment, the concentrations obtained using ELISA in Boran cattle were 214.29±61.02, 80.83±5.23 and 7.07±1.21 ng ml^{-1} after 15 minutes, one hour and one day respectively (n= 5, pre-challenge pharmacokinetics, Chapter 6). From the above results, it appears that marked differences in the concentrations occurred after 24 hours of treatment unlike in the i.v.-treated cattle in which differences were observed as early as five minutes following treatment.

Following i.m. treatment of infected cattle, marked variations were

observed in the mean peak serum homidium concentrations. Although the peak concentrations were markedly higher using ELISA, the concentrations obtained 24 hours following treatment were similar between the two methods. Marked differences were however, observed after 24 hours of treatment, similar to the above findings in non-infected cattle. The observation of accelerated rate of elimination of the drug during the first one week of treatment after which it reverted back to normal on the disappearance of trypanosomes from the circulation was similar using either the radiometric or the ELISA method.

In comparing the pharmacokinetic parameters in non-infected cattle following i.v treatment between the methods, a ten-fold difference in the AUC values was obtained (Tables 8.7 and 8.8). Compartmental pharmacokinetic analysis of the i.v. data obtained using ELISA showed a mean residence time of 63.39±7.51 hours which was significantly lower than the value of 222.8± 9.7 hours obtained in the present study using the radiometric method, suggesting that drug molecules detected by the radiometric method reside for longer periods in the animals following treatment and that all the released drug from the tissue depots three weeks following i.v. treatment could possibly be homidium metabolites.

Following i.m treatment of non-infected cattle, the extrapolated AUC values obtained using the radiometric method were higher than those obtained using the ELISA method. Similar observations were made in the MRT values. However, these variations were only slightly significant ($p \geq 0.05$, Table 8.9). Interestingly, the ten-fold differences observed in the serum drug concentrations after 24 hours of treatment have not been reflected in any of the pharmacokinetic parameters such as the extrapolated AUC values (Table 8.7) in which only slightly significant differences were reported. This interesting observation suggests that the interaction of the drug with the host under normal conditions would be similar as demonstrated by similar serum

concentrations during the first 24 hours using both the radiometric and ELISA methods to study the pharmacokinetics.

Pharmacokinetic parameters obtained in infected cattle were markedly different between the two methods. Whilst the AUC value in infected cattle of 3.11 μg.h ml^{-1}, was similar to 3.37±0.87 μg.h ml^{-1} obtained in non-infected cattle using the radiometric method, a value of 1.67 ng.h ml^{-1} was obtained in infected cattle using ELISA. Table 8.10 shows a comparison of pharmacokinetic parameters in non-infected and infected cattle between the radiometric and the ELISA (Chapters 4 and 5) methods following i.m. treatment. The approximately ten-fold difference in the serum drug concentrations between the radiometric and the ELISA methods have, similarly not been reflected in the extrapolated AUC values following treatment of infected cattle in which only a two-fold difference in the AUC values between the methods was observed. (Table 8.10). The results however, suggest an appreciable amount of drug metabolism by the trypanosomes. The results demonstrated that 50% of the drug molecules reaching the systemic circulation could possibly be metabolites, undetected by the ELISA method used. These metabolites were detected by the radiometric method which determined total radioactivity rather than the intact drug. The detection of homidium levels for longer periods in non-infected and *T. congolense*-infected cattle following i.m treatment suggests that all the drug detected using ELISA could be attributed to the injection site depot.

The detection limit of the radiometric method used in the present study was one ng which was equivalent to 169 dpm (corrected for background). This activity was four times the background count. Because of the uncertainties in the determination of such low counts, all samples with low radioactivity were counted for a longer period until the counter computed CVs obtained were below 5%. The results of the present study showed that the highest CVs recorded were 2.5%. Similarly, with the ELISA, the homidium concentrations

were extremely low after 24 hours of treatment. To be able to distinguish these low drug levels from the background, the limit of detection of the assay was measured by assaying several replicates of the zero standard and calculating the mean and the standard deviation. The mean minus three standard deviations (mean - 3SD) read in dose on the standard curve was taken as the limit of detection of the assay. Due to the extremely low concentrations involved, the mean - 3SD was used inorder to increase the confidence limits to the detection of the low serum concentrations for over long periods following treatment.

It has been established in work reported in Chapters 6 that low homidium concentrations can protect cattle from trypanosome infection for periods ranging from 120 to 144 days following treatment in the presence of a homidium-susceptible trypanosome populations. Whilst levels of approximately 2 ng ml^{-1} were detected in circulation up to four weeks following i.m. treatment with ^{14}C homidium, concentrations detected using ELISA ranged between 0.15 and 0.4 ng ml^{-1}. Also, whilst no drug was detectable within approximately three weeks of treatment in the i.v.-treated cattle, serum homidium levels were approximately ten-fold higher after 24 hours of i.m drug administration. These findings suggest the presence of homidium metabolites in serum detectable by the radiometric but undetectable by the ELISA procedure which may have been specific for homidium.

In light of the observations made in the present study, investigations need to be carried out to determine the identity of homidium metabolites and whether they possess any trypanocidal activity since it appears that out of the total homidium levels in circulation, only 10% could be intact homidium. Despite the differences observed between the two techniques in the AUC and MRT values following intramuscular drug administration to uninfected cattle, the calculated MAT and F values were markedly similar except for one animal in the ^{14}C homidium group.

So far there are no reports on the detection of homidium metabolites in serum of treated animals possibly due to the unavailability of sensitive and accurate analytical procedures for homidium. The availability of the homidium ELISA reported in Chapter 3 and used in the studies reported in the present Chapter and in Chapters 4 and 5 should make investigations into homidium metabolites possible.

The major route of excretion was in the faeces via bile. Fourteen days after intravenous injection, the fractions of the dose excreted through urine and faeces were 22.6±2.8% and 53.4±2.7% respectively. Higher drug levels were observed in urine of cattle after i.v. treatment than after i.m. treatment suggesting that the kidneys play an important role in the elimination of the initial high levels of homidium following iv. treatment. Drug levels in tissues accounted for approximately 20% of the i.v. dose given with high levels in the liver, kidneys, lungs and tongue. Due to the high levels in the tongue it appears as though there could have been some salivary excretion of the drug in addition to the other routes mentioned although this drug would ultimately end up in the faeces. Drug levels were however not determined in the salivary glands. Decline in drug levels in the kidneys was significant compared to the liver between 14 and 21 or 28 days following treatment, showing persistence in the liver over longer periods after either i.v. or i.m. treatment.

Coolbear and Midgley (1986) in their work on the uptake of homidium by two trypanosomatid flagellates *Crithidia fasculata* and *Leptomonas seymouri* found that homidium transport could not be saturated over a large concentration range. Results of the present study showed that there was non-attainment of equilibrium between free homidium concentrations in host tissues and plasma in cattle following drug administration at the recommended therapeutic dose (1 mg kg^{-1}) also suggesting covalent binding of the drug molecules to tissue proteins. Homidium was found to be eliminated relatively slowly and if the drug movement was by simple diffusion, there would be an

equilibrium established between free homidium concentrations in tissues and plasma with similar rates of movement into and out of tissues. The observation of non-attainment of equilibrium would suggest that transport into host cells was unsaturated and would possibly be similar to the observations made by Coolbear and Midgley (1976) in the trypanosomatid flagellates.

Homidium has been shown to intercalate with DNA and this property has made the compound be used widely as a biochemical probe (Newton, 1976). Studies on the nature and extent of binding of the drug was not covered by the present study but previous work has shown that binding of ethidium *in vivo* may involve more than simple intercalation with DNA (Mahler and Bastos, 1974). Results of their study on the uptake of ^{3}H-ethidium by *Saccharomyces cerevisiae*, showed that ethidium was associated with mitochondrial DNA and accounted for about 2.3% of the total ethidium bound to DNA with evidence that it might be a metabolic derivative of homidium formed within the mitochondrion which then becomes covalently bound to mitochondrial DNA. Mattern (1976) and MacGregor and Johnson (1977) similarly demonstrated that ethidium bromide interacts with nucleic acids not only by a reversible intercalation reaction but also through an irreversible, enzyme mediated binding.

The present study has demonstrated that the drug concentrations persist in tissues for long periods of time. It is this persistence of the bound homidium concentrations in the cells that could possibly explain the widespread nature of homidium resistance in trypanosomes and why this can increase several fold within a very short period of constant exposure even in the presence of extremely low concentrations in circulation. It is possible (due to the persistence of the drug in the liver) that toxic levels could build up when high doses of homidium are administered as demonstrated by Unsworth (1954a).

Since the lymphoid system is actively involved in the early events following trypanosome infection, the levels of the drug in the lymph nodes

could be of some practical significance and in susceptible animals would contribute to determining whether an infection becomes established. Luckins and Gray (1979) demonstrated that trypanosomes first appear in the lymph nodes before they are detected in the general circulation and Akol and Murray (1982) observed that the development of a chancre and enlargement of the lymph nodes after a tsetse fly bite occur simultaneously. This could play a significant role in the prophylactic treatment. Low MRT values have been associated with reduced prophylactic period (Chapter 6).

Using a chemical assay developed by Bratton and Marshall in 1939, MacGregor and Clarkson (1971) reported rapid excretion of a 15 mg kg^{-1} dose of homidium bromide given intraperitoneally to mice and that 50-55% of the dose was found to be excreted in the bile 16-18 hours after drug administration. Between 20-25% of the drug recovered was unchanged whereas 75-80% was in the form of monoacetyl amino derivatives. Kandaswamy and Henderson (1963) in their studies in mice reported that 51% of the dose given was excreted unchanged within 24 hours while Gilbert and Newton (1982), working with cattle found 2 acid labile compounds in urine represented 46% of the total activity eluted and neither were mono- or diacetyl amino conjugates. Results from this laboratory (unpublished) have confirmed the presence of monoacetyl derivatives in rat faeces within the first 24 hours of drug administration during which time none were found in cattle indicating the difficulties associated with extrapolating results from one animal species to another brought about by differences in their rates of metabolism.

Owing to the fact that homidium remains in tissues for long periods of time, it is highly important that bioavailability studies be carried out to determine the tissue levels that would be safe in animal products destined for human consumption. The effect of low level intake over prolonged period should be examined for purposes of determining the minimum acceptable levels.

In conclusion, the use of the radiometric method showed detectable pasma/serum concentrations up to four weeks following either i.v. or i.m.-treatment of non-infected cattle. Drug concentrations in both plasma and serum from the same animal were markedly similar. No binding of homidium to red blood cells was demonstrated since concentrations were markedly lower than those obtained in plasma or serum of the same animal. Extensive extravascular distribution of the drug following i.v. treatment was demonstrated. Bioavailability of the i.m. dose was 50% showing that half of the dose given did not reach the systemic circulation. Drug levels were shown to remain in tissues for long periods of time.

Following treatment of infected cattle, an increased rate of drug uptake/elimination by trypanosomes was demonstrated within the first 3 days of treatment. This rate reverted back to normal within one to two days following disappearance of trypanosomes from circulation. However, the mean residence time was higher than that observed in non-infected cattle.

The use of both ELISA and radiometric methods made possible a number of tentative conclusions to be made from the present study, on the assumption that the ELISA method detected mainly intact homidium and the radiometric measured total radioactive species in solution which may have included metabolites. These tentative conclusions are: (a) that extensive homidium metabolism occurs in cattle following either i.v. or i.m. treatment; this was observed from as early as five minutes following i.v. treatment (b) that failure to detect homidium in serum of i.v.-treated cattle within approximately three weeks using ELISA suggests that the low concentrations observed for over 10 following i.m. treatment (Chapter 4) could be almost wholly be attributed to the injection site drug depot (c) that the approximately ten-fold difference in the plasma/serum homidium concentrations observed using both ELISA and the radiometric methods following i.m. treatment of cattle suggests higher levels of metabolites than intact homidium (d) that

although high drug levels were demonstrated in the tissues for as long as 4 weeks following either i.v. or i.m. treatment the extensive metabolism of the drug could suggests a reduction in the toxic effects of homidium following treatment at the normal doses.

The above findings suggest that further investigations are required to determine the nature of these metabolites and their contribution, if any, to the development of drug resistance and the observed period of prophylaxis since their levels appear to be ten-fold higher than those observed using ELISA.

CHAPTER NINE

COMPARATIVE PHARMACOKINETICS OF HOMIDIUM AND ISOMETAMIDIUM

INTRODUCTION

Both homidium and isometamidium have been used extensively in the control of animal trypanosomiasis. Homidium is mainly used as a chemotherapeutic drug, although reports have appeared showing that it can offer protection of several months. Isometamidium is used both as a chemotherapeutic and a chemoprophylactic drug. Several studies have been reported showing that isometamidium can confer protection for up to six months (Chapter 1, Section Two). Homidium and isometamidium belong to the phenathridinium group of compounds. In fact, isometamidium contains a homidium moiety in its structural formula (Appendix B). Thus, both drugs share structural similarities.

The shared structural similarities would suggest similarities, to some extent, in their mode of action. In the field, both drugs are usually administered at a dose rate of 1 mg kg^{-1} b.w. Also, trypanosomes which are resistant to homidium have been shown to be cross-resistant to isometamidium due to the structural similarities. These have discussed in detail in Chapter 1, Section Two.

However, the major differences in the two drugs as far as field use is concerned, is in the period of prophylaxis observed following exposure of cattle to natural tsetse challenge after i.m. treatment of cattle. (Dolan et al., 1992; Stevenson et al., 1995). The period of protection afforded by isometamidium was approximately two-fold higher than that afforded by homidium and yet in terms of productivity, homidium appeared to perform better (Dolan et al., 1992) and was cost-effective compared to isometamidium despite more treatments given (Stevenson et al., 1995). It was on the basis of these findings that formed the basis of the present comparative study of the pharmacokinetics of both drugs.

STUDY OBJECTIVES

1. To measure the levels of radioactive drug in the plasma of non-infected cattle treated with 6-^{14}C isometamidium by intravenous (i.v.) injection and to determine the pharmacokinetic parameters.

2. To measure the levels of radioactive drug in the plasma of non-infected cattle treated with 6-^{14}C isometamidium by intramuscular (i.m.) injection and to determine the pharmacokinetic parameters.

3. To measure the levels of radioactive drug in the plasma of *T. congolense*- infected cattle treated with 6-^{14}C isometamidium by intramuscular (i.m.) injection and to determine the pharmacokinetic parameters.

4. To compare plasma drug concentrations and pharmacokinetic parameters between non-infected and infected cattle following treatment with 6-^{14}C isometamidium.

5. To compare plasma drug concentrations and pharmacokinetic parameters obtained following i.v. treatment of non-infected cattle with 6-^{14}C isometamidium with those obtained after similar treatment with 6-^{14}C homidium reported in Chapter 8.

6. To compare plasma drug concentrations and pharmacokinetic parameters obtained following i.m. treatment of non-infected cattle with 6-^{14}C isometamidium with those obtained after similar treatment with 6-^{14}C homidium reported in Chapter 8.

7. To compare plasma drug concentrations and pharmacokinetic parameters obtained following i.m. treatment of *T. congolense*-infected cattle with 6-^{14}C isometamidium with those obtained after similar treatment with 6-^{14}C homidium reported in Chapter 8.

MATERIALS AND METHODS

Animals

Seven healthy male Boran castrates weighing between 120 and 145 kg, were purchased from a trypanosome-free area of Central Province, Kenya, and housed in a fly-proof barn for three weeks to acclimatise to laboratory conditions before the start of experiments. At the start of each experiment the animals were placed in individual metabolic cages. Their management before and during the experiments was as described in Chapter 2.

Infection of cattle

A drug-sensitive trypanosome population (*T. congolense* IL 1180) was used. The inoculum given and the procedures followed for the infection of cattle were as described in Chapter 8.

Radiolabelled isometamidium, dosage and administration

The drug used for treatment was 6-^{14}C isometamidium chloride of specific activity 44.53 μCi mg^{-1}. The radiolabelled drug was kindly supplied by May and Baker, Dagenham, U.K., through the International Atomic Energy Agency. A 2% w/v solution was freshly prepared in sterile water immediately prior to use. The drug was administered as a single bolus at 1 mg kg^{-1} body weight by either i.v. or i.m. route.

Experimental design

The cattle were divided into three groups. The first group of three non-infected animals was treated with 6-^{14}C isometamidium by i.v. injection; the second group of two non-infected animals were treated by i.m. injection; and the third group of two animals were treated by i.m. injection approximately three weeks following trypanosome infection. Following drug administration, levels of radioactivity were measured in duplicate in plasma from all treated cattle.

Sample collection

Pre-treatment plasma samples

Immediately prior to treatment, 10 ml blood samples were collected from all the seven animals by venipuncture into EDTA-coated vacutainers (Becton, Dickinson, Coventry, UK). Plasma was separated by centrifuging the blood at 1200 g for 30 minutes and stored at -20°C until required.

Plasma samples for radioactive drug analysis

Ten millilitre blood samples were collected by venipuncture into EDTA-coated vacutainers from all the animals immediately prior to treatment and at the following intervals following treatment: 5, 10, 15, 30 and 45 minutes, 1, 2, 4, 6, 8, 12 and 24 hours, thereafter daily for 30 days, and three times a week to the end of the experimental period which varied between 21 and 60 days for the i.v.-treated cattle and between 60 and 120 days for i.m.-treated cattle. Blood samples were collected from cattle following i.m. drug administration but omitting the 5 and 10 minute sample collections. Plasma was separated as already described and stored at -20°C until required.

Sample preparation and determination of radioactivity

Procedures followed in sample preparation and radioactivity determination were as described in Chapter 8.

Data analysis

Determination of radioactivity and computation of coefficients of variation (CVs) by the counter in-built mechanism were as described in Chapter 8. During radioactivity determination, samples with low radioactivity were counted for at least 30 minutes until low (CVs) between the counts were attained. The cut-off point for acceptable CVs was 5%. The limit of detection was taken as four times the background count which was equivalent to 1 ng radiolabelled drug, determined as described in Chapter 8.

All concentrations were expressed in nanograms per ml or per gram sample (Mean±SD), calculated from the radioactivity measured. This did not take into account the metabolic state of the drug.

Statistical evaluation

The mean and standard deviations (SD) were calculated for the replicate determinations of the radioactive drug using Lotus 1-2-3 Software (Release 4). Comparison of pharmacokinetic parameters between the various treatment groups was carried out using the student's t-test at both 95 and 99% confidence limits.

Pharmacokinetic evaluation

The pharmacokinetic evaluation was carried out following the procedures described in Chapter 8 for 6-^{14}C homidium.

RESULTS

Non-infected cattle treated with 6-^{14}C isometamidium by i.v. injection

Plasma 6-^{14}C isometamidium concentrations

The mean (±SD) plasma drug concentration-versus-time plot obtained following treatment of non-infected cattle with 6-^{14}C isometamidium is given in Fig. 9.1. Five minutes following i.v. treatment, the mean (±SD) plasma 6-^{14}C isometamidium concentration was 4458±834 ng ml^{-1}. This concentration declined exponentially to 33.9±1.9 ng ml^{-1} at 24 hours, 10.1±0.2 ng ml^{-1} at 7 days and 5.4±0.9 ng ml^{-1} at 21 days. The exponential decline was especially rapid during the first 24 hours following treatment.

Pharmacokinetics

The pharmacokinetic parameters obtained following i.v. treatment of cattle with 6-^{14}C isometamidium at a dose rate of 1 mg kg^{-1} are shown in Table 9.1. The data were best described by a three-compartmental model with half-lives of 0.13±0.02, 1.22±0.19 and 173.2±94.8 hours for $t½\alpha$, $t½\beta$ and $t½\gamma$, respectively. The value for Cp0 was 5979±2000 ng ml^{-1} and those for Vc and Vss were 0.18±0.02 and 13.8±7.4 l kg^{-1}, respectively. The value for the AUC was 9923±922 ng.h ml^{-1}, giving an MRT of 82.3±11.1 hours. The values obtained for k_{12} and k_{21} were 1.15±0.12 and 0.026±0.002 per hour, respectively, and those for k_{13} and k_{31} were 2.24±0.24 and 1.86±0.66 per hour, respectively. The similarities in the rate constants k_{13} and k_{31} demonstrated that an equilibrium was attained between the free drug in plasma and in tissues.

Fig. 9.1

Mean (±SD*; n = 3) plasma isometamidium concentrations in non-infected Boran cattle treated with 6-^{14}C isometamidium by i.v. injection at a dose rate of 1 mg kg^{-1} b.w.

