STUDIES ON CHEMOPROPHYLAXIS OF TRYPANOSOMIASIS USING A COMPETITIVE ENZYME IMMUNOASSAY FOR THE DETECTION OF ISOMETAMIDIUM AND HOMIDIUM IN CATTLE AND SHEEP

A Thesis submitted for the Degree of Master of Veterinary Medicine in the Faculty of Veterinary Medicine of the University of Glasgow.

by

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Declaration

I declare that the work of this thesis is entirely my own except where acknowledged and confirm that it has not been submitted to this or any other University for award of a degree.

Dedication

I dedicate this thesis to Gillian, Mwamba, Mwiinga, Mulenga and my late Father.

ABBREVIATIONS

ASVEZA	Assistance of Veterinary Services to Zambia
CDI	Carbodiimide
CEIA	Competitive Enzyme Immunosorbent Assay
CP	Crude Protein
CSF	Cerebro-spinal Fluid
CV	Coefficient of Variation
CVRI	Central Veterinary Research Institute
EDTA	Ethylenediamine Tetra-acetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EU	European Union
GE	Gross Energy
HBR	Homidium Bromide
HPLC	High Performance Liquid Chromatography
HRP	Horseradish-peroxidase
IFAT	Indirect Fluorescent Antibody Test
ILRAD	International Laboratory for Research on Animal Diseases
ILRI	International Livestock Research Institute
IM	Intramuscular
ISMM	Isometamidium
NHS	N-hydroxysuccinamide
NBS	Normal Bovine Serum
NRS	Normal Rabbit Serum
ODA	Overseas Development Administration
PBS	Phosphate Buffered Saline
PBSG	Phosphate Buffered Saline with Glucose
PBST	Phosphate Buffered Saline with Tween
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
QC	Quality Controls
RBC	Red Blood Cells
RTTCP	Regional Tsetse and Trypanosomiasis Control Programme
S.E.M	Standard Error of the Mean
SD	Standard Deviation
SRD	Slow Release Device
TMB	3,3',5,5' tetramethylbenzidine

STATISTICS

The Student "t" test for paired samples and Analysis of variance (Anova) were used to determine any variation and differences between the two groups. Values of $P < 0.05$ were considered significant. These tests were conducted using Excel and Minitab computer programmes.

Summary

This thesis describes studies to improve the ELISA method for determining the concentrations of isometamidium and homidium in the serum of treated animals, and the use of the improved ELISA to monitor drug levels in the field and laboratory.

Chapter One is a general review of the relevant literature.

Chapter Two describes experiments that were conducted to improve the competitive enzyme immunoassay for the detection of isometamidium in the sera of treated animals. Improvements were required to this assay to reduce inter-sample variation of sera obtained from untreated animals. The use of a new conjugate isometamidium-horseradish-peroxidase produced using the n-hydroxysuccinamide-carbodiimide linkage and modifications to the assay to a sequential saturation competitive enzyme assay, successfully solved the problem of intersample variation. Following the successfull modifications to the isometamidium detection ELISA similar improvements were made to the homidium detection ELISA.

Chapter Three describes the use of the new competitive enzyme immunoassay to measure the concentrations of isometamidium in the sera of 35 treated cattle reared under traditional management in a field situation in Zambia. Breakthrough trypanosome infections in the individual cattle were monitored and related to the isometamidium concentration levels at the time of trypanosome detection. One of the 10 breakthrough infections occurred when relatively high drug levels were present and this was taken to indicate that the breakthrough infection may have been drug resistant. The work in this

section showed that trypanocide detection ELISAs are a useful tool for determining the drug levels in individual cattle sera and can provide indirect evidence of drug resistant trypanosomes when used in combination with parasitological monitoring methods.

Chapter Four describes the evaluation of the prophylactic effect of a slow release device (SRD) containing homidium bromide. The prophylatic effect of this SRD was compared with that of the normal intramuscular (i.m.) injections of isometamidium and homidium bromide. In the SRD group 50 % of the cattle remained protected up to the fourth month after treatment. In the i.m. isometamidium group 50 % of the cattle were protected up to the third month after treatment whereas in the i.m. homidium 50 % remained protected only up to the second month. The levels of isometamidium and ethidium in the serum reflected these differences in the period of prophylaxis.

Chapter Five describes an experiment to investigate influence of nutrient intake on the duration of isometamidium prophylaxis in sheep. In this experiment groups of sheep on two planes of nutrition were injected with isometamidium (0.5 mg/kg) and then challenged monthly with bloodstream forms of *T. congolense* by subcutaneous injection. The concentration of isometamidium in the sera of the animals declined at a similar rate in both groups and was below the lower limit of detection at the time of the breakthrough infections. The mean body weight and serum albumin levels of the animals on the high plane diet was significantly higher than those of the animals on the low plane diet throughout the experiment period. However the mean periods of prophylaxis were very similar in the high plane and low plane diet groups (121 $\pm$ 14 days and 117 $\pm$ 11 days respectively).

Chapter 1

General introduction and literature review

African Animal Trypanosomiasis

Introduction

Trypanosomiasis is a debilitating and commonly fatal disease of domestic cattle and small ruminants. It occurs in tropical and sub-tropical regions and is caused by infection with the protozoan parasites known as trypanosomes. In sub-Saharan Africa, the disease is spread mainly by the bite of trypanosome-infected tsetse flies. Trypanosomiasis is considered the most important infectious disease holding back the development of livestock production in much of Africa (ILRAD reports, April 1993).

Parasites

Trypanosomiasis is caused by protozoan parasites which are members of the Order Kinetoplastida, Family Trypanosomatidae and Genus *Trypanosoma.* On the basis of the site of development of the organism in the insect vector that transmits them, the genus is subdivided into two sections, the Stercoraria and Salivaria. Trypanosomes (e.g. *T. theileri*) in the Stercoraria section develop (from epimastigotes to infective metatrypanosomes) in the hindgut of the vector following ingestion of a blood meal contaminated with parasites, and infection is transmitted to the host by faecal contamination. Salivarian trypanosomes (*T. vivax, T. congolense, T. brucei brucei*) develop in the mouth parts of the tsetse fly (*Glossina* species) and are transmitted from host to host by inoculation when a fly is having a bloodmeal. In the Salivarian section there are some species that can be mechanically transmitted in the mouth parts of biting Diptera and other arthropods (Leach and Roberts, 1981).

The trypanosomiasis affecting livestock in Africa is mainly caused by *Trypanosoma congolense, T. brucei* species *and T. vivax.* These species of trypanosomes can be differentiated on the basis of their morphology on stained preparations viewed by the light microscope. Stained preparations reveal their general appearance (e.g. size, shape, flagellum, undulating membrane) and some essential internal structures (e.g. nucleus, kinetoplast, basal body) that can be used for speciation (Table 1.1).

Table 1.1 Morphological structure of pathogenic trypanosomes.
(Ref: Mulligan 1970; Hopkins, 1995)

Species	Size	Flagellum	Undulating Membrane	Shape of posterior end	Size of Kinetoplast	Position of Kinetoplast
T. brucei	11-39 μ	in slender form free flagellum	developed	bluntly pointed	small	subterminal and marginal
T. congolense	8- 24 μ	no free flagellum	not developed	round	medium	marginal and subterminal
T. vivax	18-31 μ	free flagellum	slightly developed	swollen and round	large	terminal

Trypanosomes can also be speciated in wet smears based on their movement. *T. vivax* moves fast across the field, *T. brucei* moves within a field in a circular manner and *T. congolense* normally adheres to red blood cells and moves sluggishly.

Based on the distribution in the host, Losos and Ikede (1972) divided the tsetse-transmitted trypanosomes into two groups;

i) Haematic group: these confine themselves to the blood and lymphatic systems. These include *T. vivax and T. congolense.*

ii) Humoral group: those that also invade connective tissue and body cavities. This group include *T. brucei* species.

T. congolense in the early stages of infection is capable of developing outside the circulatory system (Roberts et al., 1969; Luckins and Gray, 1978; 1979).

The distinction of trypanosomes into the above two groups is important for two reasons.

(i) The distribution of trypanosomes in the animal body leads to differences in their susceptibility to drugs (Williamson, 1976); hence identification of the trypanosome species is important for the correct choice of drug treatment.

(ii) In addition to signs like cachexia and anaemia, tissue damage is also a feature of infections from trypanosomes belonging to the subgenus *Trypanozoon.*

Vectors

Tsetse flies (Genus *Glossina*) are found only in sub-Saharan African and infest almost 10 million Km^2 of the continent. The genus *Glossina* consists of 23 species within which three main groups of tsetse are recognised on the basis of their preference for habitat. The groups are: the riverine (*palpalis* group); the forest *(fusca* group); and the savannah *(morsitans* group).

The *palpalis* group consists of *Glossina fuscipes fuscipes, G. f. martinii* and *G. f. quanzensis* (Mulligan, 1970). *G. palpalis* occupies the Congo basin, the basin of the upper Nile and rivers of West Africa.

The *fusca* group may be divided into three sub-groups. The first sub-group include: *Glossina tabaniformis, G. haningtoni* and *G. nashi.* These are confined to the Congo basin and the forests of Gabon, southern Cameroon and the Guinea coast as far west as Liberia. The second sub-group are distributed peripherally around the fly-belts of the

forest group include *G. fusca*, *G. medicorum*, *G. fuscipleuris* and *G. schwetzi*. The third sub-group include *G. longipennis* and *G. brevipalpis*.

The morsitans group include *Glossina morsitans morsitans* and *Glossina m. submorsitans*. They are found in the deciduous woodland and savannah vegetations forming a band around the rain forest of the Congo basin and the Guinea coast in West Africa (Mulligan, 1970).

The groupings simply reflect the need that all tsetse have for a humid environment and protective shade from sunlight, which the leafy vegetation provides. The arid, treeless lands limit tsetse distribution to the north, and the cool climate of increasing latitude limits the southern distribution of the fly (Connor, 1989)

The limit of the tsetse fly distribution on the northern part of the continent is the Sahara desert (15°N) and on the southern part the Kalahari desert (29°S), Natal and the tropical highlands. The individual species distribution within the flybelt is also affected by climatic factors. Climatic factors such as temperature and rainfall determines the type of vegetation to be found in the area. The optimal temperatures for tsetse are around 25°C. Sub-zero temperatures and temperatures of about 46°C will result in the death of the fly (Mulligan, 1970).

Tsetse feed exclusively on blood and transmit the parasites from one host to another during feeding. Parasites undergo a cycle of development and maturation before inoculation into a vertebrate host. If transmission is carried in this way it is called cyclical

transmission. If the hungry fly is interrupted when having a meal of blood, live trypanosomes in the blood present on the fly's mouthparts can be inoculated into a second animal as the fly completes its meal. In this case it is not cyclical transmission but mechanical because no development of the parasites has taken place in the fly. Tabanid flies transmit the parasites by mechanical transmission and no cycle of trypanosome development takes place in these flies.

The methods used for sampling of tsetse flies include:

1. Reconnaissance e.g. Black screen man fly rounds.
2. Traps.
3. Searches for resting flies.
4. Searches for puparia

1. A black screen man fly round involves two men carrying a 'screen' usually a piece of black cotton cloth, rectangular in shape and about 1.2 meters x 0.6 meters, attached along one of its long sides to a pole which is carried between the two men on their shoulders. The black cloth is baited with attractants such as acetone and phenols. The men walk along marked fly round routes and stop at frequent intervals, look about and catch any flies that land on the screen and in their vicinity with a hand-net.

Screens can also be mounted on a vehicle or a bicycle. In the case of a bicycle a screen about 0.6 meters square is hung behind the back wheel. This is covered with banding grease or other suitable slow-drying sticky substance. The bicycle is ridden through suspected areas, after which tsetse may be found stuck to the screen.

The species usually detected by this method are the palpalis group, morsitan group *(G. morsitans* and *G. swynnertoni*) and the fusca group (*G. brevipalpis, G. fuscipleuris* and *G. longipennis*). Males are more attracted to moving objects and therefore are caught more often in fly rounds. It seems their attraction to moving objects brings them into contact with teneral females which are also attracted to moving objects in search of food.

2. Traps are mainly used for studying in detail the distribution and abundance of tsetse flies. Like the black screen they are baited with attractants. They have been used in surveys for *G. pallidipes* and *G. palpalis.* The main advantage of this method is that once in position, traps will be at work throughout the hours of daylight, seven days a week. The other advantage is that they have demonstrated the presence of tsetse when other methods have failed.

Traps are devices into which tsetse flies are attracted and caught. They consists of attractants and a cage designed in such a way that the fly can easily enter but not escape. The first such design was the Harris trap by which very large numbers of *G. pallidipies* were caught in Zululand.

3. Another method used for sampling is to search for flies in their resting sites such as crevices in the bark of big trees, root holes and thickets.

4. Surveys for puparia involves searching the soil in suitable breeding sites by hand. Phelps et al. (1966), attempted to improve yields of puparia by the use of sieves, which proved useful in scooping soil from animal burrows. Another method used by Abedi and Miller (1963), was flotation in water since puparia float to the surface.

Hosts

Trypanosomses are found throughout the world and parasitise fishes, amphibia, reptiles, birds and mammals (Connor, 1989). Table 1.2 below shows the list of the vertebrate hosts of the Salivarian trypanosomes and the mode of transmission (Leach and Roberts, 1981).

Table 1.2 Distribution, Vertebrate Hosts and Mode of Transmission of Salivarian Trypanosomes. (After Leach and Roberts, 1981)

Subgenus	Species	Geographical distribution	Vertebrate host	Transmission	
				cyclical	non-cyclical
Duttonella	*T. vivax*	- West, Central and East Africa	- Wild and domestic ungulates	- *Glossina*	- biting Diptera
		- West Indies, S. America		- none	- biting Diptera
Nannomonas	*T. congolense*	-West, Central, and East Africa	- Ungulates and carnivores	- *Glossina*	- biting Diptera (rare)
	T. simiae	- West, Central and East Africa	- Mainly Suids	- *Glossina*	- biting Diptera
Pyonomonas	*T. suis*	Central Africa	Suids	*Glossina*	not known
Trypanozoon	*T. brucei*	- West, Central and East Africa	- Carnivores (some strains infective to man)	- *Glossina*	- biting Diptera (very rare)
	T. evansi	- North Africa.	- Ungulates and carnivores	- none	- biting Diptera
		- Asia, Central and South America	- Carnivores	- none	- biting Diptera
	T. equiperdum	- North and South Africa, Asia, South America	- Equines	- none	- coitus

Disease

Animal trypanosomiasis is generally known by three names;

i) Nagana in Africa south of the Sahara.

ii) Surra, caused by *T. evansi* in North Africa and Asia.

iii) Dourine, caused by *T. equiperdum*

In wild animals, such as warthog, bushbuck, kudu or buffalo, trypanosomes become established but do not produce disease. This is because these animals and the parasite have evolved together for many years so that a balanced relationship exists. These species remain as reservoirs of the parasite. In domestic animals the host/parasite relationship has not fully developed because they are relative newcomers to the tsetse-infested areas. Among domesticated animals, humpless cattle (e.g. N'dama of West Africa) of *Bos taurus* types were the first to be introduced into northern and western Africa from about 4500 BC onwards, hence they are better adapted to tsetse-transmitted trypanosomes. N'dama cattle will tolerate trypanosomes without showing obvious signs of the disease as long as they are not stressed by factors such as poor nutrition, working, lactation, parturition or other diseases. Humped Zebu (*Bos indicus*) types were introduced some 3000 years later than *Bos taurus* and reached Central and Southern Africa around 700 A.D. Goats and sheep were introduced at the same time as *Bos indicus*. In these animals the host/parasite relationship has not fully developed and these species will develop clinical disease when they are infected with parasites (Connor, 1989).

The course of the disease is variable depending on the factors associated with the host and the parasites. Generally it is characterised by the intermittent presence of parasites in

the blood and intermittent fever. The course of the infection may be acute, sub-acute or chronic. In acute cases animals develop anaemia rapidly and lose weight and body condition over a few weeks. They may die if not treated. Sub-acute or chronic trypanosomiasis runs a course of several months to one year or longer. The common sign of infection during this form, is progressive emaciation, poor general condition, lymphnode enlargement, anaemia and weakness. Animals may die or recover on their own (self-cure).

Mammalian hosts may posses natural resistance to particular species of trypanosomes. Pigs are refractory to infection with *T. vivax* (Stephen, 1966) and cattle are not likely to be infected with *T. simiae* (Roberts, 1971).

The pathology of trypanosomiasis is primarily the result of anaemia, immunological disturbance and generalised tissue damage, which produce different effects in mature and immature animals (Table 1.3).

Table 1.3 Effect of trypanosomiasis in mature and immature animals. (Connor, 1989).

	Effects
Breeding male	Semen quality and libido reduced.
Working Ox	Draught power reduced.
Breeding female	- Oestrus cycles become irregular. - Abortion is common. - Birth weights are low and calves sickly. - Weak calves result in higher neonatal mortality rates.
Young stock	- Growth rates are reduced. - Frequently stunted - More vulnerable to other diseases. - General failure to thrive.

There are indications that young animals may be less susceptible to trypanosomiasis than adults (Fiennes et al., 1946).

Diagnosis

The tests used for diagnosing African animal trypanosomiasis are generally put into two categories (Table 1.4); direct and indirect tests. Direct tests depend upon the demonstration of the trypanosomes in the peripheral blood or lymph. Indirect tests identify the product of the trypanosomes or antibody produced by the host as a specific response to the presence of the parasite or its by-products. Indirect methods are of value if the parasites are too few to be seen directly.

Clinical diagnosis

Diagnosis of trypanosomiasis can also be based on the signs that are associated with the disease. The disadvantage of this method is that these signs are not indicative of trypanosomiasis alone. For example, malnutrition and intestinal helminthiasis will normally show the same clinical signs as animals suffering from chronic trypanosomiasis.

Table 1.4 The direct and indirect tests used for diagnosing trypanosomiasis. (Hopkins, 1995)

Direct tests		Indirect tests			
Trypanosomes not concentrated	**Trypanosomes concentrated**	**Detection of antigen**	**Detection of anti-trypanosomal antibodies**	**Detection of trypanosomes**	**Detection of chromosomes**
1. Wet films of blood or lymph. 2. Giemsa stained thick and thin smears. 3. Tail tip capillary wet blood smear.	1. Intact capillary (Woo technique) 2. Buffy coat wet smear.	1. Antigen trapping ELISA.	1. Antibody trapping ELISA. 2. Indirect Fluorescent Antibody test (IFAT).	1. In vivo cultivation by inoculation of the suspect blood into rats or mice.	1. polymerase chain reaction (PCR)

Parasitological methods (Direct tests)

As mentioned above these tests depend on demonstrating the presence of the infecting organism. This is done by the examination of a sample of blood or other body fluids or tissues.

a. Wet blood film

A drop of fresh peripheral blood obtained from an ear vein or jugular vein is placed on a microscope slide and covered with a coverslip. This is examined under the microscope using x400 magnification. The trypanosomes are detected by their movement among the blood cells.

This method provides a quick and convenient means of detecting the presence of trypanosomes.

b. Wet film of lymph or cerebro-spinal fluid (CSF)

In this method lymph juice from a lymph node or CSF is placed on the slide and covered with a coverslip and examined as in the wet blood film. The disadvantage of this method is that it is difficult to obtain samples from animals, especially cattle. This method is useful mainly in the diagnosis of *T. brucei gambiense* in man.

c. Thin blood smears

A drop of flesh blood is placed on a slide which is placed horizontally on a table. Using another slide (spreader) placed at 45° to the one on the table, the drop of blood is spread into a thin layer of blood. This is dried by waving the slide in the air. After drying the slide is fixed in methanol and stained with Giemsa stain.

Thin blood films preserve the morphology of the trypanosomes and hence are useful in morphological differentiation of species.

d. Thick blood smears

Thick blood smears are prepared by putting 2-3 drops of blood onto a slide and spreading it to an area of about 1 cm in diameter with a corner of a slide. To avoid the blood smear floating off the slide during staining it is advisable to gently heat the slide during drying for about 2-3 hours at 37°C, or leaving the slide at 37°C overnight (Shute and Maryon, 1966). After drying, the slide without fixing, is stained in Giemsa stain. Lysis of the erythrocytes will occur during staining if the slide is not fixed. This makes it possible to examine a much thicker smear.

Thick smears are useful for the detection of scanty trypanosomes.

e. Intact capillary (Concentration, Woo technique)

Blood in a capillary tube is centrifuged in a micro-haematocrit centrifuge for 5 minutes. Without breaking the tube it is examined for trypanosomes at the cell/plasma interface or white layer (buffy coat) in the intact capillary (Woo and Rogers, 1974).

f. Buffy coat wet smear (Concentration technique)

After the capillary tube is centrifuged in the micro-haematocrit centrifuge, the tube is broken just a millimetre below the cell/plasma interface. A drop of the cell/plasma mixture i.e. the buffy coat and a few red blood cells are placed on the slide, covered with a coverslip and examined for trypanosomes under the microscope.

The buffy coat technique is capable of detecting trypanosomes at about 100 trypanosomes per 1 ml of blood (Woo and Rogers, 1974).

g. Rodent inoculation

Blood from a suspected animal is mixed with phosphate buffered saline glucose, pH 8.0 and inoculated into rodents intraperitoneally. The development of infection in the rodents, usually mice, is monitored in blood samples obtained by tail venesection. For *T. brucei brucei* and *T. brucei rhodesiense* this method is much more sensitive than examination of thick and thin films (Baker, Sachs and Laufer, 1967).

The rodent inoculation technique is useful when the parasitemia is scanty. One disadvantage of this technique is that some species such as *T. vivax* are not rodent infective.

Sero-immunological techniques (Indirect tests)

These tests identify the product of the trypanosomes or antibody produced by the host as a specific response to the presence of the parasite or its by-product.

a. Indirect Fluorescent Antibody Test (IFAT)

The fixed trypanosome antigen is prepared in suspension in a mixture of 80% cold acetone and 0.25% formalin (Katende et al., 1987), and used to coat the immunofluorescent slide. Test serum is added to the coated slide. The fixed trypanosomes on the slide detect antibodies in the serum samples. The test measures directly the interaction between the antigen and antibody. The preparation is examined under the UV microscope.

b. Sandwich ELISA technique for antibody and antigen detection

In the antibody detection ELISA, trypanosome antigen is adsorbed onto polystyrene microwells that capture the circulating antibodies in test serum samples.

The disadvantage of the antibody technique is that the detected antibodies persist in the animal blood for a long time after the trypanosomes have been cleared. Because of this it is difficult to differentiate a present infection from a past infection (Luckins et al., 1979)

The technical basis of the antigen detection ELISA is that the monoclonal antibody is used to coat micro-plates or polystyrene tubes. Test serum is added to the coated plates. The antigen in the serum is captured or trapped by the coated antibody. A second antibody, which is enzyme-labelled, is added and the labelled antibody will bind to the free combining sites of the captured antigen. A suitable substrate and chromogen are added and the colour change is detected visually or by spectrophotometer (Trypanosomiasis ELISA kit, Bench Protocol, Version - TRP 1.1, 1994).

c. Polymerase Chain Reaction technique (PCR)

The technique is used to amplify specific regions of an organism's DNA to levels where the DNA fragment can be identified. The amount of DNA is easily seen after staining with DNA-binding reagent when the reaction products are separated by electrophoresis in a gel.

The disadvantage of this method is the use of radioactive chemicals (ILRAD reports, October 1992).

Economic Impact of Trypanosomiasis

Cattle are highly valued for the draught power and the manure which they provide in rural areas. To a rural farmer they are a sign of wealth and represent investment and because of this they are slaughtered only for important occasion such as social ceremonies (e.g. initiation ceremonies, traditional weddings). They are also slaughtered when farmers want to realise large sums of money from the sale of meat to pay for major costs such as school fees. In most rural areas, people depend on small ruminants and poultry as a source of protein (Connor, 1989).

Direct losses caused by trypanosomiasis are due to the presence of the disease in the livestock population. They include production and reproduction losses resulting from mortality, morbidity and infertility and the cost of implementing and running trypanosomiasis control operations. The annual costs of the disease, including both production losses and control costs, are estimated to be more than US$ 500 million per annum (ILRAD, 1993).

Indirect losses are due to the risk of the disease. They include exclusion from tsetse-infested areas, reduced livestock production levels due to restricted grazing, and reduced crop production due to exclusion or limitation of draught power.

Owing to the impact that trypanosomiasis has on the rural economy there is a need to control this disease. However without the implementation of carefully planned rural development, there is a real risk that human activities and increased livestock numbers, in excess of the land's carrying capacity, will lead to irreversible degradation of the

environment. In view of this, tsetse and trypanosomiasis control activities must be done in conjunction with land use planning.

Trypanosomiasis control

Introduction

Methods of controlling trypanosomiasis may be aimed at the insect vectors or at the parasites in domestic animals.

Vector control

a. Biological control

Clearing of vegetation and game elimination

Old methods of tsetse control that had been used with varying degree of success include modification of the vegetation cover by either selective (partial clearing) or complete (total clearing) felling of trees and shrubs. By doing this the tsetse are denied a suitable habitat. Another method was the destruction (shooting) of larger wild game on which tsetse flies depend for their food. In Rhodesia (now Zimbabwe) this method proved to be successful in the 1930s (Mulligan, 1970) as large areas of the country were freed from tsetse by shooting large wild animals in the Zambesi basin. One disadvantage of the game and vegetation destruction methods is that it provoked strong opposition from conservationists.

Sterile insect technique

This is a recent concept in entomology which involves the release of large numbers of captive-bred male tsetse, made infertile by exposure to calculated dose of gamma

radiation, into an indigenous target population. The population into which the sterile males are released, must have been reduced by other methods, to levels at which sterile males can successfully compete with the wild non-sterile males. Knipling (1963) estimated that an initial 3:1 release ratio of sterile to wild males, followed by a gradually decreasing release rate could eradicate a low-density population of flies in twelve months. The concept behind this method is that all wild females mated by the sterile males will fail to breed. The desired minimum mating ratio is 1 male to each of 4 females (Dame and Ford, 1968).

Knowledge of seasonal fluctuations in feeding, dispersal, mating behaviour, reproductive status, birth and death rates and length of life of the target species is important. This helps in evaluating the fluctuations in density and reproductive viability of the target population. It also helps in estimating the relative viability of the released sterile males (Mulligan, 1970).

b. Chemical control

Methods of applying insecticides fall into two groups: (1) residual treatments applied to vegetation, food, traps and pupation sites, and (2) non-residual treatments applied by aircraft, chemical smoke generation and ground-based fogging machines.

Residual treatment

This method became available with the introduction of organo-chlorine insecticides. The only use for insecticides against tsetse before 1946 was the use of pyrethrum sprays in de-flying chambers (Mulligan, 1970). In the 1950s DDT and dieldrin were used in

ground spraying to reduce tsetse fly populations by spraying tree trunks and low branches where tsetse rest during the hottest and brightest hours of the day.

Non-residual treatment

Under this method, ultra-low-volumes of endosulphan, an organo-chloride compound with low residual effects are sequentially aerially sprayed on the infested areas using fixed-wing aircraft in open country habitat of such species as *G. morsitans*. In closed vegetation, helicopters are favoured for spraying against riverine species.

The aim of the sequential aerial spraying is to kill all adult flies with the first spray and then the new flies emerging from puparia in the four to five subsequent sprays.

The high cost and expertise needed are some of the disadvantages of this method.

Traps and targets

Traps and targets probably provide the best method of vector control for most situations. Currently targets are the method of choice for controlling tsetse in most countries.

Traps were first used for controlling tsetse by Harris (1930) in Zululand. Large numbers of *G. pallidipes* were caught using his own designed Harris trap. In 1970s serious interest in the use of traps was revived. The introduction of host odours to attract tsetse increased the efficiency of traps by up to 30 times. Trapping 4% of the population of adult females each day will reduce an isolated tsetse population by 99% in six months.

