PHARMACOLOGIC AND TOXICOLOGIC STUDIES ON BABESICIDAL DRUGS WITH SPECIAL EMPHASIS ON QUINURONIUM

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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This thesis has been submitted for examination with my approval as University of Nairobi supervisor.

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TABLE OF CONTENTS

LIST OF TABLES

PAGE

LIST OF FIGURES

PAGE

LIST OF APPENDICES

PAGE

PAGE

PAGE

PAGE

LIST OF ABBREVIATIONS

ASAT	-	Aspartate amino-transferase
BSP	-	Sulfobromophthalein
BUN	-	Blood urea nitrogen
CPK	-	Creatine phosphokinase (New name - Creatine kinase CK)
DNA	-	Deoxyribonucleic acid
EC_{50}	-	Concentration of the drug which reduces parasitemia to 50% of that in untreated controls
FAO	-	Food and Agriculture Organisation
GSH	-	Glutathione
HPLC	-	High Performance Liquid Chromatography
Hb	-	Hemoglobin
H.E.	-	Hematoxylin and eosin
i.m.	-	Intramuscular
i.p.	-	Intraperitoneal
LDH	-	Lactic dehydrogenase
LD_{50}	-	The dose of the drug that kills 50% of the population (median lethal dose)
m.p.	-	Melting point
MEM	-	Minimum Essential Media
PBS	-	Physiological saline
PCV	-	Packed cell volume (hematocrit)
RBC	-	Red blood cell count

RNA	-	Ribonucleic acid
s.c.	-	Subcutaneous
TCA	-	Trichloroacetic acid
$t_{\frac{1}{2}}$	-	Half life
WBC	-	White blood cell count
Δ Abs	-	Δ Absorbance or change in Absorbance

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ABSTRACT

PHARMACOLOGIC AND TOXICOLOGIC STUDIES ON BABESICIDAL DRUGS WITH SPECIAL EMPHASIS ON QUINURONIUM

The undesirable toxic effects of babesicidal agent quinuronium have been observed since its introduction in 1933. The main signs of salivation, defecation, lacrimation, muscular tremors and depression are thought to be associated with cholinesterase inhibition. The purpose of this project was to perform acute toxicity studies with quinuronium in laboratory animals and sheep, compare effects of quinuronium and diminazene in sheep, investigate quinuronium effects on liver function in rats and to monitor quinuronium levels in ovine plasma using an *in vitro*/*in vivo* system with *Babesia rodhaini*.

Quinuronium concentration in ovine plasma was determined by the *in vitro*/*in vivo* model system with *Babesia rodhaini*. An *in vitro* EC_{50} of 13.5ng/ml was observed for quinuronium. Plasma collected from sheep receiving therapeutic dose of 1mg/kg of quinuronium inhibited parasitemia. Quinuronium levels in ovine plasma increased to a maximum of 27ng/ml at 2 hr which remained fairly elevated up to 8 hr, thereafter the

concentration of the drug declined gradually until none was detected at 48 hr.

LD_{50} (median lethal dose) in mice intraperitoneally (i.p.) and subcutaneously (s.c.) were 4.8 and 5.4mg/kg while corresponding values in rats were 6.3 and 6.5mg/kg respectively. Clinical signs of salivation, defecation, anorexia, muscular tremors and depression were observed within 20 min in sheep following 1mg/kg of quinuronium administration s.c. In rabbits only mild signs of anorexia and depression were observed following administration of 1mg/kg of quinuronium s.c. Normal cholinesterase activity was low in rabbits compared to sheep. Quinuronium inhibited cholinesterase activity from 10 min to 24 hr in 5 sheep by 24% of the normal baseline values. The activity of cholinesterase returned to normal at 48 hr. Atropinisation partially protected against anticholinesterase activity in other 5 sheep and the activity was only inhibited by 14% of the normal baseline values.

Effects of quinuronium on hepatic function was investigated in rats. Quinuronium affected hepatic excretion of sulfobromophthalein (BSP) as measured by isolated perfused rat liver. Three groups of rats exposed to 0 (n=5), 1.5 (n=6) and 2.3 (n=6)mg/kg of quinuronium i.p. 30 min before perfusion had half life

($t_{\frac{1}{2}} \pm$ S.E.) in min of BSP elimination from perfusate of 12.5 $\pm$ 1.8, 13.7 $\pm$ 1.0 and 16.8 $\pm$ 3.3 respectively. Groups of rats receiving 0 and 2.3mg/kg had a significant difference ($p < 0.05$) in their $t_{\frac{1}{2}}$. Bile volumes decreased in both quinuronium pre-exposed groups of rats compared to controls. In another experimental study rats received 0 (n=5), 2.3 (n=6) and 5.0 (n=6) mg/kg of quinuronium daily for 2 days. A dose dependent increase in liver weight/body weight ratios were observed. Fatty liver degeneration was observed in rats receiving 5mg/kg. There was no significant depletion of hepatic glutathione (GSH) in groups of rats exposed to quinuronium. This suggested that no reactive metabolites that are dependent on hepatic glutathione inactivation are involved in quinuronium metabolism.

In another experimental study 20 sheep were used to compare the effects of quinuronium and diminazene on lactic dehydrogenase (LDH) and creatine phosphokinase (CPK) activity and blood urea nitrogen (BUN) levels in plasma. Five sheep received therapeutic dose of 1mg/kg and subsequent 5 received double (higher) dose of 2mg/kg of quinuronium. In diminazene treatments, 5 sheep received therapeutic dose of 3.5mg/kg and subsequent 5 received double (higher) dose of 7.0mg/kg respectively.

LDH activity expressed in B-B Units/ml in the two dosage levels of quinuronium increased above baseline values following administration of the drug. Activity of LDH was also elevated above baseline values in sheep receiving the two dosage levels of diminazene. CPK activity in Sigma Units/ml in the two dosage levels of quinuronium increased above baseline values following its administration with a maximum activity at 2 hr followed by a gradual decline to end of study. CPK levels in sheep receiving two dosage levels of diminazene had a similar trend compared to quinuronium treatments. BUN levels in mg/100ml increased above baseline values in both dosages of quinuronium treated animals and one animal receiving 1mg/kg had levels above the upper normal limit of 20mg/100ml from 4 hr to end of the study at 48 hr. BUN levels in sheep receiving the two dosage levels of diminazene also increased above baseline values. The increase, however, was not as high as in quinuronium treatments. Increase in BUN levels and LDH and CPK activities shows that quinuronium is more organotoxic than diminazene at therapeutic and/or above therapeutic dosages.

This study has shown that the *in vitro*/*in vivo* model system with *B. rodhaini* is a simple method that can be used to determine quinuronium levels in ovine

plasma. Quinuronium can be classified as a highly toxic compound as shown by very low median lethal dose. The study also supports the view that depression of cholinesterase activity plays an important role as the cause of death in quinuronium toxicity. The study has shown that hepatoxicity may contribute to or potentiate the cause of death in quinuronium toxicity.

CHAPTER 1
INTRODUCTION

Babesiosis is a protozoal disease of animals characterised by fever, jaundice and hemolysis. The disease is economically important throughout the world. (Todorovic et al, 1973 and Kowigshoefer, 1977). In Kenya, bovine babesiosis which is very wide spread ranks second among the tick-borne diseases and presents a lot of hinderance to livestock development (FAO, 1975). Acute babesiosis is usually seen in areas where the climate favours development of the parasite in ticks.

Babesiosis can be prevented by either tick control (Ristic, 1966), stock movement, premunity, chemotherapy, chemoprophylaxis and/or vaccination. Tick control and chemotherapy still remain superior methods for disease control. Many chemotherapeutic agents like quinuronium (Acaprin $^{(R)}$), phenamidine, diminazene (Berenil $^{(R)}$), amicarbalide (Diaprom $^{(R)}$) and imidocarb (Imozol $^{(R)}$) have been used.

Quinuronium, one of the oldest babesides has been used since 1933 (Eyre, 1966). Many studies of its toxicity in cattle (Purnell et al, 1981), sheep (Cernaianu et al, 1935; Kikuth, 1935 and Eyre, 1966) and dogs (Naude et al, 1970) have been reported. Kikuth (1935), Brown and Berger (1970) and Hashemi-Fesharki (1975 and 1977) reported it

to be not effective in controlling parasitemia. Toxic signs usually observed following administration of quinuronium include vomition, salivation, diarrhea anorexia, recumbency, muscular tremors and hypotension. These signs suggest parasympathomimetic effect of quinuronium (Eyre, 1966). Naude (1970) observed a transient elevation of serum transaminases in dogs administered quinuronium. Its liver toxicity in animals has not been investigated thoroughly. There has also been a lack of simple and sensitive method for the determination of quinuronium levels in plasma. Acute toxicity has also been inadequately reported with quinuronium. Even reports by FAO (1975) and National Council for Science and Technology (Kenya, 1980) in connection with the Kenya Government's request to undertake studies on tick-borne cattle diseases and disease study appraisals emphasized priority research on tick-borne diseases. This study has therefore investigated the use and toxicity of babesicidal drugs with emphasis placed on quinuronium which is a common babesicide often used, but toxic and lacks information on plasma drug levels. The study therefore aimed at contributing more knowledge on the following:

1) Quinuronium levels in ovine plasma as determined by an in vitro/in vivo system with Babesia rodhaini.

2) Acute toxicity with quinuronium in laboratory animals and sheep.

3) Effects of quinuronium on rat liver.

4) Comparative study of toxic effects of quinuronium and diminazene in sheep.

CHAPTER 2

LITERATURE REVIEW

2.1 GENERAL INTRODUCTION

Babesicidal drugs are useful chemotherapeutic agents against babesiosis. Some babesicidal agents like diminazene and imidocarb are also useful in the treatment of trypanosomiasis and anaplasmosis respectively. Treatment of babesiosis should be given early in the disease course to enable the drugs to assist in premunity; however larger doses produce sterile immunity (Barnett, 1965 and Adams and Todorovic, 1974). Many babesicidal drugs are available for chemotherapy - quinuronium sulfate, dimidines (diminazene, phenamidine, pentamidine and amicarbalide) and more recently imidocarb dipropionate.

The actual mode of action of babesicides is unknown. Kikuth (1935) suggested that quinuronium attacked the dividing round or oval forms of piroplasms. In a recent autoradiograph study (Irvin and Young, 1977 and 1978), radiolabelled tritiated hypoxanthine was incorporated in the deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) of the babesia parasite. Hypoxanthine is one of the purine bases which can easily be converted to guanine. Purines are required as precursors in the biosynthesis of nucleotides. It is therefore thought that babesicidal drugs interfere with the biosynthesis

of nucleic acids of the parasite. In the product manual for berenil (Hoechst, 1975) it is stated that diminazene mainly affects the fine structure and function of the cell membranes. McHardy (1982) cited Elford as proposing that imidocarb blocks entry of inositol, an essential nutrient, into the erythrocyte containing babesia parasite, apparently resulting in 'starvation' of the parasite. This mechanism would readily explain the prophylactic effect of imidocarb since the presence of very small quantities of imidocarb on the surface of the erythrocyte could make it unattractive to the parasite.

2.2 QUINURONIUM

2.2.1 PHYSICO-CHEMICAL PROPERTIES

Quinuronium sulfate-6,6' Ureylene bis (1-methyl quinolinium) bis (metho-sulfate)-($C_{23}H_{26}N_4O_9S_2$= 566.6) is a creamy to canary yellow odourless crystalline powder with a bitter taste. It is very soluble in water, and only slightly soluble in organic solvents. On heating, the aqueous solution darkens and some decomposition may occur at a melting point (m.p.) of 235^{o}C. A 5% solution in water has a pH of 2 - 3.5. Quinuronium sulfate is a quaternary ammonium compound. Quaterinary ammonium compounds are best extracted with methanol (Clarke, 1960).

2.2.2 ASSAY

Quinuronium can be identified by fluorescence on paper chromatography and ultraviolet light after spraying with either iodoplatinate spray or bromocresol green spray or potassium permanganate spray (Clarke, 1974). Spinkova and Zyka (1960) described spectrophotometric determination of quinuronium sulfate in an aqueous solution. The determination is based on coupling the primary aromatic groups (liberated by acid hydrolysis) with N-1-naphthyl)-ethylenediamine producing a red colour. Kloppel et al (1970) reported that quaternary ammonium compounds for instance quinuronium can be extracted with lipophilic anions of which he observed 6-phenyl-2, 4-dinitrophenol to be the best. Kronfeld (1959a) reported that no work had been done on bioassay of quinuronium from blood of an animal. Moffat (1975) suggested the use of SE-30 as a stationary phase for Gas Liquid Chromatographic determination of quinuronium.

2.2.3 TOXICITY

Since the introduction of quinuronium sulfate in 1933 many reports of severe toxicity in horses, cattle, sheep, pigs and dogs have been reported. In sheep signs like salivation, urination, defecation, muscular spasms, unsteadiness, collapse and occasionally death have

been reported (Cernaianu et al, 1935). He also observed a marked toxicity following administration of a second dose two weeks later. Kikuth (1935) described similar toxic signs and suggested a close resemblance to shock. Rummler and Laue (1961) and Eyre (1966) reported anti-cholinesterase activity of quinuronium in sheep. They observed protection of the animal with atropine and not pyridine 2-aldoxime methiodide (2-PAM). Eyre (1966) also noted 40% inhibition of cholinesterase activity by quinuronium, and a recovery of activity after 24 hr.

In laboratory animals Kronfeld (1959a) described signs of twitching of muscles, jumps, dyspnea, hypotension and death in rats following administration of quinuronium. He noted that adrenaline protected the animals in a parallel control trial, and attributed the cause of death to histotoxic anoxia. From the evidence of other workers (Rummler and Laue, 1961 and Eyre, 1966) the cause of death was due to hypotension resulting from excess acetylcholine circulating in the blood. Other studies reported increased motility of guinea pig intestine (Eyre, 1967) and interference of quinuronium at neuromuscular junction of rat diaphgram (Eyre, 1968). Eyre (1968) suggested that the observed inco-ordination due to quinuronium toxicity in animals could be due to its effects at the neuromuscular junction.

Naude et al (1970) reported signs of vomition, diarrhea, salivation and anorexia in dogs receiving 0.25 mg/kg of therapeutic dose and toxic dose of 1mg/kg of quinuronium. He also reported elevated serum transaminase enzymes and anemia (Hb. 9.5%). In cattle Purnell et al (1981) observed excessive salivation, anorexia, dyspnea, muscular tremors, CNS depression and recumbency within 15 minutes of quinuronium administration.

2.2.4 CLINICAL TRIALS AND PREMUNITY

Early workers cited by Barnett (1965) reported that quinuronium cleared the blood of the parasites, hence no premunity. Kikuth (1935) in a review article noted that one cattle failed to respond to quinuronium. Ryley (1957), Arifdzhanov and Bokov (1963), Riek (1964), Barnett (1965) and Purnell et al (1981) reported quinuronium to be effective in clearing cattle of babesia. Ryley (1957) and Barnett (1965), besides observed premunity with quinuronium at therapeutic dose. Beveridge (1969) in her comparative trial with imidocarb and quinuronium in chemotherapy of Babesia rodhaini of mice found quinuronium less effective than imidocarb. Brown and Berger (1970) in their trial with imidocarb in Kenya compared its effect with quinuronium and amicarbalide in splenectomised calves. Quinuronium

was found more toxic, not effective in controlling B. bigemina and relapses were observed after 12 - 19 days of treatment (Brown and Berger, 1970).

Hashemi-Fesharki (1975 and 1977) in his trial in Iran reported similar results as Brown and Berger (1970) and concluded that the recommended dose of 1mg/kg of quinuronium in sheep was ineffective in eliminating Babesia ovis. However, Purnell et al (1981) reported quinuronium to be more effective if administered at the onset of fever or hemoglobinuria; noted the absence of prophylaxis with the drug if administered before infection and observed the presence of low level parasitemia after recovery. Gray (1983) observed efficacy and premunity with quinuronium in gerbils infected with B. divergens.

2.2.5 ACTIONS AND USES

Quinuronium has a specific effect against piroplasmoses of domestic animals. It is of value in the treatment of infections due to Babesia caballi in horses, B. bovis (bovine red water), and B. bigemina (tropical red water, texas fever) in cattle, B. divergens (in European cattle), B. ovis and B. motasi in sheep, B. suis in pigs and B. canis (biliary fever, malignant jaundice, tick fever) in dogs (British Veterinary Codex, 1965).

A single dose usually produces a clinical cure within 24 hours in cases in which the disease is in early stages and the temperature is rising. A second dose may be necessary in a few instances, especially in sheep, and this is given after 24 to 48 hours of the first dose. In animals in which the disease has progressed beyond early stages, small repeated doses spread over two to three days, together with supportive treatments have caused recovery, but in such cases the drug should be used with caution. The parasites may still be harboured by the animal after clinical recovery. Quinuronium should be administered by subcutaneous route only. Kikuth (1935) and Cerruti and Fantoni (1935) reported quinuronium to be effective against Theileria dispar and Th. annulata. In theilerial infections the drug appears to attack the schizont forms. Quinuronium is also effective in the treatment of trichomoniasis in cattle.

2.3 DIMINAZENE

2.3.1 PHYSICO-CHEMICAL PROPERTIES

Diminazene diaceturate-pp'-Dimidinodiazomino benzene diaceturate tetrahydrate ($C_{22}H_{29}N_9O_6,4H_2O$=589.6) is one of the oldest dimidines used in chemotherapy of babesiosis. It is yellow odorless powder, slightly soluble in water and alcohol and very

slightly soluble in chloroform and ether. It melts at 217^{o}C with decomposition. It is extracted by organic solvents from aqueous alkaline solutions.

