FACTORS AFFECTING THE PHARMACOKINETICS, METABOLISM AND EFFICACY OF ANTHELMINTIC DRUGS

A thesis submitted for the degree of

Doctor of Philosophy

by

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To my parents

Contents

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Declaration

The contents of this thesis are the work of the author. The thesis has not been submitted previously to any university for the award of a degree. The following publications are based in full or in part on the work contained in this thesis:

1. Benchaoui, H. A., Scott, E. W. & McKellar, Q. A. (1993) Pharmacokinetics of albendazole, albendazole sulphoxide and netobimin in goats. *Journal of Veterinary Pharmacology and Therapeutics*, **16**, 237-240.

2. Benchaoui, H. A. & McKellar, Q. A. (1993) Effect of early treatment with rafoxanide on antipyrine clearance in sheep infected with *Fasciola hepatica*. *Xenobiotica*, **23**, 439-448.

3. Benchaoui, H. A. & McKellar, Q. A. (1993) Determination of rafoxanide and closantel in ovine plasma by High Performance Liquid Chromatography. *Biomedical Chromatography*, **7**, 181-183.

4. Benchaoui, H. A. & McKellar, Q. A. (1994) Potentiation of fenbendazole: pharmacokinetic and efficacy assessments of a drug combination in sheep. *Proceedings of the 6th International Congress of the European Association for Veterinary Pharmacology and Toxicology*, pp. 252 (**07**).

5. Benchaoui, H. A. & McKellar, Q. A. (1994) Synergistic compositions containing benzimidazole anthelmintics and methylenedioxyphenyl compounds. International patent application Nº PCT/GB94/00193.

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Summary

The plasma disposition of albendazole, albendazole sulphoxide, and netobimin were investigated in goats following oral administration at the dose rate of 7.5 mg/kg. Netobimin and albendazole were not detectable at any time following drug administration. Plasma levels achieved by the anthelmintically active metabolite, albendazole sulphoxide (ABSO), were not significantly different following administration of albendazole (AUC= 54.40 μg.h/ml; Cmax= 2.38 μg/ml) and albendazole sulphoxide (AUC= 63.04 μg.h/ml; Cmax= 2.77 μg/ml). Following administration of netobimin, the AUC and Cmax for ABSO were 29.76 μg.h/ml and 1.35 μg/ml, respectively.

The effect of the metabolic inhibitor, piperonyl butoxide (administered intramuscularly at 0.5 g/kg bodyweight), on the pharmacokinetics of albendazole (in sheep and goats) and fenbendazole (in goats) was studied. It was found that in sheep, pretreatment with piperonyl butoxide increased the area under the curve (AUC) and the mean residence time (MRT) of albendazole sulphoxide by 77% and 50%, respectively. The AUC and MRT of albendazole sulphone were also significantly increased with piperonyl butoxide pretreatment. In goats, the pharmacokinetic parameters of ABSO were not significantly increased by piperonyl butoxide. Pretreatment of goats with the inhibitor caused more than three-fold increases in the bioavailability of fenbendazole (FBZ) and fenbendazole sulphoxide (FBSO).
The plasma disposition of piperonyl butoxide was determined in sheep and goats. Peak plasma concentrations were achieved between 10 and 15 hours. In sheep, the AUC and Cmax were 132.96 μg.h/ml and 2.92 μg/ml, respectively. In goats, the AUC and Cmax amounted to 82.10 μg.h/ml and 1.17 μg/ml, respectively.

A pharmacokinetic dose titration study was carried out with fenbendazole and piperonyl butoxide in sheep. Fenbendazole was

given at a fixed dose (5 mg/kg) and piperonyl butoxide was administered orally at 0, 15, 31, 63, 125 and 250 mg/kg. The AUC of fenbendazole (FBZ) and fenbendazole sulphoxide (FBSO) increased significantly with dose rates of piperonyl butoxide equal to or higher than 31 mg/kg. Peak plasma concentrations (Cmax) and mean residence times (MRT) were also significantly increased with the coadministration of fenbendazole and piperonyl butoxide.
Piperonyl butoxide given orally to two sheep was well absorbed resulting in an AUC of 761.2 µg.h/ml (sheep 95) and 538.4 µg.h/ml (sheep96).

The efficacy of the combination fenbendazole-piperonyl butoxide (FBZ-PB) was assessed in sheep against benzimidazole-resistant *Ostertagia circumcincta* and *Haemonchus contortus*. The percentage reduction in the total number of *O. circumcincta* worms was 7.90% (FBZ alone) and 97.8% (FBZ-PB). For *Haemonchus contortus*, the percentage reduction was 84.8% (FBZ alone) and 99.0% (FBZ-PB). Reduction in the faecal egg output, 7 days after treatment was 93.7 % (FBZ alone) and 99.6 % (FBZ-PB). Piperonyl butoxide, given alone, had no effect against these two nematode species.

The *in vitro* metabolism of fenbendazole, oxfendazole, albendazole and triclabendazole was studied using microsomal preparations and cultured hepatocytes from rat liver. The extent of metabolism was found to be in the order triclabendazole > albendazole > fenbendazole and oxfendazole. Piperonyl butoxide inhibited the S-oxidation of all the benzimidazole drugs studied. 1-aminobenzotriazole inhibited significantly the metabolism of triclabendazole in hepatocyte cultures.

The effect of the salicylanilide compound rafoxanide against immature (4 week-old) stages of *Fasciola hepatica* was investigated in artificially infected sheep. Antipyrine clearance tests together with plasma glutamate dehydrogenase (GLDH) and gamma-glutamyl transferase (γGT) activities were used to follow the evolution of flukicidal therapy with rafoxanide. Glutamate

but increased again at 12 weeks post-infection in the infected rafoxanide-treated sheep (group B). In the infected untreated sheep (group A), Antipyrine clearance decreased between 8 and 14 weeks post-infection. In the infected treated sheep (group B), plasma clearance of antipyrine remained unchanged until 10 weeks after rafoxanide treatment when it decreased from the pre-infection value of 5.09 to 3.90 ml/min.kg. Rafoxanide did not alter antipyrine disposition in uninfected sheep (group C).

Abbreviations

ABSO	albendazole sulphoxide
$ABSO_2$	albendazole sulphone
ABZ	albendazole
AUC	area under the curve
AUCobs	area under the curve for observed values
AUFS	absorbance units full scale
AUMC	area under first moment curve
AUMCobs	area under the first moment curve for observed values
BZ	benzimidazole
CBZ	cambendazole
CI	clearance index
Clb	body clearance
Cmax	Peak plasma concentration
Cp_0	plasma concentration at time zero
CV	coefficient of variation
e	exponential
e.g.	for example
epg	eggs per gram
et al.	and others
F. hepatica	*Fasciola hepatica*
FBSO	fenbendazole sulphoxide
$FBSO_2$	fenbendazole sulphone
FBZ	fenbendazole
FMO	flavin-containing monooxygenase
g	gram
g	gravity, $10^{-11}N.m/s^2$
γGT (GGT)	gamma-glutamyl transferase
GI	gastrointestinal
GLDH	glutamate dehydrogenase
h	hours
H. contortus	*Haemonchus contortus*
HPLC	high performance liquid chromatography

i.e.	that is
IM	intramuscular
IU	international units
IV	intravenous
Kg	kilogram
L	liter
Log	logarithm
M	molar
ml	mililiter
mmol	millimoles
MRT	mean residence time
mw	molecular weight
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
O. circumcincta	*Ostertagia circumcincta*
OFZ/FBSO	oxfendazole/fenbendazole sulphoxide
OH-FBZ	hydroxyfenbendazole
OH-OFZ	hydroxyoxfendazole
PB	piperonyl butoxide
PBZ	parbendazole
PI	postinfection
pmoles	picomoles
PT	posttreatment
S-oxidation	sulphoxidation and sulphonation
SD	standard deviation
SEM	standard error of the mean
	state
t1/2β	elimination half-life
TBZ	thiabendazole
TCBSO	triclabendazole sulphoxide
$TCBSO_2$	triclabendazole sulphone
TCBZ	triclabendazole
tmax	time to peak plasma concentration
uv	ultraviolet
Vc	volume of the central compartment

Vdarea	apparent volume of distribution
Vdss	volume of distribution at steady state
w/v	weight/volume
μ g	microgram
μ l	microliter
μ M	micromolar
ºC	degrees centigrade

Chapter 1

General introduction

The economic impact of parasitism is reflected in the capital invested for control and therapeutic measures. Every year, an estimated $ 1.7 billion worth of anthelmintics are administered to ruminants worldwide in order to minimise production losses caused by parasitic nematodes and trematodes. It is not surprising, considering the large anthelmintic market for livestock that the most important advance in the chemotherapy of helminthiasis, has come from the animal health industry (Horton, 1990). From the discovery of the anthelmintic properties of phenothiazine in the 1940s to the recent exploration of the endectocide properties of the milbemycins, a considerable effort has been put to improve the potency, breadth of spectrum, efficacy and safety of antiparasitic compounds. This effort has been rewarded by two major breakthroughs. The first was the discovery of thiabendazole (Brown *et al.*, 1961) which stimulated extensive research leading to the design, by chemical manipulation of the benzimidazole ring, of many other broad spectrum anthelmintic molecules. Secondly, it was discovered that the fermentation of the actinomycete *Sreptomyces avermitilis* produced compounds that were not only effective at extremely low concentrations but also had a spectrum of activity that extended to ectoparasites (Miller *et al.*, 1979). However, the launching of a novel product is always preceeded by long and increasingly prohibitive screening and testing processes. It has been estimated that it costs $ 230 million to develop a new anthelmintic molecule from discovery to the market place (McKellar, 1993) which added to the the prospect of tough competition from existing products explains the paucity of newly developed anthelmintics. There is a rationale behind the search for novel strategies which extend the lifespan or improve the activity of existing antiparasitic drugs. This will only be achieved with a more complete understanding of the pharmacological properties of existing agents. The intimate link between pharmacokinetics, pharmacodynamics and clinical efficacy is of primary importance for the understanding and possible manipulation of various factors that affect the therapeutic outcome of anthelmintic treatment.

1.1 Factors affecting the pharmacokinetics and efficacy of anthelmintic drugs

Anthelmintics currently available belong to five major chemical families: (a) benzimidazoles and probenzimidazoles; (b) imidazothiazoles

(levamisole); (c) tetrahydropyrimidines (morantel); (d) salicylanilides; (e) macrocyclic lactones (avermectins and milbemycins).
Factors which affect the pharmacokinetics and, or, efficacy of these anthelmintics are numerous and could be classified into host-related factors, parasite-related factors and exogenous factors. The latter group of factors refers to the effect of routes of administration, drug formulation, delivery systems, feeding management during treatment and drug combinations.

1.1.1 The host

The fate of most orally administered anthelmintics is determined in three major body compartments of the ruminant animal: the gastrointestinal (GI) tract, the bloodstream and the liver.

1.1.1.1 Influence of the gastrointestinal tract

The complexity of the ruminant digestive tract in comparison with that of monogastric animals creates unique problems and opportunities for the oral administration of drugs (Koritz, 1983). The rumen has indeed a remarkable influence on the absorption, distribution and metabolism of drugs administered by the oral route and may also contribute to the metabolism of parenterally administered drugs which diffuse from the plasma across the rumen epithelium. The reticulo-rumen of the sheep makes up 20% of the body volume and may contain 5-10 kg of contents (Dobson, 1967; Hofmann, 1973). When a drug suspension is deposited in the rumen, solid particles mix and distribute through the large digesta volume. This slow mixing process not only delays further passage of the drug down the GI tract but also prolongs the rate at which solubilised particles come into contact with the absorptive epithelial surface of the rumen (Koritz, 1983). The rumen can therefore act as a reservoir which lenghtens the duration of drug absorption and outflow down the GI tract. This 'reservoir effect' of the forestomach is strongly linked to the pharmacokinetics and the clinical efficacy of benzimidazole drugs in ruminants. Marriner and Bogan (1981b) found that oral administration of fenbendazole (FBZ) resulted in higher and more sustained plasma concentrations of drug and metabolites than when the anthelmintic was given by the intraabomasal route. This provided an explanation for the observation that FBZ was more effective in removing benzimidazole-resistant *Haemonchus contortus* and *Trichostrongylus colubriformis* when given into the rumen then when given into the abomasum (Kelly *et*

al., 1977). Hennessy (1993a) emphasised the role played by the adsorption of benzimidazoles (BZ) to ruminal particulate material in the extension of their bioavailability. It has been estimated that more than 90% of oxfendazole (OFZ) and metabolites associate with rumen particulate material. The period necessary for the desorption of OFZ and metabolites from particulate to fluid digesta, added to the time required for absorption extends the residence time of this benzimidazole drug in the plasma and throughout the rest of the GI tract. Drug and metabolites are released from particulate material progressively as the drug concentration in the ruminal fluid diminishes due to absorption or outflow to the lower GI tract. The physico-chemical nature of drug association with particulate material is likely to be by physical adsorption rather than specific chemical binding (Lees *et al.*, 1988).