Fig. 9.1

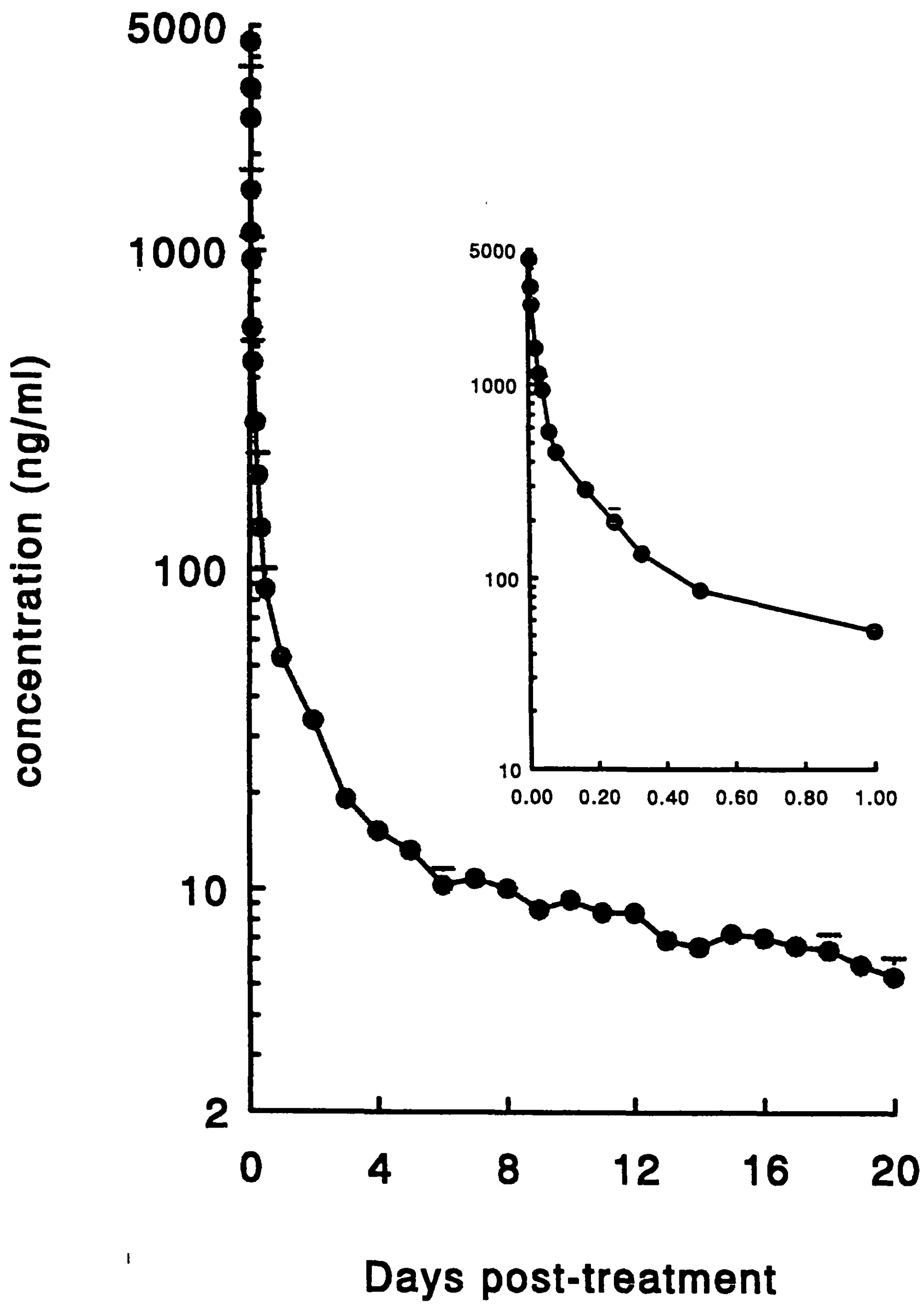


Table 9.1

Pharmacokinetic parameters of 6-^{14}C homidium and 6-^{14}C isometamidium in non-infected cattle following i.v. treatment at 1 mg kg^{-1} (mean ± SD)

Parameter	Homidium Mean ±SD (n = 3)	Isometamidium Mean ±SD (n = 3)	Significance
$t_{1/2}\alpha$ (h)	0.056±0.005	0.13±0.02	*
$t_{1/2}\beta$(h)	4.14±0.49	1.22±0.02	*
$t_{1/2}\gamma$(h)	177.1±5.3	173.2±94.8	
Cp(0) (ng ml^{-1})	1426±347	5979±2000	*
Vc (l kg^{-1})	0.75±0.22	0.18±0.02	**
AUC (ng.h ml^{-1})	5957±544	9923±922	**
AUMC (ng.h ml^{-1})	1323905±97239	450040±41800	**
Vd_{SS} (l kg^{-1})	37.8±4.6	13.8±7.4	*
k_{12} (h^{-1})	9.93±1.18	1.15±0.12	**
k_{21} (h^{-1})	1.35±0.19	0.017±0.007	**
k_{13} (h^{-1})	1.15±0.12	2.24±0.24	**
k_{31} (h^{-1})	0.026±0.002	1.86±0.66	*
MRT (h)	222.8±9.7	82.3±11.1	**

* $p \leq 0.05$

** $p \leq 0.01$

Non-infected cattle treated with 6-^{14}C isometamidium by i.m. injection

Plasma 6-^{14}C isometamidium concentrations

The mean (±SD) plasma drug concentration-versus-time plot following treatment of non-infected cattle with 6-^{14}C isometamidium is given in Fig. 9.2. Fifteen minutes following treatment the mean (±SD) plasma 6-^{14}C isometamidium concentration was 133.2±26.2 ng ml^{-1}. This declined exponentially to 21.6±5.0 ng ml^{-1} at 24 hours, and 15.1±1.7 ng ml^{-1} at 7 days. At approximately 14 days following treatment, there appeared to be a slight increase in plasma drug concentrations which were thereafter maintained at a constant level (approximately 12 ng ml^{-1} [Fig. 9.2]).

Pharmacokinetic parameters

The pharmacokinetic parameters obtained following i.m. treatment of non-infected cattle with 6-^{14}C isometamidium are given in Table 9.2. The C_{max} and t_{max} values were 160.5±1.2 ng ml^{-1} and 0.38±0.13 hours, respectively. The mean value for MRT was of 289.6±23.5 hours. The AUC value was 5841±995 ng.h ml^{-1}. In conjunction with the AUC from the i.v. study a value for bioavailabilty of 58.9±10.0% and an MAT value of 289.6±23.5 hours were obtained. The remaining pharmacokinetic parameters are given in Table 9.2.

T. congolense-infected cattle treated with 6-^{14}C isometamidium by i.m. injection

Plasma 6-^{14}C isometamidium concentrations

The plasma drug concentration-versus-time plot following treatment of infected cattle with 6-^{14}C isometamidium is given in Fig. 9.2. Fifteen minutes following treatment the mean (±SD) plasma isometamidium concentration was 56.8±30.5 ng ml^{-1}. This declined exponentially to 8.3±0.3 ng ml^{-1} at 24 hours.

Fig. 9.2

Mean (±SD; n = 2) plasma isometamidium concentrations in non-infected and *T. congolense*-infected Boran cattle treated with 6-^{14}C isometamidium by i.m. injection at a dose rate of 1 mg kg^{-1} b.w.

Fig. 9.2

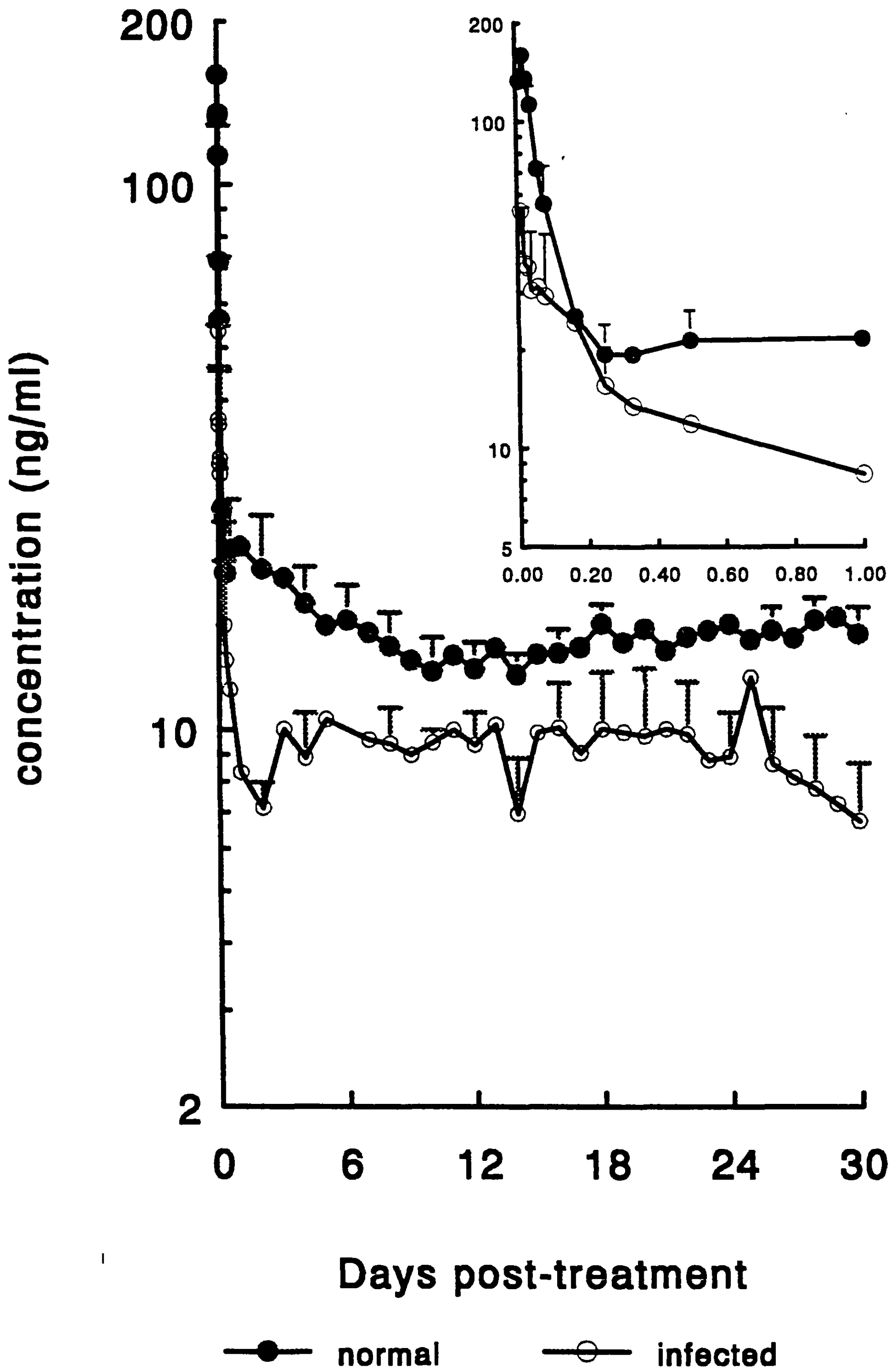


Table 9.2

Pharmacokinetic parameters of non-infected and *T. congolense*-infected cattle following intramuscular treatment with 6-^{14}C isometamidium at 1 mg kg^{-1} b.w.

Parameter	Non-infected n = 2	Infected* n = 2
t_{max} (h.)	0.38±0.13	0.25±0
C_{max} (ng ml^{-1})	160.5±1.2	56.8±30.5
$AUC_{0\text{-last}}$ (ng.h ml^{-1})	5841±995	3751±9.2
$AUC_{0\text{-}\infty}$ observed (ng.h ml^{-1})	17638±2600	100822±332668
$AUMC_{0\text{-last}}$ (ng.h^2 ml^{-1})	921313±126834	666199±20249
$AUMC_{0\text{-}\infty}$ observed (ng.h^2 ml^{-1})	5168105±1167690	35611689±11959594
$MRT_{0\text{-}\infty}$ observed (h)	289.6±23.5	352.4±2.3

* *T. congolense* IL 1180

An acceleration in the rate of drug elimination in plasma was observed during the first three days following i.m. treatment, during which time the concentrations fell below 10 ng ml^{-1}. Thereafter, the drug concentrations were elevated in both treated cattle to approximately 10 ng ml^{-1} within one to two days following the disappearance of trypanosomes from the circulation. These plasma concentrations were maintained up to approximately 25 days following treatment (Fig. 9.2).

Pharmacokinetic parameters

The pharmacokinetic parameters obtained following treatment of infected cattle are given in Table 9.2. The values for the C_{max} and the t_{max} were 56.8±30.5 ng ml^{-1} and 0.25±0 hours, respectively. The AUC value was 3751±9.2 ng.h ml^{-1}, resulting in the calculated MRT value of 352.4±2.3 hours.

Comparison between non-infected and *T. congolense*-infected cattle following treatment with 6-^{14}C isometamidium by the i.m injection

Plasma isometamidium concentrations

A comparison of the plasma 6-^{14}C isometamidium concentration-versus-time profiles between non-infected and infected cattle is given in Fig. 9.2. Fifteen minutes following treatment of non-infected cattle, the mean (± SD) plasma isometamidium concentration was 133.2±26.2 ng ml^{-1}. This declined exponentially to 21.6±5.0 ng ml^{-1} at 24 hours, 15.1±1.7 ng ml^{-1} at 7 days, after which the drug concentrations appeared to be maintained at a constant level which was approximately 10 ng ml^{-1}. Following treatment of infected cattle however, the mean plasma isometamidium concentration obtained after 15 minutes was 56.8±30.5 ng ml^{-1}. This declined exponentially to 8.3±0.3 ng ml^{-1} at 24 hours. A mean concentration of 9.6±1.0 ng ml^{-1} was attained at 7 days. An acceleration in the drug elimination rate observed

during the first three days following i.m. treatment of *T. congolense*-infected cattle was not observed in non-infected cattle given similar treatment. However, the drug concentrations appeared to be maintained at a constant level in non-infected cattle after 10 days and in infected cattle after approximately three days following i.m. treatment, although the concentrations of the radioactive drug were markedly lower in infected than non-infected cattle (Fig. 9.2).

Pharmacokinetic parameters

Following i.m. treatment of non-infected cattle, the C_{max} and t_{max} values of 160.5±1.2 ng ml^{-1} and 0.38±0.13 hours respectively, were obtained. These values were 56.8±30.5 ng ml^{-1} and 0.25±0 hours respectively following treatment of infected cattle. The mean value for MRT of 289.6±23.5 hours, for non-infected cattle was lower than 352.4±2.3 hours obtained in infected cattle. However, the AUC value of 5841±995 ng.h ml^{-1} for non-infected cattle was higher than the value of 3751±9.2 ng.h ml^{-1} obtained in infected cattle. Clearly the presence of trypanosomes at the time of treatment had an effect on the pharmacokinetics of isometamidium in cattle.

Comparison of plasma drug concentrations and pharmacokinetic parameters in non-infected cattle between 6-^{14}C homidium and 6-^{14}C isometamidium following intravenous treatment

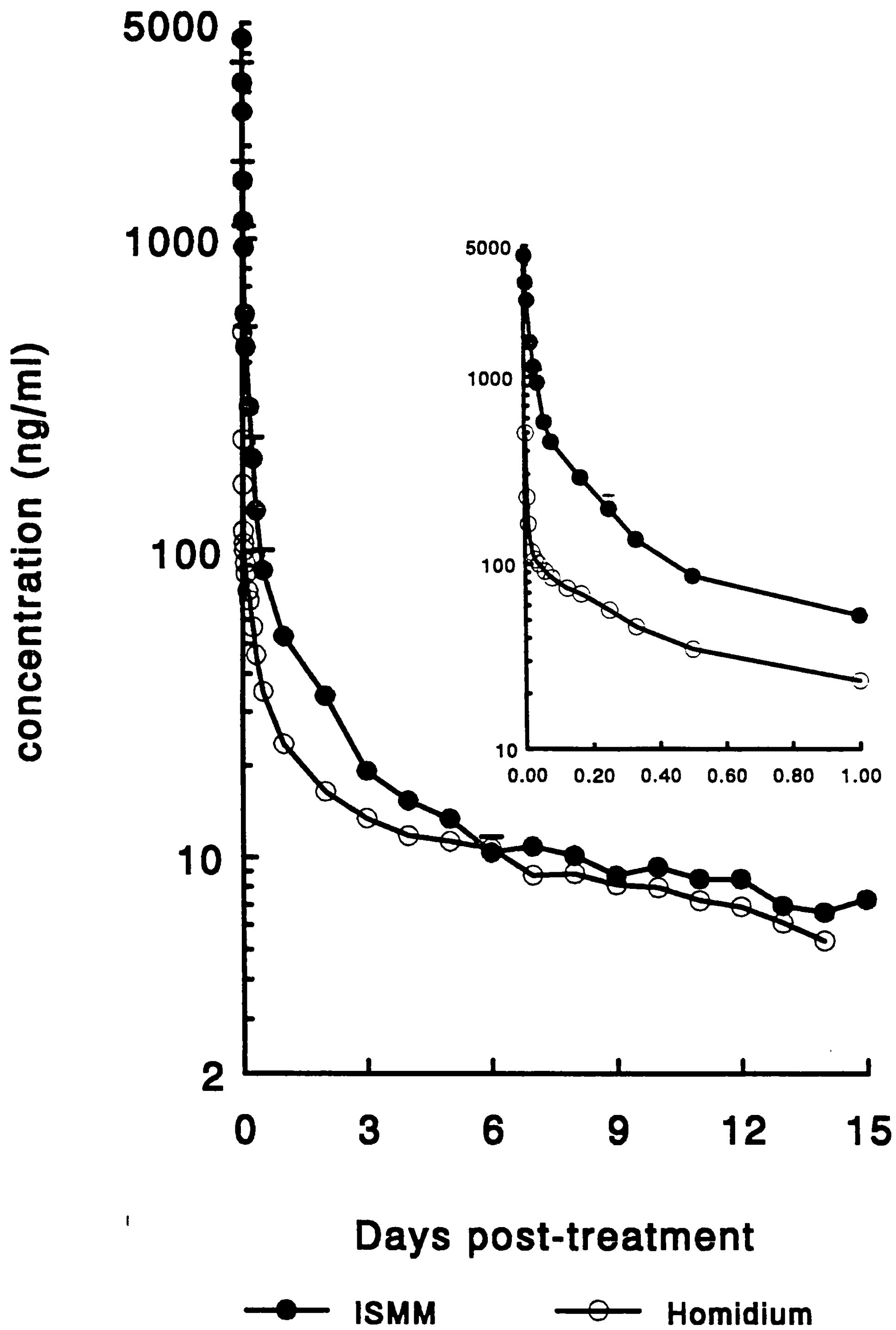
Plasma drug concentrations

The mean (±SD) plasma drug concentration-versus-time plot following i.v. treatment of non-infected cattle with either 6-^{14}C isometamidium or 6-^{14}C homidium is shown in Fig. 9.3. Both drugs showed an initial rapid exponential decline in the plasma drug concentrations. Five minutes following i.v. treatment with 6-^{14}C homidium, the mean plasma concentration was

Fig. 9.3

Mean (±SD; n = 3) plasma drug concentrations in non-infected Boran cattle after intravenous injection of 6-^{14}C isometamidium and 6-^{14}C homidium at a dose rate of 1 mg kg^{-1} b.w.

Fig. 9.3



499.6±97.9 ng ml^{-1}. Concentrations of 23.5±1.4 and 4.3±0.4 ng ml^{-1} were obtained at 24 hours and 7 days, respectively. Five minutes following i.v. treatment of cattle with 6-^{14}C isometamidium at the same dose, the mean (± SD) plasma drug concentration was 4458±834 ng ml^{-1}. This concentration declined exponentially to 33.9±1.9 ng ml^{-1} at 24 hours and to 10.1±0.18 ng ml^{-1} at 7 days. The initial drug concentrations following treatment of cattle with 6-^{14}C isometamidium (4458±834 ng ml^{-1}) were significantly higher ($p < 0.05$) than in cattle treated with 6-^{14}C homidium (500±100 ng ml^{-1}).