2.3.2 ASSAY

Early workers (Bauer, 1958 and Cunningham et al, 1964) used biological assay for determination of diminazene. Other workers have used methods like colorimetry (Raether et al, 1972), high performance liquid chromatography (HPLC) (Fouda, 1977), gas chromatography - chemical ionization mass spectrophotometry (Fouda, 1978) and more recently, radiometry using ^{14}C-labelled drug (Gilbert and Newton, 1982) and paired-ion extraction and HPLC (Aliu and Ødegaard , 1983) for determination of diminazene.

2.3.3 TOXICITY

Diminazene which belongs to the dimidines was developed about 1940, during the specific search for compounds with trypanocidal activity. They were found to be very active against babesia. A new era in the treatment of babesiosis was thus ushered in with the introduction of certain members of this group as phenamidine some decades ago and more recently diminazene.

Toxicity test in animals demonstrated brain damage in dogs, toxic effects in camels and horses, but not in cattle, rats and mice (Wade, 1977). Hutchinson and Watson (1962), Temu (1975) and Apted (1980) reported that therapeutic uses in man had not produced toxic effects and recommended diminazene as the drug of choice for early trypanosomiasis. However, Ruebush et al (1979) reported development of acute idiopathic polyneuritis following its use against B. microti in man. Dimidine poisoning produces brain lesions in dogs resembling canine cerebral babesiosis (Basson and Pienaar, 1965) and thiamine deficiency in certain carnivores (Evans et al, 1942 and Edwin et al, 1968). The lesions observed are bilateral malacia and hemorrhages of cerebral cortex, caudate nucleus and in the region of cerebellar roof nuclei (Basson and Pienaar, 1965; Losos and Crockett, 1969 and Naude et al, 1970). Fussganger and Bauer (1958) used higher doses of 30 - 35mg/kg to produce poisoning in dogs and observed tremors, nystagmus, spastic paralysis and death within 3 days. Homeida et al (1981) recently observed acute side effects of vomition, diarrhoea, histamine-like reaction, hypotension and kidney and liver damage in camels.

2.3.4 CLINICAL TRIALS AND PREMUNITY

In a clinical trial done in South Africa it was demonstrated that a dose of 4mg/kg in dogs was more effective, cheaper and safer when compared to phenamidine (Botha, 1964). Barnett (1965) reported 3.5mg/kg in cattle to be effective in eradicating babesia parasites and the animals remained immune upto 109 days. At 5mg/kg, Barnet (1965) observed a sterile cure and a susceptibility to re-infection 68 days later.

2.3.5 ACTION AND USES

Dimidines have trypanocidal, babesicidal, anti-bacterial and antifungal properties, but are mainly used, however, for the first two actions (Wade, 1977). A single dose of diminazene usually gives a clinical cure within 24 hours. For all species 3.5mg/kg is the recommended dose, but for Trypanosoma brucei in cattle the recommended dose is 7mg/kg. In trypanosomiasis, it is used mainly as curative drug. It is effective against Babesia spp. of domestic animals.

2.4 IMIDOCARB

2.4.1 PHYSICO-CHEMICAL PROPERTIES

Imidocarb dipropionate or as dihydrochloride ($C_{19}H_{20}N_6O$=421.3) is a crystalline odourless solution

which should be stored between 2^{o} to 25^{o}C. The dihydrochloride is more soluble, has neutral pH and less irritant to tissue (Haigh and Hagan, 1974).

2.4.2 ASSAY

Aliu et al (1977) described spectrophotometric and thin layer chromatographic determination of imidocarb in sheep. He observed high protein binding and high concentrations in kidney, liver, and brain. McHardy (1982) recently described an *in vitro/in vivo* system with *B. rodhaini* to determine bovine levels of imidocarb.

2.4.3 TOXICITY

Imidocarb (3,3'- bis(2-imidazdin-2-yl) is one of the most recent babesicides. Beveridge (1969), Callow and McGregor (1970), Wood (1971), Hashemi-Fesharki (1975 and 1977), Corrier and Adams (1976 and 1977) and Adams et al (1980) found toxicity of imidocarb to be acceptable within the therapeutic range (1 - 5mg/kg). However, Todorovic et al (1973) reported embarassed respiration or oral respiration, excessive salivation, muscular fasciculations, urination, defecation, inco-ordination and prostration at [illegible] doses above 5mg/kg. Todorovic et al (1973) observed that the signs were mild r with subcutaneous or intramuscular injections. Brown and Berger (1970) and

Hashemi-Fesharki (1975 and 1977) found imidocarb less toxic compared to quinuronium and amicarbalide. Corrier and Adams (1976 and 1977) and Adams et al (1980) reported signs of excessive salivation, elevated blood urea nitrogen and serum glutamic oxaloacetic transaminase levels and nephrotoxicity in goats following a lethal dose of imidocarb. Autopsies revealed renal hyperemia, hepatomegally, pulmonary congestion, hydrothorax, hydroperitoneum and hydropericardium. Histologically they observed renal tubular necrosis, and focal hepatocellular necrosis. Injection sites had focal areas of necrosis.

2.4.4 CLINICAL TRIALS AND PREMUNITY

Imidocarb has been reported to be effective in the treatment and prophylaxis of bovine babesiosis. Beveridge (1969) and Schmidt et al (1969) found imidocarb more effective than quinuronium, diminazene and amicarbalide in preventing B. rodhaini infections in mice and rats. Callow and McGregor (1970) observed that a dose of 2mg/kg of imidocarb was sufficient to eliminate B. bigemina in cattle. In other studies, Brown and Berger (1970), Todorovic et al (1973), McHardy and Simpson (1974), Adams and Todorovic (1974) Hashemi-Fesharki (1975 and 1977) found imidocarb to be effective in eradicating babesia infections of cattle

using therapeutic dose of 1mg/kg. Callow and McGregor (1970), Hart et al (1971), Roy-Smith (1971), Todorovic et al (1973), McHardy and Simpson (1974) and Kuttler (1974) observed chemoprophylaxis with imidocarb when administered before infection. Kuttler (1975) observed that when the drug was given 14 or 28 days before infection, no disease developed in calves and when the drug was given 42 days before infection there was no prophylaxis. Brown and Berger (1970) and Hashemi-Fesharki (1975 and 1977) observed that imidocarb was more effective, less toxic, caused no disease relapses, and maintained pre-immunity after treatment. However, Adams and Todorovic (1974) reported sterile immunity with larger doses of imidocarb in bovine babesiosis. Gray (1983) observed prophylaxis of B. divergens in gerbils with imidocarb.

2.4.5 ACTION AND USES

Imidocarb is currently used in chemotherapy and chemoprophylaxis of bovine babesiosis. It is also effective in the treatment of anaplasmosis (McHardy and Simpson, 1974 and Adams and Todorovic, 1974), canine ehrlichiosis (Adeyanju and Aliu, 1977; Price, 1980 and Ogunkoya et al, 1981) and Hepatozoon canis (Ogunkoya et al, 1981).

2.5 DYES

The dyes for instance acriflavin and trypan blue have been used extensively in the past for chemotherapy of babesiosis. The compounds are usually given intravenously, and hence care must be taken to ensure that none of the material is given subcutaneously since a marked reaction is usually produced (Soulsby, 1968). One single administration is usually effective. However, these compounds are not extensively used any more due to low efficacy and recurrence of relapses.

CHAPTER 3

QUINURONIUM LEVELS IN OVINE PLASMA AS DETERMINED BY AN *IN VITRO/IN VIVO* SYSTEM WITH *BABESIA RODHAINI*

3.1 INTRODUCTION

There has been a lack of simple and sensitive method for the determination of quinuronium levels in plasma. Spinkova and Syka (1960) described spectrophotometric determination of quinuronium from aqueous solution. Moffat (1975) suggested that quinuronium could be determined by Gas Liquid Chromatography using SE-30 as a stationary phase. *Babesia rodhaini* (mouse and rat strain) cultures have been used to screen compounds showing activity against babesiosis (Beveridge, 1953 and 1969 and Ryley, 1957). Irvin and Young (1977) reported babesicidal compounds inhibited incorporation of tritiated hypoxanthine by *Babesia* parasites *in vitro*. McHardy (1982) demonstrated an *in vitro/in vivo* model system using *B. rodhaini* for monitoring imidocarb levels in bovine plasma.

The present study was conducted to test this *in vitro/in vivo* model in sheep and thereafter use the system to monitor quinuronium levels in ovine plasma following injection of therapeutic dose.

3.2 MATERIALS AND METHODS

3.2.1 PARASITES/MICE

Infected red blood cells (RBC) with B. rodhaini cryopreserved at -79°C were passaged twice in male albino mice after thawing in water at 37°C. The parasites were obtained from Dr. A.D. Irvin, I.L.R.A.D., Kabete, Kenya. Mice were obtained from Veterinary Research Laboratories, Kabete, Kenya.

3.2.2. CULTURES

Blood from mice with approximately 40% parasitemia was withdrawn using alsevers solution (pH 6.0). It was diluted with heparinized Eagles' modified Minimum Essential Media (MEM) to give mixtures of 2×10^8 infected RBC. Infected RBC (2×10^8) were incubated for 2 hours at 37°C in a set of tubes with known concentrations of quinuronium. After incubation the erythrocytes were spinned and washed twice with heparinized MEM, and then suspended in 1ml of heparinized MEM. Thereafter, 0.1ml containing 2×10^7 infected RBC were injected intraperitoneally in groups of 5 mice per concentration. The mice used were approximately of the same age and weight. The course of infection was monitored by stained smears from the tail using Giemsa stain. Percentage of infected erythrocytes was evaluated on day 5. A standard reference curve was prepared from table I in which quinuronium levels were extrapolated.

Plasma (100µl) collected at various time intervals after injection of therapeutic dose (1mg/kg s.c) of quinuronium sulfate (Acaprin, Bayer AG, Leverkusen) into two sheep, was added to 2 X 10^8 infected RBC mixtures and incubated as above. The procedure was repeated and % parasitemia on day 5 was evaluated.

3.3 RESULTS

The activity of quinuronium on an *in vitro*/*in vivo* system with *B. rodhaini* is shown in Table 1. Untreated control mice had parasitemia of 87% on day 5. An *in vitro* EC_{50} of 13.5ng/ml was observed.

Plasma from the two sheep showed more than 50% inhibition of parasitemia at 1hr, with a maximum inhibition of 2hr. At 24 hr, the inhibition of parasitemia by plasma from the two animals was minimal, and at 48hr there was no inhibition of parasitemia when it was comparable to 0 time (pre-treatment) plasma collection. The corresponding calculated quinuronium levels (ng/ml) are shown in Fig. 1. Quinuronium concentration in plasma increased to a maximum mean of 27ng/ml at 2 hr which remained fairly elevated upto 8hr. Drug levels in plasma disappeared gradually until none was detectable at 48 hr.

3.4 DISCUSSION

The *in vitro*/*in vivo* model system described in this study is a simple inexpensive method that shows it can be used to determine quinuronium levels in plasma. The spectrophotometric method of Spinkova and Zyka (1960) is expensive and lacks sensitivity. The use of gas liquid chromatography using SE-30 as a stationary phase as suggested by Moffat (1975) has yet to be described to compare it with other methods.

Inhibition of parasitemia in an *in vitro*/*in vivo* system observed with quinuronium correlates with other reports (Irvin and Young, 1977 and 1978 and McHardy, 1982). Other babesicides like diminazene, imidocarb and amicarbalide have also been reported to inhibit parasitemia *in vitro* (Irvin and Young, 1977 and 1978 and McHardy, 1982). In the present study an *in vitro* EC_{50} of 13.5ng/ml was observed. McHardy (1982) observed an *in vitro* EC_{50} of 28ng/ml (0.028mg/L) for imidocarb using calf plasma. At 2 hr, the inhibition of parasitemia by plasma was maximum and plasma quinuronium levels was 27ng/ml which was above the observed *in vitro* EC_{50}. The present results demonstrate that plasma from sheep treated with quinuronium or metabolites contained therein have babesicidal activity. This suggests that quinuronium is metabolized slowly or eliminated unchanged and/or the active metabolites resemble the parent compound.

Depression of cholinesterase activity as an indicator of the presence of quinuronium in ovine plasma *in vivo* upto 24 hr (Eyre, 1966) also supports the hypothesis that metabolite or metabolites in plasma are eliminated slowly and/or quinuronium is eliminated unchanged.

Plasma quinuronium levels showed a rapid uptake of the drug to a maximum peak at 2 hr. Quinuronium is a quaternary ammonium compound which probably binds to plasma proteins. Its rapid distribution is difficult to explain, however, for a large molecule to be distributed a special transport mechanism is probably involved to enable it to cross cell membranes and capillary wall and/or it is distributed in plasma water (Klaassen,1980). The concentration of quinuronium started to decline 2 hr after injection and was below EC_{50} at 24 hr. At 48 hr, the drug was undetectable, and this may account for the resumption of normal cholinesterase activity observed after 24 hr in sheep *in vivo* following administration of therapeutic dose of quinuronium (Eyre, 1966). McHardy (1982) in his *in vitro*/*in vivo* model could not detect imidocarb levels in calf plasma at 72 hr. The rapid disappearance of quinuronium in plasma may be due to polar nature of the compound which may enable it to be eliminated mainly by urinary excretion.

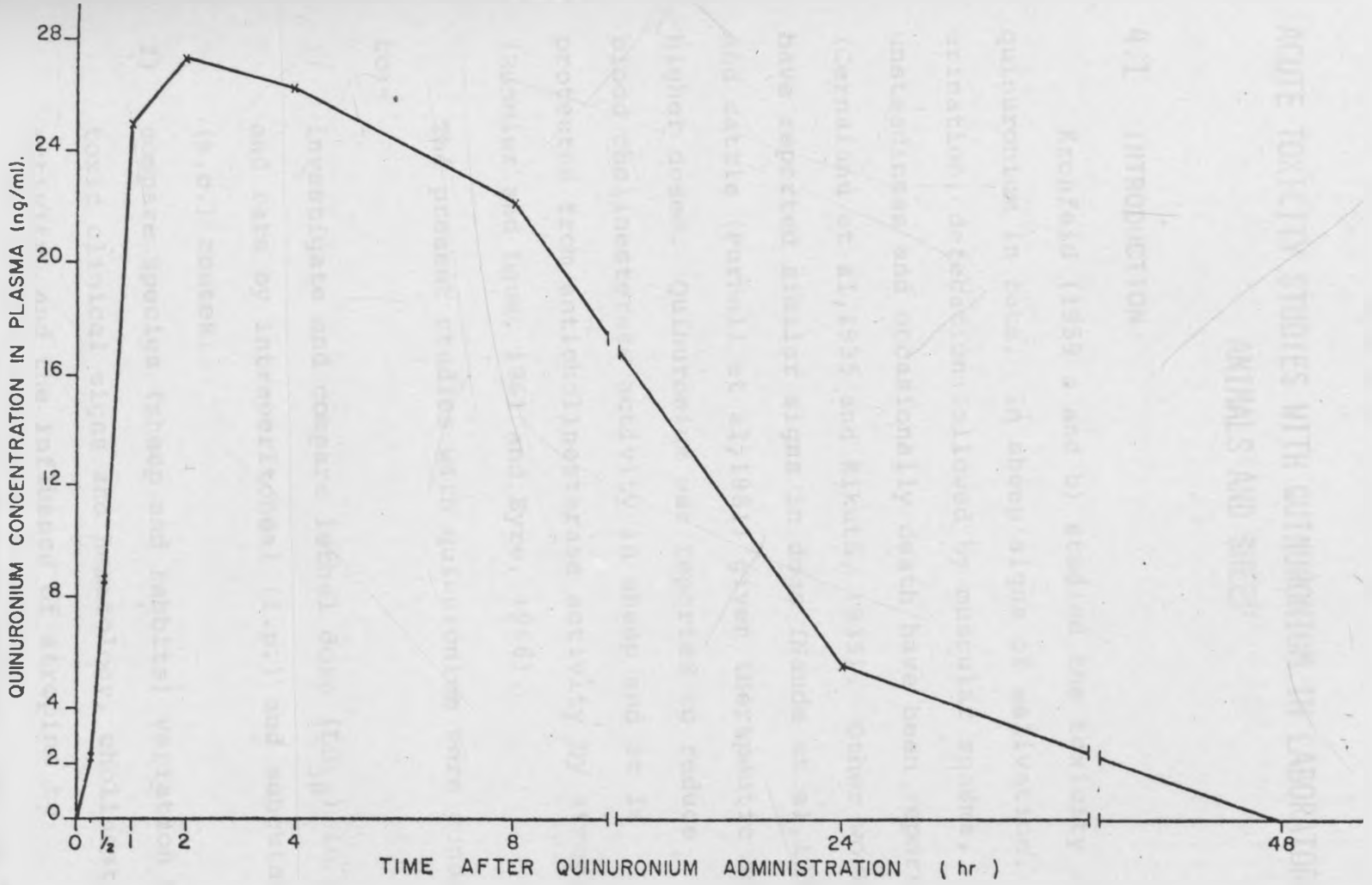
This study supports previous reports of the use of *B. rodhaini* as a useful organism in screening antibabesials.

The *in vitro*/*in vivo* model described here is simple and can be used to monitor quinuronium levels in plasma. The investigation also suggests that a second therapeutic dose of quinuronium may be administered between 24 and 48 hr if there is a clinical indication.