The oral administration of liquids or suspensions can sometimes stimulate the reflex of oesophageal groove closure causing a rumen bypass which diverts the drug preparation from the oesophagus to the abomasum. This reflex has been incriminated in instances of erratic anthelmintic treatment failures. Prichard and Hennessy (1981) found that partial or complete oesophageal groove closure occured in 42% of the sheep treated with an oral OFZ preparation and caused a reduction in OFZ bioavailability and efficacy. In goats, a higher incidence of the rumen bypass reflex occurs following administration of an oral drench (Sangster *et al.*, 1991). In cattle, failure to remove inhibited larvae of *Ostertagia ostertagi* has also been linked to the rumen bypass reflex (Duncan *et al.*, 1977); this was later supported by McEwan & Oakley (1978). There is no unanimity, however, on whether the rumen bypass effect could alter the overall disposition of orally administered anthelmintic drugs in a manner that would be significant enough to compromise the therapeutic outcome. Ngomuo and coworkers (1984) did not find a significant difference in the area under the plasma concentrations-time curve (AUC) of OFZ and its metabolites following oral or intraruminal administration to cattle. Bogan & Marriner (1987) consider that the problem has been overrated. They argued that the reflex is more likely to occur with large volume drenches than with small volumes and even if the reflex occurs, most of the drug (in suspension) would remain spread along the oesophageal groove and would eventually be deposited in the rumen or the omasum rather than quickly directed to the abomasum. A biphasic pattern of absorption occurs and results in a typical early peak plasma concentration; this

initial onset cannot always be spotted on meaned values since it occurs at different times in different animals.
In order to minimise the risks of oesophageal groove closure, it is recommended that small volumes of drug preparation (less than 10ml) are administered and also that the drench is delivered on the back of the tongue rather than in the anterior part of the buccal cavity (Gibson,1980; Hennessy, 1993b).

Absorption from the rumen, as from most body compartments, occurs by passive diffusion of the lipophilic unionized fraction of the drug. The extent of passage depends on the rumen/plasma pH gradient and the pKa of the drug and is somewhat restricted by the physical nature of the rumen wall which is composed of stratified squamous epithelium. To be absorbed, a drug needs first to be dissolved in the containing fluid; BZ are poorly soluble at ruminal pH (6.5) except for thiabendazole (TBZ) and cambendazole (CBZ) (McKellar & Scott, 1990). The rapid absorption and excretion of TBZ explains the relatively short residence time of this drug in the body and its lower efficacy in comparison to less soluble and, consequently longer residing BZs (Prichard *et al.*, 1978; Prichard *et al.*, 1981); FBZ, OFZ and also albendazole (ABZ) most likely owe their prolonged residence time to their low solubility in ruminal fluid and tendency to associate with digesta particulates. Diffusion also occurs from the bloodstream into the rumen. This has been demonstrated for levamisole administered subcutaneously (Bogan *et al.*, 1982) but not for ivermectin given by the same route (Bogan & McKellar, 1988).
The role of the rumen as a metabolising compartment of foreign compounds is well documented. The ruminal biodegradation of the antibiotic chloromphenicol prohibits its oral administration to ruminants (Theodorides *et al.*, 1968; Davis *et al.*, 1972). There is *in vivo* and *in vitro* evidence that ivermectin is degraded to some extent by the ruminal microflora. In sheep, the bioavailability of ivermectin following intraruminal administration is 75% lower than after intraabomasal administration and *in vitro* incubation of ivermectin in ruminal fluid was followed by its gradual disappearance from the incubate (Prichard *et al.*, 1985b). This may also explain the absence of subcutaneously administered ivermectin from the ruminal fluid (Bogan & McKellar, 1988). The nitro group of the flukicide nitroxynil is reduced by the ruminal microflora and therefore this drug is only active when administered parenterally (Arundel, 1985). Nevertheless, bioconversion

by the ruminal flora can sometimes be beneficial in terms of bioavailability and efficacy of anthelmintics. The conversion of the probenzimidazole drug netobimin into its active form albendazole is determined by the ruminal microflora (Delatour *et al.*, 1986). Also, the reduction in the rumen of albendazole sulphoxide (ABSO) (Lanusse *et al.*, 1992) and fenbendazole sulhpoxide (FBSO) (Marriner, 1980., Beretta *et al.*, 1987) back to their respective parent compounds ABZ and FBZ, may enhance their antinematode action since the binding affinity to nematode tubulin is higher with thioether parent compounds than with the sulphoxide metabolites (Lubega & Prichard, 1990).

Orally administered anthelmintics and metabolites that reach the abomasum originate from two sources; those in digesta that flows from the rumen and those in gastric secretion (Hennessy, 1993a). The plasma/abomasum pH gradient is larger than the plasma/rumen pH gradient. Lipophilic nonionised molecules migrate down their concentration gradient to the side of the barrier membrane where higher ionisation occurs creating the so-called ion-trapping phenomenon. For exemple, the pKa of ABSO is 7.8; at plasma pH there will be a greater proportion of this molecule in the nonionised form which promotes its passage from plasma to different tissues (Lanusse & Prichard, 1993; 1994). The greater plasma/abomasum gradient creates a strong ionic trapping effect which explains the higher concentration of ABSO found in the abomasum in comparison with plasma and other GI compartments (Lanusse *et al.*, 1993a). High concentrations of the parent compound ABZ and the sulfone metabolite were also found in the abomasal fluid. Hennessy (1993a) found 10-14% of an OFZ dose in the abomasal fluid and suggested that most of this fraction originated from gastric secretions rather than outflow from the rumen. It is surprising that no drug was found in the abomasal fluid of sheep following administration of ivermectin even at 10 times the recommended dose (Bogan & McKellar, 1988). It was however present in the abomasal mucus, which may account for the antiparasitic action of this drug against abomasal nematodes.

In the intestine, distribution of BZ metabolites is highly dependent on biliary secretion (Hennessy, 1993a) with relatively low proportions arriving from the upper GI tract or from the systemic circulation. Studies in cattle showed that only 28% of an orally administered FBZ dose flowed through the pylorus wheras 52% of the dose reached the ileocaecal

junction through biliary recycling (Prichard *et al.*, 1981). In sheep, 63% of the dose was secreted as biliary metabolites following administration of OFZ (Hennessy *et al.*, 1985). To quantify the enterohepatic recycling of FBZ metabolites, Hennessy and coworkers (1993d) infused unconjugated metabolites of FBZ into the duodenum. The rapid postinfusion appearance of free (unconjugated) metabolites in plasma and their resecretion in bile toghether with a rapid decline of biliary metabolite concentrations at the end of the infusion indicate the existence of metabolite reabsorption from the upper small intestine. This exchange could be crucial for the anthelmintic action against pathogenic nematodes located in the mucosal surface of the upper small intestine such as *Trichostrongylus colubriformis*. Following infusion of conjugated biliary metabolites of OFZ, recirculation continued several hours after cessation of the infusion suggesting that conjugated biliary metabolites may be deconjugated and absorbed at sites distal to the upper small intestine. It is postulated that these metabolites, mostly hydroxylated, are deconjugated by the intestinal microflora and then reabsorbed from this site contributing to the anthelmintic action of FBZ and OFZ against parasites of the large intestine. Hydroxylated metabolites have been shown to have a higher activity than the parent FBZ in binding to nematode tubulin (Lacey *et al.*, 1987).

Like the rumen, the ileum also contains a microflora that reduces sulfoxides into their parent compounds and converts netobimin into ABZ. Sulfoxidation also occurs to a minor extent in the ileum (Lanusse *et al.*, 1992).

Given the influence of the GI tract in the disposition of anthelmintics and considering the important structural and physiopathological changes that GI parasitism causes in this body compartment, researchers investigated the effect of GI parasitic burden on the pharmacokinetics of anthelmintic drugs. Prichard (1980) first reported a decrease in abomasal FBZ concentrations in treated sheep that were infected with *Ostertagia circumcincta* and *Trichostrongylus colubriformis*. The decrease was proportional to the severity of the parasitic gastroenteritis. Marriner *et al.* (1985) reported a lower abomasal and systemic availability of FBZ and metabolites in sheep infected with *O. circumcincta*. Bogan *et al.* (1987) showed a 25% decrease in the bioavailability of OFZ in goats infected with the same nematode. Similarly, a 32% and 59% reduction in the systemic availability of OFZ was observed in sheep and goats respectively, after

mixed infection with *H. contortus* and *T. colubriformis* (Hennessy *et al.*, 1993a) Debackere *et al.* (1993) have shown that, given at the same infection dose (50,000 L_3) to sheep, *T. colubriformis* had a greater impact on the bioavailability of febantel metabolites than *O. circumcincta* although higher infection rates (100,000 L_3) of either parasite did not cause a greater decrease in bioavailability (Landuyt *et al.*, unpublished data). Infection with *Nematodirus battus* had no effect on the pharmacokinetics of levamisole, ivermectin, netobimin or albendazole (McKellar *et al.*, 1991; 1993b). Changes in abomasal pH, altered GI motility and digesta flow rate, leakage of plasma drug across the damaged GI mucosa are among the causes that have been implicated to explain the pharmacokinetic changes observed. Marriner *et al.* (1985) have shown an increase in the abomasal pH from 2.2 to 6.5 in sheep infected with *O. circumcincta* and suggested that elevated abomasal pH reduces the ion trapping effect and the degree of solubilisation of poorly soluble drugs thus reducing parasite exposure and drug absorption. However, in the study carried out by Hennessy *et al.* (1993a), the abomasal pH did not change but abomasal flow rate did tend to increase in both sheep and goats. It is likely that an increased fluid flow rate through the GI tract reduces the residence time of drug at the absorption sites and leads to impaired uptake. Gastroenteric plasma leakage may also contribute to reducing the systemic bioavailability of anthelmintic drugs. The absence of pharmacokinetic changes during *Nematodirus* infection has been attributed to the low level of infection used in the studies.

These changes in bioavailability are trivial with regard to the anthelmintic efficacy against GI parasitism, since clinical trials are conducted on animals infected with GI parasites, however, they might be crucial when the animals are simultaneously infected with liver fluke and lungworm.

1.1.1.2 Effect of plasma protein binding

Following absorption, the blood serves as the tissue in which drug molecules are conveyed to various parts of the body. Within the bloodstream, a fraction of most drugs binds reversibly to protein, usually albumin, and the remainder (unbound drug) undergoes simultaneous distribution, biotransformation and excretion processes (Baggot, 1977a). It is also the unbound fraction that is pharmacologically active. The degree of protein binding of a drug may therefore be important in

determining its pharmacokinetic pattern as well as the duration and intensity of its action.

The majority of benzimidazole compounds show a plasma protein binding of less than 50% (Lanusse & Prichard, 1993). For ABZ metabolites, the proportion of bound drug amounted to 50.5% of the total plasma metabolites for up to 26 hours after administration, and this proportion increased to 80-100% only after 34 hours postadministration at which time, the major plasma metabolite was the inactive sulphone (Hennessy, 1985). However, two halogenated BZ have been shown to bind strongly to plasma protein ; triclabendazole (TCBZ) whose sulphoxide and sulphone metabolites are more than 99% bound to plasma albumin (Mohammed-Ali *et al.*, 1986; Hennessy *et al.*, 1987); and luxabendazole (LBZ) which is 95% bound to plasma proteins (Steel and Duwel, 1987). The extensive plasma protein binding of triclabendazole metabolites and luxabendazole reduces their distribution in the body and prolongs their elimination half-lives in comparison to other benzimidazoles. In sheep, the total TCBZ metabolite area under the plasma-concentration time curve is nearly 7 and 20 times higher than that of the total ABZ and FBZ metabolites, respectively (Marriner & Bogan, 1980; Marriner and Bogan, 1981b; Hennessy *et al.*, 1987). This difference in the extent of plasma protein binding may account for the high flukicidal action of triclabendazole and luxabendazole against both immature and mature stages of *Fasciola hepatica* (Boray *et al.*, 1983; Dorchies *et al.*, 1983; Duwel, 1987). Hennessy (1985) demonstrated that flukes are unlikely to be exposed to the anthelmintic action of TCBZ metabolites via the bile since he failed to extract biliary metabolites from the flukes recovered from treated animals and also because flukes, like cestodes, are likely to lack enzymes that enable them to hydrolize conjugated biliary metabolites (Hennessy *et al.*, 1987). However, plasma TCBZ metabolites were extracted from liver flukes, showing that the haematophagous behaviour of *F. hepatica* determines the mechanism of drug uptake. The flukicidal activity of TCBZ and LBZ may be promoted by their long residence time in the bloodstream but does not explain the specificity of the former to liver flukes and not to other blood-sucking helminths such as *Haemonchus contortus* from which plasma metabolites were also extracted following exposure (Hennessy, 1985). A likely explanation for the narrow spectrum of activity of TCBZ has recently emerged from a study on the structural and electronic features of the active metabolite triclabendazole sulphoxide (TCBSO) (Lipkowitz & McCraken, 1991). It appears that the

shape of the substituent at position 2- of TCBSO is nonplanar, all other broad-spectrum BZ anthelmintics regardless of substituent at position 2- (methyl carbamate or thiazolyl) are flat. The second distinguishing feature is the net atomic charge on the substituent at position 2-; it is an order of magnitude larger than the net atomic charge on the other BZ anthelmintics.