Targets are sheets of cloth usually black or blue sometimes with netting attached, which are impregnated with insecticides such as deltamethrin. To attract flies they are baited with animal odours such as acetone, phenols and octanol. Flies that make contact with this cloth or land on it, pick up a lethal dose of insecticide.

The Regional Tsetse and Trypanosomiasis Control Programme (RTTCP) a regional programme covering, Malawi, Mozambique, Zambia and Zimbabwe is trying to use this method to control tsetse fly in the common flybelt of these countries. The programme is using a density of 4 targets per square kilometre.

Cattle sprayed with insecticides are sometimes used as mobile targets. In 1984 trials began in Zimbabwe to test the efficacy of the pyrethroid deltamethrin ("Decatix", Cooper (Zimbabwe) LTD) and since then has been widely used to control tsetse in that country. The insecticide is used both as a dipwash or pour-on.

Trypanotolerant Livestock

Trypanotolerant cattle such as the N'dama can be reared in infested areas. This breed can tolerate levels of infection with trypanosomiasis that other breeds can not withstand. Chemotherapy is used to treat the few cases that occur. However these breeds are largely restricted to West Africa.

Parasite control using drugs

a. History of the trypanocidal drugs

Potassium antimony tartrate (tartar emetic)

The history of trypanocides date as far back as the early part of this century. The first active substances found were dyes and later arsenical and antimonal compounds. Plimmer and Thompson (1908) tested potassium antimony tartrate (tartar emetic) in laboratory rodents and showed that *T. brucei* and *T. evansi* could be eliminated. Bevan (1928) and Curson (1928) confirmed the efficacy of the use of the tartar emetic against *T. congolense* and *T. vivax* infection in cattle.

The use of the tartar emetic continued until the early fifties when it was discontinued because of high mortality.

Suramin

Suramin, a synthetic compound was the next to be developed in Germany during the 1914-18 war, but only became available in 1920. Knowles (1925) experimentally showed its efficacy against *T. equiperdum* infections. He also showed that naturally occurring *T. evansi* infections in camels could be treated with this drug.

Suramin continues to be used to date.

Antimosan

As a result of continued research on potassium antimony tartrate, antimosan, an analogous sulfonated pyrocatechol derived from trivalent antimony was developed and

Parkin (1931, 1935 b) found it to be effective against *T. congolense* and *T. vivax* but less effective against *T. brucei.*

Styrylquinolines

These were demonstrated by Browning et al., (1926) to be active trypanocides against organisms of *Trypanozoon* subgenus. However they did not act rapidly on trypanosomes and were systemically toxic. They also produced serious reaction at the site of injection.

Surfen C

Surfen C, a 4-aminoquinoline derivative was reported by Jensch (1937) to have marked activity against *T. congolense*, but in field trials in Africa it produced unacceptable toxic reactions.

Phenanthridines

Phenidium chloride a phenanthridine was reported by Browning et al (1938) to be active against the T*rypanozoon* subgenus. The drawback with this drug was its low solubility and narrow therapeutic index.

Dimidium bromide, another phenanthridine, which was readily soluble was shown by Carmichael and Bell (1944) to be active against *T. congolense* in the field. This drug had its own disadvantages in that it produced photosensitisation and local reactions (Evans, 1948). Drug resistance had also become widespread by 1952.

Quinapyramine

As a result of restructuring the surfen C molecule, a bis-quarternary salt (quinapyramine dimethosulfate) was synthesized by Barrett et al (1953) and was demonstrated experimentally by Curd and Davey (1949) to be active against trypanosomes pathogenic to livestock in Africa. Its efficacy was also demonstrated in the field by Davey (1950). However in Equidae this drug caused severe systemic reactions.

A mixture of quinapyramine dimethosulfate (3 parts) and the insoluble quinapyramine chloride (4 parts) was introduced as a prophylactic.

Serious drug resistance to quinapyramine soon emerged and it is no longer in use.

Homidium

Homidium bromide (an aminophenanthridium compound) was developed from dimidium bromide and was reported by Warkins and Woolfe (1952) to be effective against *T. congolense* and *T. vivax* at 1 mg/kg body weight. This drug was less toxic (Ford et al., 1953 a.b) than dimidium bromide.

As a result of the availability of homidium and quinapyramine dimethosulphate, mass treatment of cattle in contact with tsetse was made possible as demonstrated in Northern Nigeria where 641,000 cattle were treated in 1957-58 as compared with only 45,000 treated in 1951-52 (Mulligan, 1970). Resistant strains of *T. congolense* began to be identified and by 1966 Jones-Davies and Folkers reported widespread resistance. Despite this problem the drug, generally known as Ethidium®, is still used in many countries.

Diminazene aceturate

Fussganger (1955) reported trials with a new aromatic diamidine by the name of diminazene aceturate (marketed as Berenil®). A dose of 3.5 mg/kg body weight was reported to cure *T. congolense* and *T. vivax* infection. A much higher dose was required to treat *T. brucei* infections.

It had the added advantages of having a wide therapeutic index and being babesicidal. MacLennan (1968) reported that it virtually replaced both quinapyramine and homidium in Northern Nigeria.

Pyrithidium bromide

Pyrithidium bromide, synthesised by substituting the pyrimidyl moiety of quinapyramine into a phenanthridine resembling phenidium was reported by Watkins and Woolfe (1952). This was withdrawn, in 1985 (Connor, 1989), due to the emergence of drug resistance.

Isometamidium

The next drug to be marketed was isometamidium. It was synthesised from homidium as a compound named metamidium, (Wragg et al.,1958). Metamidium consisted of four isomers. The active component that was isolated in pure form (Berg, 1963) from metamidium was marketed as Samorin®

Melarsomine

Melarsomine (Raynaud et al., 1989) is the most recently introduced trypanocide used in domestic livestock. This was synthesised by combining melasen oxide with cysteamine in 1985 by E. Friedheim. It is active against members of the *Trypanozoon* subgenus (Raynaud et al., 1989), particularly *T. evansi.* It is marketed as Cymelarsen®.

The search for new drugs for controlling animal trypnosomiasis has been limited by the high cost of screening, development and production, and the anticipated low levels of profit from marketing trypanocides as the disease affects the poorest nations.

b. Classification of drugs

Drugs can be classified according to their chemical structure (Table 1.5.) and properties or various aspects of their biological activities (Mulligan, 1970).

Table 1.5 Characteristics of drugs used for the treatment and prophylaxis of Animal Trypanosomiasis. C = curative, P = prophylactic, i.m. = intramuscular, i.v. = intravenous, s.c. = subcutaneous, Dose is in milligrams per kilogram of body weight

(Leach and Roberts, 1981)

Drug showing chemical grouping	**Type**	**Animal**	**Field use infection**	**Dose**	**Toxic effects**	**Comments**
Homidium bromide and homidium chloride **(phenanthridine)**	C	Cattle, small ruminants, horses, pigs	*T. conglense* *T. vivax*	1.0 i.m.	Local tissue damage if given s.c. ; liver damage when given at high dosage	The bromide is soluble only in hot water, whereas the chloride is soluble in cold water. Either salt can be used alternatively with diminazene aceturate to reduce the incidence of drug resistance.
Diminazene aceturate **(aromatic diamidine)**	C	Cattle, small ruminants, dogs	*T. conglense* *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 i.m.	Similar to those of homidium.	The drug is less toxic than homidium. It is soluble in cold water and is heat sensitive. Prophylaxis lasts 2-4 months, depending on the challenge. The dose should be determined by the intensity of challenge. Higher doses may be given for drug resistant infections.
	C	Cattle	*T. congolense* *T. vivax*	0.25-1.0 i.m.		

c. Mode of drug action

Trypanocides currently used to control tsetse-transmitted trypanosomiasis differ in their mode of action.

Diminazene aceturate

Diminazene aceturate is an odourless, yellow powder with a solubility of 1:14 in water at 20°C and slightly soluble in alcohol, ether and chloroform. A dose of 3.5 mg/kg body weight given intramuscularly will eliminate infections with *T. congolense* and *T.vivax* but 5 mg/kg is required to eliminate *T. brucei* infections (Fussganger and Bauer, 1960). It is also active against *Babesia* species in cattle, sheep, horses and dogs (Kuttler,1981).

Diminazene aceturate is a curative drug and after injection is rapidly metabolised and excreted through the kidney. This characteristic of quick clearance, reduces the risk of resistance developing through prolonged exposure of parasites to sub-lethal levels of the drug.

Diminazene aceturate has selective inhibitory action on kinetoplast DNA synthesis (Newton and Le Page, 1967, 1968).

Homidium

Homidium bromide and homidium chloride belong to the phenanthridine class and are equally active *in vivo* (Leach and Roberts, 1981). At a dose rate of 1 mg/kg body weight given intramuscularly, they are both used as therapeutic agents against *T. congolense* and *T. brucei.*

Though eliminated rapidly from the body, its elimination is slower than diminazene and because of this, it has some prophylactic activity varying from 2 to 19 weeks against field challenge (Leach et al., 1955; Dolan et al., 1990, 1992).

Homidium blocks the synthesis of nucleic acid through intercalation between DNA base pairs, inhibition of RNA polymerase and DNA polymerase and incorporation of nucleic acid precursors into DNA and RNA (Kinabo, 1993).

Isometamidium

Isometamidium chloride is a phenanthridine-aromatic amidine marketed as both a therapeutic and prophylactic agent. The recommended dose range for prophylactic purposes is 0.5-1.0 mg/kg body weight. A dose rate of 1.0 mg/kg body weight confers prophylaxis to cattle for 2-22 weeks (Kirkby,1964; Pinder and Authie, 1984; Whitelaw et al., 1986; Peregrine et al., 1991). Variation in drug susceptibility between different trypanosome populations appears to be the major factor determining the duration of prophylaxis (Peregrine et al., 1991).

After intramuscular injection a local tissue reaction occurs at the injection site. This encapsulates the drug and over a period of weeks the drug leaks into the blood stream to provide protection. The mode of action on trypanosomes is similar to the one described for homidium i.e. blockage of nucleic acid synthesis.

d. Drug resistance

Introduction

The appearance of resistant strains of trypanosomes has been associated with the extensive and prolonged usage of the few drugs (diminazene, homidium, isometamidium, quinapyramine and suramin) available on the market. Because of this some of the drugs such as quinapyramine have been withdrawn (Ndoutamia et al., 1993).

Natural resistance

"Natural resistance of a trypanosome strain or species to a drug is that variation in drug sensitivity shown by trypanosomes that is not dependent on previous exposure to the drug concerned" (Leach and Roberts, 1981). For example at a recommended dose rate homidium is more effective against *T. vivax* than against *T. congolense*. In this situation *T. congolense* will show a higher rate of relapse infection than *T. vivax* (Leach and Roberts, 1981).

Development of resistance

Under-dosing is thought to be the main cause of drug resistance in the field. Under-dosing occurs when the concentration of trypanocides in the animal body is below the curative level.

Under-dosing can happen in several ways (Whiteside, 1961):

i) Through incorrect estimation of body weight or incorrect dosage when large numbers of animals are involved.

ii) Irregular treatment or termination of treatment while animals are still at risk.

Cross-resistance

The similarities in chemical structure between drugs account for some of the factors that favours development of cross-resistance (Mulligan, 1970). The modification of common receptor sites on trypanosomes during development of resistance may be the important factor (Mulligan, 1970). The pattern of cross-resistance as analysed by Whiteside, 1961 is as shown below (Table 1.6).

Table 1.6
Cross-resistance patterns of the drugs used in cattle trypanosomiasis. (after Leach and Roberts, 1981)

	Response of trypanosomes to:				
Trypanosomes resistant to	Quinapyramine	Homidium	Prothidium	Metamidium	Diminazene
Quinapyramine	R	++	+	+	++
Homidium	+	R	+	+	0
Prothidium	+	++	R	0	?
Metamidium	+	++	+	R	0
Diminazene	0	0	0	0	R

R : direct resistance.
+ : cross-resistance to curative dose
++ : cross-resistance to higher dose
0 : no cross-resistance

As a result of cross-resistance analysis, Whiteside (1960a) developed the concept of "sanative" pairs of drugs. A "sanative" pair being that pair of curative drugs which do not induce resistance to each other when used alternatively. Homidium and diminazene and isometamidium and diminazene are examples of two such sanative pairs.

Methods used to identify drug resistance

(a) Field data

It is possible to distinguish the incidence of new infections by collecting parasitological data once every two weeks from sentinel animals treated with only diminazene aceturate.

(b) Large animals

Large animals can be used to characterise the drug-resistance phenotype of trypanosome populations (Gray and Roberts, 1971, Codjia et al., 1993). Field isolates are inoculated into large animals (cattle or goats) under laboratory conditions. At the peak of parasitaemia the animals are treated with a drug and monitored for relapse infection for 180 days. The advantage of this is that data generated in this way is directly applicable to the field. The disadvantage is the purchase and maintenance of large animals is very expensive. It also takes up to six months to have definitive data (Peregrine, 1994). In addition large numbers of isolates cannot be screened.

(c) Laboratory animals

Mice and rats are normally used in conventional drug sensitivity tests. Mice are infected with approximately 1 x 10^5 trypanosomes by intraperitoneal inoculation. Drug is administered at the first detection of trypanosomes. The mice are then monitored for 60 to 90 days for relapse.They have the advantage over large animals in that they are not as expensive to purchase and maintain. However results obtained from rodent experiments may not be highly predictive for cattle (Sones et al, 1988). It also takes about 30-90 days

to confirm permanent cure (Sones et al., 1988) and not all field isolates grow in laboratory rodents (Hawking, 1963).

(d) In Vitro cultivation

The use of in *vitro* systems (Borowy et al., 1985) or the use of both *in vitro* and in vivo as in the drug incubation infectivity test (DIIT) (Kaminsky et al., 1990, 1993) may be used for characterising the drug sensitivity of trypanosome populations. However, using this test it is not possible to screen large numbers of isolates and prediction of the drug sensitivity of the trypanosome population in the host using data obtained *in vitro* is not yet possible (Kaminsky, 1990).

(e) Use of assays to determine the concentration levels of drug in sera

The use of assays that quantify the concentrations of the drug in the sera in combination with a trypanosomiasis diagnostic test can be used as an indirect method for the detection of drug resistance (Peregrine, 1994). The assay that will be able to rapidly quantify the concentration of the drug in large numbers of samples will be the assay of choice.

Drug detection assays

Several assays have been previously described for the detection of trypanocidal drugs (Philips et al., 1967; Perschke and Vollner, 1985; Kinabo and Bogan, 1988) but all have serious shortcomings.

(a) The High Performance Liquid Chromatography (HPLC) as described by Perschke and Vollner (1985) is used to analyse trypanocide drugs in sera or plasma of treated animals. The drugs that can be detected by this method include, homidium bromide, homidium chloride, isometamidium chloride, quinapyramine chloride and quinapyramine sulphate.

The steps followed in this assay are: separation of plasma from blood cells by centrifugation, pre-concentration on small silica gel columns, elution with acidified solvent and finally HPLC measurement with UV or fluorescence detection.

A large volume of serum, up to 10 ml is used in the assay. The lower detection limits are 5 ng/ml serum for homidium, 3 ng/ml serum for quinapyramine and 20 ng/ml for isometamidium.

The disadvantage of this method is that it requires large volumes of samples and because the assay is based on acidic cleavage of isometamidium to homidium, the test will not distinguish between homidium and samorin if homidium is in the sample (Perschke and Vollner, 1985).

(b) A radio-immunoassay method for isometamidium (ISSM) measurement was described by Kinabo and Bogan (1988). The assay used antibodies raised in sheep immunised against the drug cross-linked to human serum albumin. The radiolabelled isometamidium (^{14}C-ISMM) was used as a radioligand. Using this test the limit of detection of isometamidium in serum without prior extraction was 5.8 pmol/tube.

The limitations of this assay was that it was not very sensitive and it required the use of radioactive drug.

(c) Solid-phase extraction and ion-pair reversed-phase HPLC for analysing isometamidium in serum and tissues was also developed by Kinabo and Bogan (1988) and involved the hydrolysis of samples enzymatically and cleaning on a solid-phase system (C8 Bond Elute column). After this step the drug was chromatographed by an ion-pair reversed-phase technique using heptane sulphonate as a paired-ion and triethylamine as a counter-ion reagent. Detection was by fluorescence at 593nm. The lower limit of detection as demonstrated by Kinabo and Bogan (1988) was 10 ng/ml of serum and 500 ng/g of tissue.

(d) An enzyme-linked immunosorbent assay (ELISA) for the detection of isometamidium in bovine serum was developed and validated by Eisler et al. (1993). In this assay the liquid-phase isometamidium (isometamidium in the sample) competes with solid-phase bound isometamidium-protein conjugate for biotinylated sheep anti-isometamidium IgG. The specific IgG is detected by streptavidin-peroxidase using tetramethylbenzidine for colour development. The more the colour development the less the free isometamidium in the serum of the treated cattle. Calibration of the assay is done by four-parameter logistic curve-fitting. The same procedure is followed for the homidium ELISA assay.
The lower limit of detection of isometamidium is about 0.1 ng/ml of serum.
The assay is the most precise, sensitive and reproducible technique that can be used for the quantification of the drug in bovine sera. The assay may be used in the development of rational chemoprophylactic drug regimens and as an indirect method for detecting isometamidium resistance in the field (Eisler et al., 1993).

Trypanocidal delivery systems

Introduction

Several of the few trypanocides that are currently used to control trypanosomiasis in domestic animals, produce local toxicity at injection sites (Peregrine, 1994). Furthermore many of these drugs only offer short periods of prophylaxis. In order to prolong the prophylactic activity and reduce local toxicity at the injection sites, different formulations (carrier or delivery systems) have been produced and evaluated. These include: suramin complexes, dextran complexes, liposomal formulations, carrier erythrocytes and homidium and isometamidium polymers (Peregrine, 1994).

Suramin complexes (suraminates)

At normal physiological pH, suramin is an anion whereas diminazene, homidium, isometamidium and quinapyramine are cations. When either of these cationic drugs is mixed with suramin a precipitation (suramin complex) is formed. Williamson (Mulligan,1970) formulated complexes in this way and showed that there was considerable reduction in the toxicity of the cationic drug component for rats by a factor varying from 3x to 33x. In the first prophylactic trial in cattle Desowitz, (1957) observed similar reductions in toxicity. "Depot" formulation at the injection site enhanced prophylactic activity in small animals. In cattle homidium-suramin complexes offered the longest period of protection. After a single dose of 10 mg/kg of homidium-suramin complex, a minimal period of protection of thirteen months was obtained in small numbers of experimental cattle (Desowitz, 1957). The use of this complex was not recommended as a result of the reaction at the injection site if given by the subcutaneous

route in cattle (Stephen, 1958). A dose rate of 40 mg/kg body weight of quinapyramine-suramin-complex was recommended for *T. simiae* infection in pigs (Stephen, 1966). However there have been no recent reports of the use of suramin-complexes.

Dextran complexes

This delivery system involves complexing trypanocides with dextran sulphate (molecular weight 500 000). James (1978) and Aliu and Chineme (1980) complexed isometamidium with dextran sulphate and in their studies they found that the toxicity of the drug for rodents was greatly reduced. No definitive conclusion can be made about the prophylactic activity of the complexes because the activities varied greatly between the studies (Peregrine, 1994).

Liposomal formulation

Liposomes are small phospholipid vesicles used as carriers of therapeutic agents (McKellar, 1994). In order to reduce local toxicity and prolong the prophylactic effect of diminazene, homidium and isometamidium, Fluck and Hopkins (1987) produced multi-lameller liposomes into which the drugs were incorporated. They evaluated these formulations in cattle in the field and observed a small increase in the prophylactic effect of diminazene and homidium. Local toxicity was reduced when the drugs were administered in these formulations. Standardisation of these formulations was a problem and because of this further evaluation has been retarded (Peregrine, 1994).

Carrier Erythrocytes

Red blood cells (rbcs) are osmotically active and because of this, pores in their membrane can be opened and closed (Deloach and Wagner, 1988). This allows entry and entrapment of exogenous molecules into the rbcs. In studies to prolong the prophylactic effect of homidium, Deloach (1985) utilised this principle to encapsulate a maximum of 5 mg of the drug into 10^{10} rbcs. This means that 20 ml of packed rbcs containing the drug would contain sufficient drug for a 100 kg animal (Deloach, 1985). The method seems impractical for field use and no experimental trials in ruminants have been reported (Peregrine, 1994).

Homidium and isometamidium polymers

De Deken et al. (1989) produced and evaluated the prophylactic effect of a slow release cylindrical polymeric device (SRD) in rabbits. The SRD measured 1 cm in length, 1.7 mm in diameter, weighed 27.1 $\pm$ 1.3 mg and contained 25 % Ethidium. These cylindrical polymeric devices were implanted subcutaneously in the shoulder region. The 11.25 mg/kg SRD-homidium protected the rabbits against seven challenges with *T. congolense* during a period of more than 300 days while the prophylactic activity of intramuscular injection was less than one month. Only very slight or no inflammation occurred at the site of the implanted rods.

In a similar experiment, using a different polymer matrix and a lower dosage of homidium bromide (1 mg/kg), Geerts et al. (1993) showed that 95% (15 out of 16 rabbits) remained protected by the SRD until three months post implantation.

Microscopical examination of the tissue from implantation sites showed very slight tissue reaction in most of the rabbits.

This thesis describes a number of studies to:

1. Evaluate and improve a competitive enzyme immunoassay for the detection of isometamidium in bovine serum.

2. The use of an ELISA to measure isometamidium concentrations in the sera of treated cattle underfield conditions and as an indirect method for monitoring drug resistance at North Nyamphande, Zambia.

3. Evaluate the prophylactic effect of a slow release device containing homidium bromide in cattle under field conditions.

4. Influence of the plane of nutrition on the duration of isometamidium prophylaxis in sheep.

Chapter 2

Competitive enzyme immunoassay for the detection of isometamidium in Bovine serum; an improved assay using anti-isometamidium hyperimmune rabbit serum and isometamidium-horseradish peroxidase conjugate produced using a n-hydroxysuccinamide-carbodiimide linkage

INTRODUCTION

Several analytical methods have been developed for measuring concentrations of trypanocidal drugs in serum or plasma. These include the spectrophotometric method of Philips et al., (1967), high performance liquid chromatography (HPLC) (Persch and Vollner, and Kinabo and Bogan, 1988) and radio-immunoassay (Kinabo and Bogan, 1988). All these methods have their limitations. The spectrophotometric method is not sensitive in that it cannot determine isometamidium concentrations lower than 1 μg/ml in plasma. Perschke and Voller's (1985) HPLC method is not specific, in that it is an indirect method in which isometamidium has to be converted to homidium before detection. It also requires a large amount of serum (about 1 ml per sample) on the chromatographic column. The limitation of the HPLC method as described by Kinabo and Bogan (1988) is that the lower limit of detection of isometamidium is 10 ng/ml in serum and about 400 ng/g in wet tissue. The radio-immunoassay (Kinabo and Bogan, 1988c) is even less sensitive in that the lower limit of detection of isometamidium is 29 ng/ml of serum.

The competitive enzyme immunoassay (CEIA), developed by Eisler et al., (1993,1996) has several advantages over the earlier methods. This ELISA utilised anti-isometamidium hyper-immune sheep serum, and an isometamidium-horseradish-peroxidase (HRP) conjugate produced using a carbodiimide (CDI) linkage. More recently, attempts have been made to adapt the assay for anti-isometamidium hyperimmune rabbit serum and isometamidium-HRP produced using n-hydroxysuccinamide (NHS)-CDI linkage. Using these reagents a potentially more sensitive assay was obtained, where the amount of

isometamidium resulting in 50% competition was slightly lower (1.1 ng/ml) than that using the earlier reagents (2.2 ng/ml) and cross-reactivity with homidium was reduced (M. C. Eisler, personnel communication). The only disadvantage of the ELISA using the later reagents was the excessive inter-sample variation observed among the sera from untreated animals. This variation was due to the sample 'matrix' i.e. the serum containing the analyte. High inter-sample variation due to the matrix effect is a potential problem for all such direct non-extraction methodologies, but was particularly pronounced in this case. When control sera from cattle which had not been treated with any trypanocidal drug were analysed using the competitive ELISA, samples showed very low optical densities compared with values obtained using buffer alone, which in effect amounted to false positive results for isometamidium.

In this chapter, several experiments were conducted to investigate the cause of the variation in the competitive ELISA using anti-isometamidium hyperimmune rabbit serum, and isometamidium-n-hydroxysuccinamide-horseradish peroxidase conjugate among negative sera collected from untreated animals are described. These included: (a) heating the sera to which EDTA (as an anticoagulant) was added for 10 minutes at 100^0C; if the interfering factor was a protein it could have been denatured by this high temperature, (b) dialysing the sera before use which would have demonstrated whether the interfering factor was one of low molecular weight, (c) using normal rabbit serum as a blocking agent in a sample diluent buffer, which might indicate whether the interfering factor interacted with the rabbit hyperimmune serum, and (d) use of a sequential saturation procedure in which the sera and conjugate were added to the coated microtitre plate at different times.

MATERIALS AND METHODS

Bovine sera

Sera from untreated cattle

Sera were obtained from 20 healthy adult Boran cattle at Kapiti Ranch situated in the Athi plains, a tsetse free area of Kenya, and from 20, 1 - 3 month old Boran calves born at Kapiti Ranch and reared at the International Livestock Research Institute (ILRI), Kenya. None of these animals had ever received treatment with either isometamidium or homidium. Sera were also obtained from 24 Mashona cattle from Makuti on the Zambezi escarpment, an area of low tsetse challenge in Zimbabwe (Eisler, et al., 1996). None of these cattle were believed to have received treatment with either isometamidium or homidium.

Sera from isometamidium-treated cattle

Seven Boran cattle at International Livestock Research Institute (ILRI), Kenya, were treated with isometamidium chloride by deep intramuscular injection of a 1 % aqueous solution, into the middle third of the neck at a dose rate of 0.5 mg/kg body weight. One week later blood samples were collected from a jugular vein and were incubated (without anticoagulant) for four hours at 37°C to maximise clotting. They were then incubated at 4°C overnight and centrifuged at 1500 RPM for 15 minutes, after which sera were removed from the clots and aliquoted and stored at - 70°C.

Rabbit sera

Normal rabbit serum

Normal rabbit serum obtained from rabbits at ILRI, Nairobi small animal unit was kindly provided by Mr. Alfred Adema.

Anti-isometamidium hyperimmune rabbit serum

Anti-isometamidium hyperimmune rabbit serum was kindly provided by the Department of Veterinary Physiology at the University of Glasgow. The hyperimmune rabbit serum had been obtained by immunisation of two rabbits with isometamidium conjugated to porcine thyroglobulin using the following procedure. Primary immunisation was by subcutaneous injection of isometamidium-thyroglobulin conjugate emulsified in Freund's complete adjuvant (FCA), at two different sites (right side of the neck and right flank). Three booster immunisations of isometamidium-thyroglobulin conjugate emulsified in incomplete Freund's adjuvant (FICA) were given 2½, 7 and 18 weeks later. Twelve days after the final booster, blood was collected for the separation of hyperimmune serum, and stored at - 20°C until required for use in the isometamidium ELISA.

Isometamidium conjugated to horseradish peroxidase by an N-hydroxysuccinamide-carbodiimide linkage

Isometamidium conjugated to horseradish peroxidase by an N-hydroxysuccinamide-carbodiimide linkage was kindly provided by the Department of Veterinary Physiology at the University of Glasgow, and had been prepared as follows. A solution of 2 mg N-hydroxysuccinamide (Sigma) dissolved in 100 µl 1 mM sodium acetate was added to a solution of 10 mg of isometamidium chloride dissolved in 250 µl 1 mM sodium acetate,

to which 125 µl pyridine (Sigma) was added. The resultant solution was mixed well and allowed to stand at room temperature for 2 minutes. A solution of 10 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide dissolved in 250 µl of 1 mM sodium acetate was then added and mixed well.