TABLE I: EFFECTS OF INCREASING QUINURONIUM CONCENTRATION ADDED TO THE *IN VITRO*/*IN VIVO* SYSTEM WITH *B. rodhaini* AS DETERMINED BY % PARASITEMIA (± SD) IN MICE

Quinuronium concentration (ng/ml)	% Parasitemia on day 5 (n = 5)
0	87 (13.4)
2.5	74 (9.6)
5	58 (8.0)
10	46.6 (9.9)
20	24.8 (11.7)
40	12 (3.1)
80	4.7 (3.3)
160	0.6 (0.7)

FIG. 1 MEAN PLASMA CONCENTRATION OF QUINURONIUM (ng/ml) IN TWO SHEEP AFTER SUBCUTANEOUS ADMINISTRATION OF THERAPEUTIC DOSE (1mg/kg).


QUINURONIUM CONCENTRATION IN PLASMA (ng/ml).
28
24
20
16
12
8
4
2
0
0
½
1
2
4
8
24
48
TIME AFTER QUINURONIUM ADMINISTRATION (hr)

CHAPTER 4

ACUTE TOXICITY STUDIES WITH QUINURONIUM IN LABORATORY ANIMALS AND SHEEP

4.1 INTRODUCTION

Kronfeld (1959 a and b) studied the toxicity of quinuronium in rats. In sheep signs of salivation, urination, defecation followed by muscular spasms, unsteadiness and occasionally death have been reported (Cernaianu et al,1935 and Kikuth, 1935). Other workers have reported similar signs in dogs (Naude et al,1970) and cattle (Purnell et al,1981) given therapeutic and higher doses. Quinuronium was reported to reduce blood cholinesterase activity in sheep and it is protected from anticholinesterase activity by atropine (Rummler and Laue, 1961 and Eyre, 1966).

The present studies with quinuronium were conducted to:-

1) investigate and compare lethal dose (LD_{50}) in mice and rats by intraperitoneal (i.p.) and subcutaneous (s.c.) routes.
2) compare species (sheep and rabbits) variation of toxic clinical signs and hematology, cholinesterase activity, and the influence of atropine to counteract anticholinesterase activity in the two species.

4.2 MATERIALS AND METHODS

4.2.1 ANIMALS

One hundred male albino mice approximately 20g were used in the study. The same number of male albino rats approximately 200g were used in the study. They were bought from the Veterinary Research Laboratories, Kabete, Kenya. Mice and rats were offered mice and rat pellets (Unga Ltd., Nairobi, Kenya) and water ad libitum.

Sixteen white male New-Zealand rabbits 2 - 3kg were used in the study. They were obtained from the same source as the mice and rats. The rabbits were offered rabbit pellets (Unga Ltd., Nairobi, Kenya) and water ad libitum.

Fourteen male black-head Persian sheep approximately 9 months old were used in the study. They were obtained from one breeder in Nairobi region. They were offered hay and pellets and water ad libitum. The animals were not exposed to any known acetylcholinesterase inhibitor.

4.2.2. DETERMINATION OF LD_{50} IN MICE AND RATS

The mice and rats were randomly divided into 5 groups of 10 animals each. Groups 1 to 5 of mice and rats received 2.5, 3.3, 4.4, 5.7 and 7.6mg/kg body weight of quinuronium sulfate (Acaprin, Bayer AG, Leverkusen)

i.p. or s.c. The lowest dose of 2.5mg/kg was fixed after a pilot trial in which all the mice and rats tested survived following i.p. injection of quinuronium. The successive 4 doses were fixed using a geometrical factor (R) (Buck and Osweiler, 1976a) of 1.32. Mice and rats received the drug at 0800hr and thereafter observed for number of surviving or dead at the end of 13 hr period (Boulus et al, 1970). LD_{50} values were determined using the method of Wagner and Johnson (1970).

4.2.3 DRUG TREATMENTS TO RABBITS AND SHEEP

Four rabbits which served as controls (group 1) received 1ml of physiological saline. Group 2 (n=5) received 1mg/kg of quinuronium s.c. and group 3 (n=5) received 1mg/kg of atropine (Eyre, 1966) intramuscularly (i.m.) 30 min before treatment with quinuronium as in group 2. Sheep were also divided into three groups - 1 (control, n=4), 2 (n=5) and 3 (n=5). They received similar drug treatments and dosages as in the rabbits.

4.2.4 BLEEDING

Heparinized blood samples were taken for cholinesterase determination from the marginal ear and the jugular veins of the rabbits and sheep respectively. Blood

was taken at 0, 1/6, 1/2, 1, 2, 4, 8, 24, 48 and 72 hr designated hr interval. In sheep additional blood (5 - 8ml) was taken for hematologic determinations.

4.2.5 HEMATOLOGY

Hematologic parameters-total white blood cell count (WBC), red blood cell count (RBC), hematocrit (PCV) and hemoglobin (Hb) were determined at the designated time intervals in sheep. WBC and RBC were enumerated according to the standard method using Coulter electronic counter (Coulter Electronics, Inc., Hialeah, Fl.) PCV was done using the microhematocrit method. (Schalm et al,1975).

4.2.6 CHOLINESTERASE DETERMINATION

Whole blood cholinesterase activity was determined in rabbits and sheep according to the procedure of Ellman et al (1961) with some modifications. Samples were read at 410 nm using spectrophotometer (Bausch and Lumb, Rochester, New York). The unit of measurement for cholinesterase values was ΔAbs. change, where Δ Abs. = the difference between the 1st reading and the 2nd reading after 5 min interval. The results were reported as % of '0' baseline values for each animal as its own control (Shmidl et al,1982).

4.2.7 DATA ANALYSIS

Data was analysed by paired 't'-tests (Snedecor

and Cochran,1971). The significance of activity of whole blood cholinesterase and hematologic parameters were tested among and between groups of rabbits and sheep at a probability level of 0.05.

4.3 RESULTS

4.3.1 LD_{50} IN MICE AND RATS

The number of dead mice and rats at the end of 13 hr period are depicted in Table II. LD_{50} for quinuronium in mice were 4.8 and 5.4mg/kg for i.p. and s.c. routes respectively, while the corresponding values in rats were 6.3 and 6.5mg/kg.

4.3.2 CLINICAL OBSERVATIONS

In sheep toxic clinical signs of salivation, defecation, depression and muscular spasms were observed after 15 min. Thereafter, they were anorexic for 4 hr. Clinical signs in sheep subsided after 6 hr and there were no deaths. In rabbits only anorexia and depression were observed after 30 min. These lasted for 2 hr after which they regained their appetite. No deaths were observed. Sheep and rabbits pretreated with atropine showed only mild signs of anorexia and depression for about 30 min.

4.3.3. HEMATOLOGY

The hematologic parameters - WBC, RBC, PCV and

Hb in sheep showed no significant differences between and within the three groups during the period of study. The values remained normal within the species range.

4.3.4 CHOLINESTERASE ACTIVITY IN VIVO

In sheep there was a tendency towards lower cholinesterase activity from 10 min to 24 hr after administration of quinuronium. The mean reduction was 24% of the normal '0' baseline value; the difference, however, did not reach a level of significance. The activity of the enzyme returned to normal at 48 hr. In sheep pretreated with atropine there was a lesser mean reduction of cholinesterase activity by 14% of the normal '0' baseline values between 10 min to 2 hr interval (Table III).

In rabbits cholinesterase activity was even less affected between and within the three groups during the period of study (Table IV). The normal activity of cholinesterase in rabbits was about 50% lower than in sheep.

4.4 DISCUSSION

According to the classification of Loomis (1974), the values for LD_{50} observed in this study classifies quinuronium as highly toxic. LD_{50} values for rats i.p. and s.c. routes observed in this study were about

four times higher than those reported by Kronfeld (1959b). Kronfeld used only four groups of rats. Values for LD_{50} in mice by i.p. and s.c. routes and a comparison of LD_{50} in these animals by these routes have not been reported. The cause of death in quinuronium toxicity has been controversial. Kronfeld (1959b) in an *in vitro* study with rat liver tissues suggested that quinuronium toxicity was due to histotoxic anoxia. Rummler and Laue (1961) and Eyre (1966) in their studies concluded that the cause of death was due to anticholinesterase activity. Other observations have shown that quinuronium affects liver function.

Clinical signs of salivation, defecation, anorexia and muscular spasms observed in sheep are consistent with other reports (Cernaianu et al, 1935; Kikuth, 1935 and Eyre, 1966). The rabbit was observed to have low normal whole blood cholinesterase activity compared to sheep. This is due to variation in species (Buck and Osweiler, 1976b). However, quinuronium did not inhibit cholinesterase activity significantly in the rabbit *in vivo*. Eyre (1966) reported an inhbition of cholinesterase activity in rabbit whole blood *in vitro*. The whole animal nature in this study may explain the cause of difference in the findings.

This study shows that recommended dosages of quinuronium should not be exceeded due to its high toxicity. It also shows that rabbits are relatively resistant to quinuronium toxicity and may support the view that anticholinesterase activity plays an important role in quinuronium toxicity.

TABLE II: NUMBER OF MICE AND RATS RECEIVING 2.5 TO 7.6mg/kg OF QUINURONIUM DEAD AFTER 13 HR PERIOD

	Mice		Rats	
Dosage (mg/kg)	number dead (i.p.)	number dead (s.c.)	number dead (i.p.)	number dead (s.c.)
2.5	0/10	0/10	0/10	0/10
3.3	0/10	0/10	0/10	0/10
4.4	4/10	0/10	0/10	0/10
5.7	9/10	4/10	3/10	4/10
7.6	10/10	6/10	10/10	6/10

TABLE III: MEAN WHOLE BLOOD CHOLINESTERASE ACTIVITY (± SD) EXPRESSED AS % OF 'O' NORMAL BASELINE VALUES IN CONTROL AND 'TEST' SHEEP

Time after drug administration (hr)	Control (n = 4)	1mg/kg of quinuronium (n = 5)	1mg/kg of quinuronium and prior atropinisation (n = 5)
0	100	100	100
1/6	84 (15)	74 (17)	86 (14)
1/2	90 (22)	76 (20)	85 (13)
1	90 (27)	71 (11)	85 (13)
2	88 (21)	77 (15)	88 (4)
4	91 (26)	78 (22)	102 (34)
8	88 (19)	83 (7)	103 (30)
24	96 (35)	80 (8)	101 (23)
48	101 (27)	103 (12)	96 (27)
72	100 (12)	101 (9)	95 (22)

TABLE IV: MEAN WHOLE BLOOD CHOLINESTERASE ACTIVITY (± SD) EXPRESSED AS % OF 'O' NORMAL BASELINE VALUES IN CONTROL AND 'TEST' RABBITS

Time after drug administration	Control (n = 4)	1mg/kg of quinuronium (n = 6)	1mg/kg of quinuronium and prior atropinisation (n = 6)
0	100	100	100
1/6	100 (23)	92 (46)	101 (28)
1/2	98 (36)	93 (33)	101 (38)
1	114 (38)	85 (36)	97 (20)
2	102 (34)	87 (41)	79 (27)
4	83 (15)	102 (38)	84 (31)
8	98 (28)	91 (35)	84 (29)
24	110 (39)	89 (43)	100 (29)
48	108 (40)	109 (43)	120 (59)
72	113 (70)	117 (72)	119 (60)

CHAPTER 5

EFFECTS OF QUINURONIUM ON RAT LIVER

5.1 INTRODUCTION

Since the introduction of quinuronium there have been many reports of toxicity in horses, cattle, sheep and dogs. Naude et al (1970) reported occasional vomition, diarrhoea, anorexia and prominently raised serum transaminase levels in dogs administered therapeutic dose of 0.25mg/kg of quinuronium. Relationship between pre-exposure of quinuronium and sulfobromophthalein (BSP) clearance has not been reported. BSP clearance is widely used to assess hepatic function in animals (Cornelius, 1970). Hepatic glutathione (GSH) conjugates reactive metabolites from a compound; its depletion suggests formation of reactive metabolites from that compound (Mitchell et al,1973).

Isolated perfused rat liver technique offers a convenient versatile model where single reactions and their kinetics can be followed (Nagashima and Levy, 1968 and Bahr, Sjoeqvist and Levy, 1970) in the liver independent of humoral and nervous regulation. Drugs that are either too toxic or have very low concentrations in the blood, can be assayed using isolated technique where higher concentrations will predominate in the perfusate and bile. In the case of BSP uptake and disposition by the liver, a marked decrease of its transport maximum has

been observed in intact *in situ* organ compared to isolated liver technique where there is an increase in transport maximum (Stirnemann et al, 1970).

The present investigations with quinuronium were conducted to evaluate:-

1) Its effects on the hepatobiliary uptake and disposition of BSP by isolated perfused rat liver following its pre-exposure.

2) Its effects on liver to body weight ratios, histopathology and possible depletion of hepatic glutathione following its administration in rats.

5.2 MATERIALS AND METHODS

5.2.1 EFFECTS OF QUINURONIUM PRE-EXPOSURE ON HEPATIC EXCRETION OF SULFOBROMOPHTHALEIN (BSP) AS MEASURED BY ISOLATED PERFUSED RAT LIVER

5.2.1.1 Animals

Male albino rats (Veterinary Research Laboratories, Kabete) were maintained in separate animal facilities away from any known enzyme inducers or inhibitors. The bedding used in the cages were made from saw dust. The animals were provided with rat pellets (Unga Ltd., Nairobi, Kenya) and water *ad libitum* until used for an experiment or liver donor. Animals used as blood donors

weighed 250 - 300g, whereas those used as liver donors weighed 200 - 250g. The rats were divided into control (group 1, n=5), low quinuronium dosed (group 2, n=6) and high quinuronium dosed (group 3, n=6) which respectively received 0, 1.5 and 2.3mg/kg of quinuronium sulfate intraperitoneally 30 min before sacrifice.

5.2.1.2. Drugs and chemicals

Quinuronium sulfate (Acaprin(R)) was obtained from Bayer Veterinary Divison, East Africa Ltd. Sulfobromophthalein was manufactured by Koch-Light Labs., Ltd. and bovine albumin by Sigma Chemicals, St. Louis, MO., U.S.A. Heparin, sodium chloride, sodium bicarbonate, potassium chloride, calcium chloride, magnesium chloride and sodium biphosphate were manufactured by British Drug House, Poole, England. All the chemicals were analytical grade. The salts were used for preparing Krebs-Ringer solution.

5.2.1.3 Experimental procedure

Surgical procedures were performed as described by Mehendale (1976 and 1977) using methoxyflurane, (Penthrane(R), Abott Labs., North Chicago, Ill., U.S.A.) for anesthesia. The common bile duct was cannulated with PE-50 tubing. The rats were heparinized with 150 units of sodium heparin by injecting the solution into the ilio-lumbal vein posterior to the right kidney. The

site of injection was clamped with a hemostat. The portal vein was cannulated and isolated liver was connected to the circulating perfusate, the lobes were then orientated properly to facilitate perfusion. The total time from abdominal incision to removal of the liver was approximately 10 min.

The technique and apparatus (Fig. 2) used in the isolated perfused rat liver experiments were modifications of Miller (1973) and Mehendale (1976 and 1977). Livers were perfused with 30 - 45ml of 30% rat blood obtained by mixing heparinized whole rat blood with two parts of Krebs-Ringer bicarbonate buffered solution (pH 7.4) containing 1.5g bovine albumin per 30ml perfusion fluid. The perfusion was allowed 30 min for equilibration after which BSP was introduced in the perfusate at the dose of 12mg/kg body weight. Perfusion flow rate was maintained at 6ml/min. The temperature of the perfusate and liver were maintained at 37^{o}C. The perfusate was oxygenated in the wire mess oxygenator with a humidified mixture of O_2/CO_2 (95:5). Samples of 1ml perfusate were withdrawn at 0, 5, 10, 15, 30, 45, 60, 75, 90 and 120 min interval. Bile was collected at 0, 15, 30, 45, 60, 75, 90 and 120 min interval. The volume of bile was noted.

5.2.1.4 Assays of BSP in perfusate and bile

Perfusate and bile were analysed for BSP concentration using the procedure described by Kutob and Plaa

(1962) with minor modifications. Cell free perfusate (200µl) and bile (10µl) samples were used in assays. BSP concentration was estimated using spectrophotometry (Bausch and Lomb, Rochester, N.Y., U.S.A.) at 580nm. Absorbance were converted into concentrations using previously prepared standard curve.

5.2.1.5 Statistical analysis

Half life ($t_{\frac{1}{2}}$) of BSP in perfusate were calculated by logarithmic curve fitting in a HP-97 standard Pac calculator (Hewlett Packard, Cupartino, CA., U.S.A.).

The data was analysed by analysis of variance (Snedocor and Cochran, 1971). The significance of the disappearance rates of BSP in perfusate and bile, and the total bile volume were tested among groups at a probability level of 0.05.

5.2.2 EFFECTS OF QUINURONIUM INDUCED HEPATOTOXICITY OF RATS

5.2.2.1 Animals and drug treatments

Male albino rats approximately 150 - 200g (Veterinary Research Labs., Kabete) were maintaned on rat pellets (Unga Ltd., Nairobi, Kenya) and water ad libitum. The bedding used in the cages were made from saw dust. Rats were randomly divided into control or group 1 (n=5), 2 (n=6) and 3 (n=6) receiving 0.5ml of physiological

saline (PBS), 2.3mg/kg and 5mg/kg of quinuronium subcutaneously daily for 2 days respectively.

5.2.2.2 Liver weight/body weight ratios

The livers were carefully isolated from abdominal cavity after anesthetizing the rats lightly with diethyl ether. Livers were blotted to remove blood, thereafter weighed to determine liver weight/body weight ratios.

5.2.2.3 Histopathology

A cross section of the median lobe of the liver was removed from each rat. The tissues were fixed in 10% neutral formalin. They were trimmed and paraffin sections of the specimens were stained with hematoxylin and eosin (HE). Liver injury was assessed under light microscope.