Of all anthelmintic products available, the salicylanilide derivatives (rafoxanide, closantel, oxyclozanide) and also the nitrophenol flukicidal compound nitroxynil, are probably the drugs with the longest residence time in the bloodstream. This is due to their strong plasma protein binding which is more than 99% for salicylanilides (Mohammed-Ali & Bogan, 1987), and 98% for nitroxynil (Alvinerie *et al.*, 1991). It has been suggested that the long elimination halflife of some salicylanilides (2-3 weeks) may reflect turnover of plasma albumin to which they are bound since the plasma albumin turnover is about 16.6 days (Holmes *et al.*, 1968). For these drugs, the plasma should therefore be considered more as a storage compartment than as a drug-vehicle to other tissues (Michiels *et al.*, 1988). This creates an ideal situation for anthelmintic action against blood sucking parasites whereby the cidal drug is concentrated in the parasite feeding source without the compromising consequence of high tissue residue levels. Indeed, the very high degree of plasma protein binding of salicylanilides may explain the observation that in well bled out carcasses relatively low tissue residues are found and standard withdrawal periods are generally shorter than might be predicted from their plasma pharmacokinetics (Mohammed-Ali, 1985; Michiels *et al.*, 1988; McKellar & Kinabo, 1991).

1.1.1.3 Effect of hepatic drug-metabolism

Biotransformation tends to convert xenobiotics into less lipid soluble and more polar metabolites for easier and faster elimination. A certain degree of lipid solubility is a prerequisite for drug transformation by the hepatic microsomal enzyme system (Baggot, 1977b); hydrophilic drugs and the majority of metabolite products are sufficiently polar to be eliminated. Classically, drug biotransformation is subdivided into two phases. In phase I (nonsynthetic) hydrophobic molecules are rendered more water soluble by the introduction of polar functional groups. Phase II (synthetic) reactions primarily consist of conjugation with water soluble substances, such as glucuronic acid, sulphate, etc. Phase I and II

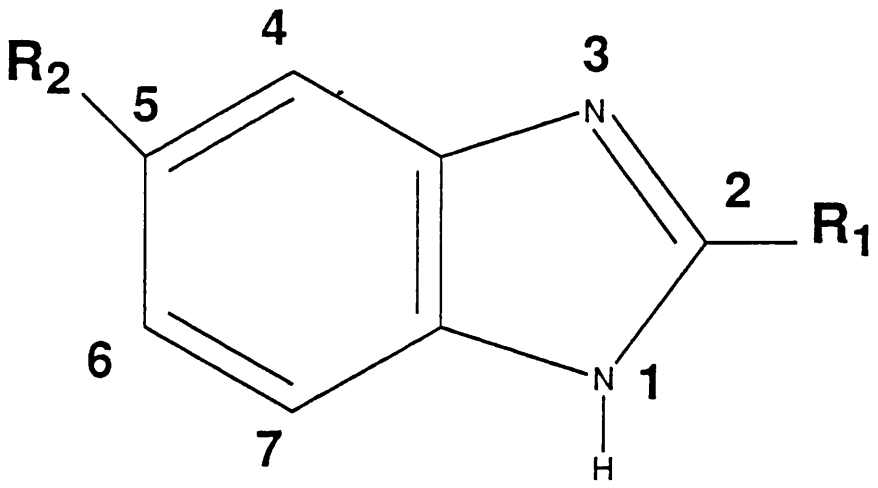

Figure 1.1 Primary substituent positions of the benzimidazole anthelmintics.

enzyme systems are predominantly localized in the liver and to a minor extent in other tissues. Metabolites formed through these reactions may be inactive, less active or occasionnally more active or more toxic than the parent molecule

Overall, BZ anthelmintics are extensively metabolized in all mammalian species studied. As a common metabolic feature of BZ compounds, parent drugs are short-lived and metabolic products predominate in plasma and all tissues and excreta of the host as well as in parasites recovered from BZ-treated animals (Delatour & Parish, 1986; Lanusse & Prichard, 1994). The primary metabolites are usually the products of phase I oxidation or hydrolysis and are more polar than the parent drug. Phase II conjugation reactions also take place whereby oxidized and/or hydrolized BZ metabolites are conjugated with glucuronide and/or sulphate to increase their polarities and facilitate their biliary and urinary excretion.

The metabolism of BZ's usually involves the substituent on position 5- of the nucleus (Figure 1-1) which undergoes a variety of reactions catalyzed by hepatic mixed function oxidases. Phase I type reactions such as hydroxylation, S-oxidation, reduction have been shown to occur at this position. Hydrolytic decarboxylation of the carbamate group at position 2- to form aminoderivatives is also a common reaction (Gottschall *et al.*, 1990). However, metabolic reactions involving the BZ ring itself such as the 5-hydroxylation of TBZ are unusual. Following oral administration of this BZ to sheep, goats and cattle, the predominant metabolite is 5-hydroxythiabendazole (Tocco *et al.*, 1965; Weir & Bogan, 1985) which is anthelmintically inactive (Stone *et al.*, 1965). Cambendazole (CBZ) is also oxidized to its phenolic metabolite, although this is unlikely to be a major pathway (Van den Heuvel *et al.*, 1978). BZ containing 5-keto substituents like mebendazole (MBZ) undergo a carbonyl reduction to give a secondary alcohol which in turn can be conjugated or undergo a carbamate hydrolysis into an aminoalcohol (Van den Bossche *et al.*, 1982). As a general rule hydrolysis of the carbamate group eliminates both biological activity and toxicity (Delatour and Parish, 1986).

The presence of a sulphur atom in the 5-substituent (e.g. FBZ, OFZ, ABZ, ABSO) or in the 2-substituent (e.g. TCBZ, TCBSO) group has a major influence on metabolism. Liver microsomal S-oxidation of the thiosubstituted BZ is a common metabolic pathway. FBZ, ABZ and probably TCBZ are metabolised to their respective sulphoxide and sulphone moieties by liver microsomal oxidation (Marriner, 1980; Galtier *et al.*, 1986a; Hennessy *et al.*, 1987; Souhaili-El Amri *et al.*, 1987, 1988a,b;

Murray *et al.*, 1992; Lanusse *et al.*, 1993c). The sulphoxides of albendazole (albendazole sulphoxide) and fenbendazole (oxfendazole) are also commercially available as anthelmintics. Sulphoxidation is a rapid and reversible process which forms an equilibrium with the respective sulphide, although the equilibrium tends to favour metabolism towards the sulphoxide (Lanusse & Prichard, 1993; 1994). The sulphoxide metabolites undergo a further oxidation into sulphones. In the rat and ruminants, pharmacokinetic and *in vitro* microsomal metabolism studies show that sulphonation is a rather slow and irreversible reaction (Delatour and Parish, 1986; Souhaili-El Amri et al., 1988a,b; Short *et al.*, 1988). *In vitro* studies with ABZ showed that two microsomal enzymatic systems are involved in the two S-oxidations; the flavin-containing monooxygenase (FMO) and the cytochrome P450 enzyme system. It was initially beleived that the two systems were sequéntially involved in the catalysis of the two successive reactions, i.e., the FMO is responsible for the sulphoxidation, and the cytochrome P450 for the sulphonation (Fargetton *et al.*, 1986; Souhaili-EL Amri *et al.*, 1987, 1988a,b). Recent stereoselective studies have demonstrated that sulphoxidation does also involve cytochrome P450, however, FMO is the predominant catalyzing system in ruminants whereas in monogastrics, both systems are equally involved in this reaction (Delatour *et al.*, 1994). The inducibility of the sulphonation has been shown following repeated treatments with ABZ and was corroborated by increased cytochrome P450c (also called P448 and more recently P450 IA1) activity in rat liver as a result of exposure to ABZ (Souhaili-El Amri *et al.*, 1988a,b). In addition, in human hepatoma cell lines, ABSO, and to a lesser extent ABSO2 induced the activity of cytochrome P450IA1 Similarly, the administration of OFZ to rabbits for 10 consecutive days at a dosing regime of 4.5 or 22.5 mg/kg/day increased 1.54 and 2.36-fold the total liver microsomal cytochrome P450 and more particularly the isozyme P450IA2 as demonstrated by western blotting (Gleizes *et al.*, 1991a). The treatment of cultured rabbit hepatocytes with OFZ (10mM) induced the isozymes P450IA1, IA2 and IIIA6 (Gleizes *et al.*, 1991b). This autoinduction of sulphoxide BZ metabolism could be detrimental to the clinical efficacy of these compounds since the final metabolite product, the sulphone, is generally known to be anthelmintically inactive.

The aromatic ring p-hydroxylation that leads to the formation of OH-FBZ and OH-OFZ is also an important metabolic route (Short *et al.*, 1987a,b, 1988; Hennessy *et al.*, 1993b). After administration of fenbendazole to

sheep, hydroxylated oxfendazole (OH-OFZ) was the major biliary metabolite contributing 66%, and hydroxyfenbendazole (OH-FBZ) 27%, of the total biliary metabolites characterized (Hennessy *et al.*, 1993d). Surprisingly, *in vitro* incubation of FBZ with subcellular fractions yielded OH-FBZ in all species studied except in sheep (Short *et al.*, 1988). Although OH-FBZ was detected in plasma of goats (Short., 1987), hydroxylated metabolites contribute very little to the FBZ metabolite profile in the bloodstream (Hennessy *et al.*, 1993d). They are excreted in bile and to a lesser extent, in urine, as free or as conjugated glucuronide and sulphate forms. Hennessy and co-workers (1993d) pointed out that because OH-OFZ was not measured in previous studies, faecal excretion of FBZ has been underestimated in sheep (Duwel, 1977), goats (Short *et al.*, 1987a) and cattle (Short *et al.*, 1987b). P-hydroxylation and subsequent conjugation is also an important metabolic process that preceedes biliary excretion of TCBZ (Hennessy *et al.*, 1987).

The substitution of the BZ ring in position 5- has been particularly important in determining the metabolic fate of the modern BZ drugs. This position is metabolically labile; a feature that has been used to retard the biotransformation of 5-substituted BZ anthelmintics (Lanusse & Prichard, 1994). Alterations of the substituent radical in this position markedly change the physico-chemical properties of the drug, hence its metabolism. Aromatic BZ derivatives such as FBZ, OFZ, MBZ and TCBZ require more extensive metabolism than aliphatic derivatives (albendazole, parbendazole) to achieve sufficient polarity for excretion. This may account for the longer residence times and elimination half-lives of aromatic BZ and their metabolites compared to those of aliphatic derivatives. The aromatic substitution slows the oxidation of the sulphur (FBZ, OFZ) or reduction of the keto (MBZ) linkage; further hydroxylation and conjugation result in more polar excretory metabolites which are more extensively eliminated in bile (40-60% of the dose) than in urine (5-20% of the dose) (Lanusse & Prichard, 1994). Enterohepatic recycling is more important for aromatic substituted BZ than for aliphatic BZ derivatives. When oxidised, 5-aliphatic substituted BZ (ABZ, PBZ) are sufficiently polar to be mostly excreted in urine rather than undergo conjugation and secretion in bile. Following ABZ administration to sheep only 8% of the dose was excreted in bile as free ABSO and 2OH-ABSO metabolites, and 6.3% as conjugated glucuronide and sulphate esters mainly of 2OH-ABSO and 2OH-ABSO2 (Hennessy *et al.*, 1989); 59% of the

dose was excreted in the urine of ABZ treated cattle, with ABSO being the major urinary metabolite (Gyurik *et al.*, 1981). The hydrolysis of the ABSO2 methylcarbamate group forms a polar aminoderivative; NH2ABSO2. The 2-aminosulphone metabolite appears in plasma and in abomasal and ileal fluids after the ABSO2 plasma peak (Lanusse *et al.*, 1993a) and is the longest -residing free metabolite in tissue (Prichard & Parish, 1986).