100 µl aliquots of the reaction mixture prepared above were added at room temperature, to a solution of 20 mg horseradish peroxidase in 1 ml of 1 mM sodium acetate solution, over a period of 15 minutes with constant mixing. The solution was incubated at 37°C for 20 hours, and then dialysed against phosphate-buffered saline (PBS), pH 7.4.

The resulting conjugate was purified further by passing the solution through a Sephadex PD-10 column (Pharmacia) and by mixing the coloured portion of the volume for 15 minutes with 0.5 ml of a 0.25 % (w/v) suspension of activated charcoal (Sigma) in distilled water containing 0.025 % (w/v) dextran T70 (Pharmacia, U.K). This was then centrifuged at 14,000 rpm, the supernatant removed, mixed with an equal volume of glycerol (Sigma) and stored in aliquots at -20 °C.

Trypanocidal drug-spiked bovine serum standards

Isometamidium standards

A 500 µg/ml solution of isometamidium chloride (Samorin®, Rhone-Merieux, France) was prepared by adding sterile distilled water to 50 mg Samorin® powder, to a final volume of 100 ml. The solution was mixed well and given time for the powder to dissolve completely. This stock solution was then stored in a dark bottle for a maximum of three days.

A 1/10 dilution was prepared by adding 1 ml stock solution to 9 ml double distilled water. This was further diluted by adding 45 µl of the 1/10 diluted solution into 1455 µl

normal rabbit serum (NBS) to give a concentration of 1.5 μg/ml Samorin. A 1:3 serial dilution was prepared in NBS in tubes labelled 1 to 10 by transferring 500 μl from a 1.5 μg/ml Samorin® solution into tube 1, vortex mixing and transferring 500 μl from tube 1 into tube 2, vortex mixing and repeating the same procedure through to tube 10. The pipette tips were changed after every third tube in the serial dilution. The final concentrations in ng/ml from tube 1 to 10 were:

500, 166.7, 55.55, 18.51, 6.17, 2.05, 0.686, 0.229, 0.076, 0.025

Homidium standards

Homidium-spiked bovine serum standards were prepared in a similar manner using homidium bromide (Ethidium®, Laprovet, France), except that a 1/20 rather than a 1/10 dilution was prepared from the stock solution, so that the resulting standards contained only half the drug concentrations present in the corresponding isometamidium standards.

Microplate coating and storage

96-well microplates (Immulon 4, Dynex Technologies, U.K) were coated with rabbit anti-isometamidium hyperimmune serum at a dilution of 1/10,000 in carbonate-bicarbonate coating buffer (0.1M, pH 9.2), by dispensing 100 μl per well. After dispensing, the plates were covered with adhesive plastic plate sealers (Dynex Technologies) and incubated overnight at 4°C. The following day the plates were removed from the fridge and stored with the well contents *in situ* at - 20°C for up to two months.

Competitive Enzyme Immunoassay: Equilibrium Method

The assay was conducted as described by Eisler et al., (1996), but using anti-isometamidium hyperimmune rabbit serum in place of hyperimmune sheep serum, and isometamidium-NHS-CDI-HRP, in place of isometamidium-CDI-HRP. Briefly, test sera and standards were prediluted 1/10 in PBS (Appendices 1 and 2) containing 0.05% Tween 20 (Sigma) to which isometamidium conjugate had been added. The samples were diluted in 8 x 12 racks of polypropylene tubes (Biostat Diagnotics, UK) arranged in positions corresponding to the final plate layout. Coated microtitre plates were allowed to thaw and equilibrate to room temperature before washing five times in a 1/5 dilution of PBS (Appendix 3) in distilled water containing 0.05% Tween 20, and blotted dry. Pre-diluted standards and samples were vortex-mixed in the polypropylene tubes (PPN) and the contents transferred to duplicate wells of the microtitre plates (100 µl per well) using a multichannel pipette. The plates were covered with plate sealers and the well contents mixed for 10 minutes at room temperature using a shaker-incubator. The plates were then incubated overnight at 4°C.

The following day after equilibrating to room temperature, the plates were washed five times with washing buffer as described above, and blotted dry. The colour reaction was developed by adding 100 µl per well of a solution prepared by mixing equal volumes of the two components of a commercially available sodium perborate / 3,3′,5,5′ tetramethylbenzidine substrate-chromogen system (Vetoquinol, UK), pre-warmed to 37 °C prior to adding to the plate. The plate was then incubated at 37°C in the shaker incubator for 10 minutes after which the reaction was stopped by adding 100 µl per well of 1 M phosphoric acid. The optical densities (OD) were read using the Multichannel spectrophotometer (Titertek® Multiskan PLUS Mk II) at a wavelength of 450 nm.

Heat treatment of sera with EDTA

250 µl volumes of 0.1 molar EDTA were added to equal volumes of sera in micro-centrifuge tubes (Eppendorf), and vortex mixed thoroughly. After closing and piercing the flip caps using a needle, the microcentrifuge tubes were heated at 100°C for 10 minutes using a boiling water bath. After heating, the tubes were centrifuged for 10 minutes at 16,000 rpm.

Since this heat treatment involved a 1/2 dilution of the sera, it was necessary to modify the competitive ELISA (equilibrium method) to allow for this dilution. For this reason, instead of preparing 1/10 pre-dilutions in isometamidium-HRP conjugate in polypropylene tubes, 50 µl volumes of the heat-treated sera were added directly to 50 µl volumes of isometamidium-HRP conjugate in duplicate wells of a washed, coated microtitre plate. The conjugate was diluted appropriately such that the final dilution of this conjugate in the wells would remain the same as that in the standard procedure. The final dilution of serum using this modified procedure was 1/4, rather than the 1/10 dilution obtained using the standard procedure. After overnight incubation, the colour reaction was developed in the normal way.

Dialysis of bovine serum

A suitable length of dialysis tubing (cylindrical volume 0.03 ml/mm, MW cut-off 8000) was boiled for 5 minutes in 0.1 M sodium EDTA. Sera were placed in segments of the dialysis tubing (one sample per segment), the ends of which were closed by knotting and then submerged in a 1 L of PBS diluted 1/10 in distilled water at 4°C with constant stirring. The PBS solution was changed after 2 and 4 hours, and thereafter the dialysis

was allowed to proceed overnight. The following morning the dialysed sera were removed from the tubing and tested using the standard competitive ELISA (equilibrium method).

Use of normal rabbit serum as a blocking agent for the competitive ELISA (Equilibrium Method)

1% normal rabbit serum was prepared by adding 200 µl of normal rabbit serum to 19.8 ml of PBST and vortex mixing. This preparation was used for the dilution of isometamidium-HRP conjugate, and hence subsequently for the pre-dilution of samples and standards before addition to the wells of microtitre plates in the competitive (equilibrium method).

Competitive Enzyme Immunoassay: Sequential Saturation Method

The sequential saturation (SS) method of competitive enzyme immunoassay was conducted in a manner generally similar to the equilibrium method, but with the following important differences. Standards and samples were diluted 1/10 in PBST (to which isometamidium-NHS-CDI-HRP conjugate had not been added), and incubated (100 µl per well) overnight at 4°C in microtitre plates which had been previously coated and washed five times as described above. The following day, the microtitre plates were washed five times as described above, and 100 µl of a 1/256,000 dilution of isometamidium-NHS-CDI-HRP in PBST (pre-warmed to 37°C) was added to every well. The microtitre plates were then incubated with constant shaking at 37°C for 15 minutes, and after a further five washes as described above, the colour reaction was developed using the substrate chromogen system as described for the equilibrium method.

Titration of anti-isometamidium hyperimmune rabbit serum and isometamidium NHS-CDI HRP conjugate

Checkerboard titration was used to determine the optimal dilutions for the anti-isometamidium hyperimmune rabbit serum and the isometamidium NHS-CDI-HRP conjugate. Four different hyperimmune serum dilutions were compared, and four different conjugate dilutions were compared, giving a total of 16 combinations of dilutions of these two reagents. On a single 96 well microtitre plate there were therefore 6 wells for each combination, where 3 tested negative sera and three, serum containing 1.0 ng/ml isometamidium. The plate layout used is shown in Table 2.1. The optimal combination of hyperimmune serum dilution and different conjugate dilution was considered to be that resulting in a mean OD for negative serum between 0.6 and 1.2, and the highest level of competition due to the isometamidium-spiked serum. The level of competition as a percentage was calculated for each combination of hyperimmune serum dilution and conjugate dilution as follows:

$$\text{Competition } \% = 1 - \frac{\text{mean OD for serum containing 1.0 ng/ml isometamidium}}{\text{mean OD for negative serum}} \times 100\%$$

Table 2.1 Checkerboard titration for anti-isometamidium hyperimmune rabbit serum and the isometamidium NHS-CDI-HRP conjugate.

Dilution of anti-isometamidium hyperimmune rabbit serum:

	1/2000			1/4000			1/8000			1/16000			conjugate
	1	2	3	4	5	6	7	8	9	10	11	12	dilution:
A	–	–	–	–	–	–	–	–	–	–	–	–	1/64000
B	+	+	+	+	+	+	+	+	+	+	+	+	„
C	–	–	–	–	–	–	–	–	–	–	–	–	1/128000
D	+	+	+	+	+	+	+	+	+	+	+	+	„
E	–	–	–	–	–	–	–	–	–	–	–	–	1/256000
F	+	+	+	+	+	+	+	+	+	+	+	+	„
G	–	–	–	–	–	–	–	–	–	–	–	–	1/512000
H	+	+	+	+	+	+	+	+	+	+	+	+	„

– = Normal bovine serum (no drug)

+ = Normal bovine serum containing 1 ng/ml of isometamidium

Cross-reactivity of homidium in the competitive ELISA

To investigate the cross-reactivity of homidium in the competitive ELISA, results obtained using normal bovine serum spiked with homidium were compared with those obtained using isometamidium standards. The microtitre plate layouts used are shown

below in Table 2.2. Cross-reactivity was assessed by fitting sigmoidal curves and also by comparing the concentration of each drug that resulted in 50 % inhibition of the optical density.

Table 2.2 Layout of ELISA plate. X = Normal Bovine Serum, Std = Standard

	1	2	3	4	5	6	7	8	9	10	11	12
A	X	X	Std 10	Std 9	Std 8	Std 7	Std 6	Std 5	Std 4	Std 3	Std 2	std 1
B	"	"	"	"	"	"	"	"	"	"	"	"
C	"	"	"	"	"	"	"	"	"	"	"	"
D	"	"	"	"	"	"	"	"	"	"	"	"
E	"	"	"	"	"	"	"	"	"	"	"	"
F	"	"	"	"	"	"	"	"	"	"	"	"
G	"	"	"	"	"	"	"	"	"	"	"	"
H	"	"	"	"	"	"	"	"	"	"	"	"

Isometamidium standards were added to Rows A, B, E and F. B was a duplicate of A and F was a duplicate of E. Homidium standards were added to Rows C, D (duplicate of C) , G and H (duplicate of G).

Isometamidium conjugate at a dilution of 1 : 128,000 was added to Rows A, B, C and D. Homidium conjugate at a dilution of 1 : 40, 000 was added to Row E, F, G and H.

Data Analysis

Absorbances were read using a mutichannel spectrophotometer connected to a personal computer. The program Eiaquik® (M. C. Eisler, 1995), was used for the following analyses. The means and coefficients of variation (CV) of the absorbances of duplicate

wells were calculated, and if the CV of any duplicate sample results was more than 12%, the result was rejected and the sample re-tested. The concentrations of unknown samples were calculated from their absorbances, using four-parameter logistic curves fitted to the absorbences of calibration standards. Response-error relationships (Rodbard, D., 1981) and precision profiles (Ekins, 1983) were also calculated, and finally results were appended to ASCII database files for subsequent analysis, using appropriate statistical software.

Inter-sample variation in the competitive ELISA

Inter-sample variance at zero dose was determined in the competitive ELISA by testing sera from 24 cattle in Zimbabwe which had not been treated with the drug. Each serum sample was tested in duplicate, twice on each of two plates. For each of the two plates, two pre-dilutions were prepared for every sample, and each of the two pre-dilutions was tested in two duplicate wells.

Experimental approach

Initially, normal (untreated) bovine sera were tested using the competitive ELISA (equilibrium method). The same or similar sera were then re-tested in the same assay

a) following dialysis of the serum,

b) following heat treatment with 0.1 M EDTA, and

c) using normal rabbit serum as a blocking agent.

Finally, the same or similar sera were tested using the competitive ELISA (sequential saturation method).

RESULTS

Inter-sample variation using the equilibrium ELISA method:

When sera from untreated Kapiti cattle were tested using the equilibrium ELISA method great variation in the optical densities (ODs) was observed. The ODs ranged from 0.208 to 3.332 and falsely indicated the presence of isometamidium. Below are some of the untreated samples that were tested (Table. 2.3 and 2.4). See appendices 4 and 5.

Table 2.3 Observation of variation in the optical densities and false positives (isometamidium concentration) of sera from Kapiti cattle.

Animal number	Optical density (nm)	B/Bo (%)	Drug conc (ng/ml)	Animal number	Optical density (nm)	B/Bo (%)	Drug conc (ng/ml)
1441	3.332	214.5	0.000	613	0.602	38.6	2.119
1012	3.115	200.5	0.000	1320	0.592	38.0	2.179
2890	2.318	149.1	0.000	21	0.242	15.4	7.522
721	1.136	73	0.447	607	0.449	28.8	3.330
419	1.132	72.8	0.482	852	0.424	27.1	3.626
163	1.095	70.4	0.544	1126	0.413	26.4	3.756
553	0.998	64.1	0.728	1148	0.368	23.6	4.393
1484	0.995	63.9	0.734	574	0.357	22.8	4.584
874	0.627	40.2	1.978	1344	0.241	15.3	7.560
824	0.623	40.0	1.999	217	0.208	13.2	9.006

nm = wavelength at 450 nm

B = Mean absorbances of the sample

B_o = Mean absorbance at zero dose

$$B/Bo\ (\%) = \frac{\text{Mean absorbances of the sample}}{\text{Mean absorbance at zero dose}} \times 100$$

Table 2.4 Observation of variation in the optical densities and false positives (isometamidium concentration) of sera from ILRI calves.

Animal number	Optical density (nm)	B/Bo (%)	Drug conc (ng/ml)	Animal number	Optical density (nm)	B/Bo (%)	Drug conc (ng/ml)
163	0.975	86.3	0.351	162	1.112	98.4	0.044
153	1.040	92.1	0.200	174	1.161	102.7	0.000
182	1.062	94.1	0.149	177	1.128	99.8	0.007
180	0.846	74.9	0.693	186	1.172	103.8	0.000
179	1.252	110.8	0.000	147	1.102	97.6	0.064
178	0.834	73.9	0.728	269	1.043	92.4	0.192
187	0.984	87.5	0.328	146	1.220	107.9	0.000
295	0.988	87.5	0.317	160	0.894	79.2	0.556
175	0.867	76.8	0.633	164	0.651	57.7	1.423
166	1.233	109.0	0.000	181	1.196	105.8	0.000

nm = wavelength at 450 nm

B = Mean absorbances of the sample

B_o = Mean absorbance at zero dose

$$B/Bo\ (\%) = \frac{\text{Mean absorbances of the sample}}{\text{Mean absorbance at zero dose}} \times 100$$

Effect of Dialysis on the equilibrium ELISA:

The result of the four samples dialysed showed that dialysis had no effect on the ODs of the samples. There was no significance difference in the ODs of dialysed samples and the non-dialysed samples (Table 2.5).

Table 2.5 Optical densities of samples before and after dialysis.

Animal number	Optical densities (nm) before dialysis	Optical density (nm) after dialysis
160	0.470	0.488
164	0.170	0.307
178	0.212	0.260
179	0.826	0.979

nm = wavelength at 450 nm

Effect of heat treatment with EDTA on the equilibrium ELISA:

When samples were heated with EDTA there was an increase in the optical densities of the heated samples. However the optical densities were still low (Table 2.6).

Table 2.6 Optical densities of samples before and after heat treatment.

Animal number	Optical densities before heat treatment	Optical densities after heat treatment
164	0.060	0.516
164	0.069	0.495
164	0.075	0.502
164	0.079	0.476
High pool	0.192	0.466
High pool	0.213	0.456
High pool	0.248	0.429
High pool	0.227	0.424

High pool was pooled sera from the animals that were showing high ODs.

Effect of using 1% normal rabbit serum as a blocking agent:

The three samples to which 1% normal rabbit serum was added showed no significant difference in their optical densities before and after adding the blocking agent (Table. 2.7).

Table 2.7 Optical densities of serum samples before and after adding 1% normal rabbit serum as a blocking agent.

Animal number	+ 1% normal rabbit serum	- 1% normal rabbit serum
High pool	0.423	0.423
164	0.109	0.122
1012	0.753	1.683

Checkerboard titrations for isometamidium conjugate and anti-isometamidium hyperimmune rabbit serum:

The best results were obtained at optimal dilution of 1/8,000 for the anti-isometamidium hyperimmune rabbit serum and 1/256,000 for the conjugate. At these dilutions the competition level was 37.8% and the B_o value was 0.747 (Table 2.8).

Table 2.8 Checkerboard titration results for sequential saturation ELISA.

	Anti-isometamidium hyperimmune rabbit serum							
	1/2,000		1/4,000		1/8,000		1/16,000	
Conjugate dilution	B_o	Comp (%)	B_o	Comp (%)	B_o	Comp (%)	B_o	Comp (%)
1/64,000	1.973	27.7	1.960	30.8	1.746	31.9	0.577	31.0
1/128,000	1.458	32.5	1.409	31.7	1.210	33.4	0.391	28.0
1/256,000	0.899	35.0	0.867	36.8	0.747	37.8	0.275	39.1
1/512,000	0.478	40.1	0.484	41.4	0.415	44.8	0.144	37.9

Comp = competition

B_o = Mean absorbance at zero dose

Intersample variation (mean response variances) of sera from untreated cattle:

When sera from untreated cattle were tested using the sequential saturation ELISA the variation that was observed among the untreated sera using equilibrium ELISA was no longer detected. The Percentage Coefficient of Variation (% CV) was 5.8 % (Table 2.9a and 2.9b)

Table 2.9a. Optical densities (OD) at 450 nm of 24 individual sera from untreated Zimbabwe Mashona cattle.

Animal number	Optical density (nm)	B/Bo (%)	Animal number	Optical density (nm)	B/Bo (%)
H 196	0.890	75	P 306	1.215	101.2
P 324	1.070	90.2	P 239	1.203	101.3
P 274	1.068	91.2	BC	1.237	103.4
T 523	1.142	95	P 317	1.205	103.9
T 391	1.082	95.1	P 221	1.300	104.5
P 225	1.145	95.8	P 236	1.298	105.1
P 336	1.170	97.4	P 243	1.283	105.8
P 272	1.220	98.2	J 437	1.288	106.6
P 242	1.212	98.8	194	1.292	106.7
T 478	1.146	99.1	P 228	1.311	107.5
P 238	1.203	99.9	P 229	1.315	108.0
J 156	1.219	101.1	P 235	1.308	109.1

nm = wave length at 450 nm

B = Mean absorbances of the sample

B_o = Mean absorbance at zero dose

$$B/Bo\ (\%) = \frac{\text{Mean absorbances of the sample}}{\text{Mean absorbance at zero dose}} \times 100$$

Table 2.9b Coefficient of Variation (CV) of untreated sera

Total Samples	Mean	Variance	SD	CV
24	1.20005	0.00491	0.07007	5.8 %

SD = Standard deviation

Calculations:

$$CV = \frac{SD}{Mean} \times 100\%$$

$$SD = \sqrt{\frac{\Sigma(x^2) - (\Sigma x)^2/N}{N - 1}}$$

$$\text{Variance} = \frac{\Sigma(x^2) - (\Sigma x)^2/N}{N - 1} = SD^2$$

N = Sample size

X = Individual observations

Calibration curves

The isometamidium sigmoidal curves fitted well to the four parameter logistic mode. The adjusted r^2 was > 0.98. Figure 2.1a shows a typical curve obtained using the sequential saturation ELISA. The four parameters are shown in Table 2.10a.

Table 2.10a Isometamidium assay parameters

Parameter	Value
A (B_o - background)	1.421
B	0.991
C	0.805
D (background)	0.069
B_o	1.490
10% B_o (ng/ml)	11.5
50% B_o (ng/ml)	0.976
90% B_o (ng/ml)	0.054

When the isometamidium sequential saturation assay was adapted for homidium, the curves fitted well to the four parameter logistic model (Table 2.10b and Figure 2.1b). The adjusted r^2 was > 98.

Table 2.10b Homidium assay parameters

Parameter	Value
A (B_o - background)	0.444
B	2.148
C	1.701
D (background)	0.165
B_o	0.610
10% B_o (ng/ml)	3.000
50% B_o (ng/ml)	0.638
90% B_o (ng/ml)	0.176

Figure2.1a Calibration curve for isometamidium sequential saturation ELISA

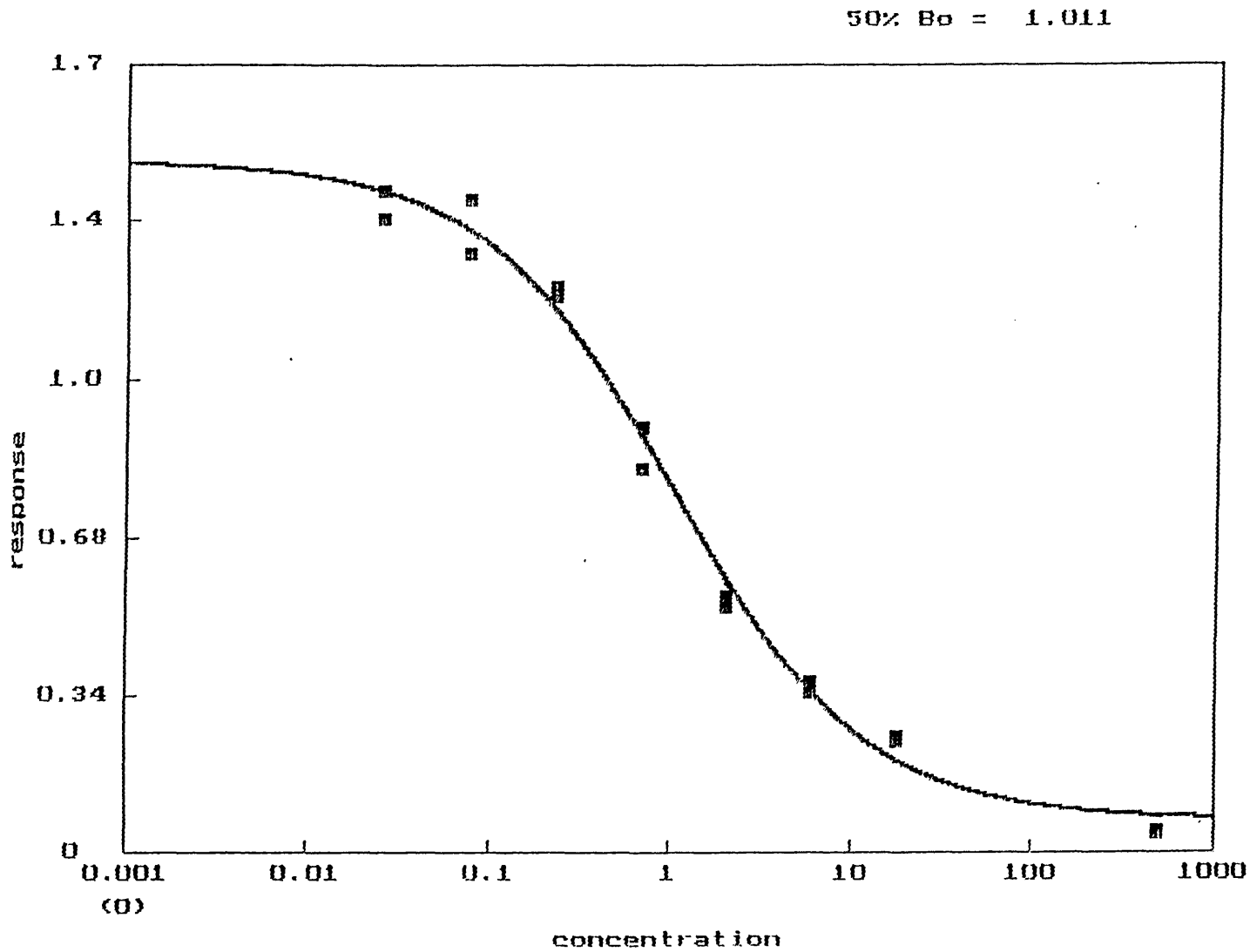
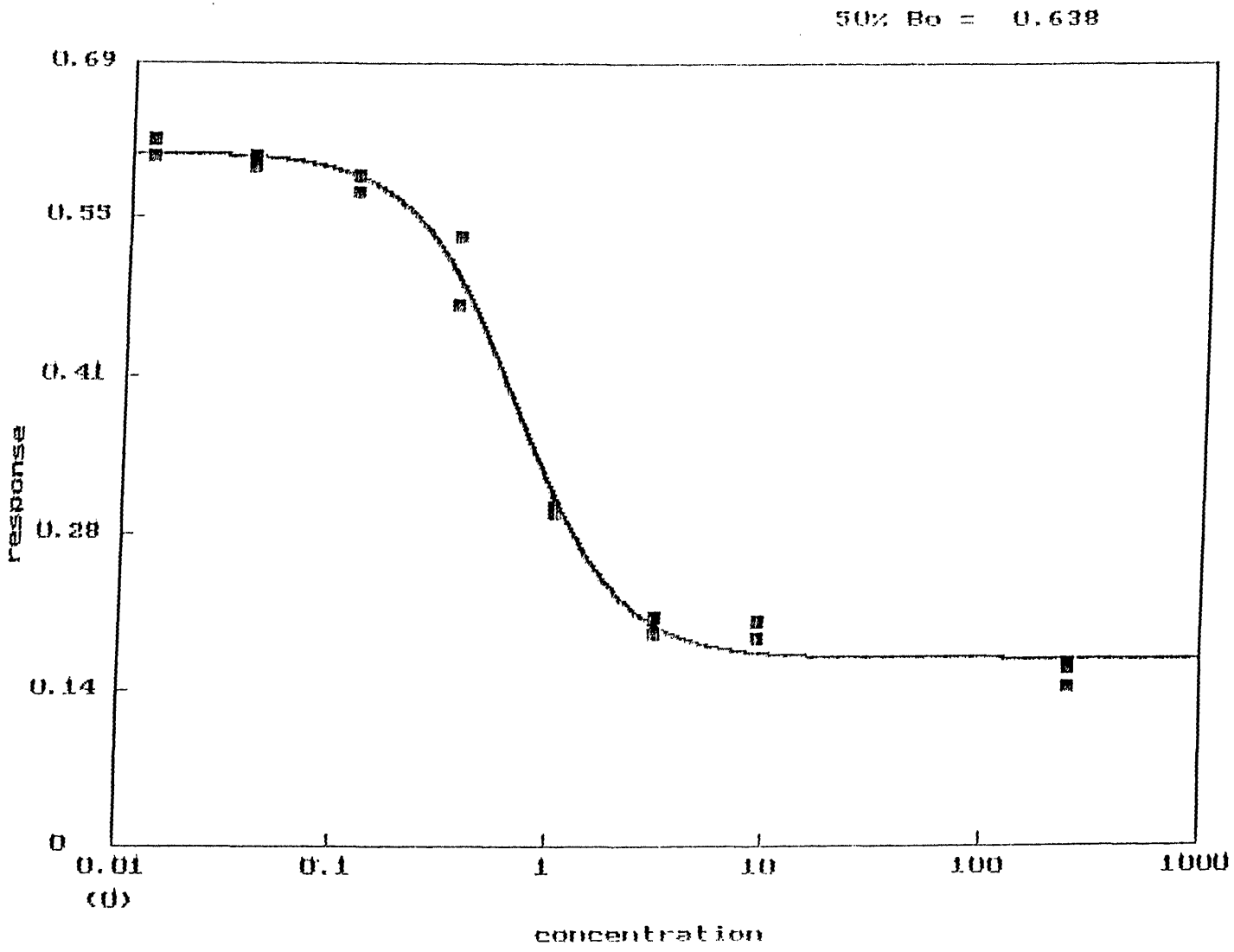


Figure 2.1b Calibration curve for homidium sequential saturation ELISA.