5.2.2.4 Hepatic glutathione

A 1g section of the lower median lobe was excised from frozen liver. It was homogenized with 10ml of 5% trichloroacetic (TCA) solution containing 5mM of disodium ethylenediaminetetracetate (EDTA) using ultra-turrax mixture for 1 minute. The clear supernatant (2ml) was recovered after centrifugation at 9,000g for 15 minutes. Hepatic GSH was determined according to the method of Buttar et al (1977) with some modifications

using a spectrophotometer (Bausch and Lomb, Rochester, N.Y.) at 410nm.

5.3 RESULTS

5.3.1 EFFECTS OF QUINURONIUM PRE-EXPOSURE ON HEPATIC EXCRETION OF SULFOBROMOPHTHALEIN AS MEASURED BY ISOLATED PERFUSED RAT LIVER

5.3.1.1 BSP in perfusate

There was a significant decrease in the rate of disappearance of BSP from the perfusate in group 3 compared to group 1 (Fig. 3, $P < 0.05$). Half lives of BSP from the perfusate ($t_{\frac{1}{2}} \pm$ S.E.) of groups 1, 2 and 3 were 12.5 $\pm$ 1.8, 13.7 $\pm$ 1.0 and 16.8 $\pm$ 3.3 min respectively. The difference in half lives between groups 1 and 2, and between 2 and 3 was not statistically significant. Groups 1 and 3 had a significant difference in their $t_{\frac{1}{2}}$.

5.3.1.2 BSP in bile

The BSP excretion rate in groups 2 and 3 was significantly lower than in group 1 ($P < 0.05$). There was no significant difference ($P > 0.05$) in the amount of BSP excreted in group 2 compared to group 3. From 0 to 45 min (Fig. 4) there was a rapid uptake and distribution of BSP in the liver tissue; it was therefore after 45 min that excretion of BSP in bile was observed.

5.3.1.3 Bile volume

The volume of bile excreted during experimental period was significantly decreased ($p < 0.05$) in groups of rats pre-exposed to quinuronium as compared to control rats (Fig. 5). There was, however, no significant difference betwen the pre-exposed groups of rats.

5.3.2 EFFECTS OF QUINURONIUM INDUCED HEPATOTOXICITY OF RATS

5.3.2.1 Clinical observations

Rats that were administered quinuronium showed signs of muscular tremors, jumps, enlarged and hyperemic eyeballs, lacrimation, depression and anorexia within 15 min of drug administration. One rat receiving 5mg/kg died at 36 hr after drug administration.

5.3.2.2 Liver weight/body weight ratios

A dose-dependent increase in liver weights relative to body weights due to quinuronium administration was observed (Table V). For example at a dose of 5mg/kg the liver weight/body weight ratio was elevated two fold when compared to control.

5.3.2.2 Histology

Control rats showed normal liver histology (Fig. 6). Focal fatty degeneration (Fig. 7) was observed in 3 rats receiving 5mg/kg quinuronium. Fatty degenerative

changes occurred peripherally around the hepatic lobule.

5.3.2.4 Hepatic GSH

The relationship between quinuronium dosages and hepatic GSH Absorbance is depicted in Table V. There was no significant differences ($P > 0.05$) in hepatic GSH among the three groups at the end of the study.

5.4 DISCUSSION

5.4.1 EFFECTS OF QUINURONIUM PRE-EXPOSURE ON HEPATIC EXCRETION OF SULFOBROMOPHTHALEIN AS MEASURED BY ISOLATED PERFUSED RAT LIVER

Isolated liver perfusion technique enables the liver which is the main site of metabolism to be studied thoroughly without interference with other organs (Garattini et al, 1973) and hence evaluate the effects of the drug or compound on its function.

A significant decrease in the rate of disappearance of BSP from the perfusate was observed in the high quinuronium dose group. Naude et al (1970) reported liver dysfunction in dogs administered therapeutic dose of quinuronium. He reported slightly or prominently raised serum transaminase levels.

In other studies with quinuronium Rummler and Laue

(1961) and Eyre (1966) reported inhibition of cholinesterase activity with quinuronium when therapeutic dosages were used in animals. Quinine and quinidine which have slight structural analogy with quinuronium have also been reported to inhibit cholinesterase activity in human plasma (Ellman et al,1961). The observed decrease in the rate of disappearance of BSP from the perfusate as measured by isolated perfused rat liver due to pre-exposure to quinuronium indicates that quinuronium dosages above 2.3mg/kg affects liver function and supplements other effects of quinuronium already reported. This observation may suggest an inhibition of the uptake and elimination of BSP by quinuronium or hepatic injury by the latter. Drugs may cause an inhibition by interfering with the protein synthesis of hepatic microsomal drug metabolizing enzymes. Alternatively, one drug may alter the rate of delivery of another drug to its site of elimination (Baggot, 1977). Excretion of BSP in bile decreased significantly in group 3 compared to other groups. This observation was comparable to decreased rate of disappearance of BSP from the perfusate in the same dosage group. BSP is rapidly excreted into bile and this has been used to assess hepatic functions in animals (Cornelius, 1970). The metabolism of quinuronium is not known. It is possible that quinuronium may cause either a decreased bile flow or a decreased active transport of BSP across

hepatocytes and/or a decreased conjugating capability of the liver, all of which could be done to hepatic failure by quinuronium. Acute hepatic injury due to exposure of a chemical could involve lipid accumulation in hepatocytes, cellular necrosis or hepatobiliary dysfunction (Plaa, 1980). Thus the observed decrease in hepatobiliary function resulted in a longer biological $t_{\frac{1}{2}}$ of BSP in group 3 and supports the hypothesis of Klaassen (1980). Mehendale (1977) reported a decreased biliary excretion of imipramine following pre-exposure to kepone. The rate of BSP excretion depends on species variation. Rabbits and rats are good biliary excretors of BSP (Klaassen and Plaa, 1967 and Williams, 1971) compared to dogs and cats.

Bile volume was significantly decreased in groups of rats pre-exposed to quinuronium. A reduction of bile volume and a decrease in the rate of excretion of BSP in bile observed in this study was consistent with the finding of Klaassen (1968) and Hart et al (1969). Some drugs like chlorpromazine and other phenothiazine derivatives (Smetana, 1963) produce cholestatic reaction, biliary statis in the canaliculi, impaired functions and increased levels of hepatic enzymes. The inhibitory mechanism of drugs on bile production is obscure. It is thought that metabolites of the drugs are excreted via

bile and this may be associated with a decrease in volume or eventual stasis. The metabolites may cause a decrease in permeability of the canaliculi membranes and hence a slowed bile flow rate (Mehendale, 1979).

5.4.2 EFFECTS OF QUINURONIUM INDUCED HEPATOTOXICITY OF RATS

Clinical signs of muscular tremors, jumps, depression, anorexia, lacrimation and death observed following administration of quinuronium in rats correlates with other reports in rats (Kronfeld, 1959a) and sheep (Rummler and Laue, 1961 and Eyre, 1966).

Increased liver to body weight ratios observed following administration of quinuronium in rats compares with those reported following administration of an antihypertensive agent pargyline in rats (DeMaster et al, 1982). Increase in liver weights following administration of a drug or a chemical is thought to be associated with an increase in de novo synthesis of hepatic microsomal metabolizing enzymes (Rammer and Merker, 1965 and Jones and Fawcett, 1966) and/or severe liver congestion (DeMaster et al,1982).

Liver injury due to chemicals has been recognized as a toxicologic problem for many years. Hepatic changes of focal fatty degeneration observed in rats receiving 5mg/kg fits into Popper and Schaffner's (1959) morphological classification of "zonal hepatocellular

alternations without inflammatory reaction". Depletion of hepatic GSH has been reported following administration of paracetamol in mice (Mitchell et al, 1973), rats (Prescott, 1980) and pargyline in rats (DeMaster et al, 1982). Mitchell et al (1973), Ginsberg et al (1982) and DeMaster et al (1982) also observed centrilobular hepatic necrosis besides depletion of hepatic GSH. In this study only early degenerative changes involving loss of cellular membranes were observed. Hepatic GSH conjugates reactive electrophiles released from metabolism of their compounds by cytochrome P-450 (Mitchell et al, 1973; Neal, 1980 and Ginsberg et al, 1982). This reaction is catalysed by glutathione S-transferase. Hepatic centrilobular necrosis is observed when over 70% of the normallyoccurring hepatic GSH stores are depleted (Mitchell et al, 1973) hence the unconjugated active metabolites become covalently bound to hepatic proteins in the centrilobular region of the liver (Jollow et al, 1973 and Jollow, 1980). The results from glutathione study suggest that no active metabolites that are dependent on hepatic GSH are involved in quinuronium metabolism. Other compounds associated with both hepatic necrosis and fatty degeneration are for instance carbon tetrachloride, chloroform and pyrrolizidine alkaloids (Plaa, 1980). Cycloheximidine, puromycin and emetin have been for instance reported to be associated with

fatty degeneration only (Plaa, 1980). It is likely that fatty degeneration observed was due to unchanged drug or non-reactive metabolites acting directly on hepatocytes. Fatty changes observed was far much less pronounced than those reported for other chemicals. Earlier observations (see Results 5.3.1) showed that quinuronium effects hepatic dysfunction in rats. Triglyceride is the main lipid that accumulates in fatty liver (Lombardi, 1966). Its accumulation is thought to be associated with an imbalance between the rate of its synthesis and release by hepatocytes into systemic circulation.

The present results show that quinuronium causes hepatobiliary dysfunctions and histologic changes of fatty degeneration. Reactive metabolites that require inactivation by hepatic glutathione are not involved in its metabolism.

TABLE V: MEAN (± S.D.) LIVER WEIGHT/BODY WEIGHT RATIOS AND HEPATIC GSH VALUES FOR CONTROL AND QUINURONIUM TREATED RATS.

Quinuronium dosage mg/kg	Liver weight/body weight ratio	Hepatic GSH (Abs.)
Saline	0.03 (0.01)	1.29 (0.05)
2.3	0.04 (0.01)	1.20 (0.18)**
5	0.06 (0.01)*	1.28 (0.12)**

* $P < 0.05$ vs saline treated group

** $P > 0.05$ vs saline treated group

FIG. 2 : APPARATUS USED IN LIVER PERFUSION EXPERIMENTS.

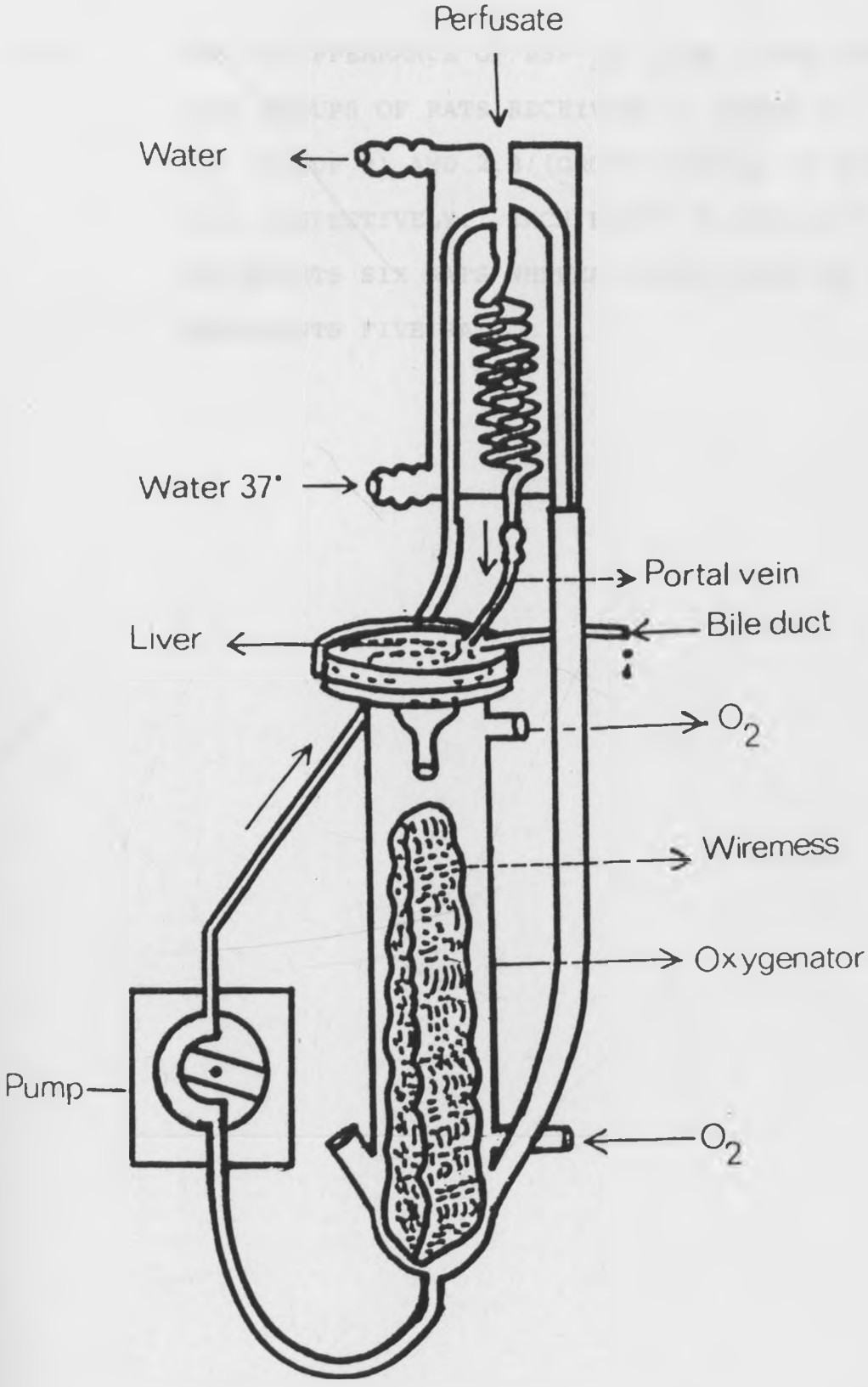

Perfusate
Water
Water 37°
Portal vein
Bile duct
Liver
O_2
Wiremess
Oxygenator
Pump
O_2

FIG. 3 : THE DISAPPEARANCE OF BSP IN VITRO LIVER PERFUSATE FROM GROUPS OF RATS RECEIVING 0 (GROUP 1), 1.5 (GROUP 2) AND 2.3 (GROUP 3)mg/kg OF QUINURONIUM RESPECTIVELY. EACH POINT IN GROUPS 2 AND 3 REPRESENTS SIX RATS WHEREAS EACH POINT IN GROUP 1 REPRESENTS FIVE RATS.

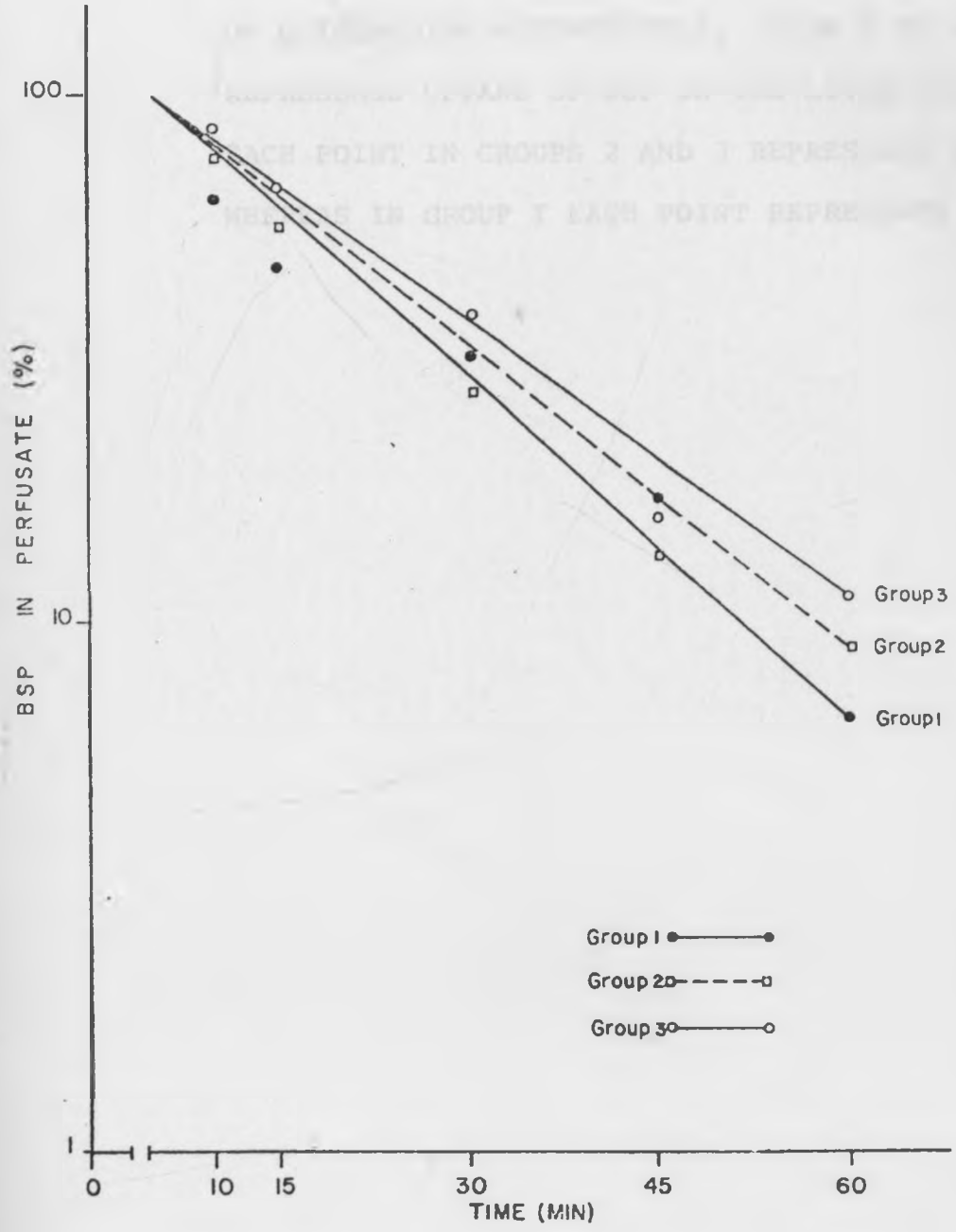

100
10
1
BSP IN PERFUSATE (%)
0
10
15
30
45
60
TIME (MIN)
Group 3
Group 2
Group 1
Group 1
Group 2
Group 3

FIG. 4: EXCRETION OF BSP IN BILE COLLECTED FROM IN VITRO PERFUSED LIVER FROM GROUPS OF RATS RECEIVING 0 (GROUP 1), 1.5 (GROUP 2) AND 2.3 (GROUP 3) mg/kg OF QUINURONIUM RESPECTIVELY. FROM 0 TO 45 MIN REPRESENTS UPTAKE OF BSP IN THE LIVER TISSUE. EACH POINT IN GROUPS 2 AND 3 REPRESENTS 6 RATS WHEREAS IN GROUP I EACH POINT REPRESENTS 5 RATS.