Recently, the chiral behaviour of albendazole sulphoxide and fenbendazole sulphoxide have been described and an enantioselective biotransformation has been suggested (Delatour *et al.*, 1990a, b; 1991; 1994; Benoit *et al.*, 1992). Two enantiomers of these sulphoxides have been identified and interestingly in sheep, goats and cattle, following administration of the parent sulphide, the plasma concentration of the isomers is never a racemate and as soon as the sulphoxide metabolite is detected there is predominance of the (+) enantiomer. Following administration of ABZ for instance, the ratio +/- starts at 75/25 (zerotime) and keeps changing in favour of the (+) isomer to reach a value of 95/5. The ratios were closer to a racemate after administration of a synthetic racemic mixture of the sulfoxide. The proposed stereoselective biotransformation suggests that sulfoxidation by the flavin monooxygenase enzyme system (FMO) produces more (+) than (-) whereas the sulfonating reaction, which is governed by the cytochrome P450 enzyme system, uses more of the (-) enantiomer as a substrate.

Liver metabolism of levamisole consists of a sulfoxidation, hydroxylation and sulphonation followed by a glucuronoconjugation. Metabolites are mainly eliminated in the urine (Nielson & Rasmussen, 1983; Labourel, 1984). The metabolism of the (L) and (D) forms of tetramisole has also been shown to be enantioselective (Labourel, 1984).
Salicylanilides are not extensively metabolised. Following administration of closantel, 90% of the dose was excreted unchanged and 98% of the plasma concentration was attributed to the parent compound. Urinary excretion is negligable (< 0.5%) and the only relatively efficient clearance route appears to be the excretion of mainly the unmetabolised drug in bile (Michiels *et al.*, 1987). The main metabolic pathway for closantel was reductive monoiodination.
For ivermectin, the major liver metabolite in sheep, cattle and rat is 24-hydroxymethyl-H2B1a which is further conjugated to fatty acids and

esters and deposited in the fat tissue. The major route of excretion is the bile (Shuet-Hing Lee Chiu & Lu, 1989).

Wide differences in metabolic capacities often exist, even among species phylogenetically related (Van't Klooster., 1992). Differences in the pharmacokinetic of BZ (Weir & Bogan, 1985; Bogan *et al.*, 1987; Sangster *et al.*, 1991; Hennessy *et al.*, 1993a), levamisole (Gillham & Obendorf, 1985), closantel (Hennessy *et al.*, 1993b) and clorsulon (Sundlof *et al.*, 1991; Sundlof & Whitlock, 1992) were found between sheep and goats and were attributed to a faster metabolism and, or, elimination in goats. Lower BZ bioavailability and faster elimination half-lives were also found in cattle in comparison to sheep (Weir & Bogan, 1985; Lanusse & Prichard, 1994).
Liver disease and parasite-mediated liver damage with alteration of the liver enzyme activities could affect biotransformation and bioavailability of anthelmintic drugs. Reduced enzymatic activity of various liver microsomal oxidases have been reported in *Fasciola hepatica*-infected rats (Galtier *et al.*, 1983; 1985a,b) and sheep (Galtier *et al.*, 1986b), which could lead to altered patterns of drug metabolism and clearance (Facino *et al.*, 1984; 1985; Tufenkji *et al.*, 1987; 1988; 1991) (See Chapter 6).

1.1.2 The parasite

The site of predilection, feeding habits and evolutive stage of a parasite, as well as its degree of susceptibility to the anthelmintic used are important factors which condition the clinical efficacy.

1.1.2.1 Location, feeding habits and stage

A primary condition for efficacy is the access of the antiparasitic moiety to the habitat of the parasite and this, at a sufficient concentration and for a sufficient length of time. Morantel is effective against most adult GI nematodes but since it is only distributed within the GI tract and is not systemically available, any possibility of action against adult lungworms is excluded. Oxibendazole is only active in its unchanged form and because it undergoes first-pass metabolism in the liver, it is active against GI helminths but not against *Dictyocaulus* (Delatour & Benoit, 1987). Ivermectin is effective against the major GI nematodes and lungworms of sheep and cattle; depending on the nematode, however the dose of ivermectin required for high efficacy varies (Egerton *et al.*, 1981;

Wescott & LeaMaster, 1982). In general abomasal parasites (*O. ostertagi, O. circumcincta and Trichostrongylus axei*) are killed at a lower dose than are the small intestinal parasites (*Cooperia onchophora, C. curticei, T. colubriformis and Nematodirus spp*). One explanation would be that, although phylogenetically related, nematodes of the abomasum have an inherently higher susceptibility than the nematodes of the small intestine. Another putative explanation supported by Bogan *et al* (1988b) and Bogan & McKellar (1988) relates the difference to a possible recovery and reestablishment of small intestinal parasites. Nematodes paralysed in proximal areas of the small intestine may recover before they have been expelled from the small intestine, and tend to reestablish distally, whereas for abomasal nematodes, a shorter passage time through their predilection site precludes such a recovery. This could explain why the majority of adult *C. curticei* recovered from ivermectin treated sheep were more distally located in the small intestine than those found in control animals (Bogan *et al.*, 1988b). Similar findings were reported in calves infected with *C. onchophora* (Bogan & McKellar, 1988).

The feeding mechanism of a parasite is a crucial factor for the uptake of antiparasitic compounds. Anthelmintic drugs are taken in by the parasite either orally or via the cuticle. The oral route is important in haematophageous helminths, and it is well documented that *F. hepatica* and *H. contortus* are particularly susceptible to anthelmintic drugs which strongly bind to plasma protein. The transcuticular pathway in parasite drug uptake is also very important and it is suggested that molecular size and lipophilicity are important criteria for anthelmintic compounds to cross the collagen matrix and the lipoid hypocuticular tissue (Verhoeven *et al.*, 1980; Ho *et al.*, 1990, 1992, 1994).

Anthelmintic efficacy against both immature and mature stages of nematodes and trematodes is highly desirable if an efficient control of helminthiasis is to be achieved. However, adult parasites are generally less refractory to anthelmintic treatment than tissue-dwelling immature parasites. A major drawback with the use of levamisole and morantel is their poor efficacy against developing and arrested larval stages of *Ostertagia spp* and *Haemonchus spp* in sheep, goats and cattle (Cornwell & Jones, 1970; Anderson & Marais, 1972; Gibson & Parfitt, 1973; Labourel, 1984; Prichard *et al.*, 1991; McKellar *et al.*, 1993c). Similarly, thiabendazole is ineffective against arrested larvae in cattle and in sheep; the dose has to be increased to kill hypobiotic stages. Oxibendazole and

mebendazole are also ineffective against arrested larvae (McKellar & Scott, 1990).

Flukicidal action against immature *F. hepatica* has been an intriguing problem until the introduction of triclabendazole which shows a highly satisfactory action against both early immature and mature flukes. The pharmacokinetic pattern that characterises the salicylanilides complicates the assessment of their action against immature fluke; this will be discussed more extensively later (Chaper 6).

1.1.2.2 Resistance

In the past three decades, the introduction of anthelmintic drugs and their subsequent routine use for control and treatment of parasitic deseases has been chronologically followed by the emergence of resistant strains of helminths. Reports have been published of resistance in nematode parasites to all the major groups of anthelmintics including the BZ, the levamisole/morantel group, the salicylanilides and the avermectins. Resistance in a nematode population to one drug of the BZ group is likely to apply to all the BZ's (Donald, 1983). This is designated side-resistance and has been defined as resistance to a compound which is the result of a selection by another compound with a similar mode of action (Prichard *et al.*, 1980). Resistance across groups is not uncommon and is designated cross or multiple resistance. Resistance in sheep and goat nematodes to the effect of BZ and other anthelmintics is undeniably of great economic importance, and although the problem has long been considered to inflict almost exclusively the southern hemisphere, there is evidence to suggest that the incidence of anthelmintic resistance is increasing in livestock in countries throughout the world, including the UK (Jackson, 1993). The prevalence of resistance in cattle nematodes to the effect of anthelmintics is low but there have been reports of BZ resistance in *T. axei* from Australia (Eagleson and Bowie, 1986) and BZ resistance in *C. onchophora* in New Zealand (Jackson *et al.*, 1987). It is not clear whether this low incidence results largely from host or parasite characteristics or simply reflects the bovine dosing regimes which may minimise the selection pressure.

For a proper understanding of the possible mechanisms involved in drug resistance, knowledge of the modes of action of the different anthelmintic classes is a prerequisite. Most available anthelmintic compounds exert their antiparasitic effects by interference with energy metabolism, neuromuscular coordination and microtubular function.

Benzimidazoles are known to affect parasite metabolism (Behm & Bryant, 1979). Studies on a cambendazole-resistant strain of *H. contortus* (Rew *et al.*, 1982) showed that, in the presence of BZ, adult parasites were able to increase their metabolism of glucose to volatile end-products, whereas catabolism of glucose was unaffected in a BZ-susceptible strain of *H. contortus*. Salicylanilides also interfere with energy metabolism as uncouplers of oxidative phosphorilation at the mitochondrial membrane through interruption of fumarate reduction into succinate. *Haemonchus contortus* resistance to salicylanilides has been reported in South Africa (Van Wyk *et al,* 1980; 1982; 1987) and in Australia (Rolfe and Boray, 1990). *H. contortus* resistant to salicylanilides may avoid the disruption of aerobic metabolism by some competitive mechanism which is still unclear (Rolfe, 1990).

The primary mode of action of the BZ is thought to consist of their binding to helminth tubulin causing a depolymerization of the microtubules and a general disruption of basic cell function (Borgers *et al.*, 1975; Friedman & Platzer, 1978). It has been observed that when given to nematode-infected animals, BZ cause microtubules to disappear from intestinal cells of BZ-susceptible but not BZ-resistant nematodes (Sangster *et al.*, 1985). Lacey and Prichard (1986) examined the total binding of ABZ, FBZ, MBZ, OFZ, and PBZ to tubulin extracts from BZ-resistant and BZ-susceptible parasites. It was found that extracts from BZ-resistant parasites bind less BZ than extracts from BZ-susceptible parasites.

Levamisole and morantel both act as cholinergic agonists which induce inflow of Na+ (Harrow and Gration, 1985), cause depolarisation of muscle membranes and muscle contraction (Aubry *et al.*, 1970). Differences in the binding of levamisole have been observed between susceptible and resistant worms (Lewis *et al.*, 1989). Resistance may be caused by a reduction in the number of cholinergic (levamisole) receptors or in the affinity of these receptors for levamisole.

Resistance to ivermectin appears to be developing rapidly throughout the world (Van Wyk & Malan., 1988; Echevarria & Trindade, 1989; Jackson *et al.*, 1992). Ivermectin exerts its anthelmintic action by irreversibly opening chloride channels in muscle membranes (Turner & Schaeffer, 1989). To date, no evidence has been put forward to explain the mechanism of resistance to ivermectin. The newly developed milbemycins have a similar mode of action to the avermectins. However, studies suggested that parasites resistant to ivermectin are not

necessarily resistant to the milbemycins (Craig *et al.*, 1992; Pankavich *et al.*, 1992). There is now evidence to contradict these suggestions; Shoop *et al* (1993) have found that 31 times and 9 times more moxidectin was required to achieve 95% clearance against ivermectin-resistant strains of *O. circumcincta* and *T. colubriformis*, respectively, than against susceptible strains. Similarly, Conder *et al* (1993) revealed that moxidectin failed to clear more than 47.2% of an ivermectin-resistant strain of *H. contortus* from jirds at a dose which invariably clears more than 98% of a susceptible strain. These workers consider therefore, that any rotational dosing programme should recognise that moxidectin, nemadectin and the avermectins belong to the same anthelmintic class.

Studies on the selection and genetics of resistance in nematode parasites suggest that resistance is polygenic and preadaptative (i.e, it arises from within the normal phenotypic range), and that there are three phases in the selection process. An initial susceptible phase is followed by an intermediate one in which heterozygous resistant individuals are common within the population and finally homozygous resistant individuals predominate within the population (Martin, 1990; Jackson, 1993). Administration of subtherapeutic doses favours survival of heterozygous resistant individuals thus increasing the rate of development of drug resistance (Waller & Prichard, 1986). The tendency to treat goats at the same dosage level as sheep may, because of pharmacokinetic differences, result in a faster development of anthelmintic resistance in goats herds. The frequency of treatments is also a major factor in the development of resistance; frequent anthelmintic interventions are likely to exert a strong selection pressure towards resistance (Waller & Prichard, 1986).