Drug concentration of treated cattle

The isometamidium levels in the 7 treated Boran cattle were determined by the sequential saturation ELISA. The results are shown in Table 2.11. The other serum samples tested were from cattle in the field trials conducted in Zambia (see Chapters 3 and 4)

Table 2.11 Isometamidium concentration of treated cattle

Animal no	B/B0 %	drug concentration (ng/ml)
BN 202	17.9	7.75
BN 204	17.6	7.95
BN 205	23.7	4.99
BN 206	19.7	6.66
BN 211	18.9	7.17
BN 219	17.8	7.79
BN 220	27.0	4.00

Cross-reactivity studies of isometamidium with homidium

When homidium spiked sera (standards) were added to a washed plate coated with anti-isometamidium hyperimmune rabbit serum and either isometamidium or homidium conjugate added, no sigmoidal curves were formed. This showed that no homidium were detected by the anti-isometamidium hyperimmune rabbit serum.

When isometamidium spiked sera (standards) were added to a washed plate coated with anti-homidium hyperimmune sheep serum and either isometamidium or homidium conjugate added, sigmoidal curves were formed. This showed that anti-homidium hyperimmune sheep serum was detecting isometamidium. Serum sample that was spiked with isometamidium concentration at 0.025 ng/ml reduced the B/Bo to 60%.

2.4 DISCUSSION

The competitive enzyme immunoassay (sequential saturation method) has proved to be a good assay for determining the drug concentrations level in the sera of cattle treated under field conditions. The fact that competitive enzyme immunoassay (sequential saturation method) can detect concentrations as low as about 0.2 ng/ml of serum indicates that the assay is very sensitive. Eisler et al. (1996) found the lower limit of detection of about 0.5 ng/ml of sera when they used competitive enzyme immunoasssay (equilibrium method). Isometamidium is normally found in low concentrations in plasma of treated animals (Kinabo and Bogan, 1988) and because of this very sensitive assays are required if low levels of drug in the animals that have been previously treated, are to be detected.

The main advantage of the competitive enzyme immunoassay (sequential saturation method) over the competitive enzyme immunoasssay (equilibrium method) is that the inter-sample variation was not observed when sera from non-treated animals were analysed. For the untreated sera the mean response variance for the sequential saturation ELISA was less than 6 % while for the equilibrium ELISA it was ~ 10 % (Eisler et al., 1996). The variation was due to the analyte in the serum. In many assays for drugs, hormones, toxins and other similar small molecules, the analyte is extracted from the matrix prior to measurement. In the assays being described here, there is no extraction step, and the analyte is qualified directly in the matrix. These assays are direct, non extraction assays. One way to reduce inter-sample variance (matrix effect) would be to employ an extraction step. Such a step would increase the complexity of the procedure, amount of equipment required and the cost. Hence the direct non-extraction approach is preferable in African laboratories.

When the equilibrium ELISA was used sera from some of the untreated animals showed very low optical densities (ODs) suggesting that they had some levels of drug. This suggests there were some factors or molecules in the sera that were interfering with the binding of the conjugate or denaturing it. This is likely to be the case because when sera and conjugate were pre-diluted in different microtubes and added on to the plate at different times, the effect was eliminated. The effect could not be eliminated by either dialysing or heating the sera. Blocking agents such as 1 % Normal Rabbit Serum also failed to solve the problem.

The main difference between the sequential saturation ELISA and equilibrium ELISA is that with the sequential saturation ELISA pre-diluted sera and conjugate are added on to the microtiter plate at different times. In brief the stages are: (a) dilution and incubation of sera overnight at 4°C. (b) dilution and addition of conjugate and incubation at 37°C for 15 minutes. (c) colour development with TMB for 10 minutes. In the equilibrium ELISA the sera and conjugate are diluted in the same micronic tube and added onto the microtiter plate at the same time.

After the isometamidium competitive ELISA was successfully modified to a sequential saturation ELISA, the method was later used for measuring homidium levels in the sera of field treated cattle (Chapter Four). The coating of the plate with anti-homidium hyper-immune sheep serum was done at an optimal dilution of 1/8,000. The conjugate dilution was at 1/40,000 in PBST.

The use of the sequential saturation ELISA to determine the concentration of the drug levels in the sera that were collected from cattle treated under field conditions (Chapter Three and Four) in Africa has proved that the assay is field applicable. A method like this

is ideal for its intended purpose because trypanosomiasis is a rural problem. Most laboratories in rural areas are not well equipped and simple assay like the drug ELISA assay is the best.

Chapter 3

Field studies using ELISA to measure isometamidium concentrations in the sera of treated cattle and use the assay as an indirect method for monitoring drug resistance at North Nyamphande, Zambia.

Introduction

In the absence of an effective vaccine, control of tsetse-transmitted trypanosomiasis in domestic cattle depends on chemotherapy, control of the tsetse fly vector (*Glossina* species) and the use of trypanotolerant animals. The commonly used drugs in cattle are diminazene aceturate, homidium bromide and chloride, and isometamidium chloride (Peregrine, 1994). These drugs have been extensively used for over 30 years and drug-resistant trypanosomes have appeared in many parts of Africa (Dolan et al., 1990; Chitambo et al., 1992; Joshua et al., 1993). Since it is unlikely that new trypanocides will be introduced onto the market in the near future, the efficacy of the drug in current use must be maintained.

In order to achieve this, control of trypanosomiasis by chemotherapy will have to be done on the basis of knowledge of the drug-susceptibility phenotypes of trypanosome populations. Various methods have been used to characterise the drug-resistance phenotypes of trypanosomes e.g. using laboratory rodents, ruminants, *in vitro* cultivation of trypanosomes in the presence of the drug and statistical analysis of field data (Peregrine, 1994). None of these methods is free of constraints; large animals are expensive to purchase and maintain, some of the trypanosomes (e.g. *Trypanosoma vivax*) are not rodent infective, and very few laboratories in Africa have the facilities necessary to screen large numbers of trypanosome isolates using *in vitro* cultivation.

In order to overcome the problems associated with these methods, Eisler et al. (1993, 1996) developed enzyme-linked immunosorbent assays (ELISA's) that are capable of determining concentrations of isometamidium in serum samples. Using these assays, large numbers of samples can be analysed within a short time. In combination with a

trypanosomiasis diagnostic test, the assay can be used as an indirect method for determining drug resistance; where cattle become infected with trypanosomes despite demonstrable circulating levels of the drug that would normally be considered protective, the infecting parasites are drug-resistant.

Eisler et al., (1994) used a competitive enzyme-linked immunosorbent assay to determine isometamidium concentrations in Boran cattle. In their experiment they concluded that trypanosomes persisting at serum isometamidium concentrations greater than 0.75 ng/ml were moderately resistant and those persisting at concentration greater than 6 ng/ml markedly resistant. In a subsequent paper (Eisler et al., 1996) they concluded that trypanosomes persisting in cattle with serum isometamidium concentration of 2.0 ng/ml may be considered moderately drug-resistant and at concentrations between 0.4 and 2.0 ng/ml a low level of drug-resistance.

The objectives of the present study were to determine whether isometamidium concentrations may be monitored effectively in individual village cattle under a chemoprophylactic regimen and to determine whether isometamidium concentrations measured in individual cattle undergoing breakthrough infections, could be used as evidence for the presence of drug resistant trypanosomes.

MATERIALS AND METHODS

Drugs

Isometamidium (Samorin®, Rhone-Merieux, France) was purchased locally in Zambia. The drug was prepared by dissolving the required quantity in sterile distilled water, just before use. To make up a 2% solution 1 gram of Samorin powder was dissolved in 50 ml of sterile water. At least 20 minutes was allowed for the drug to completely dissolve before injecting intramuscularly into the gluteal muscles of cattle using a 16 s.w.g. needle. Where the total volume of the solution was more than 15 ml per animal, it was divided into two equal volumes and injected into two sites.

Monitoring of trypanosome infections and packed cell volume (PCV)

Blood samples were collected from the peripheral ear vein, punctured by a sterile lancet, into heparinized capillary tubes. The tubes were centrifuged for 5 minutes in a microhaematocrit centrifuge (Hawksley) and the PCV were read using a PCV reader (Hawksley). After reading the PCV, buffy coat wet smears were prepared by cutting the capillary tubes a few mm below the buffy coat and examined under the microscope using the dark ground buffy coat technique (Murray et al., 1977). Thick and thin blood smears were also prepared at the crushpen using blood from the ear vein, dried away from direct sunlight and stored in a slide box for future fixing and staining with Giemsa. Only the thin smears were fixed prior to staining. Thick smears were used for screening trypanosomes while thin smears were used for species identification.

Study site

The trial was carried out at North Nyamphande Settlement Scheme village in Petauke district, Eastern Province, Zambia. The settlement scheme lies about 10 km north of Petauke, in a tsetse infested area in which many trypanosomiasis cases have been reported (Petauke District Laboratory Reports, 1994 and 1995. Unpublished). Samorin® and Berenil® have been extensively used in this area for more than ten years. The drugs are sold to farmers by employees of the Department of Veterinary and Tsetse Control Services, attached to the Belgian Government Assistance of Veterinary Services to Zambia (ASVEZA) Project. In this area Samorin® is given to the cattle at three month intervals for prophylaxis of trypanosomiasis.

Animals

Seventy Angoni (Sanga) traditional village cattle over six months old were randomly selected from a large number of cattle presented by the farmers for the trial. No nutritional supplements were given to these cattle. They were exposed to natural tsetse challenge and grazed communally on free range.

Body weight determination

A weighband (Dalton, UK) was used to estimate the weight of the animals when administering trypanocides.

Isometamidium treatment

Two weeks following the diminazene block treatment all the 35 cattle in Group 1 were treated with a 2% solution of isometamidium chloride injected in the gluteal muscles at a dose rate of 1 mg/kg body weight..

Serum sampling

Blood samples were collected from the jugular vein into plain vacutainer tubes (Becton Dickinson and Co. USA). The tubes were allowed to clot at ambient temperature before transferring them to a fridge at 4°C in the field laboratory. The following day the tubes were centrifuged and sera separated from the clot into cryogenic vials (Alpha Labs, U.K.). Separated sera were then stored in vials at - 20°C until required for ELISA.

Serum drug concentrations

The Sequential Saturation ELISA described in Chapter Two was used to determine the isometamidium levels in the sera of the cattle in the experiment. Sera for preparing quality controls (QC) were collected from 7 Boran steers at ILRI. These steers were treated with 0.5 mg/kg isometamidium by intramuscular injection. One week after treatment whole blood from individual animals was collected and separated using standard procedures. Drug concentrations in sera from individual animals and in a pool prepared from the individual sera, were determined by ELISA. The mean drug concentration for individual sera and the pool was 6.2 ng/ml. Two dilutions of the pool were prepared in normal bovine serum (NBS) for use as quality controls (QCs). QC (1/5) was prepared by adding 0.8 ml of QC serum to 3.2 ml of NBS. QC (1/2) was prepared by adding 2.0 ml of of QC to 2.0 ml of NBS. A third quality control was NBS.

Evidence for the presence of drug resistant trypanosomes

After determining the levels of isometamidium in the sera of the individual animals, the parasitological results were compared with the drug concentrations at each sampling. Breakthrough trypanosome infections in individual animals having serum drug concentrations above 0.6 ng/ml were judged to be resistant to isometamidium.

Calculation of infection rates

On every occasion of sampling, the infection rate was calculated as the number of infected cattle, divided by the number of susceptible cattle. To allow for the period of prophylaxis attributable to diminazene (Wellde et al., 1983), and the pre-patent period of infection no cattle treated with diminazene within three weeks of sampling were included in the number of susceptible cattle.

Experimental design

Two weeks before the trial, cattle were eartagged, screened for trypanosome infections and randomly allocated into two groups each of 35 animals. All the cattle were given a diminazene block treatment at a dose rate of 7.0 mg/kg body weight to clear them of any trypanosome infections before the commencement of the trial. At the beginning of the experiment (day zero) all 35 cattle in Group 1 were treated with isometamidium chloride at a dose rate of 1 mg/kg body weight. No parasitological monitoring and serum collection was done on the day of isometamidium treatment.

Group 2, consisting of 31 animals, was used as control group and received no prophylactic treatment with isometamidium.

Parasitological monitoring was conducted every fortnight following the day of prophylactic isometamidium treatment. Serum samples were collected on every occasion parasitological monitoring was conducted from cattle in Group 1 and every four weeks from cattle in the control group. Individual cattle in Group 1 and Group 2 which subsequently developed trypanosome infections received diminazene treatment at a dose rate of 7 mg/kg body weight.

RESULTS

Before commencement of the experiment (- 14 days) the infection rate was 37.14 % in Group 1 and 19.35 % in Group 2. During the 12 week period of the trial there were 10 breakthrough infections in Group 1 after treatment with isometamidium at 1 mg/kg body weight (Table 3.1). In the control group there were 19 infections during the same period (Table 3.1).

In Group 1 (n = 30) the first infection was detected in the eighth week after this group of cattle were treated with isometamidium (infection rate 3.33 %). Four more infections were detected in the 10th week (infection rate 12.12 %) and in the twelfth week 5 more infections were revealed (infection rate 18.52 %). In the control group (n = 27) two infections (infection rate 7.41 %) were detected at the first sampling (week 4). In the 4th week the infection rate was 21.41 % and in the 6th week it was 10 %. The following week it was 8.33 %. In week 10 and 12 the infection rates were 21.43 % and 40 % respectively.

Table 3.1 Number of infected cattle, number of cattle at risk, infection rate and cumulative number of infections in Group 1 and Group 2.

		Days post treatment							
	Group	- 14	0	14	28	42	56	70	84
Number infected	I	13	NS	0	0	0	1	4	5
	II	6		2	6	2	2	6	8
Number at risk	I	35	"	33	33	33	30	33	27
	II	31		27	28	20	24	28	20
Infection rates (%)	I	37.14	"	0	0	0	3.33	12.12	18.42
	II	19.35		7.41	21.43	10	8.33	21.43	40
Cumulative total number of infections	I		"	0	0	0	1	5	10
	II			2	8	10	12	17	19

Group I: Cattle treated with 1.0 mg isometamidium per kg body weight.
Group II: Cattle not treated with isometamidium
NS: No sample

The mean isometamidium concentrations in the sera of the cattle in Group 1 rose to maximum values two weeks after treatment (Table 3.2 and Fig 3.1) and thereafter started to drop gradually. Drug was still detected in the sera up to the end of the trial (week 12). Isometamidium concentrations in the sera of individual cattle at the time break-through trypanosome infections occurred are shown in Table 3.3. Out of 10 infections 6 occurred in cattle in which circulating concentrations of isometamidium were detectable by the ELISA (limit of detection 0.2 ng/ml of serum). Three infections were detected in cattle that had serum drug levels below the lower limit of detection. No serum samples was available for one animal (No 623) at the time it was found to be infected with *Trypanosoma congolense* (day 84), hence no conclusion could be reached regarding the drug sensitivity of these trypanosomes (Appendix 6).

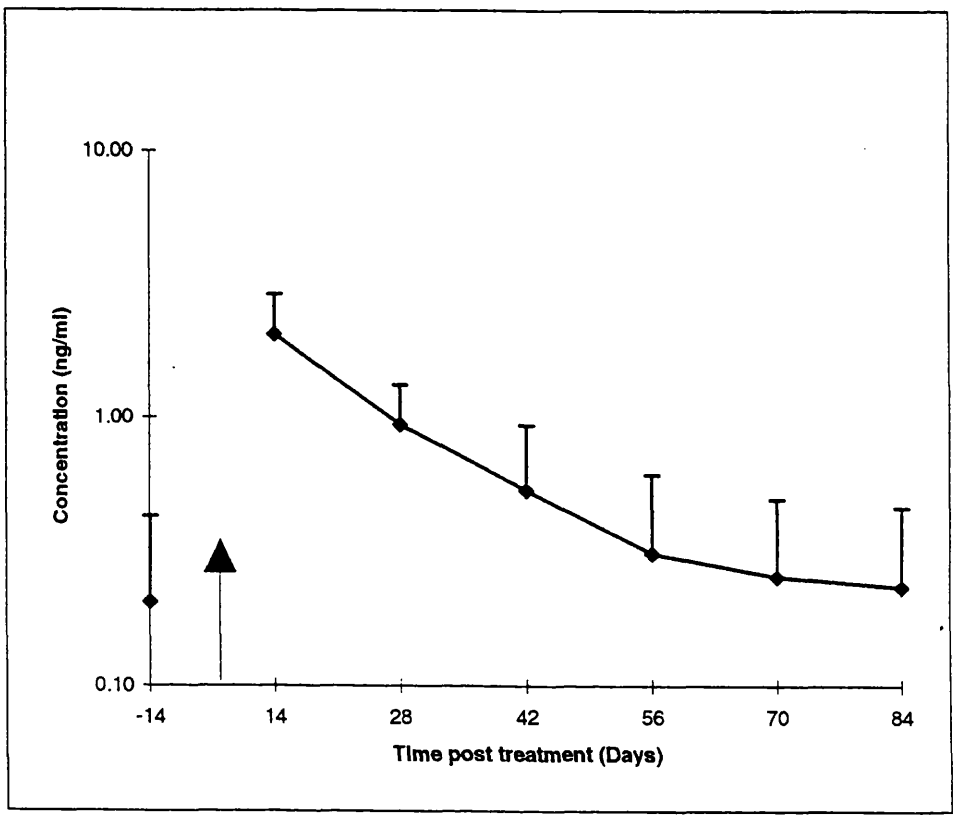
Table 3.2 Mean isometamidium concentration (ng/ml) in the sera of treated cattle. SD: Standard deviation.

	Days post treatment						
	- 14	14	28	42	56	70	84
Average	0.379	2.059	0.943	0.619	0.404	0.416	0.358
± SD	0.308	0.863	0.396	0.405	0.365	0.301	0.287
Median	0.274	1.961	0.841	0.496	0.305	0.296	0.275

Table 3.3 Drug concentrations on days breakthrough infections were detected. ND = not detected. NS = No sample. * = resistant.

Animal no	Day post treatment	Buffy coat thick/thin	Isometamidium concentration ng/ml	Resistant
889	70	*Tc*	ND	
884	56	*Tc*	0.336	
884	70	*Tc*	0.221	
612	84	*Tc*	ND	
247	84	*Tc*	ND	
614	70	*Tc*	0.376	
617	84	*Tc*	0.251	
645	84	*Tc*	0.212	
623	84	*Tc*	NS	
625	70	*Tc*	1.288	*

Figure 3.1 Mean concentration of isometamidium (ng/ml) in sera of treated cattle at North Nyamphande, Zambia. The vertical bars represent the range of the standard deviation. ↑ indicates day when the cattle were treated with isometamidium



The average PCV of the cattle in the isometamidium group increased after they were treated with isometamidium (Tables 3.4a, and Fig 3.2). PCV of infected animals were generally below 25% (Table 3.4b, 3.4c).

The PCVs of animals in Group 1 changed significantly over time ($P < 0.05$). The same was observed in the control group ($P < 0.05$). PCVs did not differ significantly between the two groups ($P > 0.05$).

Table 3.4a The mean PCV (%) of the cattle in the treatment group and control group. SD: Standard deviation.

	Days post treatment						
Group	- 14	+ 14	28	42	56	70	84
Group 1	25.09	28.06	29.19	30.12	27.93	27.82	24.87
± SD	5.32	3.56	3.76	2.93	4.33	3.17	4.22
Group 2	28.67	30.56	29.67	29.35	28.46	27.60	26.42
± SD	3.98	3.29	4.20	3.85	3.80	4.93	4.64

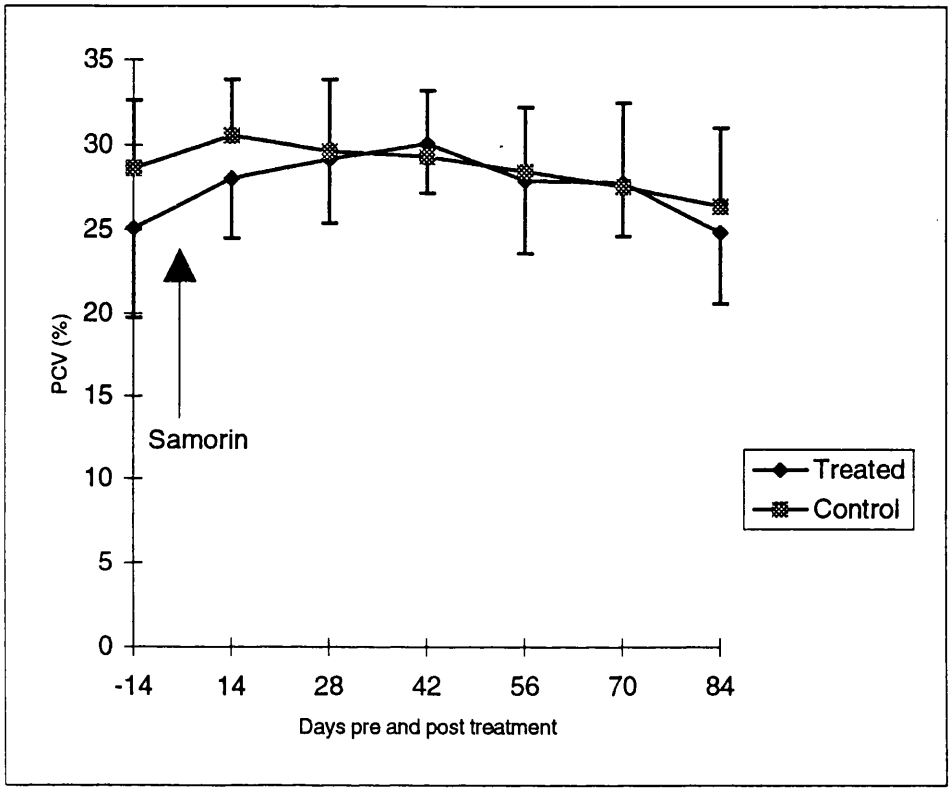
Table 3.4b The individual PCV (%) of cattle in Group 1 on the day of breakthrough infection.

Animal no	Day of break-through infection	PCV (%)	Animal no	Day of break-through infection	PCV (%)
889	70	26	612	84	32
884	56	23			
884	70	28			
247	84	23			
614	70	31			
617	84	17			
645	84	20			
625	70	22			
623	84	19			

Table 3.4c The individual PCV (%) of cattle in Group 2 on the day of breakthrough infection.

Animal no	Day of break-through infection	PCV (%)	Animal no	Day of break-through infection	PCV (%)
640	84	30	624	84	28
249	28	15	882	56	24
427	28	33	887	14	34
427	84	28	887	84	15
241	70	29	642	28	24
616	56	31	248	14	31
616	84	18	248	70	15
618	42	30	248	84	18
867	42	31	620	70	24
861	70	29	242	70	30

Figure 3.2 The mean PCV (%) of the cattle in the treatment group and control group. Vertical bars shows range of standard deviation. ↑ indicates the day cattle were treated with isometamidium.



3.4 DISCUSSION

Drug resistance has become a major problem in the control of animal trypanosomiasis. There has been an increasing number of reports of trypanocide resistance in sub-Saharan Africa in recent years (Chitambo et al., 1992; Peregrine, 1994; Joshua, 1995). Incorrect strategy i.e. irregular treatment or prolonged intervals between treatments and underdosage of the trypanocidal drugs are some of the major causes of the apparent drug resistance of trypanosomes in the field (Connor, 1989). Assays to assess drug resistance in animal trypanosomes are required for use at the herd or area level that will allow veterinary personnel to advise field staff as to which of the few trypanocidal drugs should or should not be used for the control of trypanosomiasis in those areas where particular drugs seem to lose their curative or prophylactic activity (Kaminsky, 1990). Regular monitoring of the prevalence of drug resistance in the field remains a problem as methods used to monitor drug resistance lack sensitivity and most of them are neither field applicable nor widely available.

Although the mouse sensitivity test has been widely used to characterise the drug resistance of trypanosomes populations, it has been criticised because extrapolation of drug sensitive data from small to large animals is not possible due to inconsistencies between results obtained in small animals and in large animals (Sones et al, 1988). In addition, the mouse sensitivity test may take a long time and not all field isolates develop in them. *In vitro* methods generally require considerable laboratory equipment and therefore they cannot be conducted easily in many African laboratories. There is also the problem that some isolates are slow to adapt to *in vitro* cultivation and selection may occur during the adaptation process.

The use of the drug ELISA to determine drug concentrations in the sera of treated animals has been demonstrated (Eisler et al, 1993, 1996). This assay has proved to be reliable in measuring drugs levels in the sera of treated cattle where concentrations of the drug as low as 0.2 ng/ml of serum can be detected by using this method. If the levels of drug in these treated animals are related to the occurrence of breakthrough infections, the ELISA could be used to distinguish between drug resistance and inadequate treatment.

In this field trial it was evident from the ELISA results that drug levels in individual traditional village cattle treated under field conditions could be measured successfully using the sequential saturation drug ELISA. It was also shown that when used together with the buffy coat technique and the thin and thick blood smears technique for parasitological examination, evidence of potential drug resistance could be obtained.

Eisler et al (1994) used the drug ELISA technique to determine the concentration at which three isolates of *T.congolense* which possessed different levels of resistance to isometamidium broke through in Boran cattle that were treated with isometamidium at 1 mg/ml body weight. They found that isolates which were highly resistant (IL 3893) and moderately resistant (IL 3889) broke through when the mean drug concentration in the serum of cattle was 6.1 ng/ml (range 2.8 ng/ml - 12 ng/ml). The mean drug concentration in serum of cattle infected with a susceptible isolate (IL 1180) at the time of the breakthrough was not determined, but the mean drug level one month before the first infection (1 out of 5 cattle) was 0.7 ng/ml (range 0.5 ng/ml - 0.89 ng/ml). If the half life ($t_{1/2}$) for the drug was calculated at 23.9 days in their experiment then by the time the first infection was detected the mean drug level might have been around 0.35 ng/ml

(range 0.25 ng/ml - 0.44 ng/ml). If IL 1180 is not the most isometamidium-sensitive population that has been described (Peregrine et al, 1991) then the most sensitive trypanosome isolate may be cleared at concentration even lower than the one eliminating IL 1180. Eisler et al (1994) concluded that trypanosomes occurring in the presence of serum concentration of > 6 ng/ml are highly resistant and those occurring in the presence of concentration between 0.75 ng/ml and 6 ng/ml are moderately resistant.

Recently Eisler et al., (1996) carried out an experiment to determine the relationship between serum concentrations of isometamidium in Boran cattle and protection against tsetse-transmitted challenge with IL 1180 a drug-sensitive clone and a cloned population of *T. congolense* that posssessed a moderate level of resistance to isometamidium. They concluded that trypanosomes occuring in cattle with serum isometamidium concentration of 2.0 ng/ml may be considered at least moderately drug-resistant and at concentrations between 0.4 and 2.0 ng/ml a low level of drug-resistance.

According to the observations made by Eisler et al., (1994, 1996) using IL 1180 the serum drug level that can eliminate susceptible trypanosome populations is from 0.4 ng/ml and above.