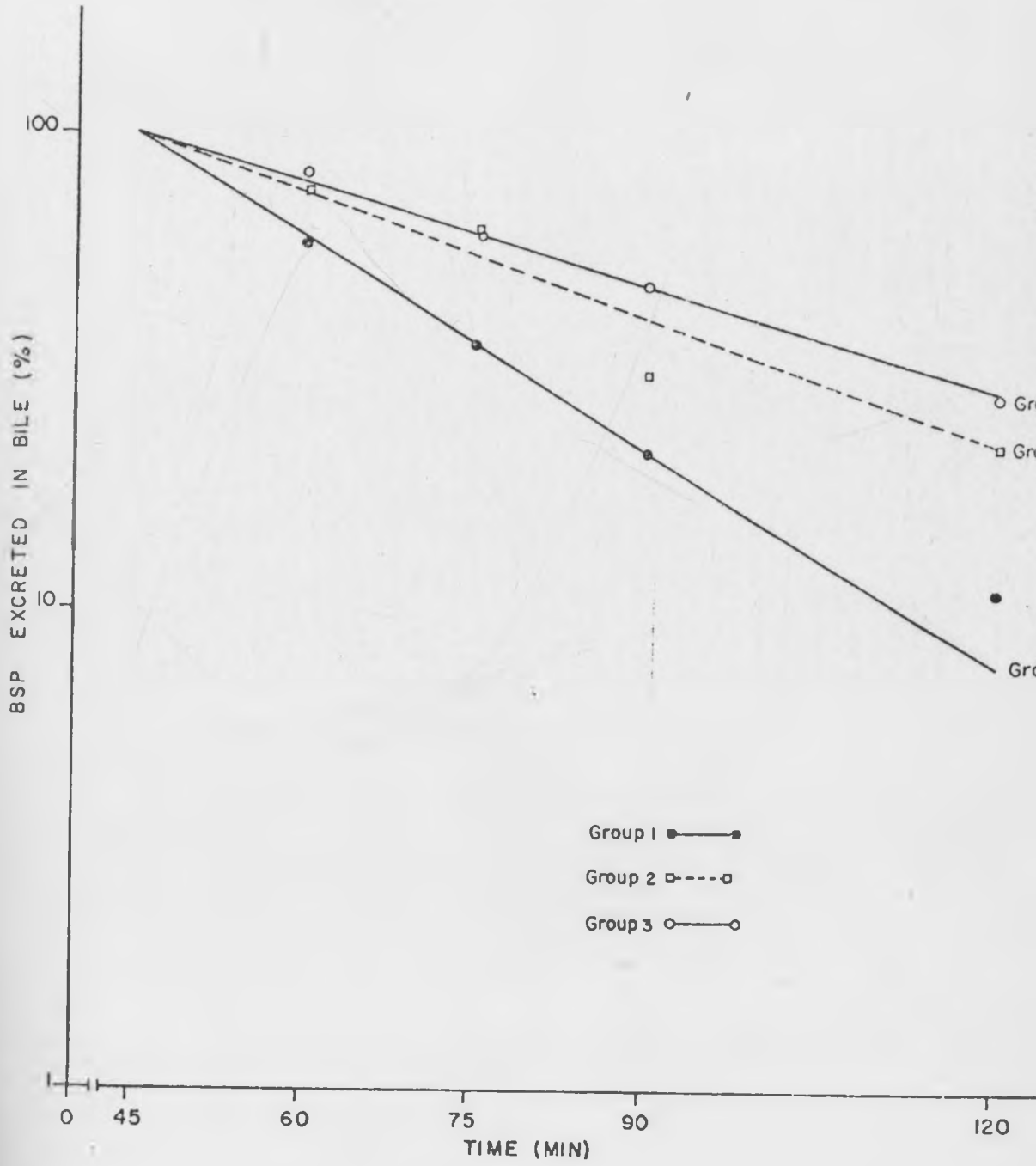

100
10
1
BSP EXCRETED IN BILE (%)
0
45
60
75
90
120
TIME (MIN)
Group 1
Group 2
Group 3

FIG. 5: CUMMULATIVE BILE VOLUME IN VITRO PERFUSED LIVERS FROM GROUPS OF RATS RECEIVING 0 (GROUP 1), 1.5 (GROUP 2) AND 2.3 (GROUP 3) mg/kg OF QUINURONIUM RESPECTIVELY.

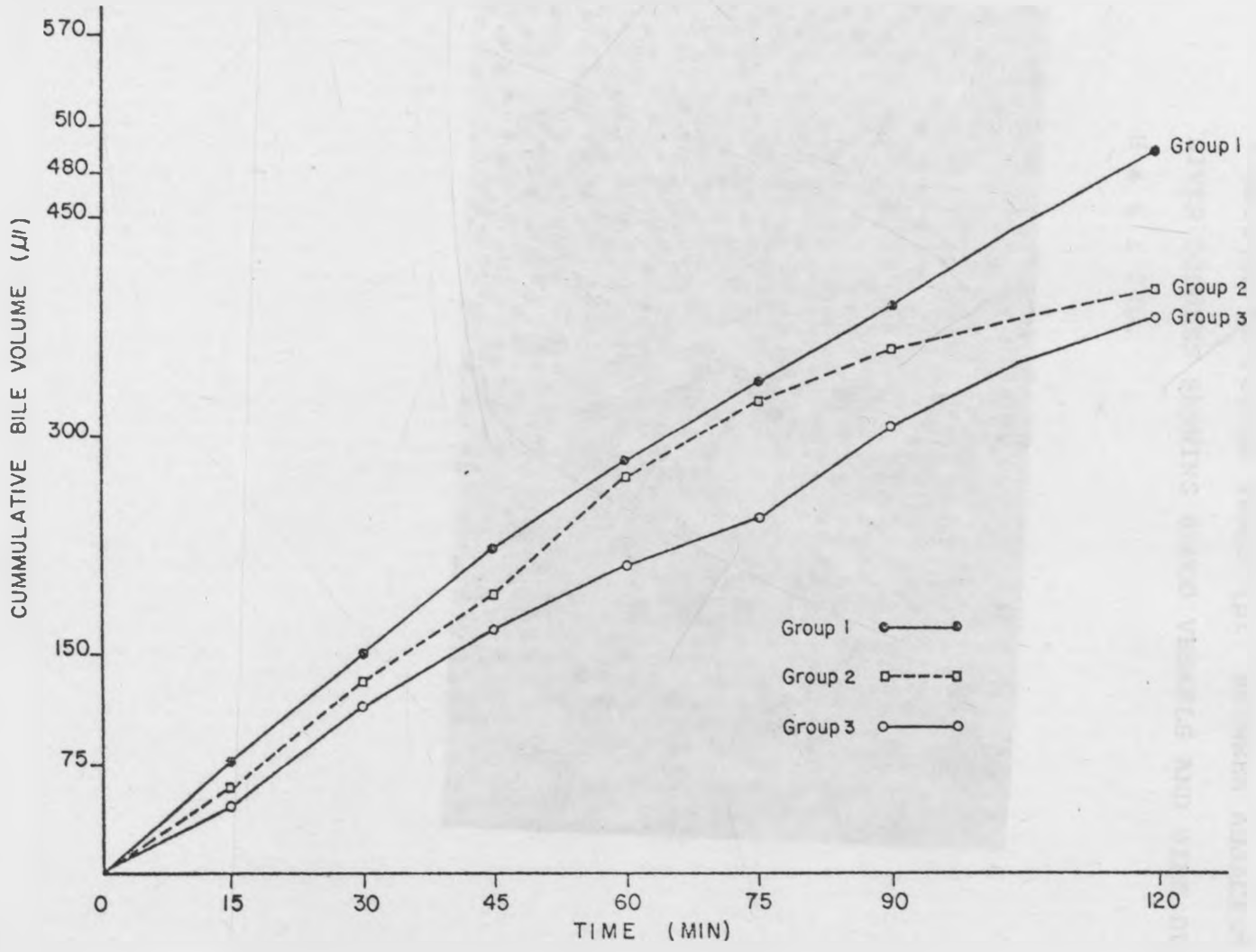

570
510
480
450
300
150
75
CUMMULATIVE BILE VOLUME (µl)
0
15
30
45
60
75
90
120
TIME (MIN)
Group I
Group 2
Group 3
Group I
Group 2
Group 3

FIG. 6 : NORMAL LIVER HISTOLOGY FROM CONTROL RAT RECEIVING SALINE. THE PHOTOMICROGRAPH REPRESENTS A SECTION OF PORTAL TRACK (pt) BETWEEN ADJACENT LIVER LOBULES SHOWING BLOOD VESSELS AND BILE DUCT. H & E X 200.

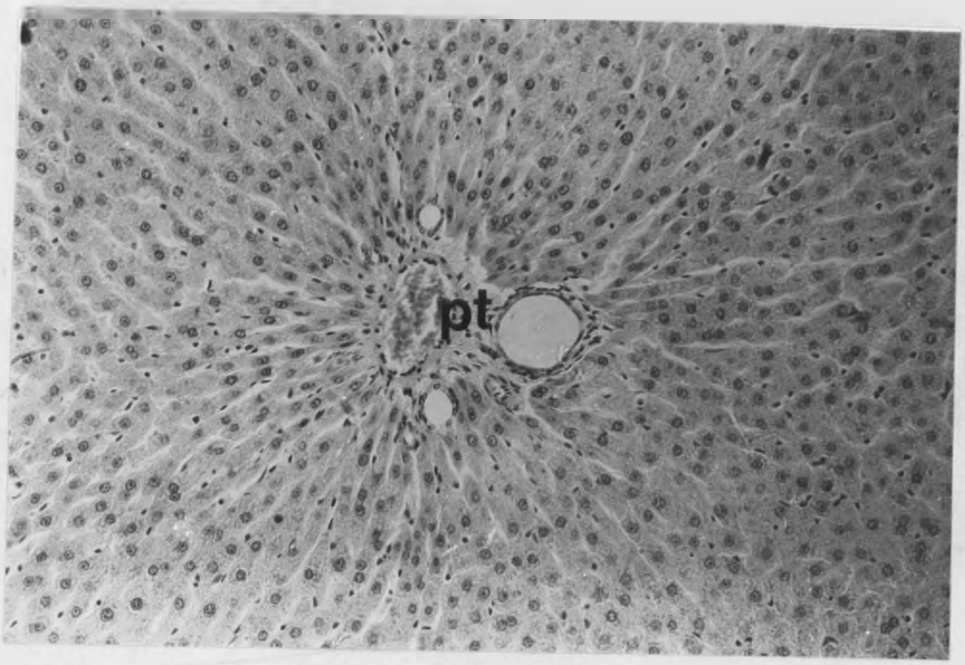
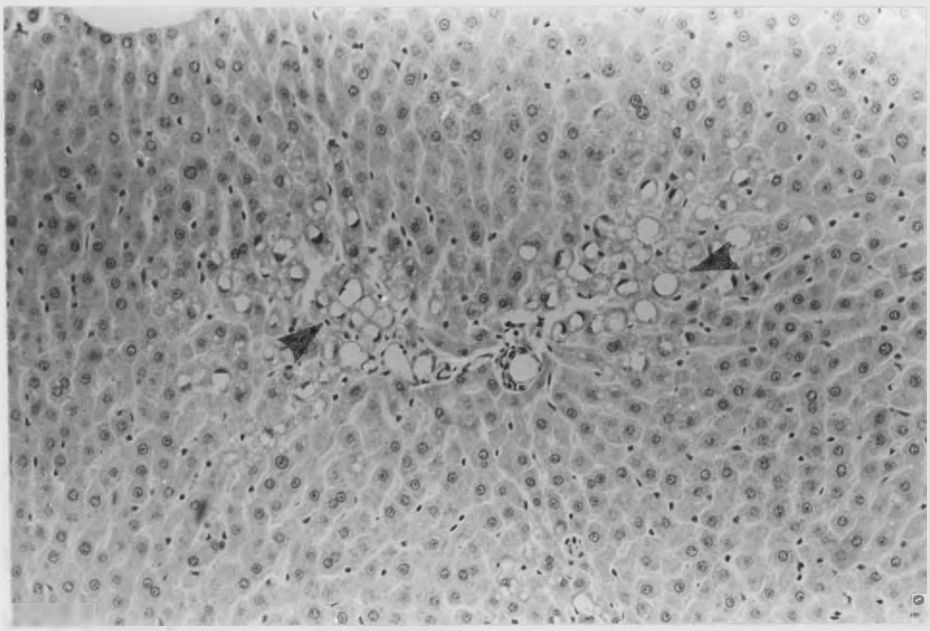


FIG. 7 : LIVER SECTION FROM ONE OF THE RATS RECEIVING 5mg/kg OF QUINURONIUM SHOWING FOCAL FATTY DEGENERATION (ARROWHEADS). H & E X 200.



CHAPTER 6

COMPARATIVE STUDIES OF THE EFFECTS OF QUINURONIUM AND DIMINAZENE IN SHEEP

6.1 INTRODUCTION

In earlier studies it was shown that quinuronium affects liver function in rats (Chapter 5.3). Other workers (Naude et al, 1970) reported biochemical changes in dogs following administration of quinuronium and dimidines. Homeida et al (1981) reported damage of liver and kidney accompanied by increased activity of aspartate amino-transferase (ASAT) in camels following administration of diminazene. There are no reports on biochemical changes following administration of quinuronium and diminazene in sheep.

The present study was conducted to investigate and compare biochemical parameters-lactic dehydrogenase (LDH), creatine phosphokinase (CPK) and blood urea nitrogen (BUN) in sheep following administration of quinuronium and diminazene.

6.2 MATERIALS AND METHODS

6.2.1 ANIMALS

Twenty male clinically healthy Persian black

head sheep approximately 20kg and 9 months of age were used in the study. They were obtained from one source in Nairobi region. They were held for two weeks to acclimatize to experimental pens in which each dose group (5 animals) were kept. The animals were offered hay, bran, salt licks and water daily. During the period of acclimatization they were routinely treated for internal parasites.

6.2.2. DRUG TREATMENTS

Twenty sheep were divided randomly into quinuronium and diminazene groups. In each drug group 5 sheep received therapeutic dose (1mg/kg of quinuronium and 3.5mg/kg of diminazene) and subsequent 5 animals received twice therapeutic dose of the babesicides (2mg/kg of quinuronium and 7.0mg/kg of diminazene). Quinuronium sulfate (Acaprin, Bayer, AG) was administered subcutaneously whereas diminazene diaceturate (Berenil, Hoechst AG) was given intramuscularly. All the animals served as their controls at 'O' (pre-treatment) time.

6.2.3 BLEEDING

Heparinized blood samples (8 - 10ml) were taken from the jugular veins of sheep at 0, 1/4, 1/2, 1, 2, 4, 8, 24 and 48 hr after drug administration.

6.2.4 BIOCHEMICAL DETERMINATIONS

Blood was centrifuged to obtain plasma, which was then frozen at -20°C pending determinations. Biochemical parameters - LDH, CPK, and BUN were determined using Sigma reagents (Sigma Chemical Co., St. Louis, MO.). LDH activity was determined according to the procedure of Berger and Broida (Sigma Technical Bulletin No. 500, Sigma, 1980) using a spectrophotometer at 546 nm. The values obtained were expressed as B-B Units/ml. CPK activity was determined according to a modified procedure of Hughes (Sigma Technical Bulletin No. 520, Sigma, 1976) using a spectrophotometer at 546 nm. The values were expressed as Sigma Units/ml. BUN was determined according to the procedure of Crocker (Sigma Technical Bulletin No. 535, Sigma, 1974) using a spectrophotometer at 546 nm. The values were expressed as mg/100ml.

6.3 RESULTS

6.3.1 CLINICAL OBSERVATIONS

6.3.1.1 Quinuronium treatment

Sheep receiving quinuronium showed signs of salivation, micturition, anorexia, depression, decreased heart rate, muscular tremors, ataxia and recumbency within 20 min. These signs were severe in those animals

receiving higher dose. Anorexia lasted for 2 hr after which they regained appetite. There were no deaths.

6.3.1.2 Diminazene treatment

Sheep receiving therapeutic dose showed only milder signs of toxicoses. Those receiving higher dose showed inappetance, depression, itchiness and reluctance to move.