Reversion to susceptibility is a very slow process and is unlikely to occur through natural selection (Martin, 1990). In a field study carried out over a 4 year period, BZ resistance did not decrease significantly in the absence of chemotherapeutic control (natural selection). A parallel study which involved treatment with levamisole over the 4-year period did reduce BZ resistance but not to a level that would have allowed reintroduction of the BZs (Martin *et al.*, 1988). Similarly, reversion to BZ susceptibility was not achieved after 6 years of counterselection with levamisole (Borgsteede & Duyn 1989).

The rapid and widespread development of anthelmintic resistance, the slow pattern of reversion to susceptibility and the unlikely introduction of anthelmintic compounds which would display novel modes of action, have stimulated an interest in developing control strategies based on the use of existing anthelmintic products.

1.1.3 Exogenous Factors

These factors refer to those parameters which are related to the therapeutic intervention. It is through these factors that a beneficial manipulation of the pharmacokinetic properties of anthelmintic drugs is to be obtained. The route of administration or delivery system selected, the feeding management adopted during anthelmintic treatment and the use of drug combinations can significantly alter the efficacy of anthelmintic compounds.

1.1.3.1 Feed Effect

The residence time of drugs in the rumen depends on the flow rate of digesta. Since the rumen volume is essentially constant, digesta residence time is inversely proportional to the quantity of food ingested (Kay, 1987). The level of food intake will therefore affect drug residence time; this is particularly true for drugs that bind extensively to digesta particulate. Following oral administration of OFZ to sheep fed a daily ration of 400 or 800 gr lucerne/wheaten chaff, OFZ availability in sheep on high compared to low intake was characterised by a more rapid rate of absorption, an earlier maximum concentration in plasma, a faster rate of elimination and overall lower systemic availability (Hennessy & Ali, 1993). The sheep on a higher daily ration had a faster digesta outflow and since OFZ associates extensively to rumen particulate material, the half-life of the drug in the rumen was similar to that of particulate digesta; the faster passage rate reduced the time for dissociation, absorption and recycling. This is in contrast with what has been found in monogastrics were administering FBZ with food led to greater bioavailability (McKellar *et al.*, 1990, 1993a).

The type of food is also an important parameter that influences the residence time of anthelmintics in ruminants. Comparative studies on the pharmacokinetics of FBZ, TCBZ, rafoxanide and ivermectin in grazing sheep and sheep fed hay and concentrate have shown that lower bioavailability of the drugs were obtained in the grazing animals. When similar groups of lambs were dosed with the gastrointestinal transit

marker chromium EDTA, a more rapid throughput of chromium by the grazing lambs and a more prolonged retention of chromium by the housed lambs, were observed (Taylor *et al.*, 1992, 1993).
These physiological and pharmacological aspects of the ruminant GIT could be exploited in order to enhance the residence time of anthelmintic drugs. Hennessy & Ali, (1993) reported that halving the feed intake for 36 hours prior to treatment significantly increased the efficacy of OFZ and ABZ against BZ-resistant *H. contortus* and *T. colubriformis*. Increase in the bioavailability of ivermectin was also obtained by reducing the feed intake.

3.2 Route of administration, formulation and delivery systems

Benzimidazoles and probenzimidazoles available, with the exception of netobimin, are poorly soluble in water and are therefore administered orally, generally as suspensions to ruminants.
Netobimin is a nitrophenylguanidine, with a sulphonic acid in its structure; a feature which enhances its water solubility and permits a flexibility of administration (Prichard *et al.*, 1991). It is formulated either as a soluble zwitterion suspension for oral administration or as an aqueous trisamine salt solution for subcutaneous injection. However, following oral or intraruminal administration in both sheep (Lanusse & Prichard, 1990) and cattle (Lanusse *et al.*, 1990; 1991) the pharmacokinetic profiles of the main plasma metabolites, ABSO and ABSO2, were significantly higher than following subcutaneous administration; this is due to a more efficient conversion of the prodrug following adminstration by the former route. These pharmacokinetic differences are reflected in clinical efficacy; a higher dose rate being required when netobimin is administered parenterally, to achieve a satisfactory removal of parasites (Duncan *et al.*, 1985; Prichard, 1987).
The aqueous solubility of netobimin has also permitted its administration via drinking water to cattle (Downey, 1987; Prichard, 1987; Jacobs *et al.*, 1988). The clinical response to drinking water treatment administered over a period of 7 days was reported to be better than the response to a single oral treatment in cattle infected with *Dictyocaulus viviparus* (Jacobs *et al.*, 1988).
Selfmedication has however advantages and disadvantages. It avoids gathering and handling of animals and it also allows the spreading of anthelmintic intake over a number of days thus exposing parasites for a prolonged length of time. The use of BZ in feed blocks may be suitable for

reducing the periparturient rise in faecal egg output near the lambing period (Bogan & Marriner, 1983; McKellar, 1988). However, considerable individual variation in feed block intake often occurs and the risk of drug resistance inherent in subtherapeutic intake is not to be underestimated (Bogan & Marriner, 1983; Donald, 1985).

Differences in the persistence and efficacy of ivermectin given orally or subcutaneously have also been investigated (Marriner *et al.*, 1987; McKellar and Marriner., 1987; Borgsteede, 1993). It has been shown that following subcutaneous administration of ivermectin to periparturient ewes, the plasma concentrations of the drug were more persistent than when the drug was administered as an oral drench and that the period of reduced faecal egg output was extended by at least one week. This has been confirmed recently by Borgsteede (1993) who found a 100% persistent effect against reinfections with *H. contortus* and *C. curticei* during a period of at least 10 days when the injectable formulation was used. Zajac *et al.* (1992) suggested that a more frequent treatment regime is necessary when the oral formulation is used.

The bioavailability and efficacy of levamisole has also been shown to be higher following subcutaneous or intramuscular injection than following oral administration (Galtier *et al.*, 1981; Bogan *et al.*, 1982). Levamisole has a relatively narrow safety margin and signs of toxicity are more likely to appear after subcutaneous injection than after drenching since plasma concentrations are higher by the former route (Prichard *et al.*, 1991).

Ivermectin and levamisole are currently available as pour-on formulations for cattle. Following pour-on administration , plasma and gastrointestinal concentrations of levamisole were lower than those observed following administration by other routes (Forsyth *et al.*, 1983). The percuteneous route has been envisaged as an alternative easy and non-invasive route for ivermectin administration in goats (Scott *et al.*, 1990); a lower bioavailability but a longer persistence of the drug were observed in comparison to the oral route. Recently, a topical formulation of ivermectin has been tested in sheep but only against blow-fly strike and lice (Eagleson *et al.*, 1993; Cramer *et al.*, 1993).

Although the introduction of controlled delivery systems for anthelmintics has revolutionised the control of internal parasites in cattle, there have been, until recently, no commercially available anthelmintic products which could be administered to sheep in a slow

release manner. The observation that small amounts of BZ delivered over an extended period of time by infusion were more efficacious than the single dosing regime has been reported by Prichard *et al* (1978) and Kwan *et al* (1988). An experimental controlled release capsule (CRC) was designed for sheep to release continuous low levels of OFZ (Anderson *et al.*, 1980) and was found effective in reducing the worm egg output over extended periods. This effect is epidemiologically beneficial since it reduces considerably the larval contamination of pastures. A similar experimental CRC was successfully tested against BZ resistant *H. contortus, O. circumcincta* and *T. colubriformis* (Le Jambre *et al.*, 1981). A commercial albendazole CRC is now available for sheep. Similar to the OFZ CRC, it is based on the Laby design which relies upon small wings extruding from the barrel of the bolus when it reaches the rumen thus preventing regurgitation or further passage of the bolus. The capsule contains 3.85 gr of albendazole in the form of tablets pushed by a spring against an orifice through which they diffuse continuously in the rumen until exhaustion for 90-100 days. The daily delivery rate is 0.5 mg/kg in a 65 kg sheep. The plasma kinetics of the ABZ metabolites were studied following the administration of the bolus and it was found that in sheep, there was more of the anthelmintically inactive ABSO2 than suphoxide and in cattle more sulphoxide than sulphone (Delatour *et al.*, 1990c). This was explained by an enzyme inducing effect of ABZ in sheep. Nevertheless, the bolus does confer parasitological control and production benefit in sheep indicating adequate concentrations of active anthelmintics (Barton *et al.*, 1990; Barger *et al.*, 1992). It is also suggested that the ABZ slow release bolus may confer activity against the establishment of incoming BZ-resistant larvae (Fisher *et al.*, 1992).; however the impact of a routine use of the CRC on the incidence of BZ resistance is still to be evaluated.

The use of liposomes and lipid-protein matrices has been investigated with the aim of improving the efficacy of BZ's. Liposomes are small phospholipid vesicles used as carriers of therapeutic agents. Incorporation of albendazole-polyvinyl pyrrolidone into lecithin cholesterol liposomes followed by intraperitoneal administration to goats infected with *F. hepatica* proved more effective than an oral administration of albendazole suspension in reducing faecal egg counts. It was suggested that the liposome formulation conferred greater efficacy on albendazole and that it could be administered more precisely and at

lower dosage rates (Yang & Fung, 1991). The use of lipid-protein matrices has recently been assessed for the administration of ABZ in a formulation which has improved efficacy. The aim of the preparation is to deliver parent ABZ which has high affinity for target nematode tubulin, to the abomasum or small intestine following oral administration. This is not normally possible since ABZ is absorbed mainly from the rumen and oxidised in the liver before it diffuses back to the abomasum. The matrix preparations have been shown to resist rumen incubation for a period of 24 hours but to release the encapsulated ABZ rapidly in the abomasum and upper small intestine. Matrix formulations result in 3-4 fold increase of ABZ abomasal concentrations compared to conventional oral suspensions (Hennessy, 1992).

1.1.3.3 Drug combinations and potentiation

The purpose of anthelmintic combinations is to broaden the spectrum of activity and, or, increase the efficacy of anthelmintic preparations. For instance, flukicidal drugs have been combined with nematodicidal drugs for simultaneous treatment of fluke and worm infestations. The prelambing dose often given to ewes to reduce or remove inhibited or recently ingested nematodes and chronic fluke burdens is an appropriate time for such combination products to be used (McKellar & Kinabo, 1991). Nevertheless, the perceived advantage of these combinations should take into account the epidemiological differences that exist between nematodes and flukes; often throughout the year one of the components is unnecessary when the other is essential (McKellar, 1993).
In countries were *H. contortus* and *T. colubriformis* are predominant parasites, a control scheme termed 'Wormkill' has been dcvelopped to contend with a drug resistance problem and reduce the frequency of treatment, using a combination of closantel and a broadspectrum anthelmintic (Dash, 1986). There is a concern however about risks of resistance development as a result of persistent declining concentrations of closantel which probably discriminate the incoming larvae in favour of closantel resistant genes (Martin, 1990).
Combinations of anthelmintics to which nematode resistance is genetically unrelated and which have similar persistence in the host, e.g. levamisole and a BZ, have also been successfully used to counter the development of resistance to the individual components (Anderson *et al.*, 1991a, b). The exposure of parasites to both drugs is thought to increase

the polygenicity of resistance and therefore to slow down its development (Martin, 1990).

Combination products have also been investigated which potentiate the effects of the incorporated anthelmintics. The coadministration of parbendazole with oxfendazole has been shown to increase the anthelmintic activity of the OFZ and to confer activity against BZ-resistant nematodes (Hennessy *et al.*, 1985; 1992). This effect is believed to be due to reduced hepatic biotransformation and biliary secretion of OFZ together with increased extrabiliary secretion of the drug. Parbendazole has been shown to cause a transient, dose related reduction in bile flow rate which explains the slower excretion of OFZ given in combination with parbendazole.

It has also been shown that the coadministration of hepatic oxidation-inhibiting compounds with netobimin alter the disposition of the metabolites ABZ, ABSO and ABSO2 (Lanusse & Prichard, 1991). Methimazole is a substrate for the flavine monooxygenase system and competes with ABZ sulphoxidation (Galtier *et al.*, 1986); metyrapone is an inhibitor of cytochrome P450 and inhibits the sulphonation reaction. Both inhibitors have been shown to improve the pharmacokinetic profiles of netobimin metabolites in ruminants (Lanusse & Prichard, 1991; 1992a,b) and have been suggested as possible potentiators for the anthelmintic activity of netobimin. Improved bioavailability of anthelmintic metabolites were also obtained when FBZ and OFZ were coadministered with methimazole (Lanusse *et al.*, 1993b). A new anthelmintic drug (AB763) based on the combination of ABZ with another parasiticide (AB762) has been recently developed and it is believed that the combination enhances the bioavailability of albendazole (Page *et al.*, 1993)

1.2 Study objectives

The aims of the studies described in this thesis were:

(1) To determine the pharmacokinetics of albendazole and related compounds in goats;

(2) To explore the effects of piperonyl butoxide (PB), an inhibitor of hepatic-drug oxidative metabolism (cytochrome P450), on the pharmacokinetics of fenbendazole and albendazole and to assess the efficacy of a combination fenbendazole-piperonyl butoxide on BZ-resistant strains of nematodes in sheep;

(3) To study the *in vitro* metabolism of ABZ, FBZ and TCBZ using rat liver microsomes and primary culture of rat hepatocytes. The effects of piperonyl butoxide on the *in vitro* S-oxidations of these benzimidazole drugs were also studied.