In the experiment carried out in Zambia the mean drug concentration at three months was 0.358 ng/ml. The result of the experiment in Zambia indicated that 1 out of 10 (10%) of the population of *Trypanosome congolense* possess at least moderate resistance at serum drug concentration of above 0.6 ng/ml. Unfortunately the drug sensitivity of this isolate could not be confirmed in mice or experimental cattle.

Overall, there were 10 trypanosome infections detected during the 12 week period following isometamidium administration. Twenty-six (26) infections occurred in untreated control cattle over the same period. Some untreated control cattle were found to be infected at every fortnightly sampling, whilst no isometamidium treated cattle were found to be infected during the first 6 weeks. Thereafter, one animal became infected over the following fortnight. Four more animals became infected over weeks 9 - 10 and 5 more became infected over weeks 11 -12.

The severity of anaemia which follows infection is affected by several factors such as the virulence of the species of trypanosomes and host factors such as age, nutritional status and breed (Murray and Dexter, 1988). In the present experiment the PCV's of the infected animals at the time of breakthrough infection were generally lower than those of the animals that were not infected. After treatment PCV's of the infected animals increased.

In conclusion, it is possible to determine drug levels in many samples from field treated animals within a short time, and use these data for monitoring drug resistance. Unlike the technique of inoculation and treatment of mice in which only rodent infective trypanosomes can be evaluated, this technique deals with all species of trypanosomes, including *Trypanosoma vivax* which is not usually infective for mice. The results of this experiment showed that there was no evidence of drug-resistant trypanosomes at North Nyamphande.

Chapter 4

Evaluation of the prophylactic effect of the slow release device containing homidium bromide in cattle under field conditions

INTRODUCTION

Homidium bromide (Ethidium®; CAMCO) is commonly administered to cattle at a dose rate of 1mg/kg body weight to treat trypanosomiasis. At this dose rate in cattle it has been shown to have prophylactic activity varying from two to nineteen weeks against tsetse field challenge (Leach et al., 1955 ; Dolan et al., 1990, 1992). The duration of prophylaxis depends primarily on the dose of the drug administered and the strain and species of parasite (Kinabo et al., 1988).

Several of the few trypanocides that are currently used to control trypanosomiasis in domestic animals produce local toxicity at the injection sites (Peregrine, 1994). Further more, many of these drugs offer only a short period of prophylaxis. In order to prolong prophylactic activity and reduce local toxicity at the injection sites produced by these drugs, different formulations (carrier or delivery systems) have been produced and evaluated. These include: Suramin complexes, dextran complexes, liposomal formulations, carrier erythrocytes and homidium and isometamidium polymers (Peregrine, 1994).

In order to determine whether the period of prophylactic effect of homidium bromide could be extended by incorporating the drug into a biodegradable slow release device (SRD) a polymer-drug complex was administered using a special needle and syringe, by the subcutaneous route above the shoulder region into the cattle.

The present study was carried out to test the prophylactic effect of the SRD at 1 mg/kg body weight in cattle under field challenge in Zambia and compare it with the

classical treatment of homidium bromide (Ethidium®) at 1 mg/kg body weight and isometamidium chloride (Samorin®) 1 mg/kg body weight given intramuscularly. The homidium-detection ELISA was used to measure serum drug levels in cattle given slow release devices and in those given by intramuscular injection. Isometamidium serum levels were measured by the isometamidium-detection ELISA (Table 4.3).

MATERIALS AND METHODS

Drugs

A cylindrical polymeric slow release device (polymer type: Rg 203, length 20-30 mm, diameter 3 mm, weight 50-75 mg) containing 25% Ethidium and 0.5% dexamethasone was developed and supplied by the University of Ghent, Department of Organic Chemistry, Section of Polymers. Preparation of SRD rods was by extrusion of a mixture of polyester and homidium bromide. This was done by adding homidium bromide to the solution of polyester in chloroform (CH_2Cl_2) and stirring for 24 hours. Films were then casted and dried under vacuum. Rods were prepared by extrusion of these films at melting temperatures (160°C).

Intramuscular injectable isometamidium chloride (Samorin®, Rhone-Merieux, France) was purchased locally and homidium bromide (Ethidium®, CAMCO) was supplied by the Central Veterinary Research Institute, Zambia.

Study area

The study was conducted at Chipopela Field Research Station, Katete District, Eastern Province of the Republic of Zambia. This station is used as a tsetse monitoring site by the Regional Tsetse and Trypanosomiasis Control Programme (RTTCP) in Zambia. The station lies between the latitude of 31° 50′ E and 32° E and Longitude of 13° 45′ S and 14°S and about 45 Km into the north-western part of Katete district. The area consist of miombo and mopani woodlands and lies in an area where *Glossina morsitan morsitan* and *Glossina pallidipes* occur (RTTCP Annual Report, 1995).

Animals

Two weeks before the trial commenced, 81 traditional Agoni (Sanga type) cattle were screened for trypanosome infection. A total number of 33 cattle were diagnosed positive for trypanosomes on both the buffy coat wet smears and Giemsa stained thin and thick blood smears. This revealed an infection rate of 40.74%. The average PCV was 24.62%. All the 81 cattle were eartagged, weighed and treated with diminazene aceturate (Berenil®; Hoechst) at a dose rate of 7 mg/kg live body weight.

The cattle were traditionally managed and grazed communally on natural pasture on free range in the tsetse infested area. No supplements were given to them. The farmers brought their cattle once every month for sampling.

Experimental design

At the beginning (day 0) of the trial 76 cattle were screened for trypanosomes and divided into four groups and treated according to their groups. All the cattle examined

were negative for trypanosomes on both the buffy coat wet smears and the thick and thin dry blood smears.

Group one Cattle in this group received ethidium in the form of a slow release device (SRD) at a dose of 1 mg/kg live body weight. These implants were injected subcutaneously in the shoulder region using a special SRD needle and syringe (Plate 1b). There were 16 cattle in this group.

Group two Cattle in this group received a dose of 1 mg/kg of isometamidium chloride by intramuscular injection (Plate 1a). There were 20 cattle in this group.

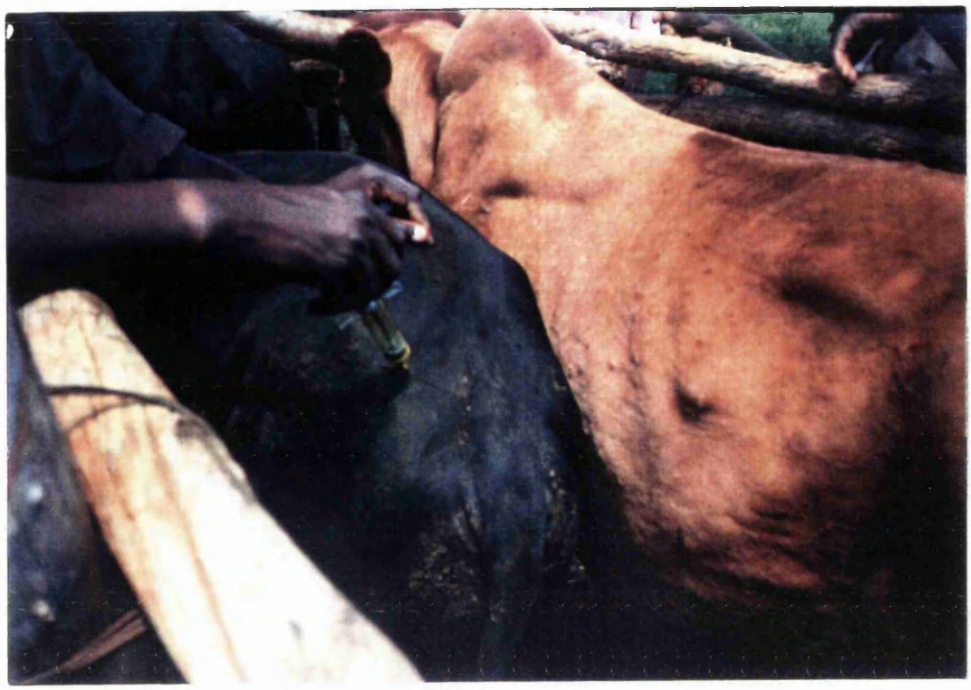
Group three This group received homidium bromide at a dose rate of 1 mg/kg by intramuscular injection. There were 20 cattle in this group.

Group four This was a control group of 20 cattle that received no treatment.

The animals were exposed to natural challenge and grazed communally on free range. Diminazene aceturate, at a dose rate of 7 mg/kg was used to treat any animal that became positive for trypanosomes during the course of the experiment.
A weighband (Dalton, U.K.) was used to estimate the weight of the animals.

Parasitological examination and packed cell volume monitoring

Cattle were screened for the presence of trypanosomes once every month. Blood samples were collected into two heparinized capillary tubes (Hawksley, U.K.) from



a

b

Plate 1. Adminstration of trypanocides in the field;
a) Intramuscular injection of homidium
b) Subcutaneous injection of a slow release device (SRD) containing homidium.

each animal from the ear vein and filled to about 10 mm from the top of the tubes. One tube was sealed immediately with cristaseal® (Hawksley, U.K.) and spun down in a microhaematocrit centrifuge (Hawksley, U.K.) for 5 minutes for packed cell volume (PCV) estimation and buffy coat examination. The second capillary tube was used for making thick and thin blood smears (Plate 2) and also acted as a spare in case of spin-out or breakage during spinning down of the first tube in the centrifuge. After making the thick and thin smears the spare tube was sealed and held in a clearly labelled capillary tray.

After spinning the tubes for 5 minutes in the centrifuge the PCV were read using the micro-haematocrit reader (Hawksley, U.K.). After reading the PCV the capillary tubes were cut with a diamond pencil 2 mm below the buffy coat and wet blood smears made on the slides.

The buffy coat wet smears were examined using the buffy coat wet smear techniques of Murry et al., (1977) at the crushpen. The thin smears were fixed in methanol and later on together, with the thick smears, stained with 10% solution of Giemsa stain, dried and examined for the trypanosomes.

Serum sampling

Each month, blood from the jugular vein of each animal was collected into plain vacutainer tubes (Becton Dickson and Company, USA). Time was allowed for the blood to clot at ambient temperature. Sera was separated from the clotted blood after spinning in the centrifuge into 2 ml cryogenic vials (Alpha Labs, U.K). Sera in the

Plate 2. Preparation of thick and thin blood smears in a field laboratory.

cryogenic vials were then stored in the freezer at - 20°C prior to analysis for drug concentrations.

Serum Drug Concentrations

A sequential saturation ELISA was used to determine the concentration of isometamidium and homidium in the sera.

This ELISA is fully described in Chapter Two.

Assessment of the sensitivity of isolates of *Trypanosoma congolense* to Isometamidium chloride and Homidium bromide in mice

Two isolates of *Trypanosoma congolense* obtained from cattle that were in the experiment, were tested in mice using the protocol as followed by Sones et al, (1988). Isolate CH01 was collected in December 1994 before the start of the experiment and isolate CH 542 in March 1995 when the first infections in cattle in the SRD group were observed. They were isolated from the cattle directly into mice and later on stabilates were made from the infected mice and stored in liquid nitrogen for further use.

In the procedure, Swiss mice were inoculated intraperitoneally with 0.2ml of diluted blood containing approximately 1 x 10^5 trypanosomes. A Neubauer counting chamber was used to count the live trypanosomes that were innoculated into the mice. Phosphate buffered saline with glucose (P.B.S.G pH 8.0, ionic strength 0.145) was used to dilute the blood.

Monitoring of blood by wet film preparations from the tail of mice followed every day until trypanosomes were detected. The mice were then treated with drugs

intraperitoneally as per treatment group (Table 4.7). After treatment they were monitored every day during the first week and then once every week for 90 days. Isolate CH01 was assessed at the CVRI in Zambia and CH 542 at ILRI in Kenya.

Monitoring of density of tsetse flies

Both traps and Black Screen Man Fly Rounds (BFR) were used to monitor fly density. Epsilon traps (Plate 3) each baited with acetone released from standard 0.5 litre brown glass bottles without a lid, plus 2 sachets of phenol were placed in the infested area where the animals under the experiment grazed. For fly rounds, a black screen cloth 2 x 1 metre suspended from a bamboo stick on the shoulders of two men was used (Plate 4). The black screen was baited with acetone in a 0.5 litre brown bottle without a lid and two sachets of phenols in two pockets on it. The two men walked and stopped at 200 metre (200 steps) intervals for 3 minutes and caught flies which rested on the screen and those following them in their vicinity with a hand net. The walking was done on permanent fly round routes in the grazing area. The fly rounds were done in the morning and afternoon every day.

Monitoring of the fly density started in December 1994 and ended in June 1995. This covered both rainy and dry seasons. The rainy season occurred from December to April and the dry season from May to June.

Plate 3. An Epsilon trap in the grazing area.

Plate 4. Preparing for a Black Screen Man Fly Round.

RESULTS

Parasitaemia

In the SRD homidium group the first infections (3 animals out of 16) in the group were observed two months after treatment. More than 50% of the infections were observed in the fourth month after treatment. In the group of cattle given i.m. isometamidium the first infections (4 out of 20 animals) were revealed two months after treatment and by the third month after treatment more than 50% of the animals were infected. In the group given i.m homidium the first infections (10 out of 20 = 50%) were observed in the second month after treatment. In the control group 8 out of 20 animals were positive one month after the beginning of the study and by the second month 50% of them were positive (Table 4.1a and Table 4.1b).

Table 4.1a Cumulative total number of positive animals in group 1 (SRD-homidium), group 2 (i.m. isometamidium), group 3 (i.m. homidium) and group 4 (control) months post treatment.

	Months post treatment						
Group	0	1	2	3	4	5	6
1	0	0	3/16	7/16	10/16	12/16	12/16
2	0	0	4/20	10/20	12/20	12/20	14/20
3	0	0	10/19	16/19	17/19	17/19	17/19
4	0	8/20	10/20	16/20	17/20	18/20	18/20

Table 4.1b Cumulative number of positive animals (%) in group 1 (SRD-homidium), group 2 (i.m. isometamidium), group 3 (i.m. homidium) and group 4 (control) months post treatment.

	Months post treatment						
Group	0	1	2	3	4	5	6
1	0	0	19	44	62	75	75
2	0	0	20	50	60	60	70
3	0	0	53	84	89	89	89
4	0	40	50	80	85	85	85

When calculating infection rates for each month, the pre-patent period (time between the infective tsetse bite and appearance of trypanosomes in the blood of the animal about two-three weeks) was taken into account. Animals that were treated with diminazene (Berenil®) the previous month were excluded from infection rate calculations. This is because exposure to therapeutic drugs such as Berenil® is one of the factors that determines the length of this period (Stephen, 1966). Mean pre-patent period in sheep on a low protein diet is between 12 and 26 days (Katunguka-Rwakishaya et al. 1995). Animals found positive and not sampled the previous month were also not included in the calculation (Table 4.2a and Tables 4.2b) because it was not known whether the infections were acquired in the previous month or during the present month for which the infection rate had been calculated.

In cattle from the SRD ethidium group, the apparent average protection period was much longer than that of the group given homidium by intramuscular injection

Table 4.2a Number of positive cattle over number of cattle at risk, months post treatment. Group 1 (SRD-homidium), group 2 (i.m. isometamidium), group 3 (i.m. homidium) and group 4 (control).

	Months post treatment					
GROUP	1	2	3	4	5	6
1	0/16	3/16	4/13	4/10	5/8	0/4
2	0/15	4/20	6/12	1/9	2/10	1/5
3	0/19	10/19	6/9	1/5	0/4	1/4
4	8/20	2/11	11/18	1/7	2/16	0/16

Table 4.2b Incidence rates (%) of the animals in the four groups, months post treatment. Group 1 (SRD-homidium), group 2 (i.m. isometamidium), group 3 (i.m. homidium) and group 4 (control).

	Months post treatment						
Group	0	1	2	3	4	5	6
1	0	0	19	31	40	62	0
2	0	0	20	50	11	20	20
3	0	0	53	67	20	0	50
4	0	40	18	61	14	12	0

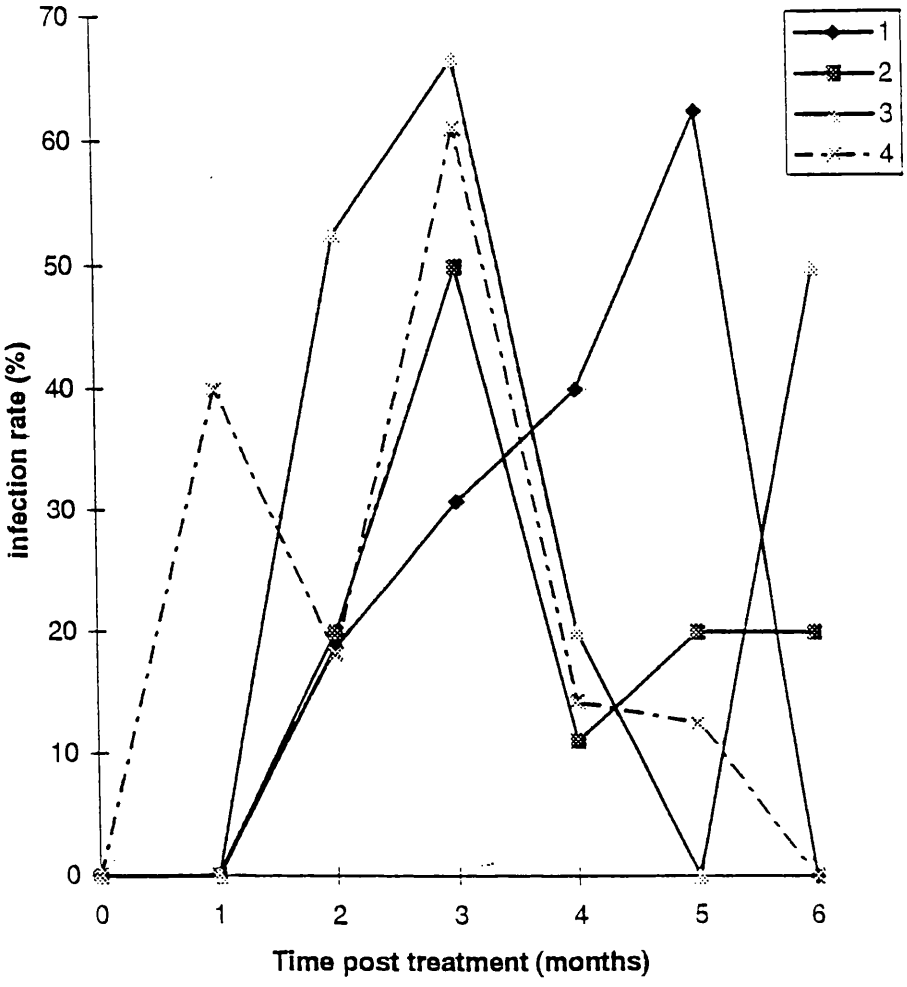


Figure 4.1 Incidence rate (%) of the animals in the four groups. 1 is SRD-homidium group, 2 is isometamidium group, 3 is i.m homidium group and 4 is control group

Inflammatory reaction

An inflammatory reaction at the SRD implantation sites was observed one month after treatment in more than 50% (9/16) of the animals. The nodules measured on average 2-3.5 cm in width and 4-5 cm in length. They persisted until the third month after treatment (Plate 5).

Drug concentrations

The isometamidium concentrations are shown in Table 4.3 and Figures 4.3a and 4.3b. Isometamidium ELISA analysis showed that the drug levels in the group given intramuscular isometamidium reached maximum levels one month after treatment and by the third month after treatment the mean level had fallen to levels of almost 0.2 ng/ml. In the SRD homidium group the drug levels reached a maximum level two months after treatment and then slowly fell until the fourth month after treatment. Levels of less than 2 ng/ml were detected in the fifth month after treatment in the SRD group . In the i.m. homidium group the maximum levels were detected one month after treatment and by the second month after treatment the levels were below the level of 0.2 ng/ml (Table 4.3 and Figures 4.3a, 4.3b). The ELISA showed that high levels of the drug were maintained above the minimal detectable level of 0.1 ng/ml (Eisler et al., 1993) for the longest time in the animals in the SRD-homidium group and the isometamidium group.

Plate 5. A nine month old calf showing an inflammatory reaction at the injection site in the shoulder region one month after subcutaneous administration of a SRD-homidium implant.

Table 4.3 Mean drug concentrations (ng/ml) per treatment group, months post treatment.
SRD-HBR = SRD-homidium, I.M ISMM = intramuscular Isometamidium
IM HBR = intramuscular homidium. SD = Standard deviation. ND denotes drug not detected.

	Months post treatment						
Group	0	1	2	3	4	5	6
SRD-HBR	0.264	0.394	0.806	0.374	0.300	ND	ND
± SD	0.276	0.153	0.245	0.133	0.091		
IM ISMM	0.347	1.242	0.580	0.236	0.375	ND	ND
± SD	0.405	0.59	0.305	0.114	0.369		
IM HBR	0.212	0.312	ND	ND	ND	ND	ND
± SD	0.132	0.211					

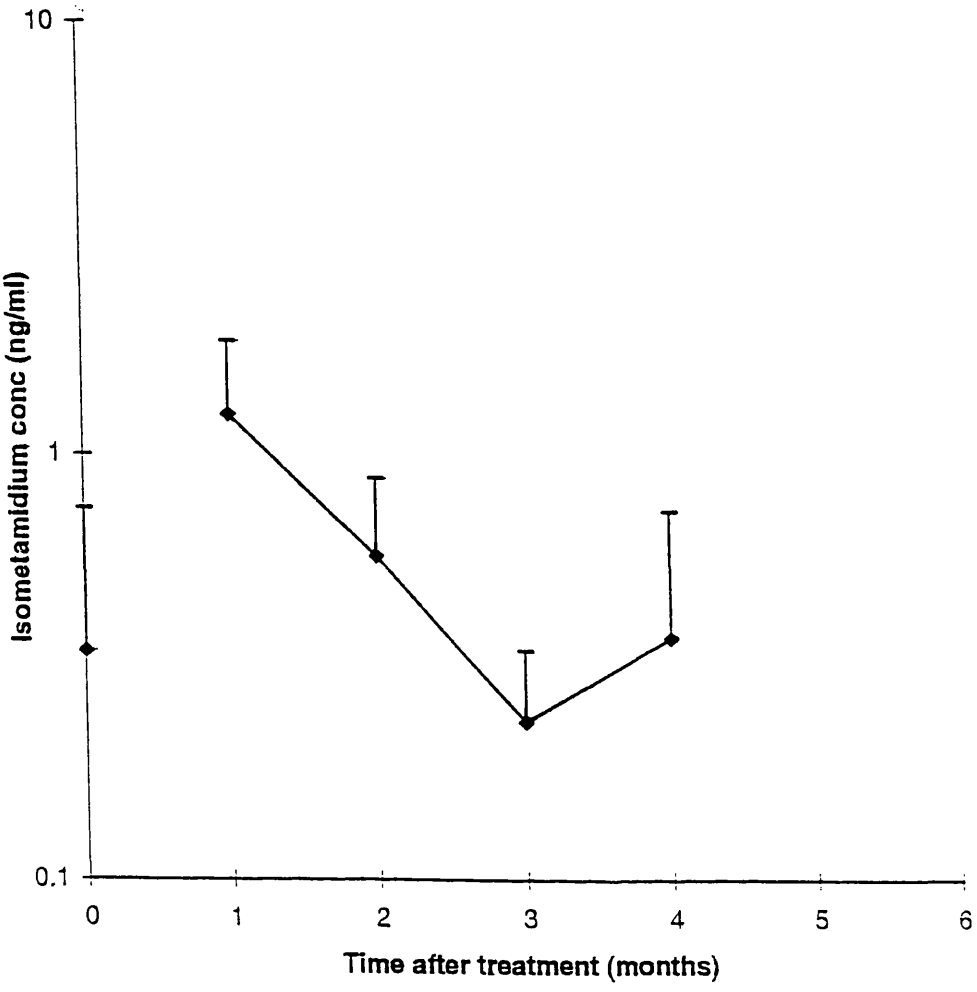


Figure 4.3a Mean isometamidium concentration (ng/ml) of the cattle treated with intramuscular isometamidium, months post treatment. The vertical bar indicates the range of the standard deviation.

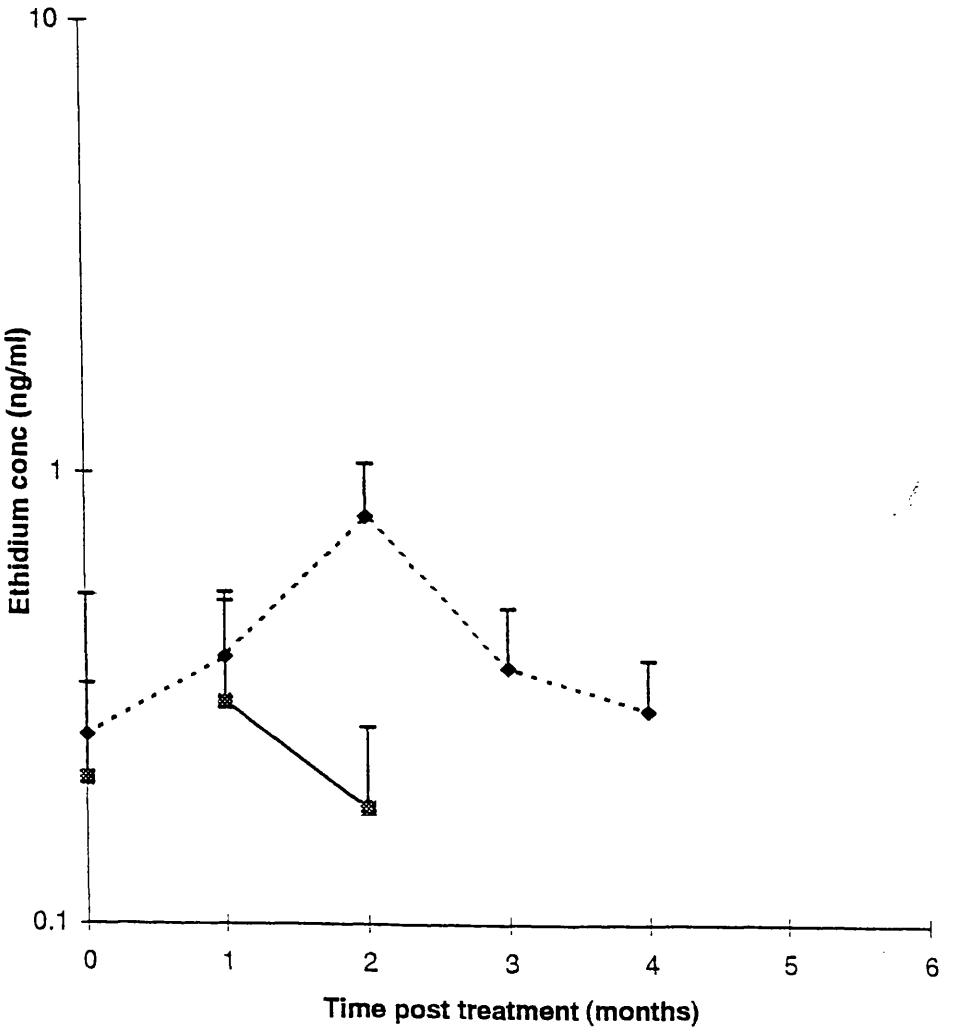


Figure 4.3b Mean homidium concentrations (ng/ml) of the cattle treated with either SRD-homidium (--------) or i.m homidium (_____). The vertical bar indicate the limit of the range of the standard deviation.

Packed Cell Volume (%)

The packed cell volume (PCV) values increased following treatment in the three groups of animals that received homidium or isometamidium and then gradually went down as the number of infection increased (Table 4.4a, b, c and Figure 4.4). In the control group the values remained almost the same during the experiment (Table 4.4a, and Fig 4.4).