Pharmacokinetic parameters

Table 9.1 shows the mean (±SD) pharmacokinetic parameters and the significance of the difference between them in non-infected cattle following i.v. treatment with either 6-^{14}C homidium or 6-^{14}C isometamidium at 1 mg kg^{-1} b.w. Most of the pharmacokinetic parameters were significantly different (Table 9.1; $p < 0.01$) between 6-^{14}C homidium-treated and 6-^{14}C isometamidium-treated cattle. The exception was the $t_{½\gamma}$ in which no significant difference was observed between the two drug compounds. Because of the rapid elimination of 6-^{14}C isometamidium the mean residence time was short (82.3±11.1 hours) compared to 222.8±9.7 hours obtained in 6-^{14}C homidium-treated cattle. Whilst the three-compartmental model fitting of the data from 6-^{14}C isometamidium-treated cattle showed an establishment of equilibrium between drug concentrations in plasma and tissues during drug distribution, the data from the 6-^{14}C homidium-treated cattle showed non-attainment of equilibrium (Chapter 8). However, the high values for the volume of distribution showed extensive extravascular distribution of both drugs following treatment.

Comparison of pharmacokinetic parameters in non-infected cattle between 6-^{14}C homidium and 6-^{14}C isometamidium following i.m treatment

Plasma drug concentrations

The mean (±SD) plasma drug concentration-versus-time plots obtained following i.m. treatment of cattle with either 6-^{14}C isometamidium or 6-^{14}C homidium is given in Fig. 9.4. The maximum plasma drug concentration (C_{max}) obtained in non-infected cattle after i.m. treatment with 6-^{14}C homidium was 159.7±77.9 ng ml^{-1} plasma, and the time at which it occurred (t_{max}) was 0.33±0.12 hours. These plasma concentrations declined exponentially to 103±3.01 and 10.8±1.4 ng ml^{-1} at one and 24 hours, respectively. Fourteen days later, a plasma 6-^{14}C homidium concentration of approximately 4 ng ml^{-1} was attained. Following treatment of cattle with 6-^{14}C isometamidium, the mean (±SD) peak plasma 6-^{14}C isometamidium concentration (C_{max}) was 160.5±1.2 ng ml^{-1} and occurred at 0.38±0.13 hours. This declined exponentially to 21.6±5.0 ng ml^{-1} at 24 hours, 15.1±1.7 ng ml^{-1} at 7 days and 12.7±1.2 ng ml^{-1} at 14 days. From approximately 10 days following treatment, isometamidium concentrations appeared to be maintained at a constant level (Fig. 9.4). It appeared as if the drug was 'infused' into the circulation from the drug depots which included the injection site. This was not observed in homidium-treated cattle given similar treatment.

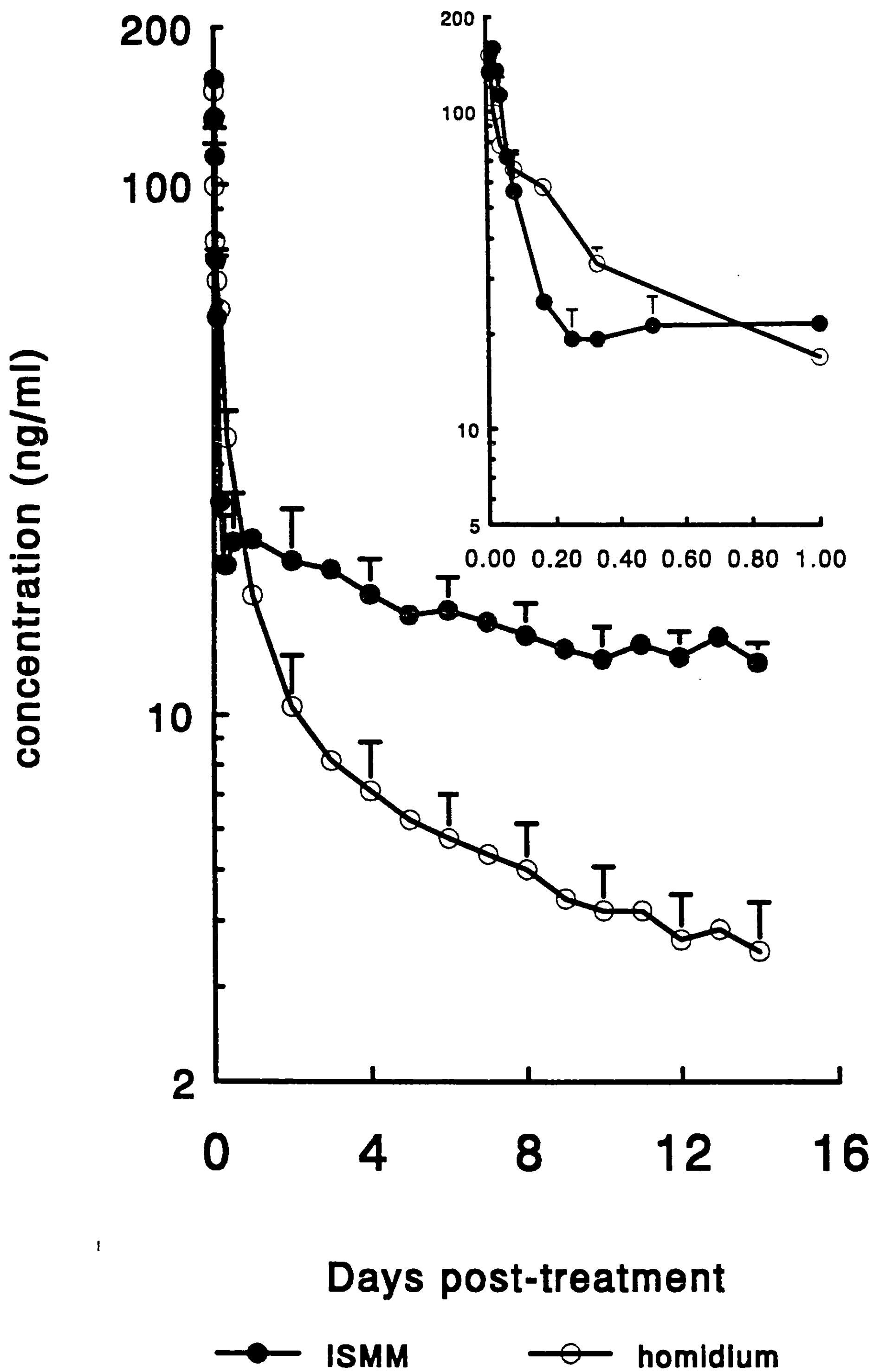
Pharmacokinetics

Following i.m treatment of non-infected cattle with 6-^{14}C homidium, an exponential decline in plasma drug concentrations was observed (Fig. 9.4). However, following i.m. treatment of non-infected cattle with 6-^{14}C isometamidium the decline in plasma drug concentrations was exponential up to approximately 10 days after treatment. In fact, the drug concentration-versus-time profile following i.m. treatment with 6-^{14}C isometamidium

Fig. 9.4

Mean (±SD; n = 2) plasma drug concentrations in non-infected Boran cattle after intramuscular injection of 6-^{14}C isometamidium and 6-^{14}C homidium at a dose rate of 1 mg kg^{-1} b.w.

Fig. 9.4



resembled one of a 'steady state infusion' of the drug due to maintenance of constant drug levels in circulation.

Due to the small numbers of animals involved, a meaningful statistical evaluation was not possible. However, a comparison of pharmacokinetic parameters in the Boran cattle between 6-^{14}C homidium and 6-^{14}C isometamidium following i.m treatment is shown in Table 9.3. The mean C_{max} and t_{max} values of 159.7±77.9 ng ml^{-1} and 0.33±0.12 hours, respectively obtained using 6-^{14}C homidium were similar to 160.5±1.2 ng ml^{-1} and 0.38±0.13 hours, respectively obtained in cattle following treatment with 6-^{14}C isometamidium. Similarly, the mean (±SD) value for MRT of 200.1±62.5 hours obtained in cattle treated with 6-^{14}C homidium was markedly similar to the MRT value of 289.6±23.5 hours obtained in the 6-^{14}C isometamidium-treated cattle. The calculated F values were also similar (Table 9.3). However, the MAT value of 207.3±33.3 hours obtained in the 6-^{14}C isometamidium-treated cattle was markedly higher than the value of 41.79±62.5 hours obtained in cattle treated with 6-^{14}C homidium.

Comparison of pharmacokinetic parameters in cattle infected with a drug-sensitive population of *T. congolense*-infected cattle between 6-^{14}C homidium and 6-^{14}C isometamidium following i.m treatment

Plasma drug concentrations

A comparison, over time, of the mean plasma drug concentration-versus-time data following treatment of infected cattle with either 6-^{14}C homidium or 6-^{14}C isometamidium is shown in Fig. 9.5. Following i.m. treatment with 6-^{14}C homidium, the peak plasma concentration was 90.6±23.3 ng ml^{-1}, which occurred 15 minutes following drug administration. Plasma drug concentrations of 56.3±6 and 8.9±2.1 ng ml^{-1} were obtained at one and 24 hours, respectively following treatment. Following treatment with 6-^{14}C

Table 9.3

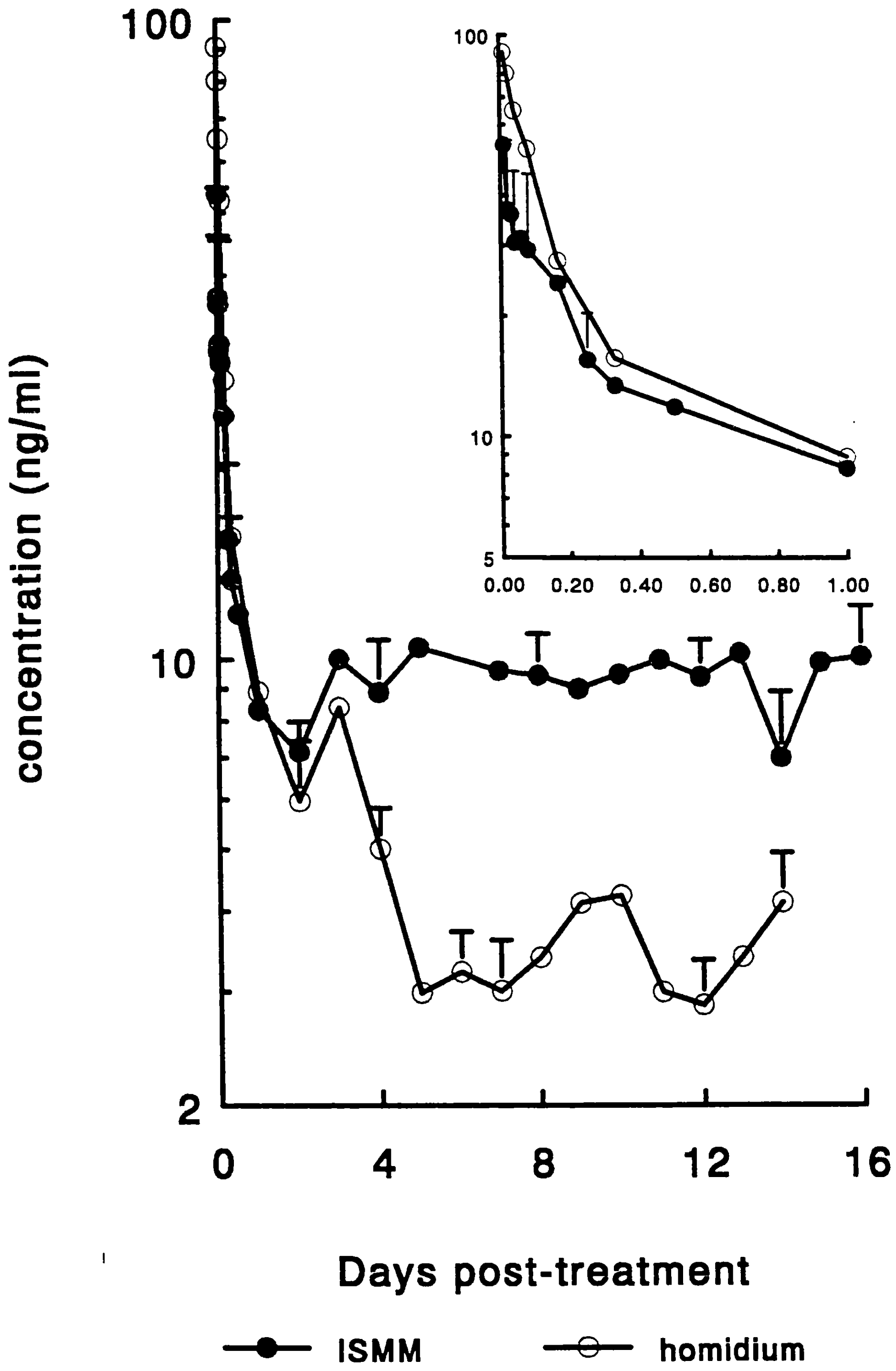
Pharmacokinetic parameters of 6-^{14}C homidium and 6-^{14}C isometamidium in non-infected Boran cattle following intramuscular treatment at 1 mg kg^{-1} b.w.

Parameter	**6-^{14}C homidium** Mean ± SD (n = 3)	**6-^{14}C isometamidium** Mean ± SD (n = 2)
t_{max} (h)	0.33±0.12	0.38±0.13
C_{max} (ng ml^{-1})	159.7±77.9	160.5±1.2
$AUC_{0\text{-}last}$ (ng.h ml^{-1})	2701±841	5841±995
$AUC_{0\text{-}\infty}$ obs(ng.h ml^{-1})	3370±873	17638±2600
$AUMC_{0\text{-}last}$ (ng.h^2 ml^{-1})	278927±89262	921313±126834
$AUMC_{0\text{-}\infty}$ obs (ng.h^2 ml^{-1})	656432±180642	5168105±1167690
$MRT_{0\text{-}\infty}$ observed (h)	200.1±62.5	289.6±23.5
MAT (h)	41.79±62.5	207.3±33.3
% F	56.6±14.7	58.9±10.0

Fig. 9.5

Mean (±SD; n = 2) plasma drug concentrations in *T. congolense*-infected Boran cattle after intramuscular injection of 6-^{14}C isometamidium and 6-^{14}C homidium at a dose rate of 1 mg kg^{-1} b.w.

Fig. 9.5



isometamidium, concentrations were 56.8±30.5 and 8.3±0.3 ng ml^{-1} at 15 minutes and 24 hours, respectively. Similarities between the two drug compounds were demonstrated in the reduced drug concentrations obtained following i.m. treatment of infected cattle when compared to the equivalent values in non-infected cattle.

An acceleration in the drug elimination rate compared to the normal rate (obtained following treatment of non-infected cattle) was observed during the first three days following i.m. treatment of infected cattle with either 6-^{14}C isometamidium or 6-^{14}C homidium. This acceleration in the elimination rate reverted to normal within one to two days following the disappearance of trypanosomes from the circulation. The acceleration in the drug elimination rate immediately following i.m. treatment of infected cattle was not observed in non-infected cattle following treatment with either of the drug compounds. However, the observed plateau in the drug concentration which can be likened to the 'steady state infusion' of the drug possibly from the injection site was observed in non-infected cattle treated with 6-^{14}C isometamidium and also in infected cattle treated with either 6-^{14}C isometamidium or 6-^{14}C homidium by i.m. injection.

Pharmacokinetics

A comparison of the pharmacokinetic parameters between 6-^{14}C homidium and 6-^{14}C isometamidium following i.m treatment of *T. congolense*-infected cattle is given in Table 9.4. The C_{max} values were low in both groups of cattle compared to those obtained in non-infected cattle. The AUC and the AUMC values obtained in the 6-^{14}C homidium-treated cattle were markedly lower compared to those obtained in the 6-^{14}C isometamidium-treated cattle. The MRT value of 352.3±112 hours obtained in infected cattle treated with 6-^{14}C homidium was similar to 352.4±2.3 hours obtained in infected cattle treated with 6-^{14}C isometamidium.

Table 9.4

Pharmacokinetic parameters of 6-^{14}C homidium and 6-^{14}C isometamidium in *T. congolense*-infected Boran cattle following intramuscular treatment at 1 mg kg^{-1} b.w.

Parameter	**6-^{14}C homidium** mean ± SD (n = 2)	**6-^{14}C isometamidium** Mean ± SD (n = 2)
t_{max} (h)	0.38±0.13	0.25±0.0
C_{max} (ng ml^{-1})	90.8±23.1	56.8±30.5
$AUC_{0\text{-}last}$ (ng.h ml^{-1})	1861±291	3751±9
$AUC_{0\text{-}\infty}$ obs(ng.h ml^{-1})	3114±40	100822±332668
$AUMC_{0\text{-}last}$ (ng.h^2 ml^{-1})	212914±42819	666199±20249
$AUMC_{0\text{-}\infty}$ obs (ng.h^2 ml^{-1})	1101889±363383	35611689±11959594
$MRT_{0\text{-}\infty}$ observed (h)	352.3±112.0	352.4±2.3

DISCUSSION

In the present Chapter, plasma drug concentrations and pharmacokinetic parameters have been determined following i.v. treatment of non-infected Boran cattle with 6-^{14}C isometamidium and also following i.m. treatment of non-infected and *T. congolense*-infected Boran cattle with the same drug. The results obtained were compared with similar groups of cattle treated with 6-^{14}C homidium reported in Chapter 8. The major comparisons centred on the (a) the distribution and elimination characteristics of the two similarly radiolabelled compounds in non-infected cattle following i.v. treatment (b) the absorption, bioavailability and elimination characteristics following i.m. treatment and (c) the effects of *T. congelense* infection on the pharmacokinetics of the two drug compounds.

Several comparisons were made of the serum drug concentrations and pharmacokinetics using the data obtained above: (a) between non-infected and *T. congolense*-infected cattle following i.m. treatment with 6-^{14}C isometamidium (b) between 6-^{14}C isometamidium and 6-^{14}C homidium (Chapter 8) following i.v. treatment of non-infected cattle, and (c) between 6-^{14}C isometamidium and 6-^{14}C homidium (Chapter 8) following i.m. treatment of either non-infected or trypanosome-infected cattle. During these comparisons, several similarities and differences in serum drug concentrations and pharmacokinetics were observed between the various groups. These included: (a) extensive distribution of both isometamidium and homidium following i.v. treatment of non-infected cattle (b) the plateau in plasma drug profiles following i.m. treatment of non-infected and infected cattle with isometamidium but not with homidium which showed an exponential decline (c) an acceleration in the elimination rate of both drugs in the presence of trypanosomes and (d) differences in the rates of release of both drugs from the injection site following i.m. treatment.

During the first 24 hours following i.v. treatment of cattle with 6-^{14}C isometamidium the drug concentrations in plasma declined rapidly (Fig. 9.1). The plasma drug concentration obtained at five minutes following treatment was over 100-fold higher than that observed at 24 hours. The low MRT values obtained following i.v. treatment suggested rapid elimination of the drug possibly saturating the elimination processes. The Vss value of approximately 14 l h^{-1} kg^{-1} was found to be larger than the normal plasma volume for cattle of approximately 35.9 ml kg^{-1} (Ruckebusch et al., 1991) suggesting extensive extravascular distribution of the drug and possible binding of the drug to tissue components including proteins. Similarities in the values obtained for rate constants k_{13} and k_{31} suggested establishment of an equilibrium between free drug in plasma and free drug in tissues.

However, following i.m. treatment of non-infected with isometamidium, a plateau effect was achieved from approximately 10 days of treatment, with maintenance of constant drug levels in what appeared like a 'steady-state infusion' of the drug possibly from the injection-site depot. The present results are in contrast with those reported by Kinabo and Bogan (1988b) in which the drug was detected by HPLC for up to two hours only, following i.m. treatment of cattle with isometamidium chloride at 0.5 mg kg^{-1} b.w. They did not detect any drug between three and 30 hours whist the present study showed approximately 10 ng ml^{-1} in circulation for over two weeks following treatment. These differences could have been due to the nature of the two techniques and their sensitivities. Whilst HPLC measured intact isometamidium whose concentrations may have been too low to detect, the radiometric method measured total radioactive drug.

Eisler et al. (1994) using a highly sensitive ELISA method were able to detect isometamidium for over 100 days following i.m. treatment of cattle. They also showed an exponential decline in the serum drug concentrations. The plateau effect in drug profiles was not observed. The serum peak drug

concentrations of between 40 and 50 ng ml^{-1} obtained using ELISA were however, higher than those reported by Kinabo and Bogan (1988b) of 20 ng ml^{-1}. Both serum peak drug concentrations were, however, lower than approximately 160 ng ml^{-1} reported in the present study.

Kinabo and Bogan (1988b) observed lesions at the injection site within one week of treatment which was characterised by structureless fibres. Their observations suggested congestive necrosis with acute inflammation. Six weeks following treatment, complete loss of muscle fibres was observed. This drug injection site depot supposedly slowly releases the drug into the circulation over long periods of time.