(4) To reach a better understanding of the flukicidal action of the salicylanilides against immature *F. hepatica* using rafoxanide as a model. A novel methodology based on the use of a pharmacokinetic marker of liver function, antipyrine, was used to assess efficacy against different stages of liver fluke.

Chapter 2

Pharmacokinetics of albendazole, albendazole sulphoxide and netobimin in goats

2.1 Introduction

Albendazole, albendazole sulphoxide and netobimin are related broad spectrum anthelmintics widely used in sheep and cattle (Campbell, 1990). In goats, the efficacy of albendazole has been assessed against gastrointestinal nematodes (McKenna & Watson, 1987; Charles *et al.*, 1989), the lungworm *Mullerius capillaris* (Helle, 1986) and the liver fluke *Fasciola hepatica* (Misra *et al*, 1989). Recently, netobimin has been shown to be efficacious against mixed infection with *M. capillaris* and Benzimidazole-resistant gastrointestinal nematodes when administered orally at 7.5 mg/kg on three successive occasions or at 10 mg/kg on two successive occasions (Cabaret, 1991). Very little information has however been provided on the pharmacokinetic behaviour of the three anthelmintics in goats (Delatour *et al.*, 1991; Hennessy *et al.*, 1993). Dosage regimens have not been clearly established in this species and often are based on knowledge acquired from studies carried out in sheep and cattle.

In the present bioequivalence study, the plasma disposition of albendazole in goats is compared to that of its synthetic metabolite (albendazole sulphoxide) and precursor (netobimin).

2.2 Materials and Methods

2.2.1 Animals

Seven (07) mixed-breed healthy goats (identification numbers G1, G3, G7, G10, G11, G12 and G13) weighing 11.5 to 30.0 kg at the beginning of the experiment were randomly allocated to two groups of two animals and one group of three animals. They were maintained indoors with hay and water provided *ad libitum* and were given concentrate pellets (Lamb Weaner, Stewarts, Larbert, UK) twice a day.

2.2.2 Drug administration and sampling procedure

Animals in each group were dosed orally with albendazole (Valbazen 2.5%; Smith Kline Animal Health Ltd.), albendazole sulphoxide (Ricobendazole 2.5%; Rycovet Ltd.) or netobimin (Hapadex 2.5%; Kirby Warrick Ltd.) in a three-way crossover design protocol. A 1-2 week washout period was allowed between crossovers. The three drug products were given at a dose rate of 7.5 mg/kg bodyweight which corresponds to 2.83×10^{-5}, 2.66×10^{-5}

and 1.78 X 10^{-5} moles of active metabolite (albendazole sulphoxide) per kilogram bodyweight for albendazole, albendazole sulphoxide and netobimin, respectively. Drug suspensions were adminstered with plastic syringes (Becton Dickinson, Dublin, Ireland) placed on the back of the tongue. Blood samples were collected in lithium heparin syringes (10 ml Monovette, Sarstedt Limited, Leicester, England) by jugular venepuncture prior to drug administration and 0.25, 0.5, 1, 2, 4, 8, 12, 24, 32, 48, 72 and 96 hours thereafter. Samples were centrifuged at 1700 *g* for 10 minutes, the plasma was removed and stored at -20 º C until analysis.

2.2.3 Drug analysis

Plasma samples were analysed by High Performance Liquid Chromatography following liquid phase extraction (samples from animals treated with albendazole and albendazole sulphoxide) and solid phase extraction (samples from animals treated with netobimin).

2.2.3.1 Standard preparation

Stock solutions (100 µg/ml) of pure standard netobimin (Schering Plough Ltd., UK), albendazole, albendazole sulphoxide and albendazole sulphone (Robert Young Ltd., UK) were prepared using methanol (Rathburn Chemicals Ltd., UK) as a solvent. These were diluted to give 20, 10, 5, 2, 1 µg/ml standard solutions for calibration and for adding to drug-free plasma samples to determine the recovery.

2.2.3.2 Liquid phase extraction

This procedure enabled the extraction of albendazole and its two metabolites albendazole sulphoxide and albendazole sulphone but was not suitable for the extraction of netobimin.

The method was adapted from the procedure described by Marriner and Bogan (1980). To 2 ml of plasma sample contained in a 50 ml ground glass tube were added 200 µl of ammonium hydroxide (NH_4OH, 0.1N), 0.2 grams of sodium chloride and 20 ml of diethyl ether (Rathburn Chemicals Ltd.). Sample tubes were stoppered and shaken for 15 minutes on a a slow rotary mixer. Fifteen ml of the ether was then transfered from the sample tube to a 50 ml thin-walled glass tube. A further 20 ml of ether were added to the sample tube, the shaking procedure repeated and 20

ml of the solvent phase removed and added to the 15 ml ether from the first extraction step. The ether was evaporated under a stream of air at 50 º C until the remaining volume was 8-10 ml. It was then transfered to a 15 ml conical tube and evaporated to dryness. the residue was reconstituted in 0.30 to 0.60 ml of methanol (depending on the amount of drug expected), placed in an ultrasonic water bath for 1 minute and finally injected onto the chromatograph.

2.2.3.3 Solid phase extraction

This procedure enabled the extraction of netobimin, albendazole, albendazole sulphoxide and albendazole sulphone and was used for plasma samples collected from goats treated with netobimin.
Samples were processed according to the method developed by Allan *et al.* (1980) and modified by Hennessy *et al.* (1985). C18 Sep Pak cartridges (Waters Chromatography Division, Milfort, USA) were used. Each cartridge was prewashed with 5 ml of methanol followed by 5.0 ml of 0.017M ammonium dihydrogen phosphate buffer (pH 5.5). Plasma (1 ml) was then applied and the cartridge was washed successively with 20 ml of distilled water, 0.5 ml of 40% methanol and 0.4 ml of 100% methanol. Netobimin and metabolites were finally eluted with 2.5 ml of 100% methanol and the elution product evaporated to a volume of 0.4 ml for analysis.

2.2.3.4 HPLC system

This comprised a solvent delivery pump (Gilson Model 802; Scotlab instrument Sales Ltd., Glasgow, UK) connected to an ODS-Hypersil (5µ) column (16 cm X 5 mm) (Shandon Southern, Cheshire, UK) and a UV detector (Model SP100 or model SP 8450, Burke Electronics Ltd, Glasgow, UK). For the quantification of albendazole, the mobile phase was a mixture methanol:0.05M ammonium carbonate (70:30, v/v) running at a flow rate of 1 ml/minute, whereas for the elution of the sulphoxide and the sulphone metabolites a mixture methanol: water (80:20) to which perchloric acid was added (1 µl of 1.1 % w/v perchloric acid per ml of solvent mixture), was used at the same flow rate. A mobile phase similar in composition (but not in proportion) to the latter was used for the elution of netobimin (47:53 methanol:water to which perchloric acid 1.1% was added at a proportion of 7 µl per ml of solvent), this solvent was run at 1.6 ml/minute. Albendazole and its two metabolites were analysed in

two separate systems and, where netobimin was to be measured, the HPLC system in which the sulphoxide and sulphone metabolites were analysed was used after washing with methanol for 20 minutes and running the appropriate solvent for 30 minutes. Absorbance was set at 0.01 AUFS (ABSO and ABSO2), 0.05 AUFS (ABZ) and 0.005 AUFS (netobimin). Wavelenghts were 292 nm for ABZ, ABSO and ABSO2, and 347 nm for netobimin. Retention times were 2.7 minutes (ABZ), 3.2 minutes (ABSO), 2.2 minutes (ABSO2) and 2.4 minutes (netobimin).

2.2.3.5 Recovery and precision

Recovery of the four compounds from plasma to which the drugs had been added was evaluated by reference to peak heights resulting from direct injection of standard solutions. The precision of extractions and chromatographic procedures was assessed by the coefficients of variation of replicate plasma samples to which known amount of drug had been added and analysed. Recoveries and coefficient of inter-assay variations are reported in Appendix A-1 and A-2. Limits of detection as assessed by a peak height of one unit were 0.01 µg/ml for ABZ and ABSO2, 0.025 µg/ml for ABSO and 0.04 µg/ml for netobimin.

The concentrations of drug (and metabolites) in unknown samples were calculated by reference to plasma samples to which known amounts of drug (and metabolites) had been added and taken through the analytical procedure.

2.2.4 Pharmacokinetic and statistical analysis

The maximum plasma concentration (Cmax) and time to maximum concentration (tmax) were determined from observed values. The area under the plasma concentration-time curve (AUCobs) and the area under the first-moment curve (AUMCobs) were calculated from observed values using the trapezoidal rule. The mean residence time (MRT) was calculated as the ratio AUMCobs/AUCobs. A non-parametric statistical test (Wilcoxon signed rank-test) was used to compare pharmacokinetic parameters obtained with different drug preparations and a value of $P < 0.05$ was considered significant.

2.3 Results

Following its oral administration netobimin was not detectable in plasma at any time; neither was its sulphide metabolite albendazole. Albendazole was also below the limit of analytical detection when given as the parent drug or when the sulphoxide form was administered. Albendazole sulphoxide and albendazole sulphone were present in the plasma, following administration of each moiety, for 32 to 48 hours (Tables 2-1 and 2-3). Fifteen minutes after drug administration, ABSO appeared in the plasma of most animals (6 out of 7) treated with albendazole sulphoxide (Table 2-1; Appendix A-7). When albendazole and netobimin were administered, albendazole sulphoxide was first detectable in only one goat (G11) at 15 minutes (albendazole) (Appendix A-3) and 30 minutes (netobimin) (Appendix A-11) postadministration, with the remaining goats showing initial detectable levels at 0.5 to 1 hour (albendazole) and 1 hour (netobimin) postadministration. Individual concentrations of albendazole sulphone following administration of albendazole, albendazole sulphoxide and netobimin are given in Appendices A-5, A-9 and A-13, respectively. The plasma concentration versus time profiles of the two metabolites albendazole sulphoxide and albendazole sulphone following administration of the three anthelmintic preparations are shown in Figure 2-1 and Figure 2-2, respectively. The pharmacokinetic parameters of both metabolites (Table 2-2 and 2-4; Appendices A-4, A-6, A-8, A-10) were not significantly different when the albendazole and the albendazole sulphoxide treatments were compared. However, the areas under the plasma concentration time curves (AUC) and maximum concentrations (Cmax) were significantly lower ($P<0.05$) following netobimin administration (Tables 2-2 and 2-4; Appendices A-12 and A-14). The mean residence time (MRT) and time to maximum concentration (tmax) were similar for the three formulations. The ratios AUC (ABSO2)/AUC (ABSO) were 0.55, 0.51 and 0.56 for albendazole, albendazole sulphoxide and netobimin, respectively.

2.4 Discussion

The anthelmintic activity of netobimin is conditioned by the bioconversion of the prodrug into albendazole (Figure 2-3). The biotransformation is exerted by the gastrointestinal microflora and consists of a reduction and a secondary cyclization to form the

Table 2-1. Plasma concentrations of albendazole sulphoxide (ABSO) (mean ± SEM) following administration of albendazole, albendazole sulphoxide and netobimin to goats at a dose rate of 7.5 mg/kg bodyweight.