Table 4.4a Mean PCV (%) of the animals in the four groups, months post treatment. Group 1 (SRD-homidium), group 2 (i.m. isometamidium), group 3 (i.m. homidium) and group 4 (control).

	Months post treatment							
GROUP	-1	0	1	2	3	4	5	6
1	26.07	29.41	28.31	31.56	30.25	25.71	22.27	30.71
± SD	8.12	6.38	5.00	6.55	5.78	4.25	6.93	3.95
2	22.16	29.25	29.4	30.30	26.60	26.60	24.43	29.80
± SD	3.70	2.86	3.36	3.26	4.39	4.05	5.32	5.16
3	25.72	27.9	32.05	30.63	31.30	24.17	27.60	30.20
± SD	5.37	5.16	5.17)	5.52	8.06	7.70	3.91	3.70
4	25.68	29.19	25.8	30.11	26.30	24.82	25.72	29.16
± SD	5.97	4.40	4.44	5.32	6.59	5.08	4.52	5.09

Table 4.4b Individual PCV(%) and drug concentrations (ng/ml) of cattle in SRD-homidium group at the first detection of infection. ND denotes not detected and NS no sample. * denotes possible drug-resistance.

Animal No	Tryps	Months post treatment	PCV (%)	Drug conc (ng/ml	Animal No	Tryps	Months post treatment	PCV (%)	Drug conc (ng/ml)
535	*Tc*	2	31	0.476	208	*Tc*	4	24	0.373
532	*Tc*	3	20	0.442	210	*Tc*	3	29	0.4
533	*Tc*	4	24	0.240	542	*Tc*	2	29	NS
538	*Tc*	5	19	ND	696	*Tv*	5	22	NS
519	*Tc*	3	28	0.538*	539	*Tc*	3	24	0.244
543	*Tc*	3	22	0.301					
207	*Tc*	2	35	1.116*					

Table 4.4c Individual PCV(%) and drug concentration (ng/ml) of the cattle in isometamidium group at the first detection of infection. ND denotes not detected. * denotes possible drug-resistance.

Animal No	Tryps	Months post treatment	PC V (%)	Drug conc (ng/ml)	Animal No	Tryps	Months post treatment	PC V (%)	Drug conc (ng/ml)
223	*Tv*	3	21	ND	537	*Tc*	5	23	ND
245	*Tc*	3	17	ND	531	*Tc*	5	22	ND
229	*Tc*	3	28	ND	221	*Tc*	6	26	ND
674	*Tc*	3	21	0.250	201	*Tc*	4	23	0.226
548	*Tc*	2	26	0.362	248	*Tc*	2	27	0.362
545	*Tc*	3	27	0.535*	546	*Tc*	3	24	0.328
536	*Tc*	2	35	0.622*	540	*Tc*	2	23	0.214

Table 4.4d Individual PCV(%) and drug concentration (ng/ml) of the cattle in im-homidium group at the first detection of infection. ND denotes not detected.

Animal No	Tryps	Months post treatment	PCV (%)	Drug conc (ng/ml	Animal No	Tryps	Months post treatment	PCV (%)	Drug conc (ng/ml)
691	*Tc*	2	31	-	225	*Tc*	2	30	0.238
690	*Tc*	4	13	ND	209	*Tc*	2	28	ND
214	*Tc*	3	28	ND	217	*Tc*	2	35	ND
200	*Tc*	3	29	ND	239	*Tc*	3	35	-
692	*Tc*	3	18	ND	219	*Tc*	3	20	ND
212	*Tc*	2	27	ND	231	*Tc*	2	29	ND
673	*Tc*	3	18	-	216	*Tc*	3	21	ND
241	*Tc*	2	40	ND	236	*Tc*	2	28	0.201
222	*Tc*	2	24	ND					

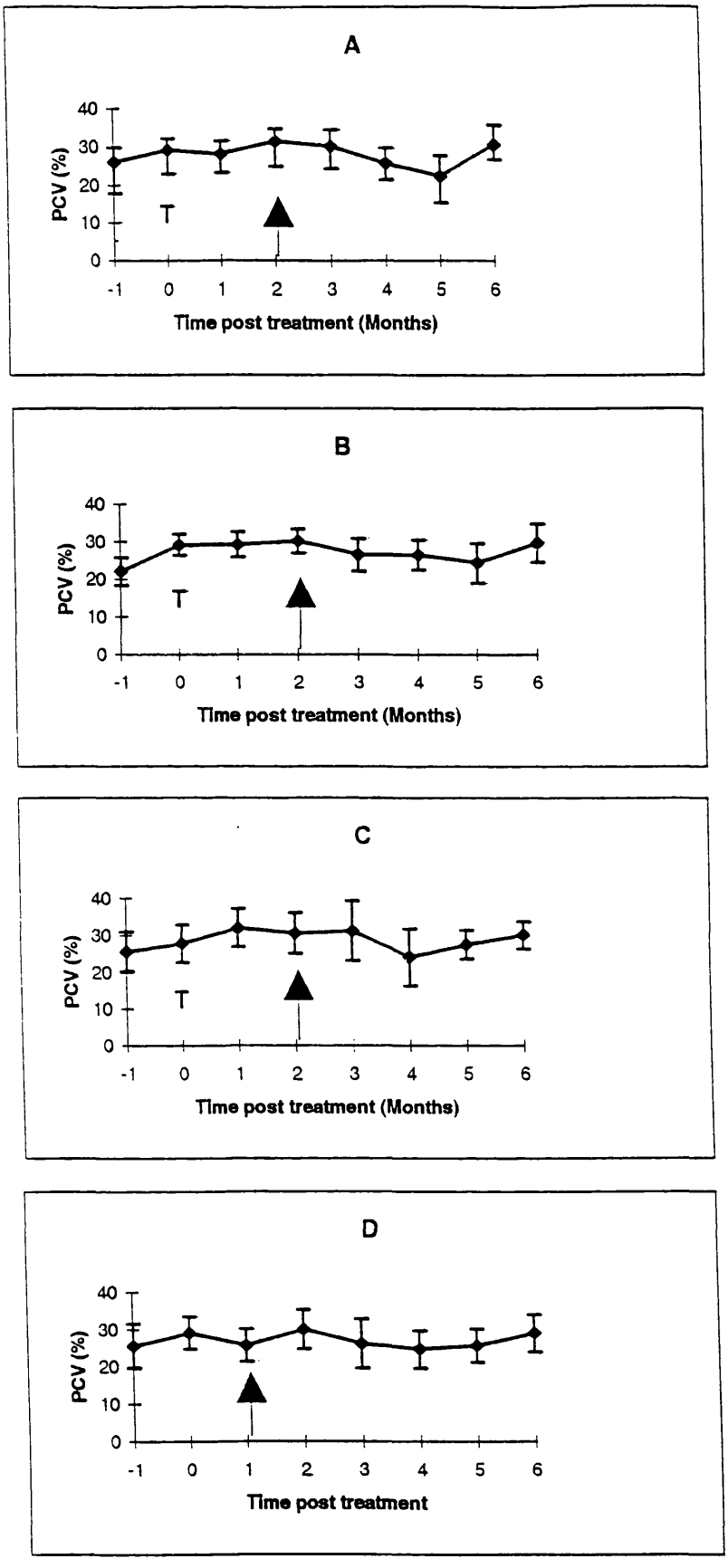


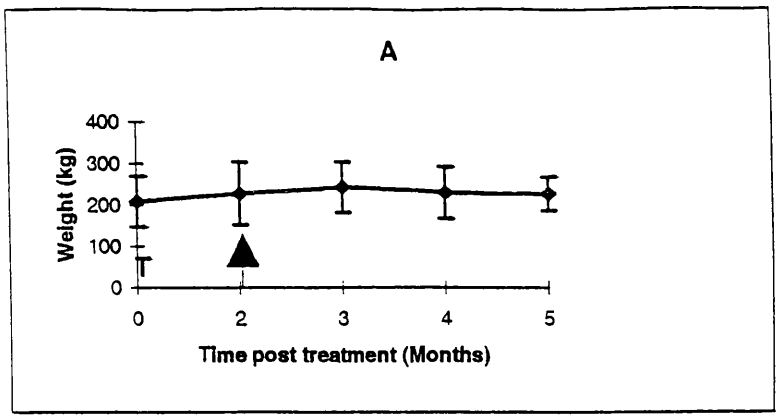
Figure 4.4 Mean PCV (%) of the animals in the four groups, (A) animals that were given SRD-homidium (B) animals that were given isometamidium intramuscularly. (C) animals that were given homidium intramusculary (D) animals in the control group. **T** denotes time of treatment. ↑ denotes time of first detection of paraasitaemia.

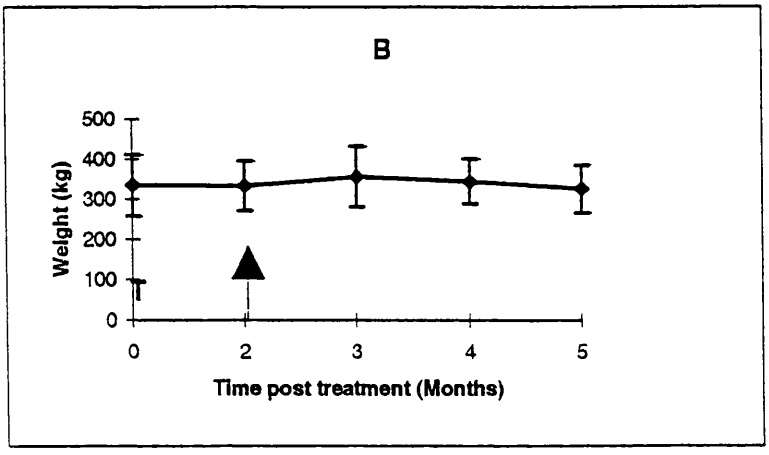
Body Weight

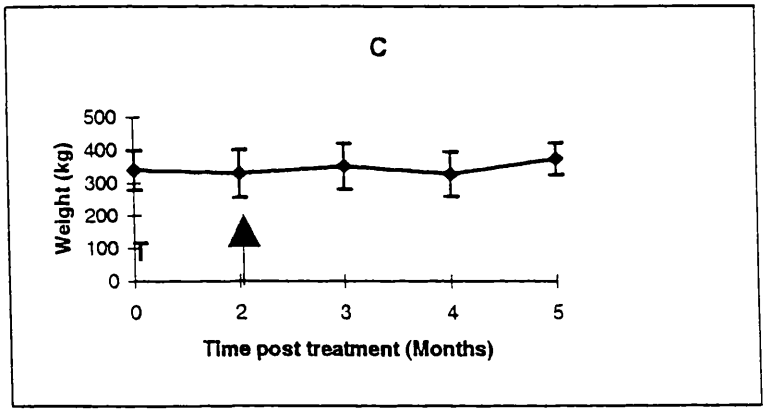
The mean body weight of the three treatment groups increased up to the third month after treatment and thereafter started to fall (Table 4.5 and fig 4.5) as the proportion of cattle with trypanosome infection increased.

Table 4.5 Mean body weight (kg) of the animals per treatment group, months post treatment. Group 1 (SRD-homidium), group 2 (i.m. isometamidium), group 3 (i.m. homidium) and group 4 (control).

	Months post treatment				
GROUP	0	2	3	4	5
1	209.29	228.56	241.87	230.71	228.18
± SD	60.80	75.69	60.91	62.32	41.19
2	336.05	334.60	357.67	346.25	327.14
± SD	76.76	61.27	75.09	56.23	59.02
3	339.00	329.58	351.58	328.33	374.00
± SD	59.82	71.94	70.12	66.98	48.27
4	316.67	320.74	320.55	304.41	292.5
± SD	53.87	44.55	53.77	54.25	56.31







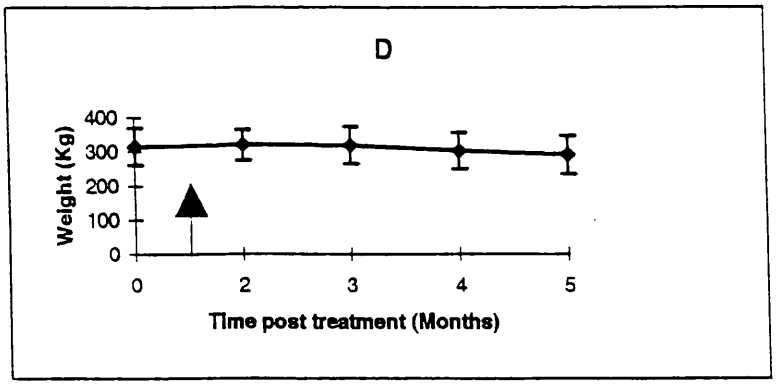


Figure 4.5 Mean body weights(kg) of the animals in the four groups, (A) animals that were given SRD-homidium (B) animals that were given isometamidium intramuscularly. (C) animals that were given homidium intramusculary (D) animals in the control group. **T** denotes time of treatment. ↑ denotes time of first detection of parasitaemia.

Tsetse monitoring

Tsetse monitoring results (Table 4.6 and Figure 4.6) showed high numbers of flies throughout the trial. There was slight drop in the number of flies caught in January 1995 (month 0) and February 1995 (month 1 post treatment) because some traps were stolen by villagers. These traps were replaced towards the end of February 1995. It is also possible that the fall in numbers might have been caused by the more dispersed nature of the tsetse population in the rainy season (Nash, 1939).

Table 4.6 Tsetse fly catches in traps and on fly rounds in the area where the cattle grazed.

	TRAPS				**BLACK SCREEN FLY ROUNDS**				
MONTH	*Glossina morsitans*		*Glossina pallidipes*		*Glossina morsitans*		*Glossina pallidipes*		TOTAL
	M	F	M	F	M	F	M	F	
DEC 94 (Month -1	9	5	0	0	185	62	0	0	261
JAN 95 (Month 0)	7	0	0	0	121	19	0	0	147
FEB 95 (Month 1)	1	22	3	0	75	38	0	0	139
MAR 95 (Month 2)	12	46	0	0	143	48	0	0	249
APR 95 (Month 3)	39	142	0	0	138	70	0	0	389
MAY 95 (Month 4)	32	114	0	0	109	72	0	0	327
JUN 95 (Month 5)	32	155	0	0	24	27	0	0	242

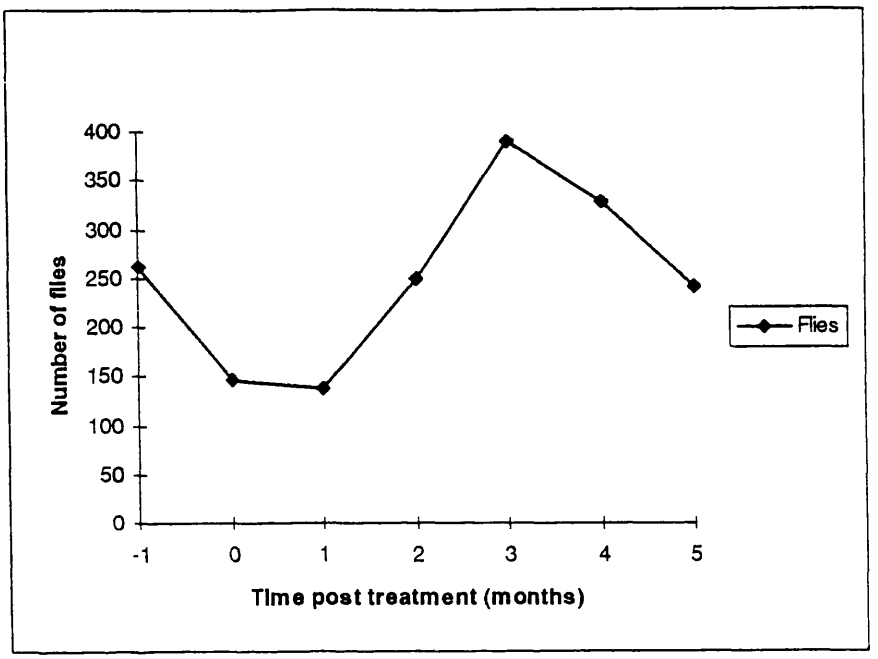


Figure 4.6 Total number of catches of flies per month in both the traps and on fly rounds.

Drug sensitivity tests

Drug sensitivity tests on the two isolates of *T. congolense* in mice showed that CD_{50} for isometamidium chloride was ≤0.01 mg/kg live body weight of the mouse. For homidium bromide it was 0.3 mg/kg live body weight whereas for diminazene aceturate was 5.8 mg/kg live body weight (Table 4.7).

Prepatent period

The mean prepatent period of CH01 was 5.5 (±0.51) days following the needle challenge and For CH 542 it was 2 (± 0) days.

Virulence and pathogenicity

All the mice (5/5) infected with CH01 and which had not received treatment (control group) died during the monitoring period. The mean number of days to death was 16 (± 4.5) days. For CH 542 the mice were euthanised with CO_2 immediately they became positive.

Response to treatment with isometamidium, homidium bromide and berenil

There was no relapse infection for CH01 after treatment with isometamidium at all the treatment dosages (0.01 mg/kg, 0.1 mg/kg, 1 mg/kg, 5 mg/kg and 10 mg/kg).

For CH 542 one out of six relapsed 24 days post-treatment with isometamidium at dose rate of 0.01 mg/kg. With homidium bromide, at 0.01 there was no effect whereas at 0.1 mg/kg three out of six relapsed six days post-treatment. In the diminazene aceturate group, four treated at 2 mg/kg, relapsed six days post-treatment and two did not respond to treatment (Table 4.7)

Table 4.7 Allocation of mice to treatment groups and the response to the drugs.

Stock and isolate	Doses (mg/kg) of ISMM/HBR/ Berenil	No effect	Temporary clearance	cure
CH01	10.0	0	0	5
Stock-ISMM	5.0	0	0	5
	1.0	0	0	5
	0.1	0	0	5
	0.01	0	0	5
CH 542	2.0	0	0	6
Isolate-ISMM	0.1	0	0	6
	0.01	0	1	5
CH 542	2.0	0	0	6
Isolate-HBR	0.1	3	3	0
	0.01	6	0	0
CH 542	50	0	0	6
Isolate-diminazene	10	0	0	6
aceturate	2	2	4	0

Stock-ISMM: Original stock tested against ISMM
Isolate-HBR: Breakthrough isolate tested against HBR
Isolate-ISMM: Breakthrough isolate tested against ISMM
Isolate-diminazene aceturate: Breakthrough isolate tested against diminazene aceturate

Discussion

The trial clearly showed that under field conditions a longer prophylactic period could be obtained by treating the cattle with homidium formulated into a slow release device (SRD). As demonstrated in this experiment a much longer protective period was offered to cattle treated with SRD than those treated with homidium given by intramuscular injection. In the cattle given homidium by intramuscular injection only 47% (9/19) were protected by the second month after treatment but 81% (13/16) were still protected in the SRD group. Whereas 50% of cattle treated with homidium by intramuscular injection remained protected for less than two months, 50% of the cattle treated with SRD remained protected for almost four months after treatment. Cattle in the isometamidium group were protected for a longer time than the cattle treated with homidium by intramuscular injection but not as long as those treated with SRD. Isometamidium forms a depot after intramuscular injection which slowly releases drug (Kinabo and Bogan, 1988). This might be the reason why the prophylactic period of isometamidium was almost the same as the period offered by the homidium SRD.

Although the SRDs were coated with 0.5% dexamethasone to prevent tissue reactions at the sites, these did occur. It appears that there was direct contact between homidium in the SRD and the surrounding tissue. This could have caused variations in the prophylactic effect in individual cattle (Geerts et al., 1993; Kageruka et al., 1996). There was less variation in the group of cattle treated with homidium given by intramuscular route. Variation was also observed in the group of cattle given isometamidium i.m. probably as a result of the inflammatory reaction that this drug causes at the injection site (Kinabo and Bogan, 1988).

Apart from inflammatory reaction at the implantation site of the SRD homidium no other side effects were observed in cattle that were given these implants. The inflammatory reaction at the implantation sites disappeared by the third month after treatment. There was no death associated with treating the cattle with SRD during or after administration of the implants.

The advantage of the SRD homidium is the protection period offered is much longer than in the case of an intramuscular injection using the same dose rate of 1 mg/kg and the implants can be easily removed from the implantation sites under the skin at slaughter. Homidium given by intramuscular injection cannot be removed from the muscles. Homidium is a mutagenic drug and because of this it is important to remove the drug before the meat is consumed (Geerts et al., 1993).

The trial also clearly demonstrated that drug detection ELISAs for isometamidium and homidium could be used to measure serum drug concentrations of treated cattle under field conditions (Appendices 7, 8 and 9). Drug concentration as low as the minimum detectable levels of 0.2 ng/ml were easily detected in the treated cattle that were in this field trial in Zambia. Using this ELISA it was possible to examine many samples at a time. In less than 2 hrs, 64 samples can be examined using this ELISA.

Using the sequential saturation ELISA, two (2) out of twelve (12) trypanosome infections that occurred in cattle that were given SDR-homidium were possibly moderately drug-resistant. These cattle had drug levels above 0.6 ng/ml at the time of

breakthrough. In the group that was given isometamidium intramuscularly two out of 14 trypanosome infections were considered moderately drug-resistant. This suggests that at Chipopela there are some trypanosome populations that are moderately resistance to homidium and isometamidium. In the group of cattle that were given homidium intramuscularly none of the trypanosome infections occurred in cattle with serum drug levels above 0.6 ng/ml.

Drug sensitivity tests in mice of the two isolates showed no resistance to isometamidium and homidium. However isolate CH 542 showed slight resistance to diminazene aceturate at the recommended single dosage rate for chemotherapy of 3.5mg/kg. The minimum curative dose (CD_{50}) for the mice was calculated at 5.8 mg/kg for diminazene aceturate.

On average in all the months during the trial more male flies were caught on fly rounds than in traps whilst more females were caught in traps than fly rounds. The results of this work agrees with the observations that were made by Bursell (1961a). Bursell observed that males' attraction to moving objects brought them into contact with teneral females which were also attracted to moving objects. Teneral females were after food as they had not yet taken their first meal and also had not mated before.

Chapter 5

Influence of the plane of nutrition on the duration of isometamidium prophylaxis in sheep.

INTRODUCTION

Isometamidium chloride (Samorin®, May and Baker) is the major chemoprophylactic drug used in domestic ruminants. At a dose rate of 0.5 mg/kg body weight it has been shown to confer complete prophylaxis for as long as 4 months in sheep under field conditions (Griffin and Allonby, 1977a). Toro et al., (1983) showed that the protection afforded by isometamidium, at a dose rate of 1.0 mg/kg against experimental infection by *T. vivax,* in sheep was 195 days.

Several factors have been suggested to have a major effect on the duration of isometamidium prophylaxis in domestic rumiants. Variation in the length of chemoprophylactic protection in the field could result from factors such as dose of the drug and strain of the parasites (Holmes and Torr, 1988). Nutritional status, concurrent diseases and breed susceptibility or tolerance could also be factors of influence (Kinabo, 1988).

The objective of the present study was to investigate whether nutritional intake and body weight influence the duration of prophylaxis afforded by isometamidium. Changes in biochemical parameters such as urea, cholesterol, albumin and β-hydroxybutyrate were also examined following treatment and at the time of breakthrough infection.

MATERIALS AND METHODS

Experimental animals

Twenty (10 pairs of twins) healthy Blackface male lamb castrates, aged 6 months old, were purchased from a hill farm in the West of Scotland. After purchase they were dosed with a broad spectrum anthelmintic, Oxfendazole (Systamex®, Pitman-Moore Ltd) at a dose rate of 5 mg/kg body weight and transferred from the farm to Cochno Research Centre, They were also vaccinated against pasteurella with Heptavac-P® (Hoechst UK Ltd) at a dose rate of 2 ml per sheep. A booster dose was given 4 weeks later. To prevent foot rot the hooves were trimmed and dipped in a 10 % w/v solution of Zinc Sulphate (Gold Hoof, Sheep Fair Products, Brecon).

Feeding and housing

The diet consisted of hay, straw and a mixture of concentrates (Table 5.1a). Straw was given only to animals assigned to the low plane diet. The concentrate was composed of a mixutre of:

1. Barley	967.5	kg/tonne FM
2. Calcium carbonate ($CaCO_3$)	10.0	"
3. Sodium chloride (NaCl)	10.0	"
4. Dicalcium phoshate (Ca_2PO_4)	10.0	"
5. Trace elements	2.5	"

Diets were mixed by hand and animals were given the rations as shown in Table 5.1a. Animals were fed in groups twice a day at 9:00 hr and 16:00 hr. The ration allowance of the high plane diet was 0.4 Kg of concentrate mixture and 0.7 Kg of hay per day.

Sheep on the low plane diet were given 0.1 Kg of concentrate mixture, 0.35 Kg of hay and 0.35 Kg of straw per day. The mean nutrient intake for each group is presented in Tables 5.1b and 5.1c.

Table 5.1a Proximate analyses (g/kg DM) of dietary ingredients.

	Barley conc	Straw	Hay
Dry matter (g/kg) FM	869.1	871.8	852.3
Crude protein	117.6	28.0	96.8
Crude fibre	42.8	456.3	347.8
Ether extract	10.3	13.6	15.3
Ash	48.0	43.8	54.3
GE (MJ/kg)	16.8	17.5	17.7

Table 5.1b Nutrients supplied g/d

Fraction	High plane	Low plane
DM	944.3	690.3
CP	98.6	47.6
CF	222.4	246.7
EE	12.7	9. 6
Ash	49.1	33.7
GE (MJ)	16.4	12.1

Table 5.1c Overall diet analyses g/kg DM

Fraction	High plane	Low plane
DM	100.0	100.0
CP	104.5	69.0
CF	235.5	357.4
EE	13.5	13.9
Ash	52.0	48.9
GE (MJ)	17.4	17.5

Dietary treatments

1. Total diet analyses

The diets were designed in such a way that the levels of crude protein was 50% greater in the high plane diet (99 versus 48 g/d in the low diet). The gross energy supplied was also higher in the high plane diet than the low plane diet (16.4 MJ v 12.1 MJ)

2. Intakes

Fibre intake (g/d) was designed to be similar in each group (220-250g crude fibre/d). Total dry matter and gross energy intake were 35% greater in the high feed animals.

Animals were housed in a large animal fly-proof isolation unit. The two groups of animals were housed separately in two large pens. Wood shavings were used as bedding.

Analyses of feed

Analyses of the feed were done using standard procedure set up by the Ministry of Agriculture, Fisheries and Food (MAFF, 1981).

All protein analyses were done using the Kjel-foss Automatic 16210 machine (A/S N Foss Electric, Denmark). Gross energy (GE) was measured using the automatic adiabatic calorimeter (GallenKamp, UK). Fibre analyses were conducted using the Fibretech Instrument (Tecator, Sweden). A muffle furnace (Gallenkamp, UK) at 500°C was used to analyse ash content. Ether extract was analysed using the electric isomantle machine (Isopad Ltd, England).

Trypanosome challenge of sheep

To challenge the sheep at monthly intervals after treatment, a population of *Trypanosoma congolense* IL 1180, known to be sensitive to isometamidium at an effective dose of 0.001 mg/kg body weight in cattle (Sones et al., 1988) was used. The inoculum given was raised in irradiated infected mice. Blood was obtained by cardiac puncture from the infected mice under deep anaesthesia at the peak of parasitaemia. Blood from the mice was pooled and the parasitaemia estimated by haemocytometer and diluted with phosphate buffered saline (PBS) containing 1.5% glucose at pH 8.0 to give a final concentration of 1×10^5 trypanosomes. This was given subcutaneously in the shoulder region as an inoculum of 2 ml. Viability and infectivity of the trypanosomes was also tested in irradiated mice by injecting the same inoculum intraperitoneally. At each monthly challenge two untreated control sheep were also inoculated to test the viability and infectivity of the trypanosomes.