Following i.m. treatment of *T. congolense*-infected cattle with 6-^{14}C isometamidium, there was an acceleration in the elimination rate of the drug in plasma (when compared to the non-infected cattle) reaching levels of approximately 7 ng ml^{-1} during the first three days of treatment from a peak concentration of approximately 60 ng ml^{-1}. Sutherland et al. (1992) in their studies on transport of isometamidium by both drug-sensitive and drug-resistant *T. congolense* observed significant drug uptake by drug-sensitive parasites. This could possibly also explain the acceleration in the elimination rate of the drug following i.m. treatment of cattle with isometamidium. The maintenance of constant drug levels from approximately three days following treatment (Fig. 9.2) could possibly be due to the drug depot at the injection site, similar to that observed in non-infected cattle.

The plasma drug concentration-versus-time profiles for 6-^{14}C isometamidium and 6-^{14}C homidium in non-infected cattle following i.v. treatment at the same dose rate (1 mg kg^{-1}) (Fig. 9.3) showed them to be similar. Both drugs showed an exponential decline in plasma concentrations. They also showed extensive extravascular distribution and possible binding to tissue components. However, the significantly higher initial plasma drug concentrations and $t\frac{1}{2}\alpha$ ($p < 0.05$) following i.v. treatment of cattle with 6-^{14}C

isometamidium and a lower value for Vdarea (Table 9.1) would suggest that isometamidium was distributed much slower than homidium. Also, homidium could possibly have more regions accessible to it and higher binding to organ tissue components than isometamidium. This possibly higher accessibility and binding to tissue components could explain the high MRT values of approximately 220 hours obtained following i.v. treatment of cattle with homidium compared to the value of approximately 80 hours obtained following similar treatment of cattle with isometamidium. Possible saturation of elimination processes following i.v. treatment of cattle with 6-^{14}C isometamidium but not with 6-^{14}C homidium was suggested.

Following i.m. treatment of non-infected cattle with both isometamidium and homidium, similar peak plasma concentrations were observed. However, the differences observed in the drug profiles could have been as a result of the approximately five-fold higher value for MAT obtained following treatment of cattle with isometamidium.

The low MAT values obtained following i.m treatment of non-infected cattle with 6-^{14}C homidium demonstrated that the release of the drug from the injection site depot was faster than that obtained following i.m. treatment with 6-^{14}C isometamidium. Interestingly, the values for MRT of approximately 200 hours and 290 hours and bioavailability of 57% and 59% following i.m. treatment with 6-^{14}C homidium and 6-^{14}C isometamidium, respectively, were similar.

Following i.m. treatment of *T. congolense*-infected cattle, plasma drug concentrations using both 6-^{14}C isometamidium and 6-^{14}C homidium showed that the concentrations were markedly reduced compared to non-infected cattle given similar treatment. This demonstrated initial rapid uptake/elimination of both drugs in a similar manner by the trypanosomes (Fig. 9.5 inset). Uptake of isometamidium by trypanosomes has been demonstrated by Sutherland et al. (1992). The demonstration by Newton and Gilbert (1982) that 80% of

homidium in circulation at 1, 8 and 12 hours was found bound to trypanosomes and the similarities in some pharmacokinetic parameters following i.m. treatment with both drugs observed in the present study would suggest that isometamidium interacts with trypanosomes in a similar manner to homidium.

One interesting observation was a 'wave-like' pattern in the plasma drug profiles following treatment of trypanosome-infected cattle with 6-^{14}C homidium (Fig. 9.5). This was not observed following similar treatment of non-infected cattle (Fig. 9.4) with the same drug compound in which a smooth exponential decline was observed. These 'wave-like' patterns tended to be less pronounced with time post-treatment. This observation could possibly suggest that the drug was released from some sites in a variable manner. Anti-trypanosome antibodies or trypanosome antigens were not determined in the present study. It would have been interesting to find out if there was a possible relationship between these parameters and the observed pattern during, possibly, the release of the drug by the disintegrating trypanosomes over time.

Pharmacokinetic parameters for 6-^{14}C isometamidium and 6-^{14}C homidium following i.m. treatment of *T. congolense*-infected cattle showed marked differences in the AUC and AUMC values (Table 9.4). However, the calculated mean MRT values of 352.3±112 hours and 352.4±2.3 hours, respectively, were strikingly similar, suggesting that the length of time the drug resided in the body following treatment of cattle with either drug compound was similar.

Although both homidium and isometamidium react with tissue at the injection-site following i.m treatment with similar rates of disappearance of the drug in plasma and similar values for bioavailability, their major difference appears to be in the rate of release of the drug from the injection site depot (differences in the MAT values). This could be related to the nature of tissue reaction at the injection site. Stevenson et al. (1995) in their comparison of

isometamidium and homidium as prophylactic drugs observed that swellings appeared at the site of injection in some of the cattle treated with isometamidium by week 51 of the one year trial and no swellings in the homidium-treated herd. This was despite the fact that the cattle treated with homidium received more treatments than those treated with isometamidium. These results by Stevenson et al. (1995) support the above observation that homidium release from the injection site depot was faster than that observed in the isometamidium-treated cattle.

The present study established the following findings after treatment of cattle with 6-^{14}C isometamidium: (a) that an exponential decline was observed following i.v. treatment of cattle with 6-^{14}C isometamidium (b) that an equilibrium was attained in the drug distribution (c) that a steady state was established and maintained by the equilibrium (d) that an extensive extravascular distribution and possible binding of the drug was demonstrated (e) that a possible first reaction could have reverted to zero order due to the saturation of elimination processes (e) that there was lack of an obvious exponential decline in the plasma drug levels following i.m. treatment of both infected and non-infected cattle and (g) that significant drug uptake/elimination by trypanosomes was observed.

The similarities between homidium and isometamidium appeared to be in (a) the exponential decline of the drugs in plasma following i.v. treatment (b) extensive distribution and possible binding of the drug (c) mean residence time and bioavailability of the drug following i.m. treatment of non-infected cattle and (d) increased rate of drug elimination during the first three days of treatment in infected cattle and similar MRT values. The above similarities in pharmacokinetics could be due to the fact that both homidium and isometamidium share structural similarities.

Major differences appeared to be in (a) demonstration of an exponential decline in drug concentrations over time, following i.m. treatment of non-

infected cattle with homidium and lack of a similar decline following treatment of cattle with isometamidium and (b) the mean absorption time values following i.m. treatment were approximately five-fold lower in homidium treated cattle. Although both drugs form depots at the injection site following i.m. treatment, the release of the drug from the depot was faster in the homidium treated cattle than in the isometamidium treated cattle.

The similarities observed in most of the pharmacokinetic parameters following i.m. treatment of both non-infected and *T. congolense*-infected cattle with both 6-^{14}C homidium and 6-^{14}C isometamidium suggests that their mechanisms of action could possibly be similar.

From the above observations, isometamidium appears to form a depot releasing small quantities of intact isometamidium into the circulation, including, possibly 'homidium-like' metabolites. Field evaluation of isometamidium as a prophylactic has been reported using the established enzyme-linked immunosorbent assay (Eisler et al., 1994). However, in light of the above observations, isometamidium prophylaxis in the field needs to be re-evaluated taking into account possible contribution of active metabolites which could include homidium which is a well established active trypanocide.

GENERAL DISCUSSION AND CONCLUSIONS

This thesis addressed a number of objectives. These included:

- the development and validation of a sensitive ELISA method for use in measuring homidium concentrations in the serum of treated cattle.

- the use of the above method to collect baseline data on homidium concentrations and pharmacokinetics in non-infected cattle for use in subsequent experiments.

- investigations into the effects of *T. congolense* infections on homidium pharmacokinetics following treatment of infected cattle.

- investigations into the prophylactic activity of homidium.

- investigations into the suitability of the ELISA method in determining serum homidium concentrations in samples collected from cattle following treatment and exposure to natural tsetse challenge.

- comparisons of serum drug concentrations and pharmacokinetics following treatment of cattle using both ELISA and the radiometric methods.

- comparisons of pharmacokinetics following treatment of non-infected and infected cattle with both ^{14}C labelled homidium and ^{14}C labelled isometamidium

These objectives were achieved and the results reported in this thesis demonstrated that:

Two ELISA methods were developed which were highly sensitive (limit of detection 0.1 ng ml^{-1}). One assay was selected for the subsequent studies. The assay was robust, highly sensitive, easy to perform, suitable for use to analyse large numbers of samples and relatively cheap.

The studies carried out in non-infected Friesian cattle showed that the decline in homidium concentrations over time was exponential and rapid during the first 24 hours following both i.v. and i.m. treatment of cattle. Following i.v. treatment of non-infected cattle, no drug was detectable in serum within approximately three weeks of drug administration. However, low serum concentrations of between 0.10 - 0.30 ng ml^{-1} were detectable in the circulation for over 10 weeks after the initial rapid fall in levels within the first 24 hours following treatment of cattle by i.m. injection.

Extending the studies from Scotland to Kenya allowed studies to be carried out using a cattle breed that is reared extensively in the trypanosomiasis endemic areas of Kenya. Also, it allowed an evaluation to be carried out on the suitability of establishing ELISA drug testing facilities in a country with endemic trypanosomiasis.

The results obtained using Boran cattle showed that the serum drug concentration-versus-time profiles between the Boran and Friesian were markedly similar. Low serum drug concentrations were detectable in serum for over 10 weeks following i.m. treatment of Boran cattle. Pharmacokinetic parameters of homidium were also similar between the two breeds. The major difference observed was the wide variability in serum concentrations between individual Boran cattle when compared to the Friesian.

It was recognised that the time intervals at which samples were collected following treatment highly influenced the estimation of the secondary pharmacokinetic parameters. As many data points as possible around the serum peak drug concentration gave better estimates of the secondary pharmacokinetic parameters especially in the determination of the

area under the curve (AUC) and the area under the moments curve (AUMC) which in turn influenced the values for the mean residence time, mean absorption time and bioavailability estimations.

Usually, the use of homidium in the field is to treat trypanosome-infected animals. In investigations on the effects of *T. congolense* infections on the pharmacokinetics of homidium, it was observed that the rate of drug elimination was accelerated in the presence of both drug-sensitive and drug-resistant trypanosome populations. This accelerated rate of drug elimination reverted to normal rate (observed in non-infected cattle) within one to two days following the disappearance of trypanosomes from the circulation in the case of drug-sensitive trypanosome population. Low serum homidium concentrations were detectable in circulation up to 90 days following treatment in such situations.

However, in the animals which were infected with a drug-resistant population, the increase in the rate of drug elimination continued until the drug was no longer detectable, within 10 days after treatment. These observations demonstrated that homidium pharmacokinetics were altered in the presence of a trypanosome infection and especially if the infection was due to a drug-resistant trypanosome population.

The significant decrease in serum homidium concentrations compared to those observed in non-infected cattle observed at five minutes following treatment of infected cattle could be as a result of significant drug uptake/elimination by the trypanosomes. Gilbert and Newton (1982) demonstrated that 80% of the drug in circulation at 1, 8 and 12 hours following i.m. treatment of *T. congolense*-infected calves with homidium was bound to trypanosomes suggesting significant drug uptake. Drug-sensitive trypanosomes would eventually be eliminated by the drug.

Besides the possibilities of drug uptake/elimination by trypanosomes, the accelerated drug elimination rate in the presence of trypanosomes could

have been as a result of any of the many pathophysiological effects associated with trypanosome infections which include fever, increased metabolic rate, anaemia, lowered serum albumin levels and tissue damage.

It is difficult to predict at this stage which of the above factors could be responsible for the changes and this requires further investigation. In trypanosome infections, fever has been associated with parasitaemia. Fever has been shown to affect the absorption, distribution and elimination of other drug compounds.

One of the current uses of homidium is as a chemoprophylactic agent against animal trypanosomiasis. Investigations into homidium chemoprophylaxis showed that the low homidium concentrations (0.10 to 0.30 ng ml^{-1}) observed in non-infected cattle were protective against infections with a drug-sensitive trypanosome population.

Following monthly trypanosome challenge (by sub-cutaneous injection) of treated cattle, trypanosomes were first detected in four out of five animals challenged with a drug-sensitive trypanosome population after the challenge at 120 days. Protection of the animals was demonstrated to be due to the presence of the homidium. Anti-trypanosome antibodies were not detected in the serum collected on each day of challenge and therefore played no role in the protection of cattle against infection. Failure of chemoprophylaxis was, however, demonstrated in the presence of the drug-resistant trypanosome population and the persistent parasitaemia had a very profound effect on serum drug concentrations. For the reasons described above, this could be due to drug uptake/elimination by trypanosomes and/or changes in host physiology including fever, increased rate of metabolism and decreased serum albumin levels.

An experiment conducted at Galana Ranch in Kenya showed that over 95% of the cattle exposed to natural tsetse challenge following homidium treatment were protected for a period of 14 weeks. Low serum homidium

concentrations were detectable for up to six weeks and were similar to those obtained in the controlled laboratory studies on homidium prophylaxis.

It was also demonstrated that the ELISA method could effectively be used to determine serum homidium concentrations in individual cattle samples from the field involving large numbers of animals on the Galana Ranch, Kenya.

The presence of drug resistant trypanosomes has been demonstrated in the work reported in this thesis as an important contributor to drug failure. Several explanations for drug failure have been given by Holmes and Torr (1988) which include drug resistance. The ease with which drugs are acquired from the drug retailers, lack of knowledge of drug preparation and administration combined with lack of weighing and diagnostic facilities and economic hardships experienced by the majority of the small scale farmers probably contribute to the development of drug resistance in the field.

Use of both ELISA and the radiometric methods for drug detection showed that the drug was undetectable after approximately three weeks following i.v. treatment of non-infected cattle using ELISA whilst approximately 4 ng ml^{-1} was detectable up to 28 days using the radiometric method. The serum drug concentrations were approximately ten-fold higher using the radiometric method than with the ELISA. One possible explanation for this difference is that the ELISA may detect only intact drug whilst the radiometric method may detect both intact and radioactive metabolites. There is also need to investigate possible metabolites of homidium using a variety of techniques. It would also be interesting to find out whether these metabolites are trypanocidal and their possible contribution to the development of drug resistance.

Comparisons between radiolabelled homidium and isometamidium showed several similarities in their pharmacokinetics in cattle. These included similarities in the mean residence time and bioavalability of the drug following

i.m. treatment. Their major difference was in the mean absorption time, the values being higher in isometamidium-treated cattle. These observations showed that the release of isometamidium from the drug depots (which include the injection site) was slower than that observed in homidium-treated cattle. This could explain the longer prophylactic periods observed in cattle treated with isometamidium compared to that observed in cattle treated with homidium.

In order to obtain the maximum benefits from the small number of trypanocides which are currently available, greater knowledge of the factors influencing their efficacy in field situations is required. This includes the problem of drug resistance. To be able to monitor the spread of drug resistance in the field, Holmes and Torr (1988) suggested the development of laboratory tests to quantify drug concentrations in treated cattle; the presence of trypanosomes in cattle with drug levels known to be trypanocidal being a valuable index of drug resistance. The highly sensitive ELISA method used in the studies reported in this thesis could be valuable in further investigations of drug resistance in the field.

In conclusion, several areas have been identified which would merit further investigations in an effort to combat animal trypanosomiasis through the more effective use of drugs. These include:

(a) Use of ELISA and other parasitological techniques to study the epidemiology of drug resistance in the field.

The detection of trypanosomes in animals with serum drug levels that have been shown to be effective against drug-sensitive trypanosomes indicates presence of drug resistance. Constant monitoring of serum homidium concentrations in treated animals including periods of high tsetse challenge (with a corresponding increase in drug use) in the field, could possibly indicate the level and spread of drug resistance.

(b) Studies on homidium uptake/elimination by trypanosomes.

Gilbert and Newton (1982) used radiolabelled homidium to study drug uptake in cattle. Their findings showed that 80% of the drug in circulation was bound to trypanosomes. Interestingly, they did not observe any differences in pharmacokinetics between non-infected and *T. congolense* cattle. Their work however, lacked a detailed pharmacokinetic evaluation. Findings of the present study suggested significant drug uptake by trypanosomes. These interesting observations merit further investigations.

(c) Studies on other factors besides drug uptake/elimination which contribute to increased elimination rate of the drug following treatment of infected cattle.

The possible factors identified in the present study were fever, increased rate of metabolism, decreased serum albumin levels and anaemia. Investigations need to be carried out in which each of the above factors would be determined as well as the serum drug concentrations following treatment of trypanosome-infected cattle. The information would assist in determining if a relationship exists between any of the above factors and the accelerated rate of drug elimination.

(d) Studies on the extent of *in vivo* homidium biotransformation including the possible identification of metabolites and their role, if any, in the chemotherapy, chemoprophylaxis and in the development of drug resistance.

Biotransformation of homidium has not been reported in cattle. The findings in the present study that there was a ten-fold difference in serum drug levels using both the radiometric and the ELISA methods suggests presence of metabolites. These meabolites need to be identified, isolated and purified before their role in chemotherapy could be investigated.

REFERENCES

Agyemang, K., Dwinger, R.H., Touray, B.N., Jeannin, P., Fofana, D. and Grieve, A.S. (1990). Effects of nutrition on degree of anaemia and liveweight changes in N'Dama cattle infected with trypanosomes. *Livestock Production Science*, **26**, 39-51.

Ainanshe, O.A., Jennings, F.W. and Holmes, P.H. (1992). Isolation of drug resistant strains of *Trypanosoma congolense* from the lower Shabelle region of Southern Somalia. *Tropical Animal Health and Production*, **24**, 65-73.

Akol, G.W.O. and Murray, M. (1982). Early events following challenge of cattle with tsetse infected with *Trypanosoma congolense*. 1. Development of the local skin reaction. *Veterinary Record*, **110**, 293-302.

Aliu, Y.O. and Odegaard, S. (1983). Paired-ion extraction and high performance liquid chromatography determination of diminazene in plasma. *Journal of Chromatography*, **276**, 218-223.

Andrianarivo, A.G., Muiya, P., Opollo, M. and Logan-Henfrey, L.L. (1995). *Trypanosoma conglense* - Comparative effects of a primary infection on bone marrow progenitor cells from N'Dama and Boran cattle. *Experimental Parasitology*, **80**, 407-419.

Ashman, P.U. and Seed, J.R. (1973). Biochemical studies in the vole, *Microtus montanus*. 11. The effects of a *Trypanosoma brucei gambiense* infection on the diurnal variation of hepatic glucose-6-phosphatase and liver glycogen. *Comparative Biochemistry and Physiology*,
45, 379-392.

Baggot, J.D. (1977). Principles of drug disposition in domestic animals. WB Saunders Co, Philadelphia.

Baldry, D.A.T. (1963). An evaluation by bioassay of the persistence of DDT deposits on riverine vegetation in Savanna vegetation zone of Northern

Nigeria and observations on the factors influencing the availability of deposits to *Glossina palpalis*. *Bulletin of Entomological Research*, **54**, 497-508.

Bauer, F. (1955a). Trypanosomen - und Babesienerkrankungen in Afrika und inre Behandlung mit dem neven Praparat Berenil. *Zeitschrift fur Tropenmedizin und Parasitologie*, **6**, 129-140.

Bauer, F. (1955b). Ergebisse der Klinischen Prufung Von Berenil. *Veternar Midizinische Nachrichten*, **3**, 152-154.

Bauer, F. (1962). The development of drug resistance to Berenil in *Trypanosoma congolense*. *Veterinary Record*, **74**, 265-266.

Bealby, K.A., Chisanga, H.K., Connor, R.J. and Rowlands G.J. (1993). A study of the effects of trypanosome infection on the fertility of female goats in the Luangwa Valley, Eastern Zambia. *International Scientific Council for Trypanosomiasis Research and Control (ISCTRC)*, 22nd Meeting, Kampala, Uganda, 22-29 October, OAU/STRC, Abstract, P.5.18.

Berg, S.S. (1960). Structure of isometamidium (M and B 4180a), 7-m-amidinophenyl-diazoamino-2-amino-10-ethyl-9-phenylphenanthridinium chloride hydrochloride the red isomer present in metamidium. *Nature*, **188**, 1106-1107.

Berg, S.S. (1963). The search for new trypanocides. VIII, Coupling of M-amidinobenzenediazonium chloride with 3,8-diamino-5-ethyl-6-phenylphenanthridinium chloride. *Journal of the Chemical Society*, 3635-3640.

Bevan, L.E.W. (1928). A method of inoculating cattle against trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **22**, 147-156.