	Mean ± SEM (n=7).		
Time (hours)	Albendazole	Albendazole sulphoxide	Netobimin
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.25	0.01 ± 0.01	0.04 ± 0.01	0.00 ± 0.00
0.5	0.07 ± 0.02	0.16 ± 0.01	0.00 ± 0.00
1	0.20 ± 0.02	0.45 ± 0.05	0.13 ± 0.02
2	0.49 ± 0.05	1.00 ± 0.09	0.46 ± 0.06
4	0.94 ± 0.09	1.89 ± 0.21	0.89 ± 0.09
8	2.01 ± 0.16	2.59 ± 0.23	1.28 ± 0.13
12	2.37 ± 0.15	2.70 ± 0.20	1.30 ± 0.13
24	1.54 ± 0.17	1.55 ± 0.21	0.77 ± 0.11
32	0.67 ± 0.20	0.64 ± 0.20	0.26 ± 0.08
48	0.01 ± 0.01	0.02 ± 0.02	0.00 ± 0.00
72	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
96	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

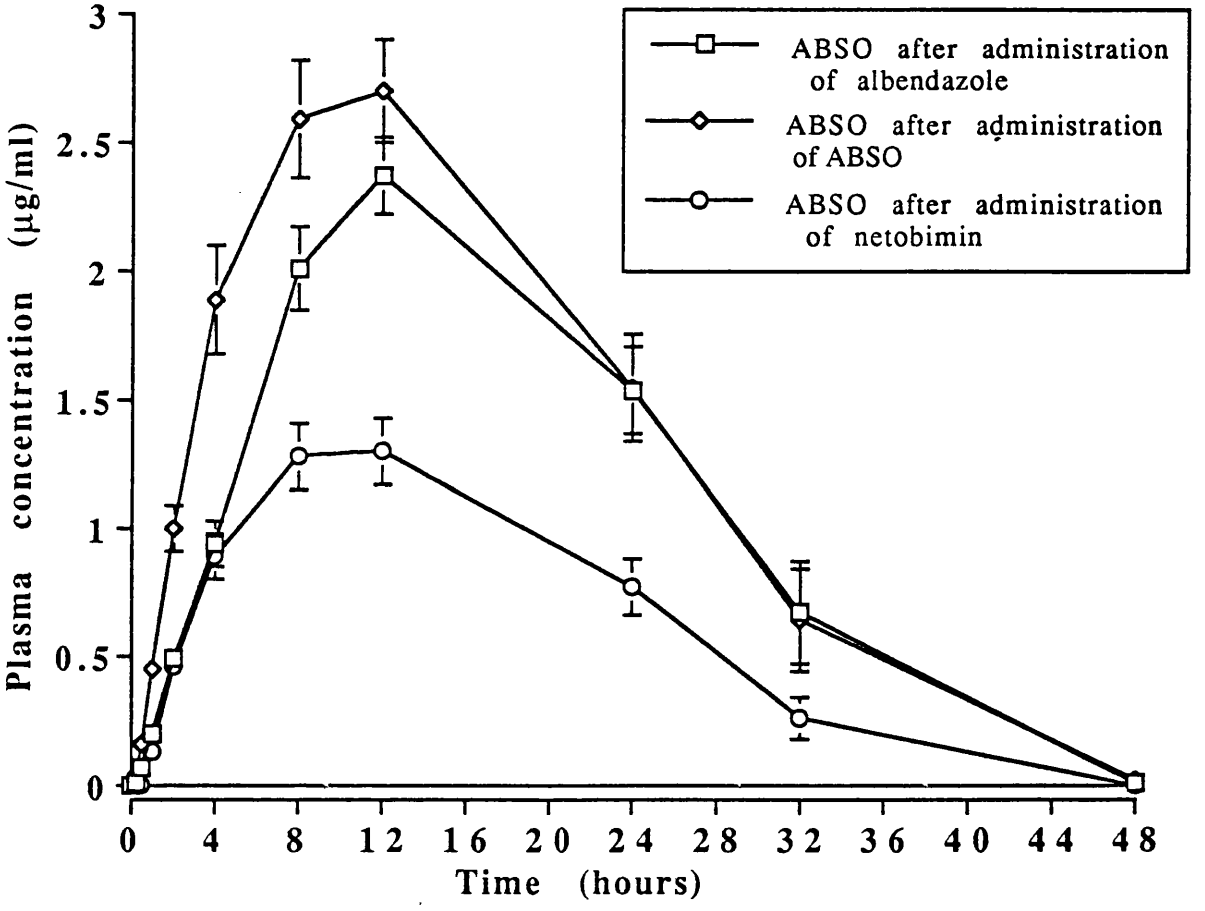

Figure 2-1. Plasma concentrations of albendazole sulphoxide (mean ± SEM) following oral administration of albendazole, albendazole sulphoxide and netobimin to goats (n=7) at a dose rate of 7.5 mg/kg bodyweight.

Table 2-2. Pharmacokinetic parameters for albendazole sulphoxide (ABSO) (mean ± SEM) following administration of albendazole, albendazole sulphoxide and netobimin to goats at a dose rate of 7.5 mg/kg bodyweight.

	Mean ± SEM (n=7)		
	ABSO		
	Albendazole	albendazole sulphoxide	Netobimin
AUC_{obs} (μg.h/ml)	54.41 ± 5.96	63.06 ± 7.16	29.75 ± 3.14*
$AUMC_{obs}$(μg.h^2/ml)	943.3 ± 145.5	1003.8 ± 163.0	459.49 ± 62.87
MRT (h)	16.96 ± 0.63	15.43 ± 0.87	15.15 ± 0.83
Cmax (μg/ml)	2.29 ± 0.12	2.77 ± 0.20	1.36 ± 0.13*
tmax (h)	11.43 ± 0.57	11.43 ± 0.57	12.57 ± 2.03

* Significantly different ($P<0.05$)

Table 2-3. Plasma concentrations of albendazole sulphone (ABSO2) (mean ± SEM) following administration of albendazole, albendazole sulphoxide and netobimin to goats at a dose rate of 7.5 mg/kg bodyweight.

	Mean ± SEM (n=7)		
Time (hours)	Albendazole	Albendazole sulphoxide	Netobimin
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.25	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1	0.01 ± 0.00	0.02 ± 0.01	0.00 ± 0.00
2	0.03 ± 0.01	0.07 ± 0.01	0.02 ± 0.01
4	0.08 ± 0.01	0.18 ± 0.02	0.08 ± 0.01
8	0.25 ± 0.02	0.41 ± 0.03	0.19 ± 0.01
12	0.50 ± 0.05	0.62 ± 0.04	0.34 ± 0.02
24	0.97 ± 0.07	1.08 ± 0.06	0.61 ± 0.03
32	1.04 ± 0.10	0.94 ± 0.13	0.49 ± 0.09
48	0.12 ± 0.11	0.10 ± 0.09	0.00 ± 0.00
72	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
96	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

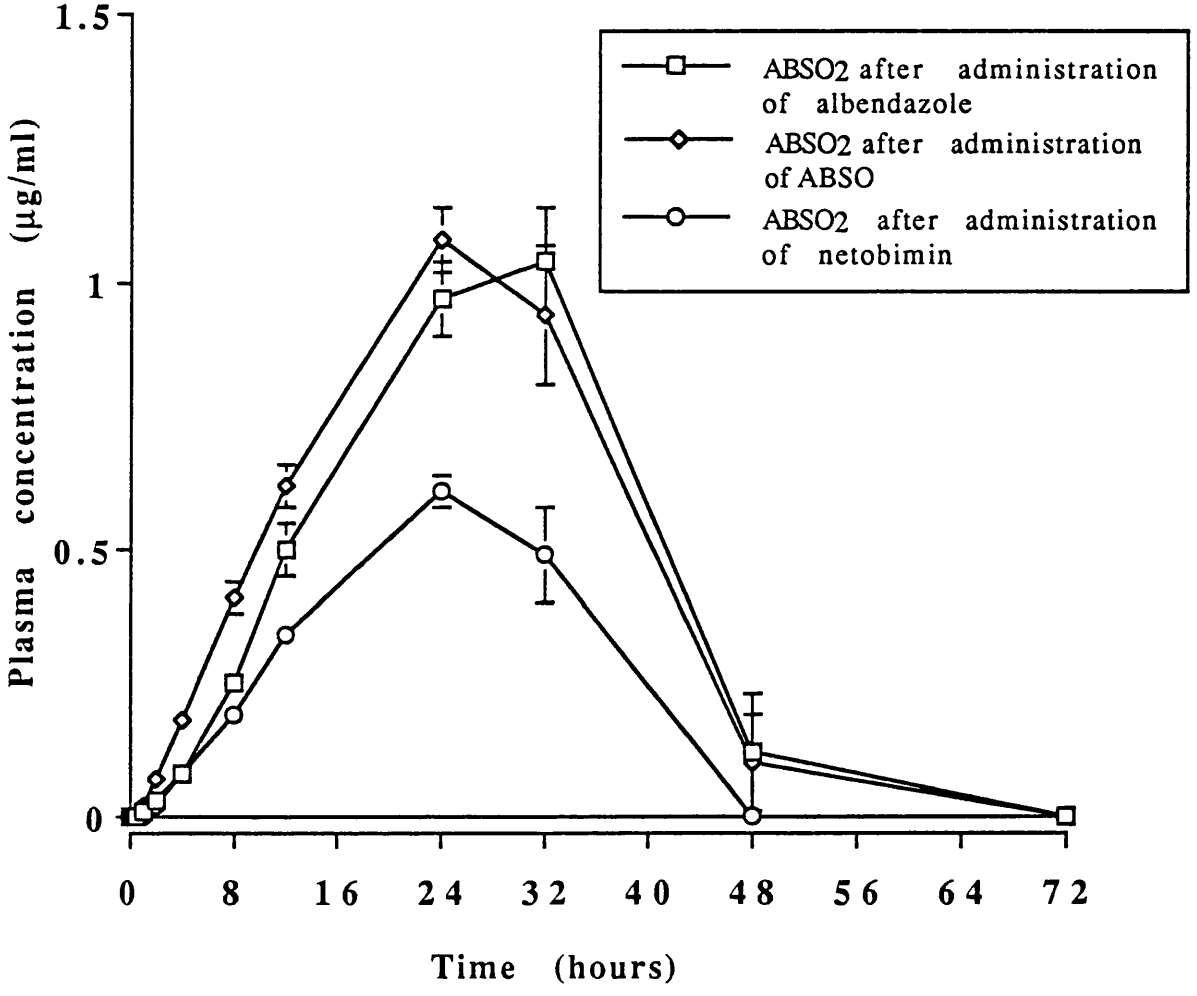

Figure 2-2. Plasma concentrations of albendazole sulphone (mean ± SEM) following oral administration of albendazole, albendazole sulphoxide and netobimin to goats at a dose rate of 7.5 mg/kg bodyweight (n=7).

Table 2-4. Pharmacokinetic parameters for albendazole sulphone (ABSO2) (mean ± SEM) following administration of albendazole, albendazole sulphoxide and netobimin to goats at a dose rate of 7.5 mg/kg bodyweight.

	Mean ± SEM (n=7)		
	ABSO2		
	Albendazole	Albendazole sulphoxide	Netobimin
AUC_{obs} (μg.h/ml)	29.85 ± 3.03	31.26 ± 2.22	15.75 ± 0.99*
$AUMC_{obs}$(μg.h^2/ml)	803.1 ± 122.9	787.67 ± 93.06	374.28 ± 31.18
MRT (h)	26.34 ± 1.32	24.78 ± 1.23	23.54 ± 0.74
Cmax (μg/ml)	1.08 ± 0.07	1.13 ± 0.05	0.63 ± 0.03*
tmax (h)	30.86 ± 1.14	28.57 ± 1.62	26.29 ± 1.48

* Significantly different ($P<0.05$)

active drug (Delatour *et al.*, 1986). Absorption of netobimin from the gastrointestinal tract has been reported in sheep following intraruminal administration of the prodrug at a dose rate of 20 mg/kg (Lanusse and Prichard, 1990). However, in the same species only trace amounts of the prodrug were detected when the dose was reduced to 7.5 mg/kg (Lanusse and Prichard, 1992) and in cattle even at 20 mg/kg, netobimin was not detectable following oral administration (Lanusse *et al.*, 1991). In the present study, netobimin was not detected in the plasma and this suggests an efficient conversion of the prodrug into the active form.

Albendazole was not detectable in the jugular blood and this is similar to results obtained in sheep and cattle (Marriner and Bogan 1980, Prichard *et al.*, 1985a; Hennessy *et al.*, 1989). Considering the rapid formation of the sulphoxide metabolite, a first-pass oxidation in the liver, and possibly in the gut mucosa, is the likely explanation for the absence of albendazole in the systemic circulation.

A delay in the appearence of the sulphoxide metabolite was observed when albendazole or netobimin were administered in comparison to the direct administration of the sulphoxide. This delay reflects the time required for the metabolic processes to occur; this was clearly shown with netobimin when the first detection of albendazole sulphoxide was observed 45 minutes later than the first appearance when the sulphoxide itself was administered.

Albendazole sulphone appeared more slowly than the sulphoxide as evidenced by the difference in the tmax of the two metabolites and this is in accordance with the slow nature of sulphonation (Souhaili-El Amri *et al.*, 1988a,b). The ratio AUC (ABSO2)/AUC (ABSO) ranging between 0.51 and 0.56 for the three drug products is consistent with that reported in goats by Delatour *et al.* (1991) (ratio= 0.56) and Benoit *et al.* (1992) (ratio= 0.59) after treatment with albendazole.