Parasitological and Packed Cell Volume (PCV) monitoring

Blood samples were collected for parasitological and PCV examination three times a week. Animals were bled from the jugular vein into heparinized vacutainer tubes (Becton Dickinson and Co., USA). At the laboratory the blood was well mixed on a blood cell suspension mixture (Luckham Denley, U.K). Two capillary tubes were filled with blood from each vacutainer tube, sealed with cristaseal (Hawksley, UK) at one end and centrifuged for 5 minutes in the microhaematocrit centrifuge. PCV were read using the PCV reader (Hawksley, UK) and the mean of the two duplicate capillary tubes was recorded. The trypanosomes were detected by the darkground

buffy coat method (Murray et al., 1977) and parasitaemia graded from 0 to 5 as described by Paris et al., 1982) as shown in table 5.3.

Table 5.3 Scoring method for estimation of parasitaemia by using darkground/phase contrast. Magnification = x400.

Score	Trypanosomes per field	Estimated parasitaemia (trypanosomes ml^{-1})
0	None	$< 10^2$
1+	1 - 3 per preparation	10^2 - 10^3
2+	4 -10 per preparation	10^3 - 10^4
3+	1 per field	5×10^3 - 5×10^4
4+	2 - 10 per field	5×10^4 - 4×10^5
5+	10 - 100 per field	$> 5 \times 10^5$
6+	> 100 per field	$> 5 \times 10^6$

Collecting of blood samples

Blood for serum was collected from the jugular vein from all the sheep into plain vacutainer tubes (Becton Dickinson and Co., USA). The blood was allowed to clot and sera separated 24 hr later. This was done after the tubes were centrifuged at 3500 rpm for 20 minutes at 4°C. Serum was removed and stored in the freezer at - 20 °C until required.

Serum drug concentration

Determination of the isometamidium serum concentration was conducted using the sequential saturation ELISA for isometamidium. This is a modified competitive enzyme immunoassay described by Eisler et al., 1996. The modified assay is fully described in Chapter Two.

The concentrations were determined at 30 minutes, 1 hour, 2 hours, 4 hours, 24 hours, 30 hours, 48 hours, 54 hours, 72 hours, 78 hours after treatment and thereafter once every week until the end of the experiment.

Biochemical assays

Serum albumin concentrations were measured by the standard BCG Succinate method using the Technicon Axon discrete-analyser (Bayer Diagnotics plc, Berkshire, UK). Serum cholesterol were estimated by the cholesterol oxidase method using the Technicon Axon discrete-analyser (Bayer Diagnotics plc, Berkshire, UK). Urea was measured by the urease endpoint method using the Technicon Axon discrete-analyser (Bayer Diagnotics plc, Berkshire, UK). β-hydroxybutyrate (BOHB) concentrations were measured by enzymatic method using the Cobas Mira discrete-analyser (Randox Laboratories, Ireland).

Body weights

The sheep were weighed once a week using a sheep weighing scale (Poldenvale Ltd, Williton, Somerset). This was done immediately after collecting blood from the animals.

Experimental design

Twenty (10 pairs of twins) Scottish Blackface Lambs were used in this study. They were divided into two groups (high plane diet group and low plane diet group) of ten animals each. One twin was allocated to the high plane (HP) diet group and the other twin was allocated to the low plane (LP) diet group. Animals were allowed to feed on

their respective diets for four weeks before commencement of the experiment. During this time they were bled three time a week for PCV analyses. They were also weighed once a week. This provided baseline data and helped the animals to get used to being handled. After four weeks on their group diets, six animals from the high plane group (HPDT) and six animals from low plane group (LPDT) were treated intramuscularly in the neck region with isometamidium at a dose rate of 0.5 mg/kg body weight. Four animals from each group acted as untreated challenge controls (HPDC and LPDC).

A month after treatment the treated animals from the two groups and one untreated animal from each control group were inoculated with *Trypanosoma congolense.* Challenge was repeated once every month until all the drug-treated animals became positive.

Data analyses

Analyses of variance (Anova) on Minitab and Excel 5 programms were used to determine any variation or differences between the two groups. Values of $P < 0.05$ were considered significant. Results are presented as means $\pm$ standard error of mean (SEM).

RESULTS

Parasitological findings

The prophylaxis period offered to the sheep on the high diet was 121 $\pm$ 14 days and that on a low plane diet was 117 $\pm$ 11 days (Tables 5.4a and 5.4b). There was no significant difference in the prophylactic period ($P > 0.05$). The prepatent period of *T.*

congolense (after the 4th challenge) in the sheep on a high plane diet was 13 ± 3 days and that on a low plane diet was 12 ± 4 days. There was no significant difference in these prepatent periods.

The mean prepatent period of the *T. congolense* in the challenged controls on a high plane diet was 8 ± 2 days and of the controls on a low plane diet was 9 ± 1 days (Table 5.4c and 5.4d) No significant difference was observed between the two groups.

Table 5.4a Prophylatic period offered by isometamidium to sheep challenged with *T. congolense* and given a high plane diet. (SD = Standard deviation. ND = not detected.)

Animal No	Date of Samorin prophylaxis treatment	Day detected positive	Prophylactic period	Number of challenge after which they became positive	Days after challenge	Samorin conc (ng/ml)	PCV (%) at break-through infection
440	12/03/96	10/07/96	112	4th	8	ND	25
446	12/03/96	19/08/96	140	5th	20	ND	25
450	12/03/96	16/07/96	112	4th	14	ND	24
458	12/03/96	7/08/96	140	5th	8	ND	32
482	12/03/96	16/07/96	112	4th	14	ND	27.5
488	12/03/96	16/07/96	112	4th	14	ND	28
		Average SD	121 (± 14)		13 (± 3)		27 (± 3)

Days of challenge
1st = 9/4/96
2nd = 7/5/96
3rd = 4/6/96
4th = 2/7/96
5th = 30/7/96

Calculation of prophylatic period:

This was the number of days from date of isometamidium chloride prophylactic treatment to the date of challenge that resulted into parasitaemia.

Table 5.4b Prophylatic period offered by isometamidium to sheep challenged with *T. congolense* and given a low plane diet. (SD = Standard deviation. ND = not detected.)

Animal No	Date of Samorin prophylaxis treatment	Day detected positive	Prophylatic period	Number of challenge after which they became positive	Days after challenge	Samorin conc (ng/ml)	PCV (%) at break-through infection
442	12/03/96	10/7/96	112	4th	8	ND	23
448	12/03/96	5/8/96	140	5th	6	ND	25.5
452	12/03/96	16/7/96	112	4th	14	ND	21
460	12/03/96	16/7/96	112	4th	14	ND	21.5
484	12/03/96	16/7/96	112	4th	14	ND	25.5
490	12/03/96	19/7/96	112	4th	17	ND	23.5
		Average	117		12		23
		SD	(± 11)		(±4)		(± 2)

Table 5.4c The prepatent period (days) of *T. congolense* in challenge control sheep given a high plane diet. (SD = Standard deviation)

Animal No	Day of needle challenge	No of challenge	Day detected positive	prepatent period	Comment
454	9/4/96	1st	17/4/96	8	terminated on 30/4/96
466	7/5/96	2nd	15/05/96	8	terminated on 28/5/95
470	4/6/96	3rd	14/5/96	10	terminated on 25/6/96
478	2/7/96	4th	8/7/96	6	terminated on 6/8/96
Average				8	
SD				(± 2)	

Table 5.4d The prepatent period (days) of *T. congolense* in the challenge control sheep given a low plane diet. (SD = Standard deviation)

Animal No	Day of needle challenge	No of challenge	Day detected positive	prepatent period	Comment
456	9/4/96	1st	19/04/96	10	terminated on 30/4/96
468	7/5/96	2nd	15/05/96	8	terminated on 28/5/96
474	4/6/96	3rd	14/06/96	10	terminated on 25/6/96
480	2/7/96	4th	10/7/96	8	terminated on 6/8/96
Average				9	
SD				(± 1)	

Serum isometamidium concentration

The mean isometamidium concentration of the treated sheep on the high plane diet reached its maximum at 4 hours post treatment whilst that for the sheep on the low plane diet was 2 hours post treatment (Tables 5.5a, 5.5b and Figure 5.1). Thereafter the concentration started decreasing. The drug concentration was measurable up to 6 weeks post treatment. After 6 weeks post treatment the drug concentrations were below the detection limit of 0.2 ng/ml of serum. There was no significant difference ($P > 0.05$) in the mean drug concentration values between the treated group on the high protein diet and the treated group on the low plane diet.

Table 5.5a Mean drug concentration (ng/ml) of the treated sheep on a high plane diet (HPDT) and on a low plane diet (LPDT) from zero to 144 hrs post treatment. (BT = Before treatment)

	Hours post treatment											
GROUP	BT	1/2	1	2	4	24	30	48	54	72	78	144
HPDT	0.03	46.82	57.80	52.92	63.15	23.46	22.58	14.74	14.86	7.83	7.17	3.24
± S.E.M	0.01	7.05	9.57	8.13	7.34	6.19	2.38	2.26	2.80	0.61	0.66	0.24
LPDT	0.03	64.80	71.62	78.60	60.81	27.75	22.97	11.78	9.96	8.31	7.34	3.40
± S.E.M	0.01	20.12	21.79	19.20	8.20	4.59	2.83	0.30	0.77	0.84	0.34	0.19

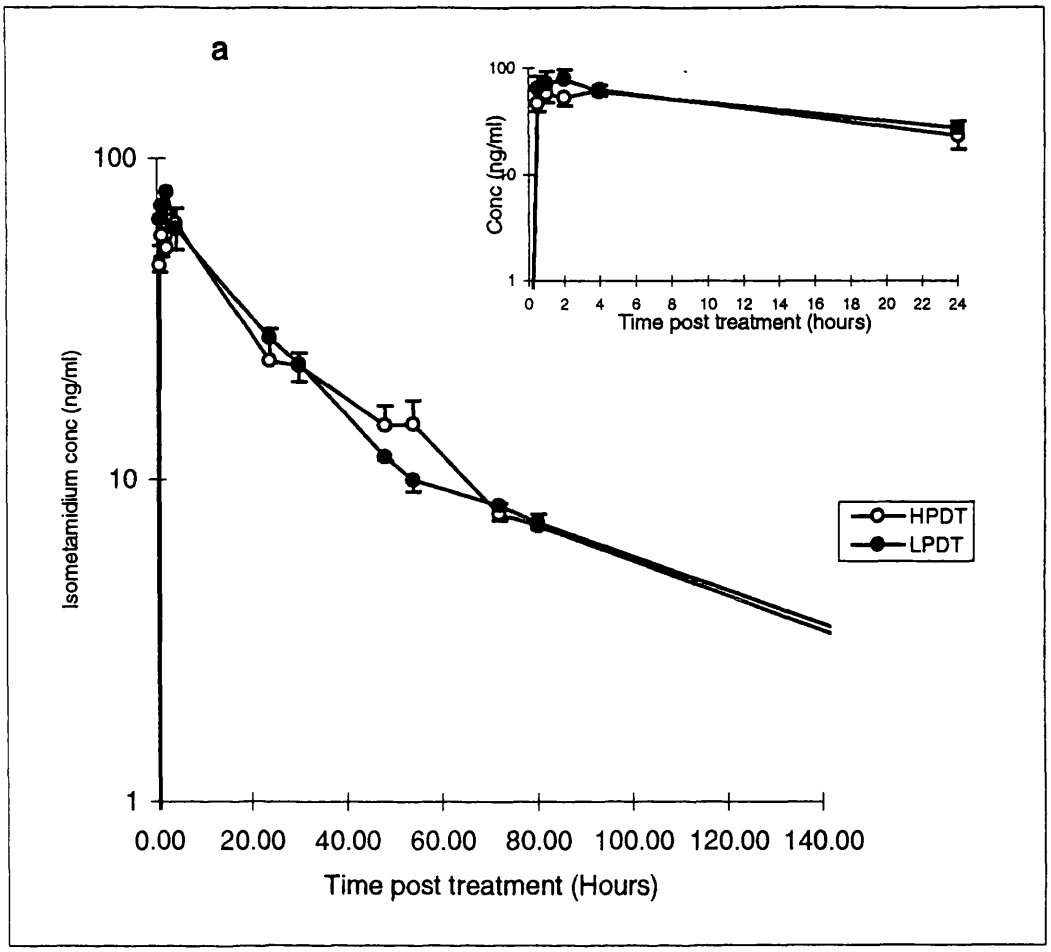
Table 5.5b Mean drug concentration (ng/ml) of the treated sheep on a high plane diet (HPDT) and on a low plane diet (LPDT) two to twenty-three weeks post treatment. (ND = Not detected)

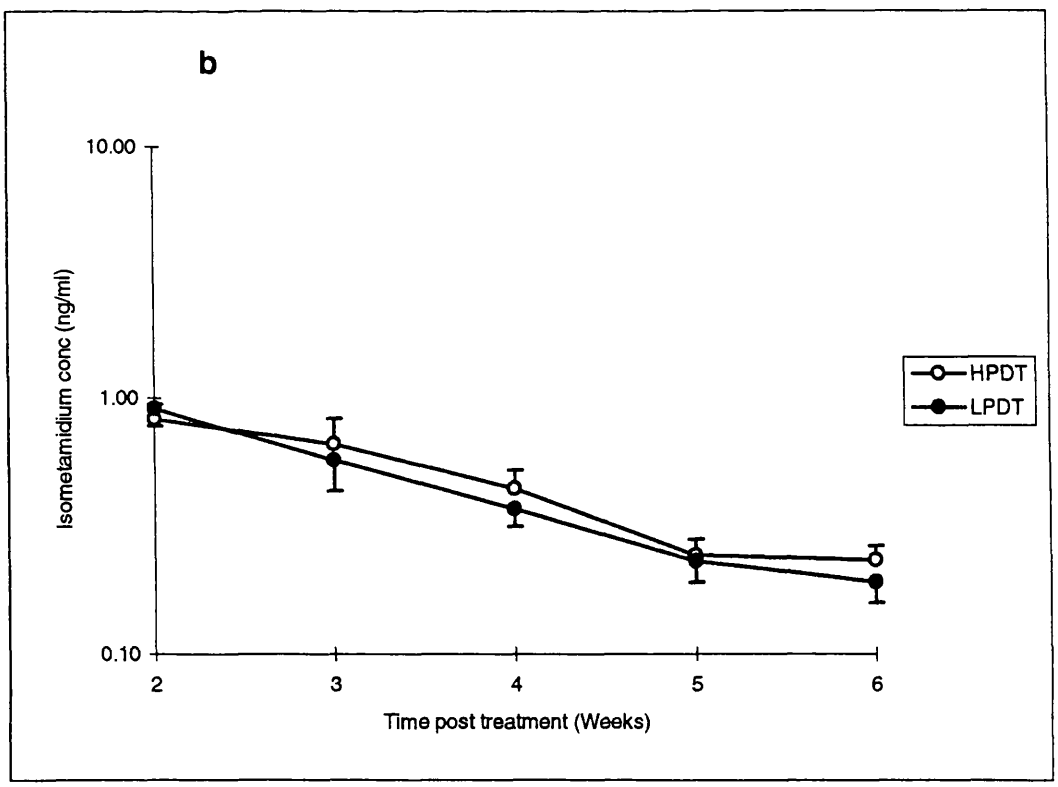
	Weeks post treatment									
Group	2	3	4	5	6	7	8	9	10	11
HPDT	0.83	0.66	0.44	0.24	0.23	ND	ND	ND	ND	ND
± S.E.M	0.12	0.17	0.08	0.04	0.03	ND	ND	ND	ND	ND
LPDT	0.91	0.57	0.37	0.23	0.19	ND	ND	ND	ND	ND
± S.E.M	0.13	0.14	0.05	0.04	0.03	ND	ND	ND	ND	ND

Continuation of table 5.5b

Weeks post treatment											
12	13	14	15	16	17	18	19	20	21	22	23
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Figure 5.1 Mean drug concentration (ng/ml) of the treated sheep on a high plane diet (HPDT) and on a low plane diet (LPDT). (a) from zero hour to 144 hrs post treatment (b) from 2 - 23 weeks post treatment. vertical bars show the limits of the range of the standard error of the mean (SEM).





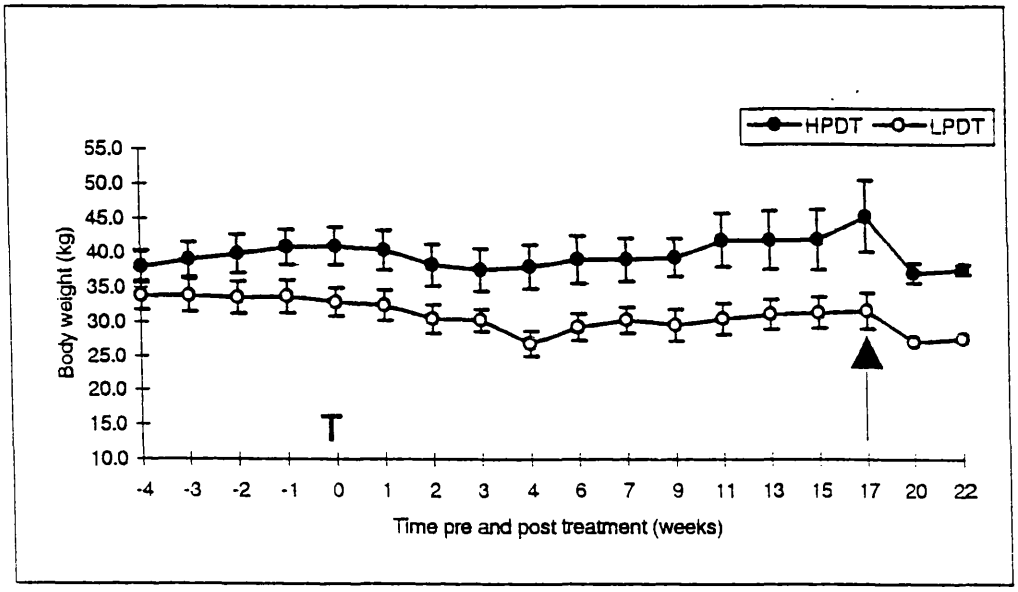
Body weight

The mean body weight of the treated sheep (HPDT) in the high plane diet group was higher than that of the sheep on a low plane diet (Table 5.6 and Figure 5.2). This remained so throughout the experiment and was significantly different. The treated sheep on a high plane diet gained 4.25 kg between the beginning of the study and the week they became parasitaemic whilst those on a low protein diet lost 1.83 kg within the same period.

Table 5.6 The mean body weight (kg) of the sheep treated with isometamidium (0.5 mg/kg body weight), maintained either on a high plane diet (HPDT) or low plane diet (LPDT) and challenged with *T. congolense*.

	Weeks post treament																	
Group	-4	-3	-2	-1	0	1	2	3	4	6	7	9	11	13	15	17	20	22
HPDT	38.0	39.0	39.8	40.8	41.0	40.4	38.3	37.5	38.0	39.0	39.0	39.3	41.8	41.8	41.9	45.3	37.0	37.5
± S.E.M	2.3	2.5	2.8	2.6	2.8	2.9	3.0	3.1	3.1	3.5	3.1	2.7	3.8	4.2	4.4	5.2	1.4	0.7
LPDT	33.8	33.8	33.5	33.7	32.8	32.4	30.4	30.3	26.8	29.3	30.3	29.5	30.4	31.1	31.3	31.6	27.0	27.5
± S.E.M	2.1	2.3	2.3	2.4	2.0	2.3	2.1	1.6	1.9	1.9	1.9	2.3	2.3	2.2	2.3	2.6		

Figure 5.2 Mean body weights of the sheep treated with isometamidium on a low and high plane diet . Vertical bars show the limits of the range of the standard error of the mean (SEM). ↑ = point of breakthrough infection. **T** = point of treatment.



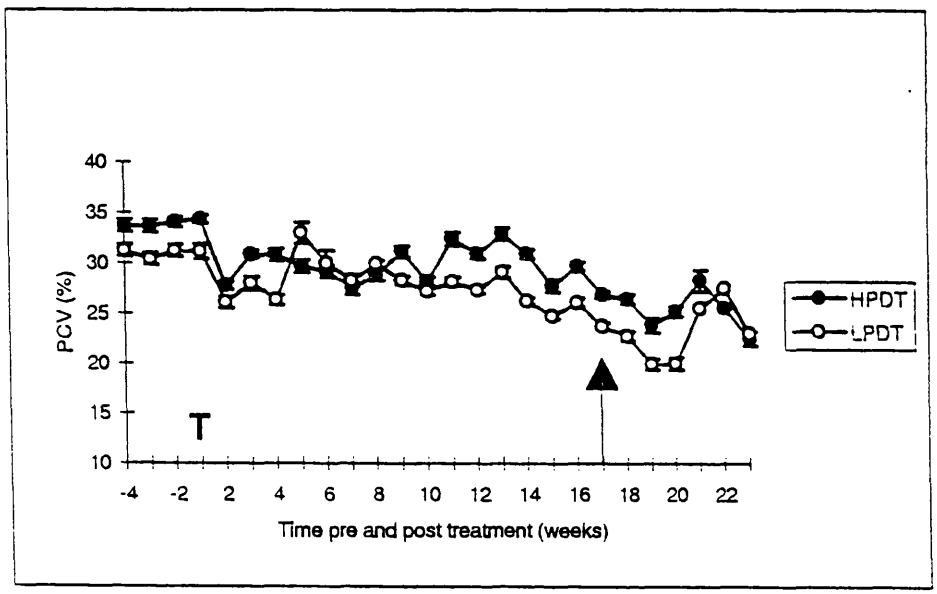
Packed cell volume (PCV, %)

There was a significant difference ($P < 0.05$) between the PCV of the treated sheep on the high plane diet and the low plane diet. The PCV of the treated sheep on a high plane diet was higher than the PCV of the sheep on the low plane diet (Table 5.7 and Figure 5.4). There was a drop in the PCV after week 17, when the drug-treated sheep in both groups became parasitaemic.

Table 5.7 Mean PCV (%) of the treated sheep on a high (HPDT) and low plane diet (LPDT).

	Weeks post treatment																						
Group	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
HPDT	33.7	27.8	30.9	30.8	29.7	29.2	27.5	28.8	31.2	28.2	32.4	30.9	32.9	30.9	27.8	29.7	26.8	26.4	23.8	25.2	28.3	25.5	22.5
± S.E.M	0.6	0.4	0.4	0.6	0.6	0.6	0.6	0.6	0.6	0.4	0.7	0.5	0.6	0.4	0.6	0.4	0.3	0.4	0.7	0.5	1.1	0.0	0.7
LPDT	31.3	26.1	28.0	26.3	33.0	30.0	28.3	29.9	28.3	27.2	28.2	27.3	29.1	26.3	24.8	26.1	23.8	22.8	19.9	20.0	25.5	27.5	23.0
± S.E.M	0.6	0.6	0.7	0.5	1.0	1.3	0.6	0.3	0.4	0.3	0.4	0.3	0.5	0.3	0.3	0.4	0.3	0.4	0.5	0.7			

Figure 5.4 Mean PCV (%) of the treated sheep on low and high plane diet. Vertical bars show the limits of the range of the standard error of the mean (SEM).
↑ = point of breakthrouh infection. T = point of treatment.



Serum cholesterol concentration

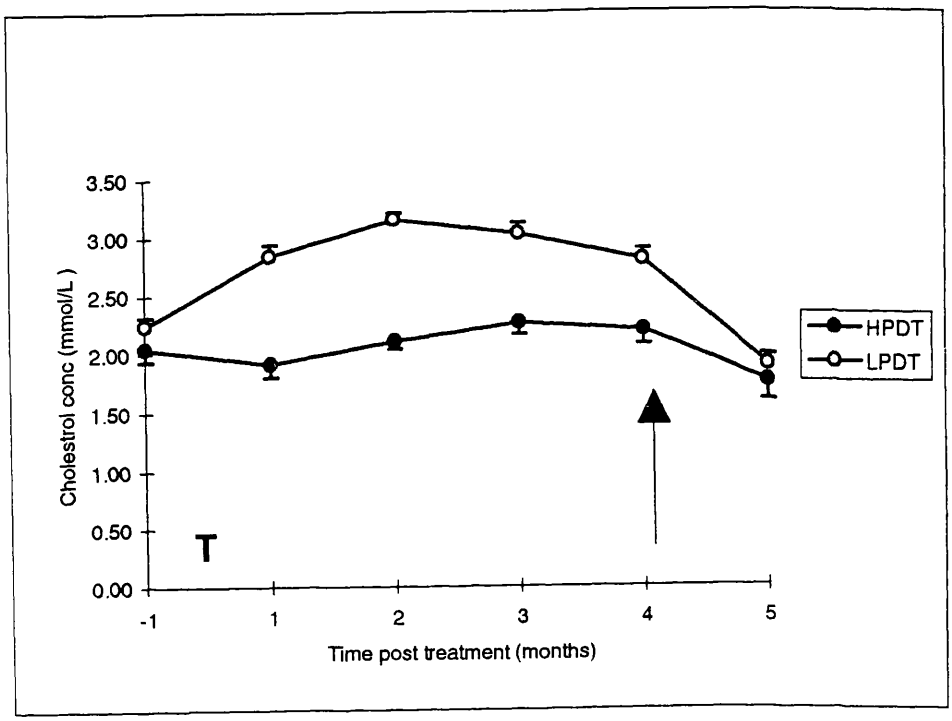
The mean cholesterol concentration of the sheep on a high plane diet was lower than the mean concentration of the sheep on the low plane diet throughout the experiment (Table 5.8, and Figure 5.5). This was statistically significant ($P < 0.05$).

Table 5.8 The mean cholesterol concentrations (mmol/L) of the treated sheep on a high (HPDT) and low plane diet (LPDT).

	Months post treatment					
Group	-1	1	2	3	4	5
HPDT	2.04	1.91	2.11	2.26	2.20	1.76
± SEM	0.11	0.11	0.07	0.10	0.12	0.16
LPDT	2.24	2.84	3.16	3.03	2.80	1.89
± SEM	0.08	0.10	0.06	0.09	0.09	0.09

Cholesterol concentration showed a significant decrease in both the treated groups of the sheep after they became parasitaemic. This happened after the 4th month post treatment.

Figure 5.4 Mean cholesterol levels (mmol/L) of the treated sheep on a low (LPDT) and high plane diet (HPDT). ↑ = point of breakthrough infection. Vertical bars show the limit of the range of the standard error of the mean (SEM). T = point of treatment.