Braide, V. and Eghianruwa, K.I. (1980). Isometamidium residues in goat tissues after parenteral administration. *Research in Veterinary Science*, **29**, 111-113.

Bratton, A.C. and Marshall, E.K. (1939). A new coupling component for sulphonilamide determination. *Journal of Biological Chemistry*, **128**, 537-550.

Browning, C.H., Cohen, J.B., Gaunt, R. and Gulbrabsen, R. (1922). Relationships between antiseptic action and chemical constitution with special reference to compounds of the pyridine, quinoline, acridine and phenazine series. *Proceedings of the Society*, **93**, 329.

Browning, C.H., Morgan, G.T., Robb, J.M.M. and Walls L.P. (1938). The trypanocidal action of certain phenanthridinium compounds. *Journal of Pathology and Bacteriology*, **46**, 203-204.

Brun, R. and Moloo, S.K. (1982). *In vitro* cultivation of animal-infective forms of a West African *Trypanosoma vivax* stock. *Acta Tropica*, **39**, 135-141.

Burnett, G.F. (1970). Control by insecticides. In: The African Trypanosomiases, Mulligan, H.W. (Editor), George Allen and Unwin, London, pp. 464-520.

Bursell, E. (1977). Chemosterilization of tsetse flies using a pressurised metepa aerosol. *Transactions of the Rhodesian Scientific Association*, **58**, 43-47.

Byamungu, M.B. and Mramba, F. (1993). Efficacy of cypermethrin high cis pour-on (Ectopor) on tsetse flies at Buhuri farm- Tanga. *International Scientific Council for Trypanosomiasis Research and Control (ISCTRC)*, 22nd Meeting, Kampala, Uganda, 22-29 October, OAU/STRC, Abstract, P.6.10.

Capbern, A., Giroud, C., Baltz, T. and Mattern, P. (1977). *Trypanosoma equiperdum*: l'etude des variations antigeniques au cours de la trypanosomose experimentale du lapin. *Experimental Parasitology*, **42**, 6-13.

Carmichael, J. and Bell, F.R. (1944). The use of a new phenanthridinium compound 1553 in the treatment of *Trypanosoma congolense* infection in cattle. *Veterinary Record*, **56**, 495-496.

Challier, A. (1982). The ecology of tsetse (*Glossina* spp.) (Diptera, Glossinidae): A Review (1970-1981). *Insect Science and its Application*, **3**, 97-143.

Challier, A. and Leveissiere, C. (1973). A new trap for capturing Glossina (*Glossina*; Diptera, *Muscidae*): a description et essais sur le terrain. Cahiers. *ORSTROM. Serie Entomologie Medicale et Parasitologie*, **11**, 251-262.

Clarke, B. and Smith, D.A. (1986). The concept of compartments. In: Introduction to Pharmacokinetics, pp. 26-34. Blackwell Scientific Publications, Oxford.

Clarke E.G.C. (1969). Isolation and identification of drugs, p. 312. Pharmaceutical Press, London.

Clausen, P.H., Sidibe, I., Bassinga, A., Richard, X., Bauer, B. and Pohlit, H. (1993). Pathogenesis and pathology of African trypanosomiasis in Baoule, N'Dama Baoule crossbred and Zebu cattle in Burkina Faso. 1. Clinical performance under high natural tsetse challenge. *Tropical Medicine and Parasitology*, **44**, 99-107.

Clausen, P.H., Sidibe, I., Kabore, I. and Bauer, B. (1992). Development of multi-drug resistance of *Trypanosoma congolense* in Zebu cattle under high tsetse challenge in the pastoral zone of Samorogouan, Burkina Faso. *Acta Tropica*, **51**, 229-236.

Codjia, V., Mulatu, W., Majiwa, P.A.O., Leak, S.G.A., Rowlands, G.J., Authie, E., d'Iteren, G.D.M. and Peregrine, A.S. (1993). Epidemiology of bovine trypanosomiasis in the Ghibe Valley, South West Ethiopia . 3. Occurrence of populations of *Trypanosoma congolense* resistant to diminazene, isometamidium and homidium. *Acta Tropica*, **53**, 151-163.

Coolbear, K.P. and Midgley, M. (1986). Characteristics of ethidium uptake by the trypanosomatid *Crithidia fasculata* and *Leptomonas Seymouri*. *Antimicrobial Agents and Chemotherapy*, **29**, 258-262.

Cunningham, I. and Honigberg, B.M. (1977). Infectivity reacquisition by *Trypanosoma brucei brucei* cultivated with tsetse salivary glands. *Science*, **197**, 1279-1282.

Cunningham, M.P., van Hoeve, K. and Lumsden, W.H.R. (1964). A bio-assay technique for the determination of trypanocidal drug levels, and its application estimating the duration of activity of Berenil in treated cattle. *Proceedings of International Congress on Parasitology*, Volume 1, Munich, pp. 301-303.

Curd, F.H.S. and Davey, D.G. (1949). Antrycide: a new trypanocidal drug. *Nature*, **163**, 89-90.

Curd, F.H.S. and Davey, D.G. (1950). Antrycide: a new trypanocidal drug. *British Journal of Pharmacology*, **5**, 25-32.

Curson, H.H. (1928). Nagana in Zululand. 13th and 14th Reports. Division of Veterinary Education and Research, Pretoria. Department of Agriculture, South Africa, pp. 309-313.

Davey, D.G. (1950). Experiments with 'Antrycide' in the Sudan and E.Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **43**, 583-616.

Desowitz, R.S. (1957). Suramin complexes. II.-Prophylactic activity against *Trypanosoma vivax* in cattle. *Annals of Tropical Medicine and Parasitology*, **51**, 457-463.

Doenhoff, M.J., Modha, J., Lambertucci, J.R. and McLaren, D.J. (1991). The immune dependence of chemotherapy. *Parasitology Today*, **7**, 16-18.

Dolan, R.B., Oketch, G., Alushula, H., Mutugi, M., Stevenson, P., Sayer, P.D. and Njogu, A.R. (1990). Homidium bromide as a chemoprophylactic for cattle trypanosomiasis in Kenya. *Acta Tropica*, **47**, 137-144.

Dolan, R.B., Stevenson, P.G.W., Alushula, H. and Oketch, G. (1992). Failure of chemoprophylaxis against bovine trypanosomiasis on Galana ranch in Kenya. *Acta Tropica*, **51**, 113-121.

Dwinger, R.H., Agyemang, K., Kaufmann, J., Grieve, A.S. and Bah, M.L. (1994). Effects of trypanosome and helminth infections on the health and production parameters of village N'Dama cattle in The Gambia. *Veterinary Parasitology*, **54**, 353-365.

Eghianruwa, K.I. and Uduebholo, M.O. (1979). Studies on isometamidium chloride (Samorin) serum and tissue concentrations in goats after intramuscular and intravenous administration. *International Scientific Council for Trypanosomiasis Research and Control (ISCTRC)*, Younde, Cameroon. OAU/STRC Publication No. **111**, pp. 222-226.

Eisler, M.C., Arowolo, R.O.A., Gault, E.A., Moloo, S.K., Holmes P.H. and Peregrine, A.S. (1994). Isometamidium concentrations in the sera of Boran cattle: correlation with prophylaxis against tsetse-transmitted *Trypanosoma congolense*. *Acta Tropica*, **56**, 39-50.

Eisler, M.C., Gault, E.A., Smith, H.V., Peregrine, A.S. and Holmes, P.H. (1993). Evaluation and improvement of an enzyme-linked

immunosorbent assay for the detection of isometamidium in bovine serum. *Therapeutic Drug Monitoring*, **15**, 236-242.

Elliot, C.T., Crooks, S.R.H and McCaughey, W.J. (1995). Development of a rapid screening test to detect β-agonist residues in bovine eye and hair. *Veterinary Record*, **137**, 643-644.

Elrayah, I.E. and Kaminsky, R. (1991). The effect of diminazene aceturate and isometamidium chloride on cultured procyclic forms of susceptible and drug resistant *Trypanosoma congolense*. *Acta Tropica*, **49**, 201-213.

Engvall, E. and Perlmann, P. (1971). ELISA. Quantitative assay of immunoglobulin G. *Immunochemistry*, **8**, 871-874.

Engvall, E. and Perlmann, P. (1972). Enzyme-linked immunosorbent assay, ELISA III. Quantification of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen coated tubes. *Journal of Immunology*, **109**, 129-135.

Engvall, E., Jonsson, K. and Perlmann, P. (1971). ELISA II. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme labelled antigen and antibody coated tubes. *Biochemica et Biophysica Acta*, **251**, 427-434.

Evans, J.T.R. (1948). *Trypanosoma congolense* infection in cattle in the Sudan. Treament with Dimidium Bromide (Phenanthridinium 1153). *Veterinary Record*, **60**, 418-420.

Fairclough, R. (1958). Preliminary observations of a new phenanthridinium chemotherapeutic activity against bovine trypanosomiasis. *International Scientific Committee for Trypanosomiasis and Research (ISCTRC)*, 7th Meeting, Brussels, OAU/STRC, pp. 51-54.

Fairclough, R. (1963a). Observations on the use of Berenil against trypanosomiasis of cattle in Kenya. *Veterinary Record*, **75**, 1107-1112.

Fairclough, R. (1963b). A comparison of metamidium, Samorin, Berenil and Ethidium bromide under field conditions in Kenya. *Veterinary Record,* **75**, 855-858.

Fauda, H.G. (1977). Determination of diminazene in plasma by high performance liquid chromatography. *Journal of Chromatographic Science*, **15**, 537-538.

Fauda, H.G. (1978). Gas chromatography chemical ionization mass spectrometric analysis of diminazene in plasma. *Biomedical Mass Spectrometry*, **5**, 72-75.

Findlay, G.M. (1930). Recent advances in chemotherapy. 1st ed. Churchill. London.

Finelle, P. and Lacotte, R. (1965). Essais des medicaments trypanopreventifs chez les anes. *International Scientific Committee for Trypanosomiasis Research*, 10th Meeting , Kampala. OAU/STRC Publication No. 97 pp. 31-33.

Ford, J. (1965). Distribution of *Glossina* and epidemiological patterns in the African trypanosomiases. *Journal of Tropical Medicine and Hygiene*, **68**, 211.

Ford, J., Nash, T.A.M. and Welsh, J.R. (1970). Control by clearing of vegetation. In: *The African Trypanosomiases*. Mulligan, H.W. (Editor). George Allen and Unwin. London, pp. 543-556.

Fussganger, R. (1955). Berenil in der Veterinarmedizin. *Veterinar Medizinische Nachrichten*, **3**, 146-151.

Fussganger, R. and Bauer, F. (1960). Investigations on Berenil resistance of trypanosomes. *Veterinary Record*, **72**, 1118-1121.

Gibaldi, M. and Perrier, D. (1982). Non-compartmental analysis based on the statistical moment theory. In: *Pharmacokinetics*, 2nd Edition, pp 409-417, Marcel Dekker, New York.

Gilbert, R.J. (1983). Studies in rabbits on the disposition and trypanocidal activity of the anti-trypanosomal drug, diminazene aceturate (Berenil). *British Journal of Pharmacology*, **80**, 133-139.

Gilbert, R.J. and Newton, B.A. (1982). Ethidium bromide; pharmacokinetics and efficacy against trypanosome infections in rabbits and calves. *Parasitology*, **85**, 127-148.

Gill, B.S. (1971). Resistance of *Trypanosoma evansi* to quinapyramine, suramin, stilbamidine and tryparsamide and analysis of cross-resistance. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **65**, 352-357.

Glasgow, J.P. and Potts, W.H. (1970). Control by handcatching and traps. In: *The African Trypanosomiases*. Mulligan, H.W. (Editor). Goerge Allen and Unwin, London, pp. 395-415.

Glover, P.E., Le Roux, J.G. and Parker, D.F. (1958). The extermination of *Glossina palpalis* on the Kuja-Migori River systems with the use of insecticides. *International Scientific Committee for Trypanosomiasis Research*, 7th Meeting, Brussels, pp. 331-342.

Goddeeris, B.M., Katende, J.M., Irvin, A.D. and Chumo, R.S.C. (1982). The indirect fluorescent antibody test for experimental and epizootiological studies on East Coast fever (*Theileria parva* infection in cattle). Evaluation of a cell culture shizont antigen fixed and sored in suspension. *Research in Veterinary Science*, **33**, 360-365.

Goodwin, L.G. and Tierney, E.D. (1977). Trypanocidal activity of blood and tissue fluid from normal and infected rabbits treated with curative drugs. *Parasitology*, **74**, 33-45.

Gray, A.R. and Roberts, C.J. (1971). The stability of resistance to diminazene aceturate and quinapyramine sulphate in a strain of *Trypanosoma vivax* during cyclical transmission through antelope. *Parasitology*, **63**, 163-168.

Gray, M.A. and Peregrine, A.S. (1993). An *in vitro* assay for drug sensitivity of *Trypanosoma congolense* using *in vitro*-derived metacyclic trypanosomes. *Acta Tropica*, **54**, 291-300.

Gray, M.A., Cunningham, I., Gardiner, P.R., Taylor, A.M. and Luckins, A.G. (1981). Cultivation of infective forms of *Trypanosoma congolense* from trypanosomes in the proboscis of *Glossina morsitans*. *Pa.·asitology*, **82**, 81-95.

Gray, M.A., Kimarua, R.W., Peregrine, A.S. and Stevenson, P. (1993). Drug sensitivity screening *in vitro* of populations of *Trypanosoma congolense* originating from cattle and tsetse flies at Nguruman, Kenya. *Acta Tropica*, **55**, 1-9.

Grootenhuis, J.G. and Olubayo, R.O. (1993). Disease research in the wildlife-livestock interface in Kenya. *Veterinary Quarterly*, **15**, 55-59.

Groothuis, D.G., van Miert, A.S.J.P.A.M., Ziv, G and Nouws, J.F.M. (1978). Effects of experimental Escherichia coli endotoxaemia on ampicillin:amoxycillin blood levels after oral and parental administration in calves. *Journal of Veterinary Pharmacology and Therapeutics*,
1, 81-84.

Guedegbe, B., Verhulst, A., van Meirvenne, N., Pandey, V.S. and Doko, A. (1992). Serological evidence of the existence of wild animal reservoirs of *Trypanosoma brucei gambiense* in the National Park of Pendjari in the Republic of Benin. *Annals de la Societe Belge de Medecine Tropicale*, **72**, 113-120.

Guimaraes, J.L. and Lourie, E.M. (1951). The inhibition of some pharmacological actions of pentamidine by Suramin. *British Journal of Pharmacology and Chemotherapy*, **6**, 513 -530.

Hargrove, J.W. (1972). Some advances in the trapping of tsetse (*Glossina* spp.) and other flies. *Journal of Ecology and Entomology*, **2**, 123-137.

Hargrove, J.W. (1977). Some advances in the trapping of tsetse (*Glossina* spp.) and other flies, *Ecolological Entomology*, **2**, 123-137.

Hargrove, J.W. (1980). Improved estimates of the efficiency of traps for *Glossina morsitans morsitans* Westwood and *Glossina pallidipes* Austen (Diptera: *Glossinidae*), with a note on the effect of the concentration of accompanying host odour on efficiency. *Bulletin of Entomological Research*, **70**, 579-587.

Harris, R.H.T.P. (1938). The control and possible extermination of the tsetse by trapping. *Acta Conv. ter. trop. Malar. Morb.*, Amsterdam, **1**, 663-677.

Hawking, F. (1963). Drug resistance of *Trypanosoma congolense* and other trypanosomes to quinapyramine, phenanthridines, Berenil and other compounds in mice. *Annals of Tropical Medicine and Parasitology*, **57**, 262-282.

Hill, J. (1965). Studies on isometamidium: The effect of isometamidium, homidium and pyrithidium on the infectivity of trypanosomes for mice. *British Journal of Pharmacology*, **25**, 628-663.

Hill, J. and McFadzean, J.A. (1963). Studies on isometamidium. Depots of isometamidium in mice and rats and their importance for prophylaxis against *Trypanosoma congolense. Transactions of the Royal Society of Tropical Medicine and Hygiene*, **57**, 476-484.

Hirumi, H. and Hirumi, K. (1984). Continuous cultivation of animal-infective bloodstream forms of an East African *Trypanosoma congolense* stock. *Tropical Medicine and Parasitology*, **78**, 327-330.

Hirumi, H., Doyle, J.J. and Hirumi, K. (1977). Cultivation of bloodstream *Trypanosoma brucei. Bulletin of the World Health Organization*, **55**, 405-409.

Hladky, S.B. (1990). Steady-state volume of distribution: AUMC. In: Pharmacokinetics, pp. 100-103. Manchester University Press, Manchester.

Hoare, C.A. (1970). Systematic description of the mammalian trypanosomes of Africa. In: *The African Trypanosomiases*. Mulligan, H.W. (Editor). Allen and Unwin, London, pp. 24-59.

Hocking, K.S., Lee, C.W., Beesely, J.S.S. and Matechi, H.T. (1966). Aircraft applications of insecticides in East Africa. XVI- Airspray experiment with endosulphan against *Glossina morsitans* Westw., *G. swynnertoni* Aust. and *G. pallidipes* Aust. *Bulletin of Entomological Research*, **56**, 737-744.

Holmes, P.H., MacAskill, J.A., Whitelaw, D.D., Jennings, F.W. and Urquhart, G.M. (1979). Immunological clearance of ^{75}Se-labelled *Trypanosoma brucei* in mice. I. Aspects of radiolabelling technique. *Immunology*, **36**, 415-420.

Holmes, P.H. and Scott, J.M. (1982). Chemotherapy against animal trypanosomiasis. In: *Perspectives of trypanosomiasis research.* J.R. Baker, Ed., Research studies press, Chichester, pp. 59-69.

Holmes, P.H. and Torr, S.J. (1988). The control of animal trypanosomiasis in Africa. Current methods and future trends. *Outlook on Agriculture*, **17**, 54-60.

Homeida, A.M., El Amin, E.A., Adam, S.E.I. and Mahmoud M.M. (1981). Toxicity of diminazene aceturate (Berenil) to camels. *Journal of Comparative Pathology*, **91**, 355-360.

Ismail, A.A. (1988). Studies on the susceptibility of the Orma and Galana Boran cattle to trypanosome infection. Ph.D. Thesis, University of Nairobi.

ILRAD Report (1990). Annual Report if the International Laboratory for Research on Animal Diseases.

Itty, P., Rowlands, G.J., Minengu, M., Ngamuna, S., van Winkel, F., and d'Ieteren, G.D.M. (1995a). The economics of the recently introduced village cattle production in a tsetse affected area. 1. Trypanotolerant N'Dama cattle in Zaire. *Agricultural Systems*, **47**, 473-491.

Itty, P., Rowlands, G.J., Morkramer G., Defly, A. and d'Ieteren, G.D.M. (1995b). The economics of the recently introduced village cattle production in a tsetse affected area. 2. Trypanotolerant cattle in Southern Togo. *Agricultural Systems*, **47**, 473-491.

Jahnke, H.E. (1974). The economics of controlling tsetse flies and cattle trypanosomiasis in Africa examined for the case of Uganda. IFO Forschungsberichte der Afrika-Studienstelle, **48.** Ifo-Institut fur Wirtschaftsforschung, Weltforum Verlag. Munchen.

Jensch H. (1937). Neue chemotherapeutika der 4-amino-chinolon-reihe. *Angewandte Chemie*, **50**, 891-895.

Jones-Davies, W.J. (1967). The discovery of Berenil-resistant *Trypanosoma vivax* in Northern Nigeria. *Veterinary Record*, **80**, 531-532.

Jones-Davies, W.J. (1968). Diminazene aceturate and homidium chloride; resistance in tsetse fly-transmitted trypanosomes in cattle in Northern Nigeria. *Veterinary Record*, **83**, 433-436.

Jones-Davies, W.J. and Folkers, C. (1966). The prevalence of homidium-resistant strains of trypanosomes in cattle in Northern Nigeria. *Bulletin of Epizootic Diseases of Africa*, **14**, 65-72.

Jordan, A.M. (1985). The vectors of African trypanosomiasis: research towards non-insecticidal methods of control. *British Medical Bulletin*, **41**, 181-186.

Jordan, A.M. (1986). Trypanosomiasis control and African rural development, Longman, London and New York.

Jordan, A.M. (1994). A systematic approach to tsetse and trypanosomiasis control. *FAO Animal Health Production* Paper No. 121, FAO, Rome.