6.3.2 BIOCHEMICAL PARAMETERS

The mean activity of LDH at 'O' baseline values or at pre-treatment time for sheep receiving therapeutic and higher dose of quinuronium were 820 and 893 B-B Units/ml respectively (Table VI). Following administration of quinuronium the activity increased gradually above the baseline values upto 1 hr. At 2 hr there was a slight decrease up to 8 hr. The activity, however, increased substantially by 50 - 100% of baseline values at 24 and 48 hr. Sheep receiving higher dose of 2mg/kg of quinuronium showed a significant increase in activity of LDH up to 30 min compared to those receiving therapeutic dose. The trend of enzymatic activity was similar in the two dosages. LDH activity of sheep receiving diminazene is depicted in Table VI. The activity of the enzyme in these animals was generally

lower even at pre-treatment time probably due to diurnal variation . Sheep receiving therapeutic dose of diminazene had an increased trend of LDH activity with peak activity at 8 hr, thereafter an elevated drop above baseline values to end of study. The activity of LDH showed a transient drop in those receiving higher dose of diminazene up to 1 hr, then an increased trend above baseline values to end of the study.

CPK activity in quinuronium treated sheep increased above baseline values following administration of the drug with a maximum activity at 2 hr in both dosage levels (Table VII). After 2 hr the activity declined gradually to levels comparable to baseline values by the end of study. The two quinuronium dosage groups were not significantly different in their activity during the period of study. CPK activity in diminazene treated animals had similar trend compared to quinuronium treated sheep from baseline values to maximum activity at 2 hr followed by a gradual drop. The decrease from 2 hr to end of study was, however, significantly different from quinuronium treated animals ($P < 0.05$).

BUN levels in plasma increased above baseline values in both dosages of quinuronium treated animals. One sheep receiving therapeutic dose had levels above 20mg/100ml from 4 hr to end of study. There was no significant difference in BUN levels in the two

quinuronium dosages. BUN levels in diminazene treated sheep had similar trend compared to quinuronium, except the mean increase was higher than quinuronium treated animals (Table VIII).

6.4 DISCUSSION

Clinical signs of salivation, micturition, anorexia and muscular tremors observed following administration of quinuronium in sheep are consistent with other reports (Kikuth, 1935; Rummler and Laue, 1961) and those observed earlier (Chapter 3.3.2). Similar signs had been reported in dogs (Naude et al, 1970) and cattle (Purnell et al, 1981). These signs are due to anti-cholinesterase effect of quinuronium (Eyre, 1966 and see also Chapter 4.3). Sheep administered higher dose of diminazene showed only mild signs of inappetance, depression and reluctance to move. There are no reports on any signs of toxicosis in sheep following administration of diminazene. Toxic signs of vomition, anorexia, CNS disturbances and death have, however, been reported in dogs administered diminazene (Basson and Pienaar, 1965; Naude et al, 1970). In camels Homeida et al (1981) reported hyperesthesia, salivation, itching and sweating following administration of diminazene. These signs ressemble histamine-like reaction or cholinergic stimulation.

Although the values for the activity of LDH were within the normal reported range of < 1800 B-B Units/ml (Kronfeld and Medway, 1969) there was an increased trend following administration of quinuronium. The activity of LDH also increased above the baseline values in diminazene treated sheep. There are no reports of increased LDH activity in sheep in the two babesicides. Increased LDH activity is associated with hepatocellular damage or necrosis and myocardial damage (Benjamin, 1978). Since quinuronium has been observed to affect liver function in rats (Chapter 5.3), it is probably that some LDH activity is of hepatic origin, even though Marquez et al (1977) and Beatty and Dokey (1983) observed more activity of the enzyme in skeletal and cardiac muscles in normal lambs.

CPK levels increased above baseline values in both quinuronium and diminazene treated animals. Increased CPK activity is associated with muscular dystrophy and myocardial damage or infarctions in humans (Okinaka et al, 1961). In animals its activity also increases in myocardial infarction, muscle necrosis or damage (Benjamin, 1978). Increased activity observed in this study was possibly due to increased muscular damage as evidenced clinically by muscular tremors and itchiness following administration of the two drugs within 2 hr.

A decline of activity after 2 hr (Table VII) was comparable with absence of muscular tremors. However, in the diminazene groups CPK declined considerably below the baseline values by the end of the study. This observation is difficult to explain. Okinaka et al (1961) had reported similar increase of CPK activity in humans following muscular activity.

BUN levels increased above baseline values (Table VIII) following administration of quinuronium and in certain cases the values were above the upper normal limit of 20mg/100ml of plasma reported for sheep (Kronfeld and Medway, 1969). Similar increases of BUN levels had been reported in dogs following administration of phenamidine (Naude et al, 1970). Increase of BUN levels in plasma or serum is associated with increased protein catabolism, decreased renal blood flow, heart congestion, hypotension and dehydration (Kronfeld and Medway, 1969 and Benjamin, 1978). Increased levels of BUN observed following administration of quinuronium and diminazene may depend on hypotensive properties of these compounds, with quinuronium being more potent.

This study suggests that quinuronium is more toxic than diminazene at therapeutic and/or above therapeutic dosage levels. It also corroborates that quinuronium is organotoxic. Quinuronium should not be given in cases of hepatic, renal and cardiovascular diseases.

TABLE VI: MEAN (± S.E.) ACTIVITY IN B-B UNITS/ml OF PLASMA LDH FROM SHEEP RECEIVING THERAPEUTIC AND HIGHER DOSAGES OF QUINURONIUM AND DIMINAZENE RESPECTIVELY.

Time after drug administration (hr)	Quinuronium		Diminazene	
	1mg/kg	2mg/kg	3.5mg/kg	7.0mg/kg
0	820 (55)	893 (93)	291 (14)	537 (44)
1/4	934 (120)	1155 (59)	333 (28)	418 (54)
1/2	1009 (90)	1356 (22)	508 (85)	389 (36)
1	1211 (116)	1280 (78)	494 (13)	530 (42)
2	999 (76)	943 (91)	374 (22)	596 (25)
4	922 (117)	1213 (66)	556 (39)	579 (54)
8	771 (114)	1350 (158)	1040 (113)	891 (56)
24	1295 (148)	1678 (68)	511 (50)	843 (67)
48	1538 (29)	1622 (53)	418 (40)	806 (66)

TABLE VII: MEAN (± S.E.) ACTIVITY IN SIGMA UNITS/ml OF PLASMA CPK FROM SHEEP RECEIVING THERAPEUTIC AND HIGHER DOSAGES OF QUINURONIUM AND DIMINAZENE RESPECTIVELY.

Time after drug administration (hr)	Quinuronium		Diminazene	
	1mg/kg	2mg/kg	3.5mg/kg	7.0mg/kg
0	46 (1.38)	39 (0.55)	43 (1.52)	47 (1.23)
1/4	47 (1.05)	48 (1.17)	54 (2.88)	68 (2.88)
1/2	53 (1.04)	52 (1.19)	61 (2.09)	73 (1.97)
1	57 (0.98)	57 (0.83)	63 (7.68)	64 (7.10)
2	62 (1.06)	66 (0.97)	60 (2.96)	75 (1.03)
4	61 (0.89)	54 (1.65)	27 (0.96)	38 (4.03)
8	52 (1.49)	48 (1.24)	38 (3.01)	26 (0.98)
24	47 (0.55)	46 (0.88)	22 (0.14)	28 (1.56)
48	46 (0.84)	41 (0.43)	17 (0.38)	22 (1.44)

TABLE VIII: MEAN (± S.E.) LEVELS IN mg/100ml OF PLASMA BUN FROM SHEEP RECEIVING THERAPEUTIC AND HIGHER DOSAGES OF QUINURONIUM AND DIMINAZENE RESPECTIVELY

Time after drug administration (hr)	Quinuronium		Diminazene	
	1mg/kg	2mg/kg	3.5mg/kg	7.0mg/kg
0	11 (0.41)	12 (0.36)	9 (0.70)	7 (0.35)
1/4	16 (0.53)	14 (0.43)	15 (0.77)	12 (0.54)
1/2	16 (0.71)	15 (0.45)	16 (1.75)	12 (0.49)
1	18 (0.41)	17 (0.50)	12 (0.41)	13 (0.73)
2	16 (0.07)	17 (0.95)	13 (0.90)	12 (0.63)
4	16 (0.86)	21 (0.79)	13 (0.70)	24 (1.60)
8	14 (1.36)	16 (0.92)	15 (1.34)	13 (0.55)
24	23 (2.18)	19 (1.33)	13 (1.25)	14 (0.87)
48	18 (0.95)	12 (0.17)	16 (1.13)	15 (1.19)

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

7.1 GENERAL DISCUSSION

Toxicity of quinuronium has been well documented. LD_{50} values for rats i.p. and s.c. routes observed in this study were about four times higher than those reported by Kronfeld (1959a). Kronfeld used only four groups of rats and a different strain. Values for LD_{50} in mice i.p. and s.c. routes have not been reported. According to the classification of Loomis (1974), the values for LD_{50} observed in this study classifies quinuronium as highly toxic. The cause of death in quinuronium toxicity has been quite controversial. Kronfeld (1959b) in an *in vitro* study with rat liver tissues suggested that death was due to histotoxic anoxia. Rummler and Laue (1961) and Eyre (1966) in their studies concluded that the cause of death was due to anti-cholinesterase activity. From the observations of low depression of cholinesterase activity in sheep (Chapter 5.3.4) and hepatotoxicity in rats (Chapter 5.3) following exposure of quinuronium, it is evident that the two factors enhance the cause of death.

Clinical signs of salivation, micturition, anorexia, depression and muscular tremors observed following

administration of quinuronium in sheep and rats are consistent with other reports in sheep (Kikuth, 1935; Cernaianu et al,1935 and Rummler and Laue, 1961) and in rats (Kronfeld, 1959a). Similar signs had been reported in dogs (Naude et al,1970) and cattle (Purnell, 1981). These signs are due to anticholinesterase effect of quinuronium (Eyre, 1966). Sheep administered higher dose of diminazene showed only mild signs of inappetance, depression and reluctance to move. There is no information on any signs of toxicosis in sheep following administration of diminazene. Toxic signs of vomition, anorexia, CNS disturbances and death had been reported in dogs administered diminazene (Basson and Pienaar, 1965 and Naude et al,1970). In camels, Homeida et al (1981) reported signs of hyperesthesia, salivation, intermittent convulsions, frequent urination and defecation, itching and sweating following administration of diminazene. These signs resemble histamine-like reaction or cholinergic stimulation. In people, a 65-year old man developed acute idiopathic polyneuritis following treatment with diminazene against B. microti (Ruebush et al,1979).

The rabbit was observed to have low normal blood cholinesterase activity compared to sheep. This was due to variation in species. Quinuronium did not inhibit cholinesterase activity significantly in the rabbit

in vivo in this study. Eyre (1966) reported an inhibition of cholinesterase activity in rabbit whole blood in an in vitro study; the whole animal nature in this study may explain the cause of disparity. In sheep there was a tendency towards a low depression of cholinesterase activity from 10 min to 24 hr after quinuronium administration. Similar anticholinesterase effect of quinuronium had been reported in sheep (Rummler and Laue, 1961 and Eyre, 1966).

Quinuronium affected liver function as indicated by decreased disappearance of sulfobromophthalein (BSP) in the isolated perfused rat liver. BSP is rapidly excreted into bile and this has been used to assess hepatic functions in animals (Cornelius, 1970). Quinuronium or its metabolites may inhibit the uptake and elimination of BSP or cause hepatic injury. The observed decrease in hepatobiliary function resulted in a longer biological half life ($t_{\frac{1}{2}}$) of BSP and a decreased bile volume in rats exposed to higher dose of 2.3mg/kg of quinuronium. Mehendale (1977) reported a decreased biliary excretion of imipramine following pre-exposure to kepone.

In sheep the effects of quinuronium and diminazene were assessed on biochemical parameters - LDH, CPK and BUN.

Although the values for LDH activity were within normal range (Kronfeld and Medway, 1969), its activity increased above baseline values following administration of quinuronium and diminazene. There are no reports of increased LDH activity in sheep with the two babesicides. Increased LDH activity is associated with hepatocellular damage or necrosis and myocardial damage (Benjamin, 1978). Marquez et al (1977) and Beatty and Doxey (1983) reported high levels of LDH in cardiac and skeletal muscles compared to soft tissues like liver, kidney and brain in normal lambs. Beatty and Doxey (1983), found LDH isoenzymes LDH_1, LDH_3, LDH_2, LDH_5 and LDH_4 in decreasing order of activity in ovine serum. In liver tissue they found LDH isoenzymes LDH_1, LDH_2, LDH_3, LDH_4 and LDH_5 in decreasing order of activity. CPK activity increased above baseline values in the quinuronium and diminazene treated sheep. The increase in activity was higher in quinuronium than diminazene treated animals. Increased CPK activity is associated with muscular dystrophy and myocardial damage (Okinaka et al, 1961 and Benjamin, 1978). BUN levels increased above baseline values following adminstration of quinuronium. Similar increased BUN levels had been reported in dogs following administration of phenamidine (Naude et al, 1970). Increased BUN levels is associated with increased protein catabolism, hypotension and dehydration

(Kronfeld and Medway, 1969 and Benjamin, 1978). Increased levels of LDH suggests that quinuronium affects liver function and musculo-skeletal muscles.

Fatty degeneration was observed in rats exposed to 5mg/kg; the cause of the lesions could have been responsible for other hepatic dysfunctions observed in perfused rat livers *in vitro* (Chapter 4.3). Liver injury due to chemicals has been recognized as a toxicologic problem for many years. Hepatic changes of fatty accumulation observed in rat livers fits into Popper and Schaffner's (1959) morphological classification of "zonal hepato-cellular alterations without inflammatory reaction". Plaa (1980) lists chemicals like carbon tetrachloride, chloroform, pyrrolizidine alkaloids, cycloheximidine, puromycin and emetin as capable of producing fatty degeneration. Accumulation of fat in hepatocytes is thought to be associated with an imbalance between the rate of fat synthesis and its release into systemic circulation (Lombardi, 1966). In the same rats that were exposed to 5mg/kg of quinuronium there was no significant depletion of hepatic GSH compared to controls. Depletion of hepatic GSH and hepatic necrosis was reported in rats administered paracetamol (Buttar et al, 1977 and Ginsberg et al, 1982) and pargyline (DeMaster et al, 1982). Hepatic GSH conjugates reactive

electrophiles released from metabolism of their compounds by cytochrome P-450 (Neal, 1980). Data obtained from hepatic GSH study in rats suggests that no reactive metabolites inactivated by glutathione are formed during the metabolism of quinuronium.

Quinuronium inhibited parasitemia in an *in vitro*/*in vivo* system with *B. rodhaini*. This observation was in agreement with those of Beveridge (1969); Irvin and Young (1977 and 1978) and McHardy (1982). An *in vitro*/*in vivo* EC_{50} of 13.5ng/ml for quinuronium was observed with *B. rodhaini*. McHardy (1982) observed an *in vitro* EC_{50} of 28ng/ml for imidocarb using calf plasma. *In vitro*/*in vivo* system described demonstrated a possible simple method for monitoring quinuronium levels in ovine plasma. McHardy (1982) reported determination of imidocarb in bovine plasma using the *in vitro*/*in vivo* model system. Inhibiton of parasitemia by plasma from sheep treated with quinuronium suggests that quinuronium is metabolised rather slowly or eliminated unchanged and/or metabolites have antibabesial activity. Previous report by Spinkova and Zyka (1960) described determination of quinuronium from aqueous solution by spectrophotometric method. Pilot trials to use the method of Spinkova and Zyka (1960) for assaying quinuronium in a biological system failed due to low sensitivity. Spectrophotometric method involves several

chemical reactions and is expensive. Recovery of quinuronium from plasma is rather difficult since it binds strongly with hydrophilic compounds due to its chemical structure.

7.2 CONCLUSIONS

The following observations and conclusions can be made from the findings of this study:-

1. In vitro/in vivo model using B. rodhaini is a simple method which can be used to monitor quinuronium concentrations in ovine plasma.

2. Whenever a second dose of quinuronium is clinically indicated, it should be administered between 24 and 48 hr.

3. Quinuronium and/or metabolites of quinuronium have anti-babesial activity as shown in the in vitro/in vivo model system.

4. Quinuronium can be classified as highly toxic as indicated by LD_{50} of 4.8 and 5.4mg/kg in mice for i.p. and s.c. and 6.3 and 6.5mg/kg in rats i.p. and s.c. routes respectively.

5. The study supports the view that depression of cholinesterase activity plays an important role as the cause of death in quinuronium toxicity.

6. Rabbits are moderately resistant to quinuronium toxicity as evidenced by lack of toxic signs and absence of anticholinesterase effect *in vivo*.

7. Quinuronium is hepatotoxic as shown by decreased disappearance of BSP in perfused rat liver and presence of fatty degeneration in rat liver. Hepatotoxicity may contribute to or potentiate the cause of death. Quinuronium may therefore not be indicated in animal patients with hepatic diseases.

8. Reactive metabolites that are dependent on hepatic glutathione for their inactivation are not formed during metabolism of quinuronium.

9. Quinuronium is more organotoxic than diminazene at therapeutic and/or above therapeutic doses as indicated by increased activities of LDH and CPK.

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APPENDICES

APPENDIX 1

Number of mice and rats receiving 2.5 to 7.6 mg/kg of quinuronium that died after 13-hour period

Dosage (mg/kg)	Rats		Mice	
	Number dead (i.p.)	Number dead (s.c.)	Number dead (i.p.)	Number dead (s.c.)
2.5	0/10	0/10	0/10	0/10
3.3	0/10	0/10	0/10	0/10
4.4	4/10	0/10	0/10	0/10
5.7	9/10	4/10	3/10	4/10
7.6	10/10	6/10	10/10	6/10

APPENDIX 2

Δ Abs. of cholinesterase activity in control or group 1 rabbits receiving 0 mg/kg of quinuronium s.c.