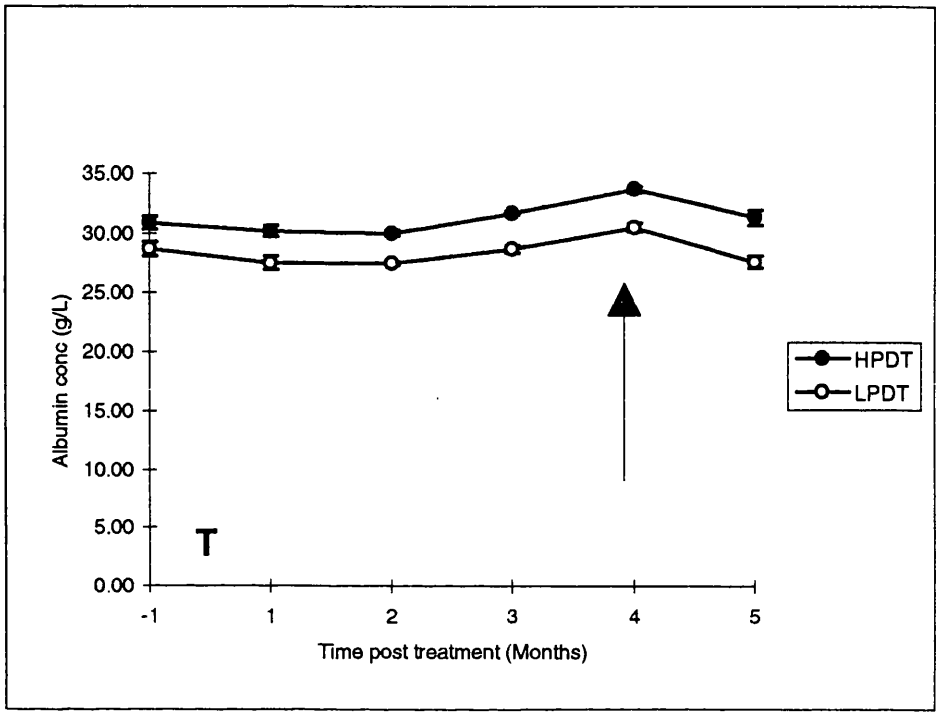
Serum albumin

The mean serum albumin concentrations in treated sheep on the high plane diet increased from 30.17 ± 0.48 g/L at one month post treatment to 33.67 ± 0.24 g/L four months post treatment. At four months post treatment the animals became positive for trypanosomes and the concentration of albumin decreased in the sheep. The albumin levels decreased to 31.33 ± 0.63 g/L (Table 5.9 and Figure 5.6). The mean serum albumin concentration in the treated sheep on the low plane diet showed an increase from 27.50 ± 0.56 g/L one month post treatment to 30.50 ± 0.37 g/L four months post treatment. A decrease in the mean albumin concentration was also observed in this group when they became positive for trypanosomes and at five months post treatment the mean concentration dropped to 27.60 ± 0.52 g/L. The mean albumin concentration was higher in treated sheep on a high plane diet than those on a low plane diet and this was statistically significant ($P < 0.05$).

Table 5.9 Mean albumin concentrations (g/L) of the treated sheep on a high plane (HPDT) and low plane diet (LPDT).

	Months post treatment					
Group	-1	1	2	3	4	5
HPDT	30.83	30.17	30.00	31.67	33.67	31.33
± S.E.M	0.54	0.48	0.25	0.10	0.24	0.63
LPDT	28.67	27.50	27.50	28.67	30.50	27.60
± S.E.M	0.62	0.56	0.17	0.30	0.37	0.52

Figure 5.5 Mean albumin concentration (g/L) of the treated sheep on a high (HPDT) and low plane diet (LPDT). ↑ = point of breakthrough infection. Vertical bars show the range of the standard error of the mean (SEM). T = point of treament.



Urea concentration

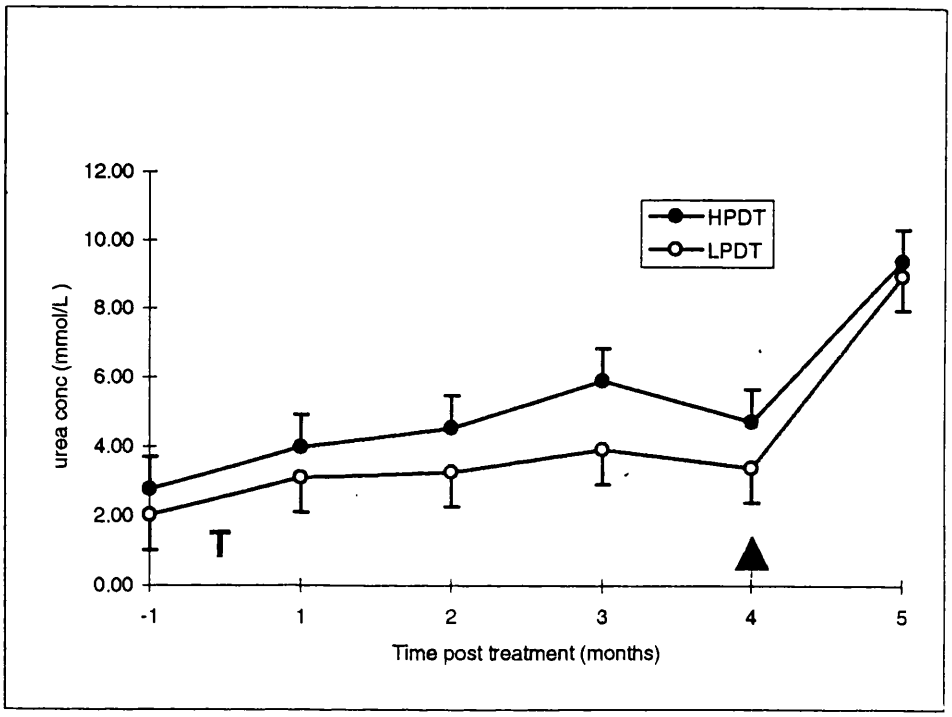
The mean urea concentration of treated sheep on the high plane diet was higher than that of the sheep on the low plane diet. Statistically there was no difference ($P > 0.05$).

Table 5.10 Mean urea concentrations (mmol/L) of the treated sheep on a high plane (HPDT) and low plane diet (LPDT).

	Months post treatment					
Group	-1	1	2	3	4	5
HPDT	2.77	3.98	4.53	5.90	4.73	9.40
± S.E.M	0.07	0.21	0.33	0.16	0.04	0.11
LPDT	2.02	3.10	3.27	3.92	3.40	8.96
± S.E.M	0.09	0.10	0.43	0.20	0.25	0.38

After the sheep became positive for trypanosomes the mean concentration of urea in both treated groups increased significantly. This occured between month 4 and month 5 post treatment (Table 5.10 and Figure 5.8).

Figure 5.6 Mean urea concentration (mmol/L) of the treated sheep on a high (HPDT) and low plane diet (LPDT). ↑ = point of breakthrough. Vertical bars show the limit of the range of the standard error of the mean (SEM). T = point of treatment



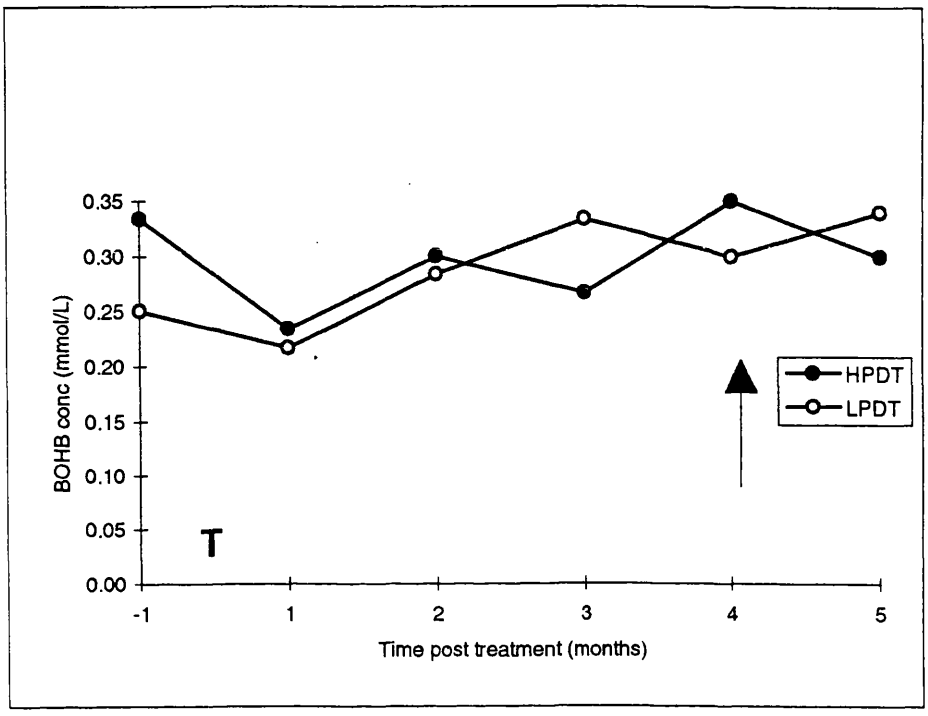
β-hydroxybutyrate (BOHB)

The mean concentration of β-hydroxybutyrate (mmol/L) fluctuated in both treated sheep on either high or low plane diet throughout the experiment (Table 5.11 and Figure 5.9). Statistically there was no significance difference ($P > 0.05$) in the concentration of BOHB between the sheep on the high diet and those on the low plane diet.

Table 5.11 Mean BOHB concentrations (mmol/L) of the treated sheep on a high plane (HPDT) and low plane diet (LPDT).

	Months post treatment					
Group	-1	1	2	3	4	5
HPDT	0.33	0.23	0.30	0.27	0.35	0.30
± S.E.M	0.01	0.01	0.02	0.02	0.01	0.01
LPDT	0.25	0.22	0.28	0.33	0.30	0.34
± S.E.M	0.02	0.01	0.02	0.01	0.00	0.01

Figure 5.7 Mean BOHB concentration (mmol/L) of the treated sheep on a high (HPDT) and low plane diet (LPDT). ↑ = point of breakthrough infection. Vertical bars show the limit of the range of the standard error of the mean (SEM). T = point of treatment.



DISCUSSION

In the present experiment nutritional intake and body weight had no influence on the isometamidium prophylaxis period in the sheep challenged with *T. congolense*. The mean prophylaxis period offered to the sheep on a high plane diet was 121 $\pm$ 14 days while for the sheep on a low plane diet was 117 $\pm$ 11 days. Nor was there an effect on the prepatent period to parasitaemia in both control groups. The prepatent period of the trypanosomes in the control group on the high plane diet was 8 $\pm$ 2 days while those on the low plane diet was 9 $\pm$ 1 day.

Diet had no influence on the isometamidium concentration in the sheep on either high plane diet or low plane diet. Statistically there was no difference ($P > 0.05$) between the mean drug concentration of the sheep on the high plane diet and that on the low plane diet, suggesting that pharmacokinetics were not influenced by the dietary level of protein and gross energy. The mean drug concentrations at the time of the breakthrough infection in both groups were below the lower limit of detection of 0.2 ng/ml. This shows that the trypanosomes used in this experiment were not resistant to isometamidium. This is in agreement with the observation that was made by Sones et al., (1988). Results of their studies in cattle showed that the trypanosomes were sensitive to isometamidium at 0.001 mg/kg body weight.

The mean PCV of the animals on a high plane diet was higher than that of the sheep on a low plane diet indicating that dietary plane had some influence on the PCV values. Trypanosome infection also had an influence on the PCV values. After the

breakthrough infections the mean values decreased in the sheep in both groups. Katunguka-Rwakishaya et al., (1992,1993) observed similar results in sheep given the high and low plane diet and infected with *T. congolense*.

Diet had an influence on the body weights. The mean body weight of the sheep on a high plane diet was higher than that of the sheep on a low plane diet and was statistically significant ($P < 0.05$) throughout the experiment. Body weight did not influence the prophylatic period of the sheep either on a high or low plane diet.

Diet had an influence on cholestrol levels. The levels of cholestrol of the sheep on the low plane diet was higher than that of the sheep on the high plane diet and was statistically significant ($P < 0.05$). Trypanosome infection did have an effect on the amount of cholesterol in the serum. When the animals became parasitaemic the levels of cholestrol dropped in both groups. Trypanosomes depend on cholesterol found in the blood of their host for their energy (Gillet and Owen, 1987). The appearance of the trypanosomes indicated that they were taking up the cholesterol for their energy requirements. In the treated animals on a high plane diet the mean concentration dropped from 2.20 mmol/L to 1.76 mmol/L one month after trypanosomes breakthrough. In the low plane group the concentration dropped from 2.28 to 1.89 mmol/L within the same period.

There was a dietary effect on the concentration of albumin in the two treated groups. The high plane diet group had higher concentration of albumin than the low plane

group. Trypanosomes had an effect on the concentration of albumin. When trypanosomes were detected, the concentration of albumin in both groups decreased.

Urea concentration in both treated groups remained statistically similar, suggesting that dietary intake did not influence the mean concentrations of urea. Trypanosome infection did have an influence on the concentration of urea. After breakthrough infection the urea concentration increased in both treated groups. This results suggests that protein catabolism was increased in the infected sheep. There was no dietary or trypanosome infection influence on the concentration of β-hydroxybutyrate. Throughout the experiment the mean values of β-hydroxybutyrate of the two groups fluctuated.

In conclusion this study has shown that the prophylactic period conferred by isometamidium is not influenced by the dietary intake of the sheep, despite dietary influences on PCV, body weight and the serum concentrations of urea, cholesterol and albumin concentrations.

CONCLUSION

The competitive enzyme immunoassay using isometamidium-horseradish-peroxidase conjugate produced using n-hydroxysuccinamide and modification to the assay to a sequential saturation CEIA has proved to be a useful development to the assay for measuring isometamidium and homidium concentrations in sera of treated cattle and sheep. The ELISA also proved that in combination with other parasitological diagnostic methods it can be used as an indirect method for monitoring drug-resistant trypanosomes in treated animals.

Formulating homidium bromide into slow release devices (SRD) prolonged the prophylactic effect of homidium in the cattle kept under natural tsetse challenge, at Chipopela in Zambia. The homidium-SRD protected 50 % of the animals up to four months post treatment whereas 50 % of the animals given homidium by intramuscular injection were protected only up to two months post treatment.

The sheep experiment showed that the plane of nutrition had no effect on the duration of isometamidium prophylaxis, despite effects on the PCV, body weight, and serum concentrations of urea, albumin and cholestrol.

RECOMMEDATIONS

1. From the results obtained in these studies it is recommended that the competitive enzyme immunosorbent assay (CEIA) in combination with other parasitological diagnostic methods be used to monitor drug resistance in the field.

2. The studies carried out at North Nyamphande and Chipopela showed that by the third month after treatment the serum levels of isometamidium are below 0.4 ng/ml. It is recommended that the intervals between drug administration be re-assessed and perhaps reduced.

3. Further work on the homidium-SRD is justified since the prophylaxis period of homidium may be usefully extended by such devices.

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Appendices

Appendix 1

Preparation of Phoshate buffered saline (PBS x 10) pH 7.4 for ELISA

1. Sodium chloride (NaCl) 80g

2. Sodium hydrogen phosphate (Na_2HPO_4) 14.8g

3. Potassium hydrogen phosphate (KH_2PO_4) 4.3g

Dissolve the above salts in 1litre deionised water.

Appendix 2

Preparation of Phosphate buffered saline , pH 7.4, containing 0.05% Tween 20 (PBST) for ELISA

This is prepared by mixing the following solution thoughly:

1. 900 ml of deionised water

2. 100 ml PBS X 10

3. 500 µl of Tween 20

Appendix 3

Preparation of washing buffer pH 7.4 for ELISA

1. 4.9 litres of deionised water

2. 100 ml of PBS x 10

3. 2.5 ml of Tween 20

Mix the above solutions throughly in 5 liter container.

Appendix 4

Optical densities (OD) wavelength (absorbances at 450 nm) of 24 individual sera from untreated Zimbabwe Mashona cattle, obtained using sequential saturation assay.
Plate A.

ID	OD 1a	OD 1b	OD 2a	OD 2b	Mean 1	Vars 1	Mean 2	Vars 2	Mean 1& 2	Vars 1& 2
P324	1.098	1.041	1.126	1.016	1.070	0.00162	1.071	0.00605	1.070	0.00000
P274	1.103	1.033	1.143	1.050	1.068	0.00245	1.097	0.00432	1.082	0.00041
T523	1.150	1.134	1.149	1.077	1.142	0.00013	1.113	0.00259	1.128	0.00042
T391	1.147	1.017	1.232	1.121	1.082	0.00845	1.177	0.00616	1.129	0.00447
P225	1.176	1.114	1.153	1.106	1.145	0.00192	1.130	0.00110	1.137	0.00012
P336	1.179	1.160	1.208	1.079	1.170	0.00018	1.144	0.00832	1.157	0.00034
P272	1.192	1.247	1.094	1.132	1.220	0.00151	1.113	0.00072	1.166	0.00567
P242	1.205	1.218	1.153	1.118	1.212	0.00008	1.136	0.00061	1.174	0.00289
T478	1.214	1.078	1.233	1.179	1.146	0.00925	1.206	0.00146	1.176	0.00180
P238	1.262	1.144	1.205	1.135	1.203	0.00696	1.170	0.00245	1.187	0.00054
J156	1.245	1.192	1.236	1.129	1.219	0.00140	1.183	0.00572	1.201	0.00065
P306	1.212	1.218	1.223	1.153	1.215	0.00002	1.188	0.00245	1.202	0.00036
P239	1.228	1.178	1.212	1.193	1.203	0.00125	1.203	0.00018	1.203	0.00000
BC	1.246	1.227	1.244	1.194	1.237	0.00018	1.219	0.00125	1.228	0.00015
P317	1.231	1.179	1.316	1.208	1.205	0.00135	1.262	0.00583	1.234	0.00162
P221	1.335	1.265	1.235	1.125	1.300	0.00245	1.180	0.00605	1.240	0.00720
P236	1.341	1.255	1.191	1.206	1.298	0.00370	1.199	0.00011	1.248	0.00495
P243	1.316	1.250	1.246	1.214	1.283	0.00218	1.230	0.00051	1.257	0.00140
J437	1.321	1.255	1.274	1.210	1.288	0.00218	1.242	0.00205	1.265	0.00106
194	1.356	1.228	1.283	1.198	1.292	0.00819	1.41	0.00361	1.266	0.00133
P228	1.272	1.349	1.235	1.251	1.311	0.00296	1.243	0.00013	1.277	0.00228
P229	1.346	1.284	1.247	1.250	1.315	0.00192	1.249	0.00000	1.282	0.00221
P235	1.308	1.308	1.313	1.253	1.308	0.00000	1.283	0.00180	1.296	0.00031

ID = Identity of the animal OD = Optical densities Vars = Variances

Appendix 5

Optical densities (OD) wavelength (absorbances at 450 nm) of 24 individual sera from untreated Zimbabwe Mashona cattle. Plate B.

ID	OD 1a	OD 1b	OD 2a	OD 2b	Mean 1	Vars 1	Mean 2	Vars 2	Mean 1 & 2	Vars 1 & 2
P306	1.067	1.083	0.934	0.979	1.075	0.00013	0.957	0.00101	1.016	0.00702
T523	1.114	1.023	1.034	0.934	1.069	0.00414	0.984	0.00500	1.026	0.00357
P324	1.104	1.104	1.027	0.996	1.094	0.00022	1.012	0.00048	1.053	0.00336
P272	1.072	1.107	1.086	0.996	1.090	0.00061	1.041	0.00405	1.065	0.00118
P336	1.107	1.067	1.070	1.038	1.087	0.00080	1.057	0.00051	1.071	0.00054
P239	1.160	1.171	1.051	1.027	1.166	0.00006	1.039	0.00029	1.102	0.00800
P274	1.147	1.172	1.086	1.035	1.160	0.00031	1.061	0.00130	1.110	0.00490
P242	1.145	1.155	1.087	1.063	1.150	0.00005	1.075	0.00029	1.113	0.00281
P243	1.094	1.204	1.104	1.101	1.149	0.00605	1.103	0.00000	1.126	0.00108
H196	1.177	1.115	1.124	1.087	1.146	0.00192	1.106	0.00068	1.126	0.00082
P236	1.172	1.154	1.091	1.091	1.163	0.00016	1.091	0.00000	1.27	0.00259
P317	1.189	1.181	1.089	1.063	1.185	0.00003	1.076	0.00034	1.131	0.00594
P225	1.230	1.109	1.164	1.042	1.170	0.00732	1.103	0.00744	1.136	0.00221
BC	1.114	1.156	1.154	1.137	1.135	0.00088	1.146	0.00014	1.140	0.00006
J156	1.234	1.130	1.116	1.089	1.182	0.00541	1.103	0.00036	1.142	0.00316
P238	1.203	1.198	1.048	1.130	1.201	0.00001	1.089	0.00336	1.145	0.00622
P221	1.223	1.205	1.108	1.093	1.214	0.00016	1.101	0.00011	1.157	0.00644
P228	1.211	1.203	1.198	1.061	1.207	0.00003	1.130	0.00938	1.168	0.00300
P235	1.199	1.182	1.154	1.141	1.191	0.00014	1.148	0.00008	1.169	0.00092
J437	1.258	1.136	1.160	1.130	1.197	0.00744	1.145	0.00045	1.171	0.00135
194	1.234	1.242	1.191	1.145	1.238	0.00003	1.168	0.00106	1.203	0.00245
P229	1.291	1.286	1.200	1.178	1.289	0.00001	1.189	0.00024	1.239	0.00495

Appendix 6

Concentration (ng/ml) of isometamidium in individual sera of treated cattle at North Nyamphande. ND: not detected. NS : No sample.

Animal No	- 14 days	+ 14 days	28 days	42 days	56 days	70 days	84 days
863	ND	1.504	0.921	ND	ND	0.296	ND
866	0.276	2.503	0.998	0.394	ND	ND	ND
864	0.422	1.627	1.111	ND	ND	ND	ND
869	0.354	1.45	0.733	0.261	ND	ND	ND
886	0.388	4.19	1.661	NS	ND	ND	0.248
884	ND	1.252	1.381	NS	0.336	0.221	0.377
883	ND	2.156	0.841	0.36	ND	NS	ND
881	ND	1.47	0.947	0.445	0.228	NS	NS
610	0.236	1.71	0.499	NS	ND	ND	NS
612	ND	2.019	0.946	0.496	0.276	ND	NS
426	ND	0.917	0.643	0.284	0.448	ND	ND
428	0.413	2.638	1.468	0.388	0.306	ND	NS
429	ND	2.066	NS	NS	ND	ND	0.338
871	0.221	1.828	1.284	0.574	0.204	0.421	0.248
870	ND	3.066	0.834	ND	0.28	ND	ND
247	ND	0.942	0.788	0.516	0.226	ND	ND
246	0.205	1.44	0.547	NS	0.204	0.232	0.298
872	ND	1.154	0.708	0.777	0.519	10.957	ND
860	0.208	2.602	1.256	0.918	NS	NS	NS
862	ND	3.034	NS	0.618	NS	0.45	ND
614	ND	4.422	1.676	1.584	1.812	0.376	0.308
880	0.233	1.981	0.693	1.901	0.338	0.404	0.298
617	ND	1.754	1.254	NS	NS	NS	0.251
649	ND	0.46	0.196	0.36	NS	NS	NS
648	0.271	2.495	1.262	0.379	ND	ND	ND
643	ND	2.146	0.784	0.292	ND	0.28	ND
646	ND	2.47	1.046	0.552	0.34	0.222	0.246
645	ND	1.399	0.516	ND	NS	NS	0.212
641	ND	3.505	1.984	0.671	0.304	ND	ND
623	ND	1.94	0.991	0.442	NS	ND	NS
625	ND	2.02	0.754	ND	NS	1.288	0.562
628	1.323	1.852	0.452	0.973	0.501	0.75	1.292
632	ND	1.661	0.722	0.375	0.284	0.202	ND
622	NS	NS	0.554	ND	0.447	0.263	0.236
889	ND	2.336	0.676	NS	0.227	ND	ND
Average	0.379	2.059	0.943	0.619	0.404	0.416	0.358
SD	0.308	0.863	0.396	0.405	0.365	0.301	0.287
Median	0.274	1.961	0.841	0.496	0.305	0.296	0.275

Appendix 7

Concentration (ng/ml) of homidium in the sera of individual cattle treated with SRD-homidium at Chipopela. ND: not detected. NS: No sample.

Animal No	Month 0	Month 1	Month 2	Month 3	Month 4	Month 5	Month6
207	ND	0.161	1.116	0.205	0.373	0.225	ND
208	ND	0.388	0.945	0.413	0.373	0.225	ND
210	ND	0.296	0.543	0.4	0.356	NS	NS
519	ND	NS	NS	0.538	0.472	0.313	NS
532	ND	0.362	0.442	0.24	NS	NS	NS
533	0.202	0.395	1.166	0.444	0.24	0.335	NS
534	0.504	0.371	0.769	0.4	0.411	0.257	ND
535	ND	0.273	0.476	0.226	0.187	ND	ND
538	ND	0.29	0.975	NS	0.313	ND	ND
539	0.214	NS	0.898	0.244	ND	ND	ND
542	0.267	0.359	NS	0.411	0.232	ND	ND
543	0.652	0.573	0.727	0.301	0.303	0.205	NS
676	1.002	0.777	NS	NS	NS	NS	NS
677	ND	0.486	NS	0.667	0.317	NS	NS
AVG	0.264	0.394	0.806	0.374	0.3	0.196	ND
STD	0.276	0.153	0.245	0.133	0.091	0.097	
	0.54	0.547	1.051	0.507	0.391	0.294	
MED	0.129	0.366	0.834	0.4	0.308	0.205	

Appendix 8

Concentration (ng/ml) of homidium in the sera of individual cattle treated with homidium intramuscularly at Chipopela. ND: not detected. NS: No sample.

Animal No	Month 0	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
200	0.206	0.398	0.211	ND	ND	ND	ND
206	ND	0.307	ND	ND	ND	ND	ND
209	ND	0.24	ND	ND	ND	ND	ND
212	ND	NS	ND	ND	ND	ND	ND
214	ND	0.252	NS	ND	ND	ND	ND
216	ND	ND	0.225	ND	ND	ND	ND
217	ND	0.246	ND	ND	ND	NS	NS
219	ND	0.22	0.209	NS	0.355	0.281	ND
222	ND	0.243	ND	ND	ND	NS	NS
225	0.631	0.507	0.238	ND	NS	NS	NS
231	0.322	0.211	ND	0.216	0.214	0.418	ND
236	0.369	NS	0.201	ND	NS	NS	NS
239	0.216	0.371	0.224	NS	NS	NS	NS
241	ND	249	ND	ND	ND	NS	NS
673	ND	1.056	0.453	NS	NS	NS	NS
690	ND	ND	ND	ND	ND	NS	NS
691	0.237	0.28	NS	ND	NS	NS	NS
692	ND	0.254	ND	ND	NS	NS	NS
697	ND	0.309	ND	ND	NS	NS	NS
AVG	0.212	0.312	ND	ND	ND	ND	ND
STD	0.132	0.211					
	0.344	0.524					
MED	0.191	0.252					

Appendix 9

Concentration (ng/ml) of isometamidium in the sera of individual cattle treated with isometamidium intramuscularly at Chipopela.

Animal No	Month 0	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
201	0.32	NS	1.36	NS	0.2	ND	ND
220	0.39	NS	0.52	NS	0.2	ND	ND
221	ND	NS	0.69	NS	0.2	0.385	ND
223	1.81	0.82	0.25	ND	ND	NS	NS
229	ND	0.94	0.16	ND	0.7	NS	NS
245	ND	0.56	0.35	ND	NS	NS	NS
247	0.28	NS	0.6	NS	0.2	NS	ND
248	ND	NS	0.8	NS	0.2	ND	ND
249	0.42	1.41	0.33	0.24	0.6	NS	NS
530	ND	0.87	0.3	ND	0.2	ND	ND
531	ND	NS	1.19	0.37	0.3	ND	ND
536	ND	1.35	0.62	0.26	0.2	0.284	0.217
537	ND	1.43	0.56	0.26	NS	ND	NS
540	ND	0.51	0.21	ND	0.3	ND	ND
545	ND	NS	NS	0.54	NS	0.227	NS
546	0.59	NS	0.79	0.33	ND	ND	ND
547	0.24	2.37	0.63	0.27	NS	ND	ND
548	0.69	1.12	0.36	ND	NS	NS	ND
549	ND	NS	0.6	0.22	0.2	ND	ND
674	ND	2.27	0.69	0.25	ND	NS	NS
AVG	0.35	1.24	0.58	0.24	0.4	0.182	ND
STD	0.41	0.59	0.31	0.11	0.4	0.074	
	0.75	1.83	0.89	0.35	0.7	0.255	
MED	0.18	1.12	0.6	0.24	0.2	0.151	