Kamara, D.W. and Echessah, P.N. (1994). Assessment of the socio-economic factors affecting implementation of community-based tsetse control in Busia, Kenya. KETRI Publication No. 51, 1994.

Kamau, S.W., Omukuba, J., Kiragu, J., Ndungu, J.M., Masika, P. and Wachira, P.M. (1993). Use of cypermethrin pour-on in control of tsetse and trypanosomiasis in Kenya. *International Scientific Council for Trypanosomiasis Research and Control (ISCTRC)*, 22nd Meeting, Kampala, Uganda, 22-29 October. OAU/STRC, Abstract P.6.12.

Kandaswamy, T.S. and Henderson, J.F. (1963). The metabolism of ethhidium in normal and neoplastic tissues. *Cancer Reaserch*, **23**, 250-253.

Katende, J.M., Musoke, A.J., Nantulya, V.M. and Goddeeris, B.M. (1987). A new method for fixation and preservation of trypanosomal antigens for use in the indirect immunofluorescence antibody test for diagnosis of bovine trypanososmiasis. *Tropical Medicine and Parasitology*, **38**, 41-44.

Katunguka-Rwakishaya, E., Parkins, J.J., Fishwick, G., Murray, M. and Holmes, P.H. (1993). The pathology of *Trypanosoma congolense* infection in Scottish Blackface sheep - influence of dietery protein. *Veterinary Parasitology*, **47**, 189-204.

Kellner, H.M., Eckert, H.G. and Volz, M.H. (1985). Studies in cattle on the deposition of the anti-trypanocidal drug diminazene diaceturate (Berenil®). *Tropical Medicine and Parasitology*, **36**, 199-204.

Kenya Trypanosomiasis Research Institute (KETRI) Strategic Plan, 1990-2000 (1991).

Kinabo, L.D.B. (1993). Pharmacology of existing drugs for animal trypanosomiasis. *Acta Tropica*, **54**, 169-183.

Kinabo, L.D.B. and Bogan, J.A. (1988a). Solid-phase extraction and ion-pair reversed-phase HPLC of isometamidium in bovine serum and tissues. *Acta Tropica*, **45**, 165-170.

Kinabo, L.D.B. and Bogan, J.A. (1988b). Pharmacokinetic and histopathological investigations of isometamidium in cattle. *Research in Veterinary Science*, **44**, 267-269.

Kinabo, L.D.B. and Bogan, J.A. (1988c). Development of a radioimmunoassay for isometamidium. *Veterinary Research Communications*, **12**, 375-382.

Kinabo, L.D.B. and McKellar, Q.A. (1990). Isometamidium in goats: disposition kinetics, mammary excretion and tissue residues. *British Veterinary Journal*, **146**, 405-412.

Kinabo, L.D.B. and McKellar, Q.A. (1991). Isometamidium in pigs: disposition kinetics, tissue residues and adverse reactions. *Research in Veterinary Science*, **50**, 6-13.

Kitwika, W.A.M. and Malele, I. (1993). The efficacy of alphamethrin (Dominex 100 EC) on tsetse flies at Mivumoni heifer breeding farm, Tanga. *International Scientific Council for Trypanosomiasis Research and Control (ISCTRC)*, 22nd Meeting, Kampala, Uganda, 22-29 October, OAU/STRC, Abstract P.6.11.

Knowles, R.H. (1925). Treatment of camels affected with *Trypanosoma Soudanense* with 'Bayer 205' and further observations on the formol-gel test. *Journal of Comparative Pathology*, **38**, 42-46.

Kora, S. and Bojang, M.A. (1992). Comparative studies on N'Dama and Zebu following repeated infections with *Trypanosoma congolense*. *Research in Veterinary Science*, **52**, 292-298.

Kuzoe, F.A.S. (1993). Current situation of African trypanosomiasis. *Acta Tropica*, **54**, 153-162.

Lancien, J. (1981). Description du piege monoconique utilise pour l'elimination des glossines en Republique Populaire du Congo. Cahiers. *OSTROM. Serie Entomologie Medicale et Parasitologie*, **19**, 235-238.

Leach, T.M., Karib, A.A., Ford, E.J.H. and Wilmshurst, E.C. (1955). Studies on ethidium bromide VI. The prophylactic properties of the drug. *Journal of Comparative Pathology and Therapeutics*, **65**, 130-142.

Leach, T.M. and El Karib, A.A. (1960). Prophylaxis against repeated artificial challenge with *Trypanosoma congolense*. A comparative trial of Antrycide Prosalt and Prothidium bromide. *Journal of Comparative Pathology*, **70,** 385-395.

Le Rouex, P.L. (1936). Trypanosomiasis. Annual Reports, 1936. Veterinary Department, Northern Rhodesia, Appendix C.64.

Losos, G. J. and Crockett, E. (1969). Toxicity of Berenil in the dog. *Veterinary Record,* August, p. 196.

Luckins, A.G. and Gray, A.R. (1979). Trypanosomes in lymph nodes of cattle and sheep infected with *Trypanosoma congolense. Research in Veterinary Science*, **27**. 129-131.

Luckins, A.G., Boid, R., Rae, P., Mahmoud, M.M., El Malik, K.H. and Gray, A.R. (1979). Serodiagnosis of infection with *Trypanosoma evansi* in camels in the Sudan. *Tropical Animal Health Production,* **11**, 1-12.

Luguru, S.M., Bennet, S.R. and Chizyuka, H.G.B. (1993). Observations on the incidence of bovine trypanosomiasis in cattle dipped in deltamethrin in a tsetse-infested aarea of Zambia. *Tropical Animal Health and Production*, **25**, 129-130.

MacAdam, R.F. and Williamson, J. (1972). Drug effects on the fine structure of *Trypanosoma rhodesiense*: diamidines. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **66**, 897-904.

MacGregor, J.T. and Clarkson, T.W. (1971). Metabolism and biliary excretion of phenanthridinium salts - 1. Nature of the biliary metabolites. *Biochemical Pharmacology*, **21**, 1679-1696.

MacGregor, J.T. and Johnson, I.J. (1977). *In vitro* activation of ethidium bromide and other phenanthridium compounds: mutagenic activity in *Salmonella typhimurium. Mutation Research*, **48**, 103-108.

MacLennan, K.J.R. (1957). A staining technique for the identification of trypanosomes in the thick blood films. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **51**, 302-310.

MacLennan, K.J.R. (1968). Some recent findings concerning the use of Berenil for the treatment of bovine trypanosomiasis in Northern Nigeria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **62**, 139-140.

Mahler, H.R. and Bastos, R.N. (1974). A novel reaction of mitochondrial DNA with ethidium bromide. *FEBS Letter*, **39**, 27-34.

Maina, A. (1977). The need for joint tsetse programmes across national borders. *International Scientific Council for Trypanosomiasis Research and Control (ISCTRC)* 15th Meeting, Banjul, Gambia. OAU/STRC Publication No. 110, pp. 582-584.

Maloo, S.H. (1993). Epidemiological studies of vector-bourne diseases with consequent development of preventitive medicine programmes for small-holder dairy farmers in Coastal Kenya. PhD Thesis. Department of Veterinary Medicine. University of Glasgow, pp. 278-324.

Mamman, M., Williams, D.J.L., Murphy, N.B. and Peregrine A.S. (1995). Apparent rarity of diminazene-resistant trypanosomes in goats infected with diminazene resistant population of *Trypanosoma congolense. Research in Veterinary Science*, **58**, 113-118.

Mango, C.K.A., Langley, P.A., Okedi, T., Guya, S., Otieno, D. and Omuse, J.K. (1993). Efficacy data of S-31183- pyriproxyfen on *Glossina*

pallidipes Austen population suppression in Kiboko, Machakos District. *International Scientific Council for Trypanosomiasis Research and Control (ISCTRC)*, 22nd Meeting, Kampala, Uganda, 22-29 October. OAU/STRC, Abstract P.6.16.

Masake, R.A. and Nantulya, V.M. (1991). Sensitivity of an antigen enzyme immunoassay for diagnosis of *Trypanosoma congolense* infections in goats and cattle. *Journal of Parasitology*, **77**, 231-236.

Mate, A.M. (1993). Community participation with regard to the control of *Glossina morsitans centralis* (Machado) using targets in Western Zambia. *International Scientific Council for Trypanosomiasis Research and Control (ISCTRC)*, 22nd Meeting, Kampala, Uganda, 22-29 October. OAU/STRC, Abstract 6.29.

Mattern, I.E. (1976). Mutagenicity of ethidium bromide after metabolic activation *in vitro*. *Mutation Research*, **38**, 120.

Matthiessen, P. (1983). Monitoring the enviromental impact of pesticides. *Zimbabwe Science News*, **17**, 44-45.

McGuire, T.C., Musoke, A.J. and Kurtti, T. (1979). Functional properties of bovine IgG1 and IgG2: Interaction with complement, macrophages, neutrophils and skin. *Immunology*, **38**, 249-256.

Meirvenne, N., van Janssens, P.G., Manus, E., Lumsden, W.H.R. and Herbert, W.J. (1975). Antigenic variation in syringe-passaged populations of *Trypanosoma* (*Trypanozoon*) *brucei*. II. Comparative studies on two antigenic-type collections. *Annales Societe Belge Medecine Tropicale*, **55**, 25.

Moffat, A.C. (1986): Clarke's isolation and identification of drugs: in pharmaceuticals, body fluids and postmortem materials. Pharmaceutical Press. London.

Morris, K.R.S. and Morris, M.G. (1949). The use of traps against tsetse in West Africa. *Bulletin of Entomological Research*, **39**, 491-528.

Muguwa, J., Obayl, H., Musisi, R., Tibaingana, M., Drabile, C., Magona, J., Okoth, J. and Lancien, J. (1993). The effects of supplementing trapping control with deltamethrin pour-on formulation against tsetse flies (*Glossina fuscipes fuscipes* Newstead) in Buteba sub-county, Tororo District, Uganda. *International Scientific Committee for Trypanosomiasis Research and Control (ISCTRC)*, 22nd Meeting, Kampala, Uganda, 22-29 October. OAU/STRC, Abstract P.6.9.

Munstermann, S., Mbura, R.J., Maloo, S.H. and Lohr, K.F. (1992). Trypanosomiasis control in Boran cattle in Kenya: a comparison between chemoprophylaxis and a parasite detection and intravenous treatment method using isometamidium chloride. *Tropical Animal Health Production*, **24**, 17-27.

Murilla, G.A. and Kratzer, R.D. (1989). Sorbent extraction and high performance liquid chromatography of diminazene aceturate in bovine plasma and tissues. *International Scientific Council for Trypanosomiasis Research and Control (ISCTRC)*, 20th Meeting, Mombasa, Kenya. OAU/STRC Publication 115, pp. 347-353.

Murray, M. and Dexter, T.M. (1988). Anaemia in bovine African trypanosomiasis. A review. *Acta Tropica*, **45**, 389-432.

Murray, M., Murray, P.K. and McIntyre, W.I.M. (1977). An improved parasitological technique for the diagnosis of African trypanosomiasis. *Transactions of the Royal Society of Medicine and Hygiene*, **71**, 325-326.

Murray, M. and Morrison, W.I. (1980). Pathogenesis and pathology of African trypanosomiasis in domestic livestock. *Report of the Expert Consultation on Research on Trypanosomiasis*, 1-5 October pp. 70-75, FAO, Rome. Appendix VIII.

Mutayoba, B.M., Eckersall, P.D., Cestnik, V., Jeffcoate, I.A., Gray, C.E. and Holmes, P.H. (1995). Effects of *Trypanosoma congolense* on

pituitary and adrenocortical function in sheep: changes in the adrenal gland and cortisol secretion. *Research in Veterinary Science*, **58**, 174-179.

Mwambu, P.M. (1967). Cattle trypanosomiasis survey in the area adjoining the South Busoga fly belt. *International Scientific Council for Trypanosomiasis Research and Control (ISCTRC)*, 11th Meeting, Nairobi, Kenya. OAU/STRC Publication No. 100, p. 25.

Mwambu, P.M. (1971). The effect of a block-treatment regimen, using ethidium, on cattle trypanosomiasis in an endemic area. *East African Agricultural and Forestry Journal*, April, 414-417.

Mwambu, P.M. and Mayende, J.S.P. (1971). Occurrence of Berenil resistant strains of *Trypanosoma vivax. Transactions of the Royal Society of Tropical Medicine and Hygiene*, **36**, 414-418.

Mwangi, E.K. (1993). Variation in susceptibility to tsetse-bourne trypanosomiasis among three *Bos indicus* cattle breeds in different tsetse endemic localities in Kenya. Thesis. Department of Veterinary Medicine, University of Glasgow, pp. 285-302.

Nantulya, V.M. (1989). An antigen detection enzyme immunoassay for the diagnosis of Rhodesian sleeping sickness. *Parasite Immunology*, **11**, 69-75.

Nantulya, V.M., Musoke, A.J., Rurangirwa, F.R. and Maloo, S.K. (1984). Resistance of cattle to tsetse-transmitted challenge with *Trypanosoma brucei* and *Trypanosoma congolense* after spontaneous recovery from syringe passaged infections. *Infection and Immunity*, **43**, 735-738.

Nash, T.A.M. (1970). Control by parasites and predators. In: The African Trypanosomiases. Mulligan, H.W. (editor), Allen and Unwin, London, pp. 521-532.

Ndoutamia, G., Moloo, S.K., Murphy, N.B. and Peregrine, A.S. (1993). Derivatization and characterization of a quinapyramine-resistant clone

of *Trypanosoma congolense*. *Antimicrobial Agents and Chemotherapy*, **37**, 1163-1166.

Newton, B.A. (1972). Recent studies on the mechanism of action of Berenil (diminazene) and related compounds. In: Comparative Biochemistry of parasites. Van den Bosssche, H., Ed. Academic Press, New York, pp. 127-133.

Newton, B.A. (1976). Antiprotozoal drugs as biochemical probes. In: Biochemistry of parasites and host parasite relationships. Van den Bosssche, H., Ed. Janssen Research Foundation. Elsevier/North Holland Biomedical Press, Amsterdam, pp. 459-476.

Nielsen, K., Sheppard, J., Holmes, W. and Tizard, I. (1978). Experimental bovine trypanosomiasis. Changes in serum immunoglobulins, complement and complement components in infected animals. *Immunology*, **35**, 817-826.

Njogu, A.R., Dolan, R.B., Wilson, A.J. and Sayer, P.D. (1985). Trypanotolerance in East African Orma Boran. *Veterinary Record*, **117**, 632-636.

Ogwu, D., Njoku, C.O. and Osori, D.I.K. (1985). Effects of *Trypanosoma vivax* infections on pregnancy and fertility of heifers. *International Scientific Council for Trypanosomiasis Research and Control (ISCTRC)*, 18th Meeting, Harare, Zimbabwe. pp. 191-192.

Okuna, N.M., Magona, J. and Mayende, J.S.P. (1993). Preliminary observations on the impact of trypanosomiasis on sheep and goats in an enzootic area in Uganda and the need for prophylactic treatment. *International Scientific Council for Trypanosomiasis Research and Control (ISCTRC)*, 22nd Meeting, Kampala, Uganda, 22-29 October. OAU/STRC, Abstract P.5.31.

Olaho, W.M., Mukunza, F. and Powell, C.N. (1995). Partial protection against natural trypanosomiasis after vaccination with a flagellar pocket antigen from *Trypanosoma brucei rhodesiense*. *Vaccine*, **13**, 151-154.

Opiyo, E.A., Hussain, M., Kiragu, J.M., Omuse, J.K. and Kiniiya, S. (1993). Evaluation of formulations of deltamethrin and cypermethrin for tsetse control in Kenya. *International Scientific Council for Trypanosomiasis Research and Control (ISCTRC)*, 22nd Meeting, Kampala, Uganda, 22-29 October. OAU/STRC, Abstract P.6.15.

Osaer, S., Goossens, B., Clifford, D.J., Kora, S. and Kassama, M. (1994). A comparison of the susceptibility of Djallonke sheep and West African Dwarf goats to experimental infection with two different strains of *Trypanosoma congolense*. *Veterinary Parasitology*, **51**, 191-204.

Osman, A.S., Jennings, F.W. and Holmes P.H. (1992). The rapid development of drug-resistance by *Trypanosoma evansi* in immunosuppressed mice. *Acta Tropica*, **50**, 249-257.

Otsyula, M., Kamar, K., Mutugi, M., and Njogu, A.R. (1992). Preliminary efficacy trial of Cymelarsen, a novel trypanocide, in camels naturally infected with *Trypanosoma evansi* in Kenya. *Acta Tropica*, **50**, 271-273.

Peregrine, A.S. and Mamman, M. (1993). Pharmacology of diminazene; A Review. *Acta Tropica*, **54**, 185-203.

Peregrine, A.S., Knowles, G., Ibitayo, A.I., Scott, J.R., Moloo, S.K. and Murphy, N.B. (1991). Variation in resistance to isometamidium chloride and diminazene aceturate by clones derived from a stock of *Trypanosoma congolense*. *Parasitology*, **102**, 93-100.

Peregrine, A.S., Moloo, S.K. and Whitelaw, D.D. (1987). Therapeutic and prophylactic activity of isometamidum chloride in Boran cattle against *Trypanosoma vivax* transmitted by *Glossina morsitans centralis*. *Research in Veterinary Science*, **43**, 268-270.

Peregrine, A.S., Ogunyemi, O., Whitelaw, D.D., Holmes, P.H., Maloo, S.K., Hirumi, H., Urquhart, G.M. and Murray, M. (1988). Factors influencing the duration of isometamidium chloride (Samorin) prophylaxis against experimental challenge with metacyclic forms of *Trypanosoma congolense*. *Veterinary Parasitology*, **28**, 53-64.

Perschke, H. and Vollner, L. (1985). Determination of the trypanocidal drugs homidium, isometamidium and quinapyramine in bovine serum or plasma using HPLC. *Acta Tropica*, **42**, 209-216.

Phillips, F.S., Sternberg, S.S., Cronin, A.P., Sodergren, J.E. and Vidal, P.M. (1967). Physiologic disposition and intracellular localization of isometamidium. *Cancer Research*, **27**, 333-349.

Pinder, M. and Authie, E. (1984). The appearance of isometamidium resistant *Trypanosoma congolense* in West Africa. *Acta Tropica*, **41**, 247-252.

Plimmer, H.G. and Thompson, J.D. (1908). Further results of the experimental treatment of trypanosomiasis in rats. *Proceedings of the Royal Society*, London, **80**, 1-2.

Pospichal, H., Brun, R., Kaminsky, R. and Jenni, L. (1994). Induction of resistance to melarsenoxide cysteamine (Mel Cy) in *Trypanosoma brucei brucei*. *Acta Tropica*, **58**, 187-197.

Powell, C. (1993). Experimental immunity against trypanosomiasis in rabbits. *Vaccine*, **11**, 307-310.

Raether, W., Hajdu, P., Seidenath, H. and Damm, D. (1972). Pharmakokinetische und Chemoprophlktische untersunchungen mit Berenil an Wistar-Ratten (*Trypanosoma rhodesiense*). *Zeitschrift fur Tropenmedizin und Parasitologie*, **23**, 418-427.

Randall, J.B. and Beveridge, C.G.L. (1946). A note on the toxicity of Phenanthridinium 1553 in cattle. *Veterinary Record*, **58**, 398-399.

Rogers, D.J. and Williams, B.G. (1993). Monitoring trypanosomiasis in space and time. *Parasitology*, **106**, 577-592.

Rogers, D.J., Hendrickx, G. and Slingenbergh, J.H.W. (1994). Tsetse flies and their control. *Epizooties*, **13**, 1075-1124.

Rottcher, D. and Schillinger, D. (1985). Multiple drug resistance in *Trypanosoma vivax* in the Tana River District of Kenya. *Veterinary Record*, **117**, 557-558.

Rubenstein, K.E., Schneider, R.S. and Ullman, E.F. (1972). 'Homogeneous' enzyme immunoassay. A new immunochemical technique. *Biochemical and Biophysical Research Communications*, **47**, 846-851.

Ruckebusch, Y. Phaneuf, L. and Dunlop, R. (1991). Cardiovascular System. In: *Physiology of Small and Large Animals*, pp. 99-100. B.C. Dekker Inc. Hamilton, Ontario.

Schonefeld, A., Rottcher, D. and Moloo, S.K. (1987). The sensitivity to trypanocidal drugs of *Trypanosoma vivax* isolated in Kenya and Somalia. *Tropical Medicine and Parasitology*, **38**, 177-180.