	Time of study (hr.)									
	0	1/6	1/2	1	2	4	8	24	48	72
Animal No.										
ER 9	0.06	0.055	0.05	0.065	0.05	0.04	0.04	0.05	0.055	0.045
ER 10	0.08	0.065	0.055	0.06	0.06	0.06	0.08	0.07	0.06	0.06
ER 11	0.03	0.04	0.045	0.05	0.045	0.03	0.04	0.05	0.05	0.065
ER 12	0.06	0.055	0.055	0.0638	0.06	0.055	0.055	0.0625	0.06	0.05

APPENDIX 3

Δ Abs. of cholinesterase activity in group 2 rabbits receiving 1 mg/kg of quinuronium s.c.

	Time of study (hr.)									
	0	1/6	1/2	1	2	4	8	24	48	72
Animal No.										
ER 5	0.04	0.02	0.04	0.05	0.04	0.05	0.05	0.05	0.05	0.025
ER 6	0.04	0.045	0.05	0.02	0.06	0.034	0.04	0.06	0.07	0.055
ER 7	0.062	0.055	0.05	0.05	0.05	0.04	0.04	0.025	0.08	0.09
ER 8	0.04	0.05	0.055	0.06	0.05	0.06	0.055	0.04	0.04	0.09

APPENDIX 4

Δ Abs. of cholinesterase activity in group 3 rabbits receiving 1 mg/kg of quinuronium s.c. and prior atropinisation

	Time of study (hr.)									
	0	1/6	1/2	1	2	4	8	24	48	72
Animal No.										
ER 1	0.055	0.07	0.08	0.06	0.064	0.06	0.06	0.07	0.01	0.08
ER 2	0.06	0.06	0.07	0.04	0.03	0.03	0.055	0.05	0.03	0.065
ER 3	0.07	0.045	0.045	0.08	0.05	0.03	0.03	0.05	0.07	0.025
ER 4	0.035	0.03	0.03	0.04	0.03	0.04	0.025	0.045	0.07	0.075

APPENDIX 5

sheep No. 1 (control or group 1) receiving 0 mg/kg of quinuronium s.c.

	Time of study (hr.)									
	0	1/6	1/2	1	2	4	8	24	48	72
Parameters										
Δ Abs.	0.15	0.116	0.108	0.1	0.11	0.105	0.115	0.08	0.97	0.123
WBC (X 10^3 µl)	-	-	-	-	-	-	-	-	-	-
Hb. (g/dl)	11	10.5	12	10.9	12	13.0	12.5	11.9	12.1	13
PCV (%)	29	30	31	27	29	30	29.5	31	33	30

APPENDIX 6

sheep No. 2 (control or group 1) receiving 0 mg/kg of quinuronium s.c.

	Time of study (hr.)									
	0	1/6	1/2	1	2	4	8	24	48	72
Parameters										
Δ Abs.	0.11	0.09	0.11	0.105	0.1	0.104	0.101	0.12	0.11	0.11
WBC (X 10^3 µl)	10	11	12.1	9.8	10.5	10.7	11.0	13	9.9	10.8
Hb. (g/dl)	10	9.5	11.8	12.0	13.5	14.0	13.7	12.9	13	10.9
PCV (%)	30	31	28.9	31	30	35	30	29.5	31.5	32

APPENDIX 7

sheep No. 3 (control or group 1) receiving 0 mg/kg of quinuronium s.c.

	Time of study (hr.)									
	0	1/6	1/2	1	2	4	3	24	48	72
Parameters										
Δ Abs.	0.0905	0.10	0.11	0,12	0.11	0,12	0,108	0.13	0.119	0.104
WBC (X 10^3 µl)	11	12	13,5	10.9	14.0	10,2	9.8	11.0	13.0	12.5
Hb. (g/dl)	9,9	10,9	9.5	13	12.5	11,9	10,85	12	13.5	12.8
PCV (%)	29	28.9	31	32.5	33.0	34	30	40	35	34

APPENDIX 8

sheep No. 4 (group 2) receiving 1 mg/kg of quinuronium s.c.

Parameters	Time of study (hr.)									
	0	1/6	1/2	1	2	4	8	24	48	72
Δ Abs.	0.07	0.05	0.05	0.05	0.05	0.05	0.05	0.06	0.08	0.075
WBC (X 10^3 µl)	11.15	12.00	11.15	12.15	10.45	10.50	10.70	11.25	9.40	10.00
Hb. (g/dl)	10.3	10.9	10.2	11.1	10.2	10.4	10.6	10.5	9.6	9.4
PCV (%)	31	33	31	31	30	25	25	29	26	26

APPENDIX 9

sheep No. 5 (group 2) receiving 1 mg/kg of quinuronium s.c.

	Time of study (hr.)									
	0	1/6	1/2	1	2	4	8	24	48	72
Parameters										
Δ Abs.	0,095	0.05	0,07	0.07	0.07	0.08	0.08	0.07	0.1	0.098
WBC (X 10^3 µl)	10.35	8.25	6.3	8.5	9.6	10,3	9.2	9.65	8.2	8.6
Hb. (g/dl)	11.1	8.9	6.6	8.3	10.4	10,6	9,9	8.8	9.5	10
PCV (%)	28	24	18	18	28	27	25	30	24	24

APPENDIX 10

sheep No. 6 (group 2) receiving 1 mg/kg of quinuronium s.c.

	Time of study (hr.)									
	0	1/6	1/2	1	2	4	8	24	48	72
Parameters										
Δ Abs.	0.1	0.07	0.07	0.06	0.08	0.06	0.09	0.07	0.085	0.093
WBC (X 10^3 µl)	9.0	10.6	5.4	8.6	9.95	11.95	11.0	11.7	9.95	9.4
Hb. (g/dl)	8.6	10.1	5.4	8.2	9.6	10.7	10.3	10.7	9.6	9.1
PCV (%)	24	27	14	24	26	29	26	27	26	24

APPENDIX 11

sheep No. 7 (group 2) receiving 1 mg/kg of quinuronium s.c.

	Time of study (hr.)									
	0	1/6	1/2	1	2	4	8	24	48	72
Parameters										
Δ Abs.	0.08	0.06	0.09	0.07	0.08	0.09	0.07	0.07	0.09	0.09
WBC (X 10^3 µl)	12.5	11.5	11.0	9.9	9.95	12.0	10.8	11.0	9.9	9.2
Hb. (g/dl)	11.8	10.7	10.2	10.1	9.6	10.2	10.5	10.5	9.8	9.4
PCV (%)	31	28	26	27	24	27	28	28	26	24

APPENDIX 12

sheep No. 8 (group 2) receiving 1 mg/kg of quinuronium s.c.

Parameters	Time of study (hr.)									
	0	1/6	1/2	1	2	4	8	24	48	72
Δ Abs.	0.1	0.1	0.06	0.06	0.06	0.06	0.08	0.085	0.1	0.09
WBC (X 10^3 µl)	10.8	11.2	13.05	9.9	10.0	11.05	9.9	11.75	12.0	12.98
Hb. (g/dl)	11.8	10.2	9.9	10.9	11.8	11.5	12.9	13.7	14.2	13.9
PCV (%)	33	35.5	34.8	36	35.4	34.0	30.0	27	28.5	31

APPENDIX 13

sheep No. 9 (group 3) receiving 1 mg/kg of quinuronium s.c. and prior atropinisation

	Time of study (hr.)									
	0	1/6	1/2	1	2	4	8	24	48	72
Parameters										
Δ Abs.	0.1	0.07	0.08	0.09	0.09	0.1	0.14	0.1	0.11	0.11
WBC (X 10^3 µl,	10.5	11.7	13.9	12.05	11.07	10.9	10.05	8.98	11.7	12.5
Hb. (g/dl)	11.05	12.0	13.0	13.5	12.9	11.9	9.9	10.9	12.0	11.98
PCV (%)	35	28	29	30	31.5	33	28	29.9	32	33

APPENDIX 14

sheep No. 10 (group 3) receiving 1 mg/kg of quinuronium s.c. and prior atropinisation

Parameters	Time of study (hr.)									
	0	1/6	1/2	1	2	4	8	24	48	72
Δ Abs.	0.07	0.06	0.07	0.07	0.065	0.11	0.09	0.095	0.09	0.06
WBC ($X\ 10^3\ \mu l$)	10.8	11.5	12.0	9.8	8.9	10.0	11.0	12.0	10.0	9.9
Hb. (g/dl)	12.0	10.9	9.8	9.9	10.3	11.9	12.8	13.5	12.9	13.0
PCV (%)	30	33	30	31	29	28.5	31.5	32	33	34

APPENDIX 15

sheep No. 11 (group 3) receiving 1 mg/kg of quinuronium s.c, and prior atropinisation

	Time of study (hr)									
	0	1/6	1/2	1	2	4	8	24	48	72
Parameters										
Δ Abs.	0.14	0.15	0.09	0.09	0.12	0.11	0.12	0.102	0.08	0.09
WBC ($X10^3$ µl)	11.0	12.5	11.09	13.0	10.0	9.8	10.3	10.2	9.9	10.3
Hb. (g/dl)	13.0	12.5	11.0	12.9	10.0	11.5	12.8	13.5	14.0	12.9
PCV (%)	35	34	37	30	28	27	29	31.5	33	34

APPENDIX 16

sheep No. 12 (group 3) receiving 1 mg/kg of quinuronium s.c. and prior atropinisation

	Time of study (hr)									
	0	1/6	1/2	1	2	4	8	24	48	72
Parameters										
Δ Abs.	0.1	0.08	0.09	0.085	0.09	0.09	0.095	0.1	0.085	0.12
WBC ($X10^3$ µl)	9.85	10.5	11.8	12.9	10.05	11.08	9.8	9.8	8.9	8.99
Hb. (g/dl)	10.5	11.9	12.5	13.0	14	14.0	11.0	13.0	14.0	13.0
PCV (%)	30.5	33	34.0	35	29	29.5	31.5	34.05	35	30.5

APPENDIX 17

sheep No. 13 (group 3) receiving 1 mg/kg of quinuronium s.c. and prior atropinisation

	Time of study (hr.)									
	0	1/6	1/2	1	2	4	8	24	48	72
Parameters										
Δ Abs.	0.09	0.08	0.08	0.075	0.075	0.075	0.06	0.085	0.09	0.085
WBC ($X10^3 \mu l$)	9.9	10.5	11.03	11.0	9.98	10.0	8.9	10.5	9.5	10.0
Hb. (g/dl)	11.0	12.0	13.9	13.0	12.0	11.5	12.5	13.0	14	13.5
PCV (%)	31	32	33	34	37	38	39	33	34	35

APPENDIX 18

Rat No. 1 (group 1 or control) pre-exposed to 0 mg/kg of quinuronium i.p. 30 minutes before liver perfusion

	Time of study (min.)									
	0	5	10	15	30	45	60	75	90	120
Parameter										
BSP Abs. in perfusate X 100	0.0	18.8	15.0	10.6	5.7	2.7	0.7	1.2	0.5	0.7
BSP Abs. in bile X 100	0.0	-	-	1.0	54.6	89.3	70.7	51.2	30.7	14.5
BSP (µg/ml) in perfusate	0.0	12.5	9.7	6.9	3.7	1.75	0.45	0.8	0.3	0.45
BSP (µg/ml) in bile	0.0	-	-	10.0	38.0	62.0	49.5	35.7	21.5	10.5
Cummulative bile volume (µl)	-	-	-	62	132	200	255	300	345	430

APPENDIX 19

Rat No. 2 (group 1 or control) pre-exposed to 0 mg/kg of quinuronium i.p. 30 minutes before liver perfusion

	Time of study (min.)									
	0	5	10	15	30	45	60	75	90	120
Parameter										
BSP Abs. in perfusate X 100	0.0	26.0	20.5	14.3	7.1	6.1	2.3	1.8	1.8	0.8
BSP Abs. in bile X 100	0.0	-	-	1.00	150.4	-	128.9	74.3	40.6	17.8
BSP (µg/ml) in perfusate	0.0	16.65	13.1	9.25	4.51	4.0	1.5	1.18	1.18	0.5
BSP (µg/ml) in bile	0.0	-	-	1.0	150.4	-	80.9	51.6	28.5	12.5
Cummulative bile volume (µl)	-	-	-	52	112	161	210	253	297	362

APPENDIX 20

Rat No. 3 (group 1 or control) pre-exposed to 0 mg/kg of quinuronium i.p. 30 minutes before liver perfusion

	Time of study (min.)									
	0	5	10	15	30	45	60	75	90	120
Parameter										
BSP Abs. in perfusate X 100	0.0	12.1	9.7	6.4	2.6	1.4	1.6	0.2	1.4	0.3
BSP Abs. in bile X 100	0.0	-	-	10.9	88.1	99	54.6	24.5	14.6	9.5
BSP (µg/ml in perfusate	0,0	7.84	6.3	4.15	1.7	0.9	1.05	0.11	0.9	0.18
BSP (µg/ml) in bile	0,0	-	-	7.8	61.15	68.5	38.0	17.5	10.52	6.8
Cummulative bile volume (µl)	-	-	-	114	200	290	350	421	485	613

APPENDIX 21

Rat No. 4 (group 1 or control) pre-exposed to 0 mg/kg of quinuronium i.p. 30 minutes before liver perfusion

	Time of study (min.)									
Parameter	0	5	10	15	30	45	60	75	90	120
BSP Abs. in perfusate X 100	0.0	16.1	13.9	12.3	6.5	1.4	1.9	0.2	1.7	0.9
BSP Abs. in bile X 100	0.0	-	-	0.5	74	139.2	108.3	85.2	56.7	31.5
BSP (µg/ml) in perfusate	0.0	10.5	9.0	7.98	4.2	0.9	1.2	0.11	0.11	0.55
BSP (µg/ml) in bile	0.0	-	-	5	51.2	96.4	78	59.1	39.5	22
Cummulative bile volume (µl)	-	-	-	59	121	168	215	249	283	370

APPENDIX 22

Rat No. 5 (group 1 or control) pre-exposed to 0 mg/kg of quinuronium i.p. 30 minutes before liver perfusion

	Time of study (min.)									
	0	5	10	15	30	45	60	75	90	120
Parameter										
BSP Abs. in perfusate X 100	0.0	23.6	15.0	10.0	5.2	1.8	0.9	0.7	0.7	0.0
BSP Abs. in bile X 100	0.0	-	-	3.6	198.1	-	74.8	24.4	10.1	4.4
BSP (µg/ml) in perfusate	0.0	15.1	10.5	7.0	3.37	1.18	0.55	0.45	0.45	0.0
BSP (µg/ml) in bile	0.0	-	-	2.7	138	-	51.7	17.48	7.15	3.3
Cummulative bile volume (µl)	-	-	-	85	196	297	385	468	546	698

APPENDIX 23

Rat No. 6 (group 2) pre-exposed to 1.5 mg/kg of quinuronium i.p. 30 minutes before liver perfusion

	Time of study (min.)									
	0	5	10	15	30	45	60	75	90	120
Parameter										
BSP Abs. in perfusate X 100	0.0	22	17	10.1	15.0	5.0	1.0	0.8	0.7	0.0
BSP Abs. in bile X 100	0.0	-	-	1.0	67	130	115	85	70	61
BSP (µg/ml) in perfusate	0.0	14.05	10.92	6.55	9.71	3.25	0.65	0.5	0.45	0.0
BSP (µg/ml) in bile	0.0	-	-	0.7	46.5	90	79.5	59	48.7	42.5
Cummulative bile volume (µl)	-	-	-	15	75	105	115	125	140	160

APPENDIX 24

Rat No. 7 (group 2) pre-exposed to 1.5 mg/kg of quinuronium i.p. 30 minutes before liver perfusion

Parameter	Time of study (min.)									
	0	5	10	15	30	45	60	75	90	120
BSP Abs. in perfusate X 100	0.0	26.0	18.0	14.0	9.0	3.2	3.1	2.6	3.0	2.0
BSP Abs. in bile X 100	0.0	-	-	0.0	18	174	170	122	105	62
BSP (µg/ml) in perfusate	0.0	16.65	11.5	9.1	5.83	2.08	2.05	1.68	1.95	1.3
BSP (µg/ml) in bile	0.0	-	-	0.0	12.5	120.15	119.5	84.5	72.5	43
Cummulative bile volume (µl)	-	-	-	-	-	-	-	-	-	-

APPENDIX 25

Rat No. 8 (group 2) pre-exposed to 1.5 mg/kg of quinuronium i.p. 30 minutes before liver perfusion

	Time of study (min.)									
	0	5	10	15	30	45	60	75	90	120
Parameter										
BSP Abs. in perfusate X 100	0.0	27	15.5	11.0	6.9	4.6	0.8	0.0	0.0	0.0
BSP Abs. in bile X 100	0.0	-	-	20	145	105	37	120	4.5	3.0
BSP (µg/ml) in perfusate	0.0	17.25	9.98	7.15	4.5	3.0	0.5	0.0	0.0	0.0
BSP (µg/ml) in bile	0.0	-	-	14	101	72.5	29.5	83	3.5	2.3
Cummulative bile volume (µl)	-	-	-	70	137	181	205	227	248	398

APPENDIX 26

Rat No. 9 (group 2) pre-exposed to 1.5 mg/kg of quinuronium i.p. 30 minutes before liver perfusion