Scott, J.M. and Pegram, R.G. (1974). A high incidence of *Trypanosoma congolense* strains resistant to homidium bromide in Ethiopia. *Tropical Animal Health and Production*, **6**, 215-221.

Sekoni, V.O. (1994). Reproductive disorders caused by animal trypanosomiasis - A Review. *Theriogenology*, **42**, 557-570.

Shereni, W. and Pope, A.J.R. (1993). Effects of residual deposits of deltamethrin applied by the ground spraying technique against tsetse fly (Diptera: *Glossinidae*) populations in North-Western Zimbabwe. *International Scientific Council for Trypanosomiasis Research and Control (ISCTRC)*, 22nd Meeting, Kampala, Uganda, 22-29 October. OAU/STRC, Abstract P.6.18.

Shetty, S.N. (1986). Isometamidium residue in cattle meat in Nigeria. *Bulletin of Animal Health and Production in Africa*, **34**, 264-267.

Sones, K.R., Njogu, A.R. and Holmes, P.H. (1988). Assessment of sensitivity of *Trypanosoma congolense* to isomeatmidium chloride: a comparison of tests using cattle and mice. *Acta Tropica*, **45**, 153-164.

Stephen, L.E. (1963). An attempt to produce resistance to homidium in a strain of *Trypanosoma vivax* transmitted by the tsetse fly. *Journal of Comparative Pathology and Therapeutics*, **73**, 76-83.

Stephen, L.E. (1970). Clinical manifestations of the trypanosomiases in livestock and other domestic animals. In: *The African Trypanosomiases*, Mulligan, H.W. (Editor), George Allen and Unwin, London, 774-794.

Stephen, L.E. (1986). Trypanosomiasis. A veterinary perspective. Pergamon Press. Oxford.

Stevenson. P., Sones, K.R., Gicheru, M.M. and Mwagi, E.K. (1995). Comparison of isometamidium chloride and homidium bromide as prophylactic drugs for trypanosomiasis in cattle at Nguruman, Kenya. *Acta Tropica*, **59**, 77-84.

Sutherland, I.A., Mounsey, A. and Holmes, P.H. (1992). Transport of isometamidium (Samorin) by drug resistant and drug sensitive *Trypanosoma congolense. Parasitology*, **104**, 461-467.

Swallow, B.M. and Mulatu, W. (1993). Evaluating the willingness of Ghibe Valley (Ethiopia) residents to contribute time and money to a tsetse control programme using targets. *International Scientific Council for Trypanosomiasis Research and Control (ISCTRC)*, 22nd Meeting, Kampala, Uganda, 22-29 October. OAU/STRC, Abstract 6.27.

Swallow, B.M., Mulatu, W. and Leak, S.G.A. (1993). Evaluation of pour-on by Ethiopian farmers. *International Scientific Council for*

Trypanosomiasis Research and Control (ISCTRC), 22nd Meeting, Kampala, Uganda, 22-29 October. OAU/STRC, Abstract 6.30.

Tizard, I. (1985). Immunology and pathogenesis of trypanosomiasis. CRC Press, Inc., Boca Raton, Florida.

Trail, J.C.M., d'Ieteren, G.D.M., Murray, M., Ordner, G., Yangari, G., Collardelle, C., Sauveroche, B., Maille, J.C. and Vivian, P. (1994). Measurement of trypanotolerance criteria and their effect on reproductive performance of N'Dama cattle. *Veterinary Parasitology*, **45**, 241-255.

Unsworth, K. (1954a). The curative effect of ethidium bromide against *Trypanosoma vivax* infections of Zebu cattle in West Africa, with observations on the toxicity of the drug. *Annals of Tropical Medicine and Parasitology*, **48**, 229-236.

Unsworth, K. (1954b). Further observations on the curative effect of Ethidium bromide against *Trypanosoma vivax* infections in Zebu cattle in West Africa, *Annals of Tropical Medicine and Parasitology*, **48**, 237-241.

Vale, G.A. (1974). The responses of tsetse flies (Diptera: *Glossinidae*) to mobile and stationary baits. *Bulletin of Entomological Research*, **64**, 545-548.

Vale, G.A. (1982). The trap orientated behaviour of tsetse flies (*Glossinidae*) and other Diptera. *Bulletin of Entomological Research*, **72**, 71-93.

Vale, G.A. and Hargrove, J.W. (1979). A method of studying the efficiency of traps for tsetse flies (Diptera: *Glossinidae*) and other insects. *Bulletin of Entomological Research*, **69**, 183-193.

Van Miert, A.S.J.P.A.M., van Gogh, H. and Wit, J.G. (1976). The influence of pyrogen induced fever on absorption of sulpha drugs. *Veterinary Record*, **99**, 480-481.

Van Rensberg, S.W.J. (1938). Surfen C therapy in *Trypanosoma congolense*. *Journal of Veterinary Science and Animal Industry*, **10**, 13-20.

Watkins, T.F. and Woolfe, G. (1952). Effect of changing the quarternizing group on the trypanocidal activity of dimidium bromide. *Nature*, **169**, 506.

Watkins, T.F. and Woolfe, G. (1956). Prophylaxis of trypanosome infections in cattle. *Nature*, **178**, 368.

Wellde, R.L., Deindl, E., Sadun, E., Williams, J. and Warui, G. (1974). 1. Clinical observations of experimentally infected cattle. *Experimental Parasitology*, **36**, 6-19.

Wernsdorfer W.H. (1994). Epidemiology of drug resistance in malaria. *Acta Tropica*, **56**, 143-156.

Whitelaw, D.D., Bell, I.R., Holmes, P.H., Maloo, S.K., Hirumi, H., Urquhart, G.M. and Murray, M. (1986). Isometamidium chloride prophylaxis against *Trypanosoma congolense* challenge and the development of immune responses in Boran cattle. *Veterinary Record*, **118**, 722-726.

Whitelaw, D.D., Gault, E.A., Holmes, P.H., Sutherland, E.A., Rowell, S.J., Phillips, A. and Urquhart, G.M. (1991). Development of an enzyme-linked immunosorbent assay for the detection and measurement of the trypanocidal drug isometamidium chloride in cattle. *Research in Veterinary Science*, **50**, 185-189.

Whiteside, E.F. (1960). Recent work in Kenya on the control of drug-resistant cattle trypanosomiasis. *International Scientific Committee for Trypanosomiasis Research.* 8th Meeting , Jos. Commission for Technical Co-operation South of the Sahara, London. Publication No. **62**, 141-152.

Whiteside E.F. (1962). Interactions between drugs, trypanosomes and cattle in the field. In: *Drugs, Parasites and Hosts*, pp. 116-141, Goodwin, L.G. and Nimmo-Smith, R.H. (Eds.) Churchill, London.

Whiteside, E.F (1963). A strain of *Trypanosoma congolense* directly resistant to Berenil. *Journal of Comparative Pathology*, **73**, 167-175.

WHO/FAO/OIE (1963). The economic losses caused by animal diseases. Animal Health Year Book, 284-313. FAO, Rome.

Wilde, J.K.H. and Robson, J. (1953). The effect against *Trypanosoma congolense* in Zebu of 3 new phenanthridine compounds. *Veterinary Record*, **65**, 49-51.

Williamson, J. (1957). Suramin complexes. I: Prophylactic activity against *Trypanosoma congolense* in small animals. *Annals of Tropical Medicine and Parasitology*, **51**, 440-456.

Williamson, J. (1970). Review of chemotherapeutic and chemoprophylactic agents. In: *The African Trypanosomiasis*, pp. 125-221. Mulligan H.W. (Ed.), Allen and Unwin, London.

Wilson S.G. and Fairclough, R. (1953). A preliminary note on the treatment of *Trypanosoma congolense* infections with ethidium bromide in Kenya colony. *Veterinary Record*, **65**, 201-202.

Wilson, S.G. (1960). Animal trypanosomiasis in Northern Nigeria. Symposium on Animal Trypanosomiasis, Luanda. Commission for Technical Co-operation South of the Sahara, London. Publication No. **45**, pp. 37-52.

Winrock International (1992). Assessment of Animal Agriculture in sub-Saharan Africa. Winrock International Institute for Agriculturaal Development. Morrilton. Arkansas, p. 162.

Wragg, W.R., Washbourne, K., Brown, K.N. and Hill, J. (1958). Metamidium: a new trypanocidal drug. *Nature*, **182**, 1005-1006.

Yalow, R.S. and Berson, S.A. (1960). Immunoassay of endogenous plasma insulin in man. *Journal of Clinical Investigations*, **39**, 1157.

Yamaoka, K., Nakagawa, T and Uno, T. (1978). Application of Akaike's Information Criterion (AAIC) in the evaluation of linear pharmacikinetic equations. *Journal of Pharmacology and Biopharmaceutics*,
6, 165-175.

Young, J.K. (1973). Blood sulfonamide levels in feedlot cattle with respiratory disease. *Modern Veterinary Practice*, **54**, 21.

Zhang, Z.Q., Giroud, C. and Baitz T. (1992). *In vivo* and *in vitro* sensitivity of *Trypanosoma evansi* and *T. equiperdum* to diminazene, suramin, Mel Cy, quinapyramine and isometamidium. *Acta Tropica*, **50**, 101-110.

APPENDIX A

1.0 CHEMISTRY OF CURRENT ANIMAL TRYPANOCIDAL DRUGS

1.1 Diminazene aceturate (Berenil®, Veriben®)

It is an aromatic diamine

1.1.1 Chemical names: p,p'-diaminodiazoaminobenzene diaceturate

1,3-Bis(4-amidinophenyl) triazene

1.1.2 Molecular formula: $C_{22}H_{29}N_9O_6.4H_2O$

1.1.3 Molecular mass: 587.6 as the salt

1.1.4 Structural formula: See Appendix B.

1.1.5 Description: A yellow, odourless powder. Melting point 217°C with decomposition.

1.1.6 Solubility: About 70% at 20°C in water. Slightly soluble in alcohol. Sparingly soluble in ether and chloroform. Though stable when dry, an aqueous solution in contact with air is stable for only two or three days.

1.2 Homidium bromide (Ethidium®)/Homidium chloride (Novidium®)

It is a phenanthridinium compound.

1.2.1 Chemical name: 2,7-diamino-9-phenyl-10-ethylphenanthridinium bromide/chloride anhydrate.

1.2.2 Molecular formula: $C_2H_{20}BrN_3$

$C_2H_{20}ClN_3$

1.2.3 Molecular mass: 394 as salt

412.3 as a monohydrate

1.2.4 Structural formula: See Appendix B.

1.2.5 Description: A dark purple, almost odourless, crystalline or amorphous powder with a persistent bitter taste and highly fluorescent under UV light. Melting Point 245°C with decomposition.

1.2.6 Solubility: 3.5% in water at 20°C and 10% at 100°C. A 5-6% solution in water is stable for several days at 20°C. Solubility is only 0.13% in chloroform.

1.3 Isometamidium chloride: (Samorin®, Trypamidium®)

Is a phenanthridinium compound

1.3.1 Chemical name: 7-(n-amidinophenyldiazoamino-)-7-amino-10-ethyl-9-phenylphenanthridinium chloride hydrochloride

1.3.2 Molecular formula: $C_{22}H_{25}ClN_7HCl$

1.3.3 Molecular mass: 531.5

1.3.4 Structural formula: See Section Appendix B.

1.3.5 Description: Marketed as Samorin® and Trypamidium® it is composed of 5 different isomers and the powder appears dark red in colour.

1.3.6 Solubility: Fairly soluble in water.

1.4 Melarsenoxide cyteamine (Mel Cy®)

1.4.1 Molecular mass: 501.3

1.4.2 Structural formula: See Section Appendix B.

1.4.3 Description: White crystalline powder. Contains 14.9% Arsenic.

1.4.4 Solubility: Highly soluble in water

1.5 Suramin (Antrypol®; Naganol®)

1.5.1 Chemical name: 8,8'-[carbonylbis[imino-3,1-phenylenecarbonylimino(4-methyl-3,1-phenylene)carbonyl-imino]]bis-1,3,5-naphthalenetrisulfonic acid hexasodium salt.

1.5.2 Molecular formula: $C_{51}H_{34}N_6Na_6O_{23}S_6$

1.5.3 Molecular mass: 1429.21

1.5.4 Structural formula: See Appendix B.

1.5.5 Description: White or slightly pink or cream coloured powder. Slightly bitter taste. Hygroscopic.

1.5.6 Solubility: Freely soluble in water and in physiological salt solution. Sparingly soluble in ethanol. Insoluble in benzene, ether, petroleum ether and chloroform. Aqueous solutions are neutral to litmus.

1.6 Quinapyramine (Antrycide®)

1.6.1 Chemical name: 4-amino-6-[(2-amino-1,6-dimethylpyrimidinium-4-yl)amino] quinolinium salts.

1.6.2 Molecular formula: Dimethoate $C_{19}H_{28}N_6O_8S_2$.

1.6.3 Structural formula: See Appendix B.

1.6.4 Description: Creamy white crystals from aqueous methanol. MPt 265-266.

1.6.5 Solubility: Freely soluble in water.

APPENDIX B

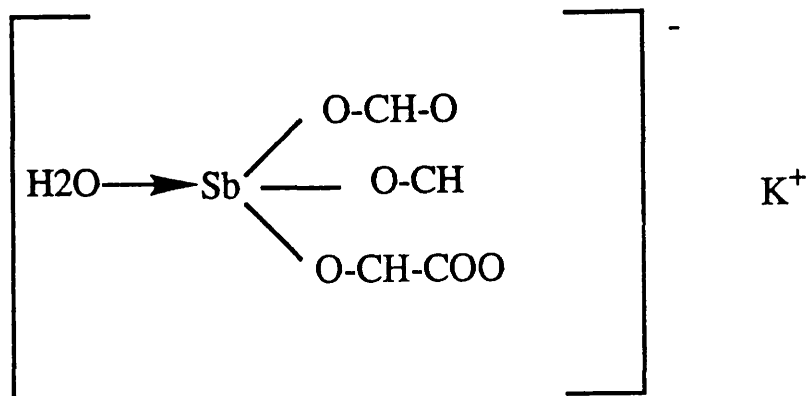


Fig. 1.1(a) Potassium antimony tartrate (tartar emetic)

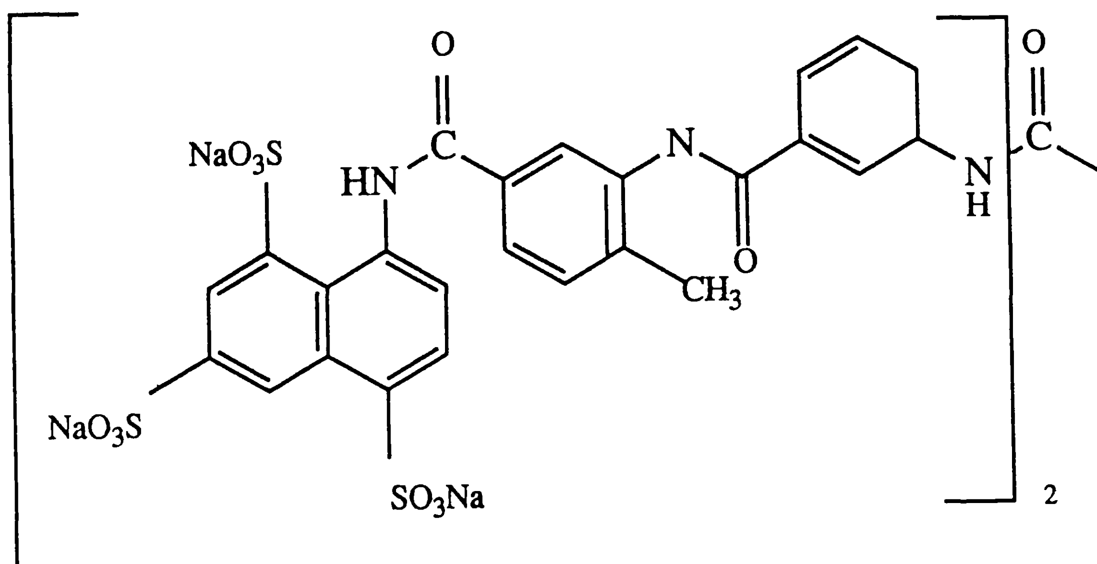


Fig. 1.1(b) Suramin

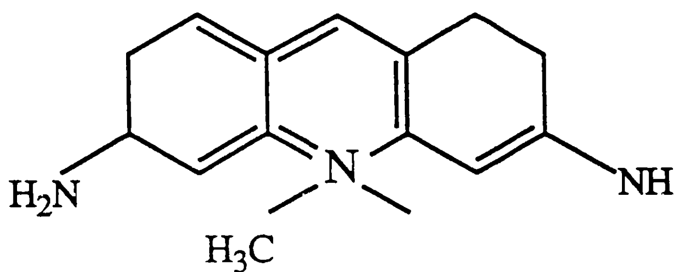


Fig. 1.1(c) Trypaflavine

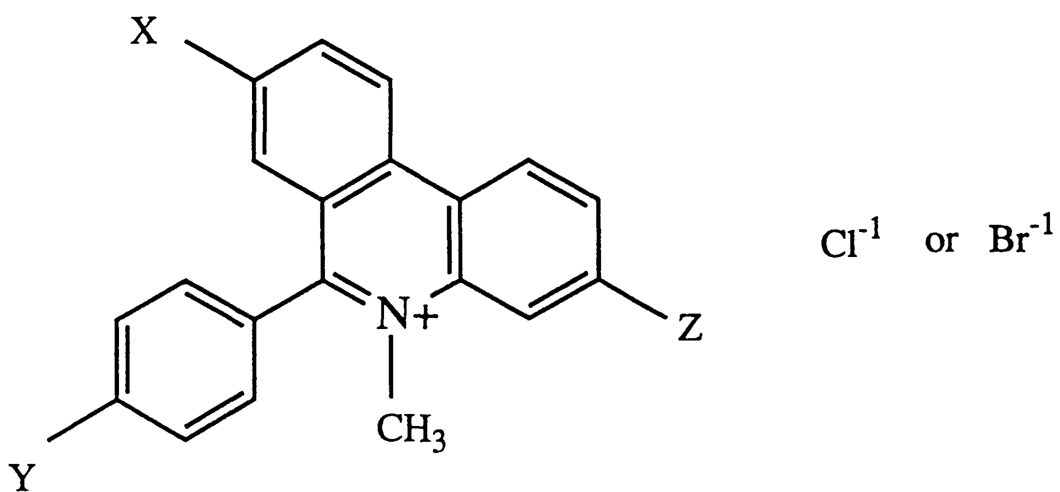


Fig. 1.1(d) Phenidium (X, Y = NH2) Z = H

Dimidium (X, Z = NH2) Y = H

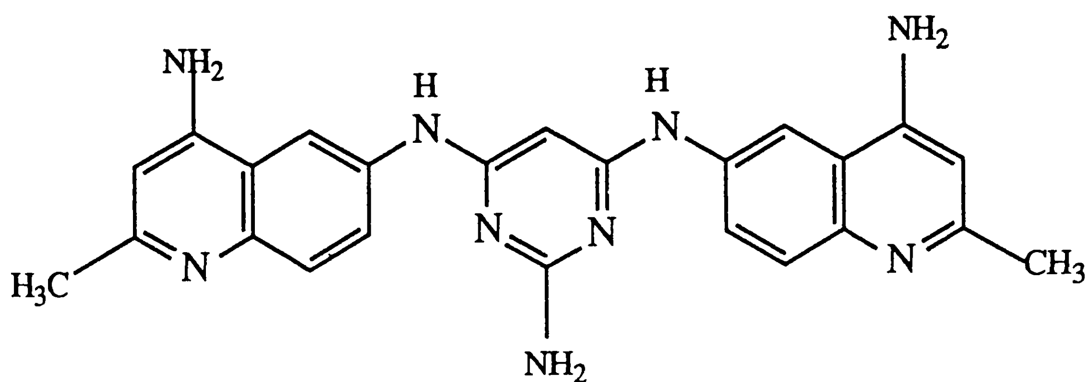


Fig. 1.1(e) Surfen C

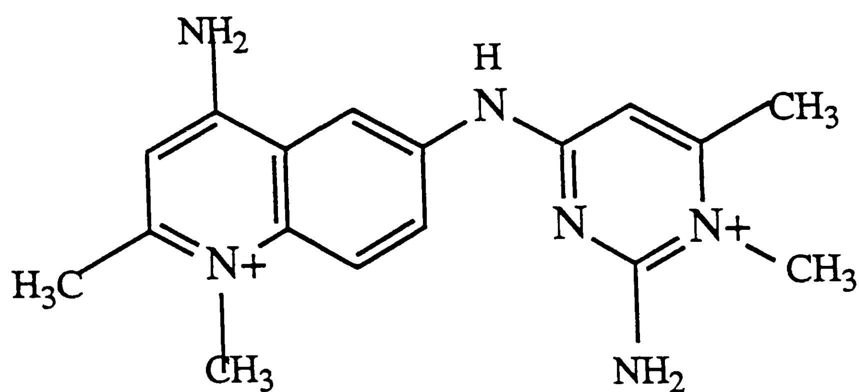


Fig. 1.1(f) Quinapyramine

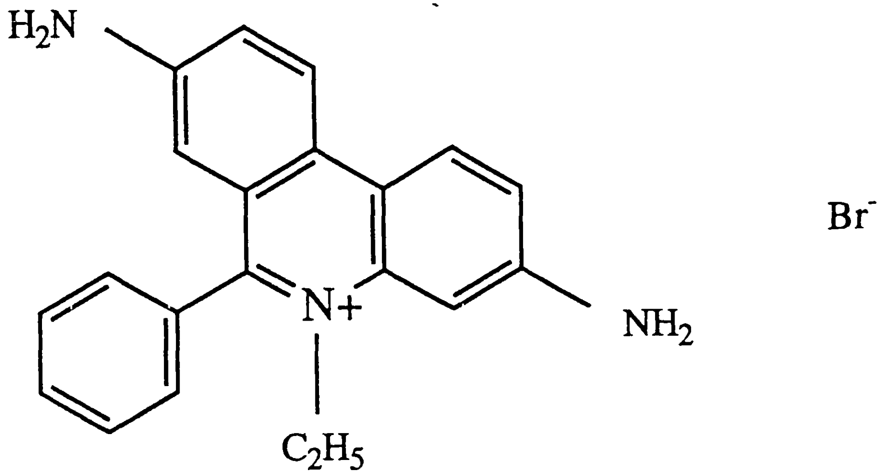


Fig. 1.1(g) Homidium bromide

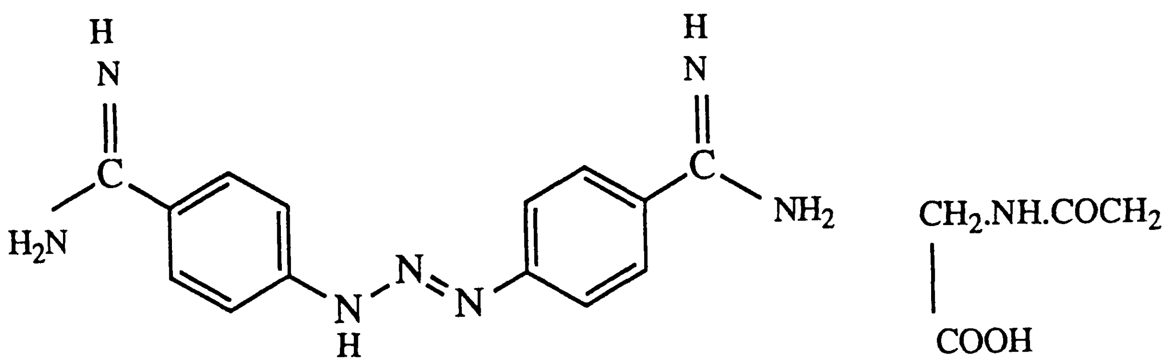


Fig. 1.1(h) Diminazene aceturate

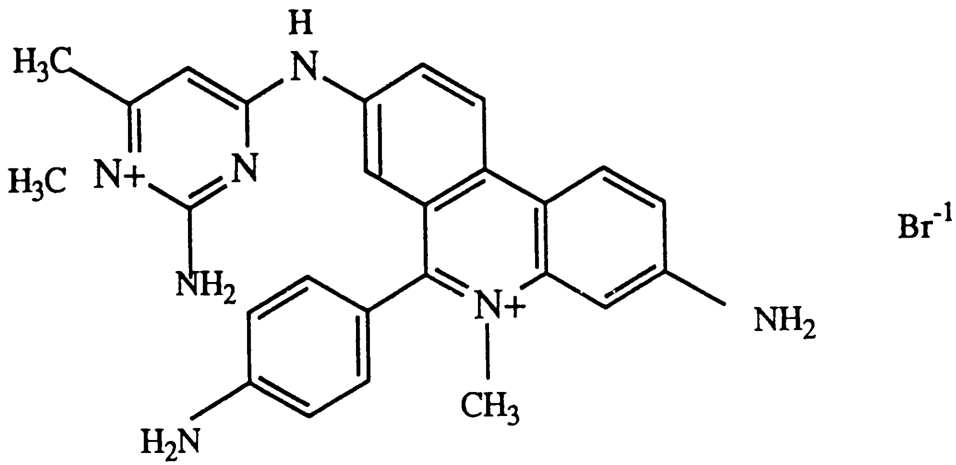


Fig. 1.1(i) Pyrithidium bromide

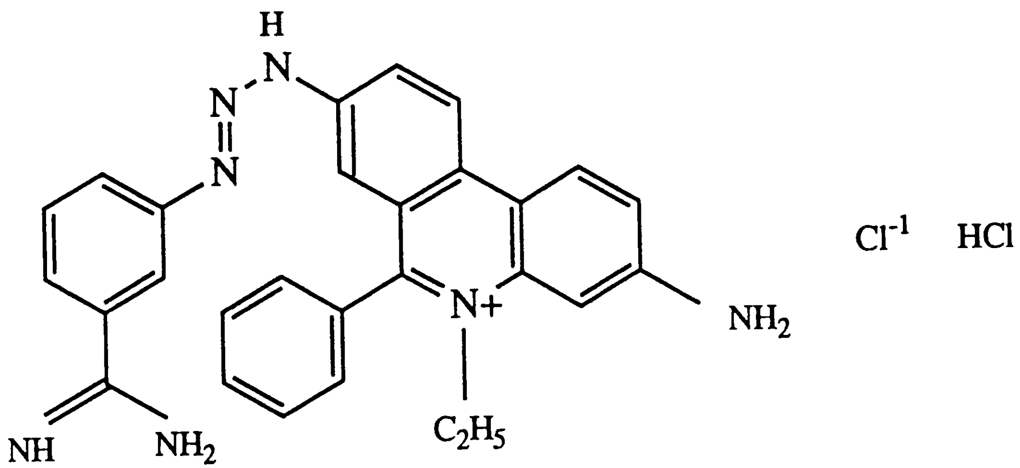


Fig. 1.1(j) Isometamidium chloride

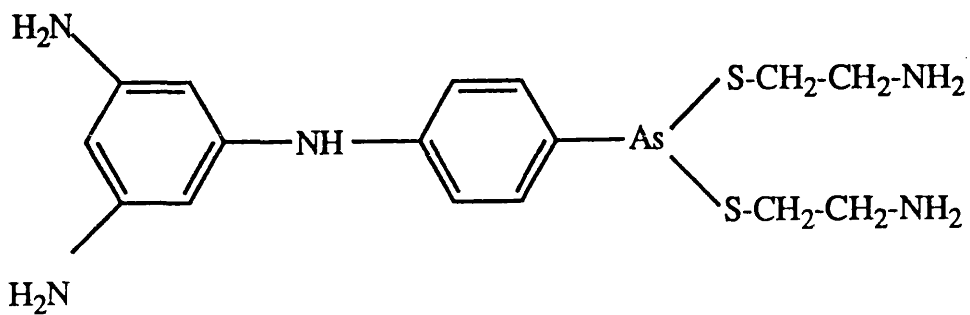


Fig. 1.1(k) Melarsenoxide cysteamine

APPENDIX C

Individual PCV (%) values in untreated control cattle following exposure to natural tsetse challenge

Animal No.	Time post-treatment													
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12	Week 13	Week 14
264	27	26	26	24	21	26	24	26	25	24	20	20	23	22
265	28	27	24	22	26	28	27	23	27	22	25	23	23	24
266	29	31	30	28	26	24	26	25	27	28	30	28	26	24
267	29	30	28	28	19	27	28	29	30	30	31	31	27	26
268	27	30	31	32	27	27	27	25	24	23	23	25	24	28
269	26	27	26	27	21	23	21	27	28	25	26	24	26	25
270	23	24	27	27	28	27	26	29	28	23	25	24	26	28
271	27	29	25	28	25	25	29	29	27	20	23	24	24	27
272	27	27	26	37	24	32	29	31	27	29	31	28	29	30
273	27	27	26	27	22	24	28	18	21	26	26	25	26	25
274	29	26	28	26	26	26	28	29	28	27	26	28	29	28
278	25	23	26	22	21	23	24	21	23	23	20	21	24	20
279	27	25	20	21	23	23	22	21	24	27	27	24	27	26
280	27	30	29	30	25	23	25	22	22	27	26	26	32	28
281	27	25	27	30	28	23	24	28	28	29	25	25	28	26
282	26	24	25	18	24	25	25	24	32	30	25	24	25	25
283	27	27	25	27	24	26	29	31	31	32	31	30	34	27
284	28	28	27	32	26	33	28	30	28	28	30	28	28	27
285	35	37	30	30	34	37	35	31	34	35	32	31	36	31
286	27	22	27	25	25	23	24	20	24	24	22	25	30	15
287	28	28	28	28	28	26	27	32	30	32	29	29	24	30
288	25	30	25	25	15	16	21	12	14	18	23	21	26	23
289	26	26	27	23	23	25	23	24	26	25	25	25	28	26
290	30	30	25	32	29	29	28	29	34	30	30	30	30	29
291	28	35	26	24	26	23	26	28	28	27	25	26	26	24
292	29	29	27	21	19	26	28	20	27	32	27	24	25	26

Individual PCV (%) values in untreated control cattle following exposure to natural tsetse challenge

293	23	23	27	25	26	27	24	19	25	24	26	27	27	23
294	26	29	25	28	25	27	27	28	27	30	26	25	27	25
295	31	31	28	22	25	28	27	28	27	27	27	24	27	25
296	29	30	29	25	23	25	29	31	28	27	29	29	27	31
297	32	27	31	33	28	35	31	26	31	28	31	31	33	28
298	25	24	26	27	22	20	19	23	23	20	23	22	26	22
299	29	32	31	37	30	31	32	32	32	30	30	32	29	29
301	25	27	21	24	24	22	19	21	24	22	19	20	25	29
302	30	35	33	32	37	30	27	28	29	28	29	30	35	33
303	28	29	26	28	29	29	27	27	25	25	28	32	26	25
304	28	26	24	28	25	23	26	25	22	27	24	21	25	23
305	25	23	28	26	25	20	22	24	26	26	25	23	26	26
306	24	24	25	24	24	25	26	32	25	28	23	23	25	24
307	28	29	22	19	23	26	25	21	24	26	27	28	26	27
308	31	33	28	27	26	22	23	25	24	25	24	25	31	26
309	28	24	18	21	25	25	18	19	23	22	16	15	24	21
310	26	21	22	23	28	15	14	24	27	25	23	23	31	23
312	26	26	22	20	21	24	25	10	18	23	21	17	30	25
313	34	30	26	31	27	28	29	30	32	26	33	28	27	29
314	31	30	28	31	28	28	31	30	31	29	35	30	31	28
315	30	32	30	21	24	28	27	30	30	33	32	26	30	29
316	31	35	25	28	29	24	28	29	28	28	31	27	30	29
317	25	27	23	24	23	24	28	27	23	25	24	17	20	18
318	30	31	31	25	26	29	29	31	30	30	28	30	28	31
319	27	31	31	25	28	28	31	26	30	30	27	32	30	30
320	30	33	28	27	24	28	28	29	32	29	29	30	32	30
321	27	26	31	30	28	29	29	30	30	27	28	24	30	26
322	25	26	26	26	25	20	24	26	27	28	28	24	27	25
323	26	24	26	27	23	26	28	22	34	29	26	24	31	28
324	25	24	23	24	26	27	22	23	22	22	23	20	28	25

Individual PCV (%) values in untreated control cattle following exposure to natural tsetse challenge

325	28	25	23	22	26	25	27	14	23	25	25	25	29	24
326	28	27	22	28	27	25	27	24	27	26	26	24	28	24
327	27	28	30	25	25	28	28	27	28	29	27	27	31	27
328	26	26	27	24	24	20	20	22	22	23	26	23	29	24
329	25	23	25	20	21	20	23	24	24	25	23	25	28	25
330	27	28	28	27	14	20	20	24	27	26	22	23	32	29
331	29	33	26	35	31	25	28	28	27	25	27	28	29	27
332	27	26	30	29	28	30	30	30	33	26	25	25	30	30
333	29	27	25	26	23	20	22	29	22	26	24	24	29	32
334	29	33	25	34	34	33	31	27	27	27	27	28	30	25
335	25	27	23	23	25	19	22	22	25	22	22	22	21	25
336	24	29	33	34	34	28	22	28	31	31	27	26	30	26
337	31	24	25	30	19	25	26	25	24	28	26	28	30	26
338	28	36	27	26	23	23	23	27	25	24	25	24	30	26
339	26	33	25	20	24	30	27	29	22	18	21	22	27	24
340	27	25	22	23	24	24	25	27	22	28	24	21	27	22
341	26	22	22	23	20	25	27	27	27	22	27	23	25	23
342	31	32	25	21	28	28	28	27	30	28	26	25	35	26
343	28	28	28	29	28	16	29	26	27	28	28	26	29	30
344	26	27	24	19	23	24	20	18	22	22	21	20	24	23
345	27	30	24	24	19	16	24	30	26	18	25	25	32	29
346	28	24	24	16	20	25	24	26	26	22	23	22	22	21
347	29	32	25	29	30	30	25	24	28	31	25	29	33	27
Mean	27.59	27.97	26.24	26.19	25.09	25.34	25.76	25.63	26.59	26.27	25.95	25.23	27.97	26.05
SD	2.26	3.57	2.94	4.34	3.90	4.13	3.56	4.46	3.69	3.49	3.40	3.59	3.21	3.14

Individual PCV (%) values in cattle following exposure to natural tsetse challenge after treatment with homidium bromide at 1 mg kg^{-1} bw

Animal No.	Time post-treatment													
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12	Week 13	Week 14
176	30	31	24	25	25	25	27	29	29	28	26	26	31	29
177	28	30	30	33	27	29	31	32	31	31	28	28	33	27
178	27	31	29	28	30	30	27	27	26	28	26	25	30	28
179	28	25	28	27	25	28	21	21	22	21	26	26	28	26
180	31	28	27	36	32	33	31	31	31	31	32	29	36	31
181	29	30	29	30	30	27	29	29	28	29	26	28	29	25
182	27	28	29	27	30	26	30	25	14	17	19	19	20	21
184	34	26	29	33	27	30	32	32	30	34	28	28	31	29
185	26	26	28	28	26	27	28	26	27	27	27	25	30	27
186	30	26	27	30	26	27	26	29	28	29	30	23	25	25
187	26	24	28	29	29	21	16	22	23	22	22	16	24	22
188	29	31	25	32	30	31	31	32	30	32	30	26	31	25
190	30	30	30	30	31	33	32	30	31	34	37	28	35	31
191	29	26	27	30	26	28	28	31	28	27	28	26	29	28
192	29	25	27	28	31	30	27	27	31	29	24	26	29	25
193	27	28	28	27	25	25	26	24	27	27	25	24	27	27
194	33	22	31	29	26	27	27	26	28	27	29	30	29	27
195	27	25	25	27	25	24	27	27	28	29	26	27	26	26
196	27	28	25	31	28	26	28	29	30	27	42	27	29	25
198	28	31	29	33	30	31	31	31	33	32	32	32	35	30
202	25	25	26	32	28	29	28	26	27	31	28	26	30	25
203	28	25	30	28	27	25	27	26	29	28	27	25	30	25
204	25	26	27	27	26	25	25	28	25	25	26	23	28	26
205	28	29	26	28	27	27	26	31	30	27	29	26	29	27
206	26	30	27	33	28	26	31	28	28	28	27	25	32	23
207	28	27	28	27	25	26	26	27	27	30	26	25	29	25
208	32	25	31	31	31	30	32	29	32	31	31	32	35	31

Appendix C.2

Individual PCV (%) values in cattle following exposure to natural tsetse challenge after treatment with homidium bromide at 1 mg kg^{-1} bw

209	30	24	26	29	27	27	29	27	27	29	30	25	32	28
210	28	25	26	30	27	27	27	29	28	27	24	22	27	27
211	30	30	30	32	30	28	30	28	35	32	29	29	31	29
212	26	25	24	27	28	27	30	28	30	30	27	27	30	27
213	26	25	26	28	26	26	24	24	27	24	28	28	29	26
214	28	30	28	28	29	27	28	31	30	27	28	28	32	30
215	28	27	28	30	29	30	29	32	31	29	30	28	35	30
216	28	29	29	30	29	28	30	33	30	29	27	25	32	29
217	29	31	30	34	29	34	32	32	34	34	33	32	40	31
218	29	27	28	30	25	26	26	10						
219	28	26	28	28	24	28	26	30	27	29	27	23	28	28
220	29	28	26	28	30	29	30	29	30	26	27	26	30	27
221	27	29	26	35	26	28	30	31	29	29	24	28	32	26
222	25	29	25	32	28	25	29	32	26	31	28	25	29	27
223	29	30	29	29	28	30	29	31	32	34	31	28	33	29
224	25	30	31	32	28	28	29	28	35	33	26	28	32	28
225	24	25	23	29	29	30	28	29	32	28	23	25	30	26
226	26	26	28	27	29	27	28	29	28	28	31	25	30	29
227	27	26	26	28	26	30	26	29	28	29	27	25	31	25
228	24	24	24	24	25	26	27	25	22	23	34	24	26	23
230	25	25	27	27	28	25	27	27	27	27	27	23	30	26
231	26	29	25	27	25	26	28	28	33	33	30	27	29	29
232	27	30	28	27	26	26	27	27	28	26	26	24	28	26
233	28	29	28	29	29	27	26	27	28	30	28	28	29	30
234	24	23	20	23	26	23	27	27	26	25	25	24	24	25
235	29	28	27	29	27	19	23	28	26	27	27	26	28	28
236	27	28	25	28	25	20	27	26	27	29	29	24	31	25
237	33	31	28	32	24	30	32	31	33	31	32	31	35	33
238	31	31	28	33	23	29	30	31	32	34	32	33	35	35
239	25	22	24	25	22	21	23	24	24	25	24	21	27	20

Individual PCV (%) values in cattle following exposure to natural tsetse challenge after treatment with homidium bromide at 1 mg kg^{-1} bw

240	24	25	23	27	27	25	24	26	25	25	24	23	25	25
241	25	25	25	27	27	23	27	25	23	24	25	24	29	29
242	25	25	27	30	28	27	28	21	25	29	30	25	25	27
243	28	30	28	29	30	29	28	27	34	32	25	34	30	28
244	28	28	27	29	28	26	26	29	26	25	25	23	26	25
245	24	23	26	26	29	24	26	26	26	27	26	24	27	27
246	29	26	28	34	28	27	27	26	27	26	23	25	25	26
247	29	29	27	30	28	27	27	27	30	28	27	27	31	27
248	29	27	26	25	29	23	26	28	30	30	28	28	28	30
249	23	19	20	22	21	22	22	24	16	16	19	15	20	20
250	24	24	26	28	26	29	27	31	34	32	28	28	31	26
251	25	27	26	30	26	26	27	29	27	28	26	29	30	27
252	27	29	25	29	30	28	29	31	30	31	32	27	31	28
253	28	22	24	31	25	27	29	28	30	30	26	29	31	28
254	26	27	24	27	28	24	24	23	31	25	26	23	25	25
255	25	27	28	26	22	24	24	27	27	28	27	23	26	26
256	26	25	26	26	26	25	24	26	25	28	24	24	28	26
257	27	25	26	27	27	23	24	25	27	28	24	24	29	25
258	29	30	31	37	35	37	31	32	34	33	33	31	35	29
259	29	31	30	27	29	28	26	31	31	30	27	30	31	27
260	26	26	27	29	29	27	26	29	32	27	28	23	31	29
262	27	28	26	29	29	28	28	26	30	29	25	32	34	27
263	25	24	26	30	22	27	24	30	30	30	32	28	36	28
Mean	27.45	27.03	26.91	29.05	27.34	26.99	27.39	27.81	28.46	28.35	27.61	26.10	29.73	27.00
SD	2.24	2.64	2.22	2.78	2.43	3.00	2.76	3.36	3.65	3.40	3.52	3.33	3.47	2.57

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