	Time of study (min.)									
	0	5	10	15	30	45	60	75	90	120
Parameter										
BSP Abs. in perfusate X 100	0.0	28	20.5	10	4.6	3.8	0.8	0.0	0.0	0.0
BSP Abs. in bile X 100	0.0	-	-	4.0	150	85	80	54	36	25
BSP (µg/ml) in perfusate	0.0	17.87	13.1	6.5	3.0	2.5	0.5	0.0	0.0	0.0
BSP (µg/ml) in bile	0.0	-	-	3.0	105	59	55.5	37.5	25	17.5
Cummulative bile volume (µl)	-	-	-	88	166	263	353	440	506	569.5

APPENDIX 27

Rat No. 10 (group 2) pre-exposed to 1.5 mg/kg of quinuronium i.p. 30 minutes before liver perfusion

	Time of study (min.)									
	0	5	10	15	30	45	60	75	90	120
Parameter										
BSP Abs. in perfusate X 100	0.0	29	19.5	14	7.5	6.0	1.55	0.4	3.0	4.0
BSP Abs. in bile X 100	0.0	-	-	14	130	95	65	32	13.5	5.0
BSP (μg/ml) in perfusate	0.0	18.58	12.45	9.1	4.85	3.9	1.0	0.25	1.95	2.6
BSP (μg/ml) in bile	0.0	-	-	10	90	65.8	45.2	22.5	9.5	3.7
Cummulative bile volume (μl)	-	-	-	35	75	107	145	174	205	227.5

APPENDIX 28

Rat No. 11 (group 2) pre-exposed to 1.5 mg/kg of quinuronium i.p. 30 minutes before liver perfusion

	Time of study (min.)									
	0	5	10	15	30	45	60	75	90	120
Parameter										
BSP Abs. in perfusate X 100	0.0	27.0	20.5	16.0	7.65	4.85	2.6	0.0	0.0	1.0
BSP Abs. in bile X 100	0.0	-	-	45	150	120	70	35	15	5
BSP (µg/ml) in perfusate	0.0	17.25	13.1	10.2	5.00	3.17	1.7	0.0	0.0	0.65
BSP (µg/ml) in bile	0.0	-	-	31.4	105	83.0	48.7	24.5	10.5	3.7
Cummulative bile volume (µl)	-	-	-	93	181	308	389	494	573	638.5

APPENDIX 29

Rat No. 12 (group 3) pre-exposed to 2.3 mg/kg of quinuronium i.p. 30 minutes before liver perfusion

	Time of study (min.)									
	0	5	10	15	30	45	60	75	90	120
Parameter										
BSP Abs. in perfusate X 100	0.0	40	33	27	16	8	3	3	1	2
BSP Abs. in bile X 100	0.0	-	-	0.1	120	100	100	90	120	60
BSP (µg/ml) in perfusate	0.0	25.47	21.05	17.25	10.2	5.2	1.95	1.95	0.65	1.3
BSP (µg/ml) in bile	0.0	-	-	0.1	83	166	138.4	125	83	41.8
Cummulative bile volume (µl)	-	-	-	44	87	129	157	193	220	264

APPENDIX 30

Rat No. 13 (group 3) pre-exposed to 2,3 mg/kg of quinuronium i.p. 30 minutes before liver perfusion

	Time of study (min.)									
	0	5	10	15	30	45	60	75	90	120
Parameter										
BSP Abs. in perfusate X 100	0.0	25	13	4	-	0.3	0,3	0,5	1.0	1.0
BSP Abs. in bile X 100	0,0	-	-	-	62	110	95	27	5.8	2.0
BSP (µg/ml) in perfusate	0,0	14.78	8,4	2,6	-	0,18	0,18	0.3	0,65	0.65
BSP (µg/ml) in bile	0,0	-	-	43,0	152,2	65,8	18,8	4.3	3.0	1.5
Cummulative bile volume (µl)	-	-	-	103	205	278	350	414	479	594

APPENDIX 31

Rat No. 14 (group 3) pre-exposed to 2.3 mg/kg of quinuronium i.p. 30 minutes before liver perfusion

	Time of study (min.)									
	0	5	10	15	30	45	60	75	90	120
Parameter										
BSP Abs. in perfusate X 100	0.0	27	24	17	8	3.1	2	1	0.5	0.0
BSP Abs. in bile X 100	0.0	-	-	7.0	120	115	75	52.0	33	21
BSP (µg/ml) in perfusate	0.0	17.25	15.35	10.92	5.2	2.1	1.3	0.65	0.3	0.0
BSP (µg/ml) in bile	0.0	-	-	5.0	83	79.5	52.0	36.3	23.0	14.7
Cummulative bile volume (µl)	-	-	-	-	-	-	-	-	-	-

Rat No. 15 (group 3) pre-exposed to 2.3 mg/kg of quinuronium i.p. 30 minutes before liver perfusion

Parameter	Time of study (min.)									
	0	5	10	15	30	45	60	75	90	120
BSP Abs. in perfusate X 100	0.0	42	39.0	35.0	23.0	14.0	9.0	6.0	3.0	1.0
BSP Abs. in bile X 100	0.0	-	-	2.0	120	150	150	120	100	58
BSP (µg/ml) in perfusate	0.0	26.74	24.85	22.42	14.78	9.08	5.85	3.9	1.95	0.65
BSP (µg/ml) in bile	0.0	-	-	1.5	83	105	105	83	69.2	40.2
Cummulative bile volume (µl)	-	-	-	34	85	139	189	233	268	331

APPENDIX 33

Rat No. 16 (group 3) pre-exposed to 2.3 mg/kg of quinuronium i.p. 30 minutes before liver perfusion

	Time of study (min.)									
	0	5	10	15	30	45	60	75	90	120
Parameter										
BSP Abs. in perfusate X 100	0.0	39.0	32.0	26.0	12.0	4.0	1.5	0.0	0.5	0.0
BSP Abs. in bile X 100	0.0	-	-	0.1	70	115	130	63	35	15
BSP (µg/ml) in perfusate	0.0	24.85	19.53	16.65	7.78	2.6	0.98	0.0	0.3	0.0
BSP (µg/ml) in bile	0.0	-	-	0.1	48.5	79.5	90	43.7	24.5	10.5
Cummulative bile volume (µl)	-	-	-	45	85	145	200	240	284	356

APPENDIX 34

Rat No. 17 (group 3) pre-exposed to 2.3 mg/kg of quinuronium i.p. 30 minutes before liver perfusion

	Time of study (min.)									
	0	5	10	15	30	45	60	75	90	120
Parameter										
BSP Abs. in perfusate X 100	0.0	23.0	22.0	19	15	1	60	5.0	3.0	2.0
BSP Abs. in bile X 100	0.0	-	-	5	100	110	100	90	110	80
BSP (µg/ml) in perfusate	0.0	14.78	14.05	12.4	9.71	6.5	3.9	5.32	1.95	1.3
BSP (µg/ml) in bile	0.0	-	-	3.5	69.2	76.1	69.2	62.5	76.1	55.5
Cummulative bile volume (µl)	-	-	-	94	149	197	249	298	348	446

APPENDIX 35

Half lives of BSP disappearance from perfusate in isolated perfused rat livers from control and quinuronium pre-exposed rats

Rat No.	mg/kg of quinuronium	$t_{\frac{1}{2}}$ (min)
1	0	11.88
2	0	16.43
3	0	15.19
4	0	13.25
5	0	5.86
6	1.5	11.95
7	1.5	16.48
8	1.5	11.64
9	1.5	11.18
10	1.5	14.03
11	1.5	16.66
12	2.3	14.66
13	2.3	7.73
14	2.3	13.84
15	2.3	24.32
16	2.3	11.62
17	2.3	28.51

APPENDIX 36

Rats administered 0.5 ml of physiological saline (PBS) s.c. daily for 2 days

Animal No.	Liver weight/ body weight ratio	Hepatic glutathione (GSH) Abs.	Liver histology
GR 1	0.03	1.30	Normal
GR 2	0.03	1.21	Normal
GR 3	0.03	1.35	Normal
GR 4	0.03	1.30	Normal
GR 5	0.05	1.30	Normal

APPENDIX 37

Rats administered 2.3 mg/kg of quinuronium s.c. daily for 2 days

Animal No.	Liver weight/ body weight ratio	Hepatic glutathione (GSH) Abs.	Liver histology
GR 6	0.038	1.2	Normal
GR 7	0.045	0.9	Normal
GR 8	0.035	1.3	Few fatty degene-rations
GR 9	0.05	1.3	Normal
GR 10	0.04	1.4	Normal
GR 11	0.045	1.1	Normal

APPENDIX 38

Rats administered 5 mg/kg of quinuronium s.c. daily for 2 days

Animal No.	Liver weight/ body weight ratio	Hepatic glutathione (GSH) Abs.	Liver histology
GR 12	0.05	1.3	Normal
GR 13	0.07	1.35	Fatty degene-rations
GR 14	0.077	1.25	Fatty degene-rations
GR 15	0.06	1.4	Fatty degene-rations
GR 16	0,07	1,1	Early fatty degenerations
GR 17	-	-	-

Plasma CPK (Sigma Units/ml) from sheep receiving therapeutic dose (1 mg/kg) of quinuronium s.c.

	Time of study (hr.)								
	0	1/4	1/2	1	2	4	8	24	48
Animal No.									
AO	40.5	48	50	59.5	65	66.6	60.5	45	40
861	51.5	55	56.5	60	55	55	51.5	50	49
852	39.5	40.5	45	48.5	68	63.5	40.5	45	40
879	55	47	57.8	60	65.3	59	55	50	52
891	43.5	45	53.5	56.5	59	62	54.8	45	43

Plasma CPK (Sigma Units/ml) from sheep receiving double or higher dose (2 mg/kg) of quinuronium s.c.

	Time of study (hr.)								
	0	1/4	1/2	1	2	4	8	24	48
Animal No.									
A1	37.0	49.5	52	59.8	59	59.5	55	52.3	40
893	38	39.5	45	50	65	60	49.5	40	39
A5	37	55	57	57	69	58	53.5	45	40
865	43.5	50	60	59	72	40.5	42	46	44.5
A3	38	45	57.3	60	66.5	49.5	42.0	45	40

Plasma CPK (Sigma Units/ml) from sheep receiving therapeutic dose (3.5 mg/kg) of diminazene i.m.

	Time of study (hr.)								
	0	1/4	1/2	1	2	4	8	24	48
Animal No.									
898	78	-	-	-	-	-	-	-	-
890	41	71	60.5	108.0	72	28	37	21	15
870	38	45	60.5	39.5	67.5	24	29.5	22	18
844	52	49	50.5	56.5	49.5	24	55	22	18
833	42	52	71	49.5	49.5	32	29.5	21	18

Plasma CPK (Sigma Units/ml) from sheep receiving double or higher dose (7.0 mg/kg) of diminazene i.m.

	Time of study (hr.)								
	0	1/4	1/2	1	2	4	8	24	48
Animal No.									
A4	43.5	85.0	75.0	66	73.5	18.0	21.0	21.0	18
A6	41	51.5	60.5	64.0	69.0	27.0	28.0	25	18
845	45	69	73.5	97.5	72.0	28	25	22	-
860	57	55	87.5	28	82	49.5	-	35	25
A8	47	78	69	-	78	76.5	30	38	27

Plasma LDH (B-B Units/ml) from sheep receiving therapeutic dose (1 mg/kg) of quinuronium s.c.

	Time of study (hr.)								
	0	1/4	1/2	1	2	4	8	24	48
Animal No.									
AO	610	340	740	1830	590	690	735	-	1640
861	990	1465	1400	1465	1600	1400	1425	1800	1700
852	460	290	460	460	845	385	445	665	1320
879	1490	1560	1530	1560	1080	1215	-	1800	1500
891	550	1015	915	740	880	-	480	915	1530

APPENDIX 44

Plasma LDH (B-B Units/ml) from sheep receiving double or higher dose (2 mg/kg) of quinuronium s.c.

	Time of study (hr.)								
	0	1/4	1/2	1	2	4	8	24	48
Animal No.									
A1	450	1190	1290	1320	990	1215	1340	1420	1575
893	-	-	-	-	-	-	-	-	-
A5	1360	1450	1420	1670	1190	1450	1830	1800	1800
865	845	1100	1000	915	980	845	950	2000	850
A3	920	880	1360	1215	650	1340	880	1490	1490

Plasma LDH (B-B Units/ml) from sheep receiving therapeutic dose (3.5 mg/kg) of diminazene i.m.

	Time of study (hr.)								
	0	1/4	1/2	1	2	4	8	24	48
Animal No.									
898	270	-	-	-	-	-	-	-	-
890	285	270	-	485	250	400	1320	380	550
870	220	-	340	460	400	445	800	320	-
844	300	430	800	570	385	670	530	760	320
833	360	300	385	460	460	710	1510	585	385

APPENDIX 46

Plasma LDH (B-B Units/ml) from sheep receiving double or higher dose (7.0 mg/kg) of diminazene i.m.

	Time of study (hr.)								
	0	1/4	1/2	1	2	4	8	24	48
Animal No.									
A4	610	880	525	845	665	900	755	665	990
A6	460	330	210	445	690	360	1380	915	445
845	340	300	630	450	670	800	850	-	-
860	740	410	300	380	570	285	760	600	1010
A8	537	570	690	750	900	889	710	1190	780

APPENDIX 47

Plasma BUN (mg/100 ml) levels from sheep receiving therapeutic dose (1 mg/kg) of quinuronium s.c.

	Time of study (hr.)								
	0	1/4	1/2	1	2	4	8	24	48
Animal No.									
AO	9.0	15.25	13.5	19.0	12.5	10	21.0	15.25	14.5
861	14.0	15.25	21.0	19.0	14.5	14.0	9.0	22.5	15.25
852	12.0	15.5	19.0	19.0	22.5	15.25	15.75	21.75	18.0
879	9.5	12.0	14.0	14.5	16.5	21.75	-	41.5	26.5
891	12.25	19.5	13.5	16.5	14.5	17.25	10.5	14.5	18.0

APPENDIX 48

Plasma BUN (mg/100 ml) levels from sheep receiving double or higher dose (2 mg/kg) of quinuronium s.c.

	Time of study							
	0	1/4	1/2	1	2	4	8	48
Animal No.								
A1	14.0	13.5	18.0	20.0	25.5	25.5	19	12.0
893	12.0	13.5	14.5	16.5	14.5	18.00	12.5	12.25
A5	12.0	18.0	-	19.5	17.25	24.0	22.5	12.0
865	12.5	14.0	14.5	14.5	14.5	19.0	13.5	12.0
A3	9.0	12.5	14.5	15.25	14.5	16.5	12.25	14.0

APPENDIX 49

Plasma BUN (mg/ 100 ml) levels from sheep receiving therapeutic dose (3.5 mg/kg) of diminazene i.m.

	Time of study (hr.)								
	0	1/4	1/2	1	2	4	8	24	48
Animal No.									
898	-	-	-	-	-	-	-	-	-
890	9.0	13.5	10.5	10.0	14.0	14.5	10.5	12.5	16.5
870	5.5	13.5	21.0	13.5	16.5	9.0	22.5	20.0	18.0
844	10.5	12.0	-	12.0	8.0	12.25	13.5	8.0	19.0
833	12.0	19.0	15.25	13.5	13.5	15.25	12.25	12.0	9.0

APPENDIX 50

Plasma BUN (mg/100 ml) levels from sheep receiving double or higher dose (7.0 mg/kg) of diminazene i.m.

	Time of study (hr.)								
	0	1/4	1/2	1	2	4	8	24	48
Animal No.									
A4	5.0	14.5	13.5	10.5	15.25	29.5	10.0	19.0	19.5
A6	6.25	8.0	15.75	15.25	12.25	32.0	14.0	14.0	13.5
845	8.0	13.5	10.5	12.25	13.5	20.0	17.25	10.5	-
860	9.5	10.5	12.25	9.0	13.5	12.0	12.0	-	18.0
A8	6.25	13.5	9.5	18.0	7.0	25.0	12.0	14.0	9.0

APPENDIX 51

Effects of increasing quinuronium concentration added to the *in vitro*/*in vivo* system with *B. rodhaini* as tested by percentage parasitemia in groups of 5 mice

Quinuronium concentration (ng/ml)							
0	2.5	5	10	20	40	80	160
% Parasitemia on day 5							
98	80	70	42	40	10	8	1
90	75	60	60	25	15	7.5	1.5
65	85	55	53	32	8	5	0
86	60	57	43	15	15	3	0.5
97	70	48	35	12	12	0	0

APPENDIX 52

Effects of plasma collected at various time intervals after injection of quinuronium (1 mg/kg s.c.) in sheep No. AO added to the *in vitro*/*in vivo* system with *B. rodhaini*, as tested by percentage parasitemia in groups of 5 mice

Time of plasma collection (hr.)								
0	1/4	1/2	1	2	4	8	24	48
% Parasitemia on day 5								
86	74	59	25	25	20	25	70	90
91	69	61	33	29	35	40	65	86
83	85	50	23	28	15	15	60	90
95	70	42	18	15	20	20	62	98
85	80	48	22	19	30	30	55	80

APPENDIX 53

Effects of plasma collected at various time intervals after injection of quinuronium (1 mg/kg s.c.) in sheep No. 861 added to the *in vitro*/*in vivo* system with *B. rodhaini*, as tested by percentage parasitemia in groups of 5 mice

Time of plasma collection (hr.)								
0	1/4	1/2	1	2	4	8	24	48
% Parasitemia on day 5								
85	75	60	30	30	15	35	65	85
95	80	50	25	20	20	20	70	80
82	81	68	35	20	25	30	55	98
90	67	40	20	15	30	40	60	90
78	70	51	15	20	18	25	63	79