There was bioequivalence between albendazole and albendazole sulphoxide since no major differences were noted in the pharmacokinetic parameters of the two compounds given at the same dose rate. Tiberghien & Bogan (1987) have shown a similar equipotency in sheep and cattle. The rapid sulphoxidation of albendazole in the liver together with the reduction of the sulphoxide drug into the sulphide form by the ruminal microflora

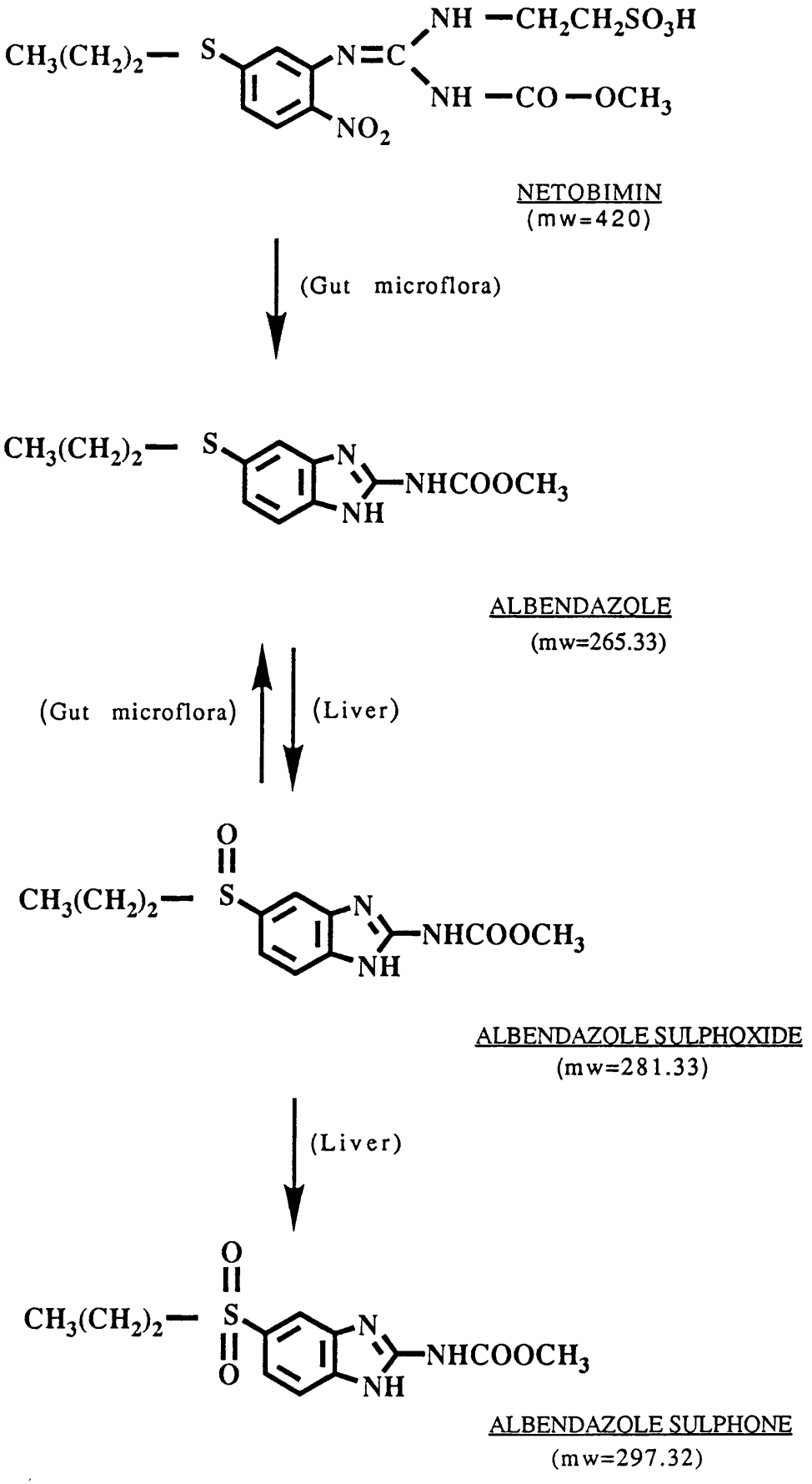

Figure 2-3. Biotransformation of netobimin

are the two phenomenon that make the fate of the two compounds indistinguishible and help to explain the similar plasma disposition observed between the parent drug and the metabolite.
The systemic availability of ABSO and ABSO2 was lower with netobimin than with the sulphide and the sulphoxide drugs. Since the molecular weight of the prodrug is higher than that of the sulphide and the sulphoxide metabolites (Figure 2-3), the dosage rate should be adjusted on the molar basis for bioequivalence. Netobimin is recommended at the same dose rate (7.5 mg/kg) as albendazole for the treatment and control of lungworm and adult gastrointestinal nematode infections in cattle but to achieve efficacy against type-II ostertagiasis in this species, the dose rate of netobimin has to be increased to 20 mg/kg bodyweight.

In conclusion albendazole and albendazole sulphoxide are bioequivalent. However, if a dose rate of 7.5 mg/kg is adopted for the use of these two compounds in goats, the adequate equivalent dosage for netobimin would be 58.9% higher (i. e. 11.9 mg/kg).

Chapter 3

Effect of piperonyl butoxide on the pharmacokinetics of albendazole and fenbendazole in sheep and goats

3.1 Introduction

Benzimidazole thioether anthelmintics are extensively metabolised into their sulphoxides which in turn are oxidized into the more polar and less anthelmintically active sulphone metabolites. Slowing down the sulphoxidation and, or, the sulphonation of these compounds could be a useful means of prolonging the residence time of the active moieties (sulphide and, or sulphoxide) in the host's body thus optimizing the exposure of parasites to their lethal action.

Piperonyl butoxide (PB) (Figure 3-1) is a synthetic methylenedioxyphenyl derivative widely used as a pyrethrin synergist (O'Brian, 1967; Haley, 1978; Wayne Ivie & Rowe, 1986) and has been shown to inhibit mixed function oxidase activity in insects as well as in mammalians (Franklin, 1977; Haley, 1978). Inhibition of the cytochrome P450 activity was observed in microsomal preparations from goats treated for three consecutive days with piperonyl butoxide (Burley & Bray, 1983).

The present study was carried out in order to investigate the effect of piperonyl butoxide as a metabolic inhibitor on the pharmacokinetics of fenbendazole in goats and albendazole in sheep and goats.

3.2 Materials and Methods

3.2.1 Animals

Six mixed breed goats and five Scottish Balckface sheep were used. They were kept indoors throughout the experimental period with hay and water provided *ad libitum* and concentrate twice a day.

3.2.2 Experimental design

3.2.2.1 Goats

Each goat received fenbendazole and albendazole alone or with piperonyl butoxide in a 4-phase randomized crossover study. A 4-week washout period was allowed between phases.

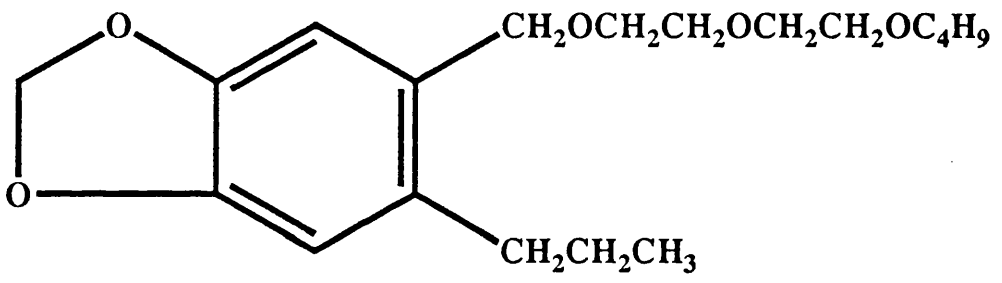

Figure 3-1. Chemical structure of piperonyl butoxide.

3.2.2.2 Sheep

Sheep were allocated to two groups (one group of three animals and another group of two animals) and were administered the drugs in a two-way crossover design protocol, i.e, in the first phase one group received albendazole alone while the second group received both albendazole and piperonyl butoxide and in the second phase each group received the alternative treatment in such a way that at the end of the experiment both treatments had been given sequentially to every animal used. A 4-week washout period was allowed between the experimental phases.

3.2.3 Drug administration and sampling procedure

Oral drenches of albendazole (Valbazen 2.5%) and fenbendazole (Panacur 2.5%) were given at a dose rate of 7.5 mg/kg bodyweight. Piperonyl butoxide (Aldrich Chemicals) was administered intramuscularly at a dose rate of 0.5 g/kg bodyweight, one hour prior to anthelmintic administration.

Blood samples were taken as previously described at 0, 0.25, 0.5, 1, 2, 4, 8, 12, 24, 32, 48, 72 and 96 hours following administration of albendazole and fenbendazole. For the measurment of piperonyl butoxide concentrations in plasma, two additional samples were taken 0.25 and 0.5 hours after piperonyl butoxide administration. Samples were stored at -20 º C and analysed for the measurement of ABZ/FBZ metabolites and piperonyl butoxide concentrations.

3.2.4 Drug analysis

3.2.4.1 Standard preparation

Standards of ABZ metabolites (Robert Young Ltd., UK), FBZ metabolites (Hoechst Ltd., Gebaude, Germany) and piperonyl butoxide (Aldrich Chemicals Ltd., UK) were prepared as described previously (see Section 2.2.3.1).

3.2.4.2 Extraction

In order to extract simultaneously piperonyl butoxide and BZ metabolites, an extraction method involving plasma precipitation was developed. Half a ml of plasma was precipitated with 1ml of acetonitrile and vortexed for 30 secondes, 4 ml of chloroform were added and the tube was shaken on a slow rotary mixer for 10

minutes. After centrifugation at 1700 *g*, 4 ml of the organic phase were transfered to a thin-walled 10 ml conical glass tube and evaporated to dryness at 50 º C under air. The residue was reconstituted with 150 µl of methanol, placed in an ultrasonic water bath for 1 minute and finally injected.

3.2.4.3 HPLC system

HPLC conditions were similar to those described in Chapter 2 (Section 2.2.3.4) except for the solvent mixture used for the elution of ABSO, ABSO2 and piperonyl butoxide. This was a mixture methanol:water (87:13) to which 160 µl of 1.1% perchloric acid per 100ml of solvent were added; the flow rate was 0.6 ml/min. The retention times were 2.50, 3.10 and 5.00 minutes for albendazole sulphone, piperonyl butoxide and albendazole sulphoxide respectively (Figure 3-2). Recoveries and precision of the method are reported in Appendices B-1 and B-2.

3.2.5 Pharmacokinetic and statistical analysis

Pharmacokinetic parameters were estimated as previously described (see Section 2.2.4).

Differences between treatments were analysed by a Wilcoxon signed rank test for data obtained in goats. Pharmacokinetic data obtained in sheep were investigated for normality using normal probability plots and correlation analysis and were then analysed by a paired Student-t test. Comparisons between sheep and goats were carried out using a Mann-Witney U-test. Differences were judged significant when $P<0.05$.

3.3 Results

3.3.1 Fenbendazole and albendazole metabolites in goats

Plasma concentrations of fenbendazole (Table 3-1, Appendix B-3 and B-5), fenbendazole sulphoxide (Table 3-3, Appendices B-7 and B-9) and fenbendazole sulphone (Table 3-5, Appendix B-11 and B-13) are plotted in Figures 3-3, 3-4 and 3-5, respectively. Mean pharmacokinetic parameters of these metabolites are shown on Tables 3-2, 3-4 and 3-6 (see Appendix B for individual values). Mean maximum concentrations (Cmax) achieved following administration of fenbendazole alone were 0.19, 0.25 and

Table 3-1. Plasma concentrations of fenbendazole (FBZ) (μg/ml) in goats following administration of fenbendazole (7.5 mg/kg) either alone or in combination with piperonyl butoxide (0.5 g/kg).

	Mean ± SEM (n=6)	
Time (hours)	Fenbendazole alone	Fenbendazole with Piperonyl butoxide
0	0.00 ± 0.00	0.00 ± 0.00
0.25	0.00 ± 0.00	0.00 ± 0.00
0.50	0.00 ± 0.00	0.00 ± 0.00
1	0.00 ± 0.00	0.01 ± 0.00
2	0.02 ± 0.01	0.05 ± 0.02
4	0.09 ± 0.02	0.14 ± 0.04
8	0.19 ± 0.03	0.32 ± 0.05
12	0.18 ± 0.02	0.37 ± 0.05
24	0.13 ± 0.02	0.40 ± 0.08
32	0.07 ± 0.02	0.31 ± 0.05
48	0.01 ± 0.01	0.14 ± 0.04
72	0.00 ± 0.00	0.02 ± 0.01
96	0.00 ± 0.00	0.00 ± 0.00

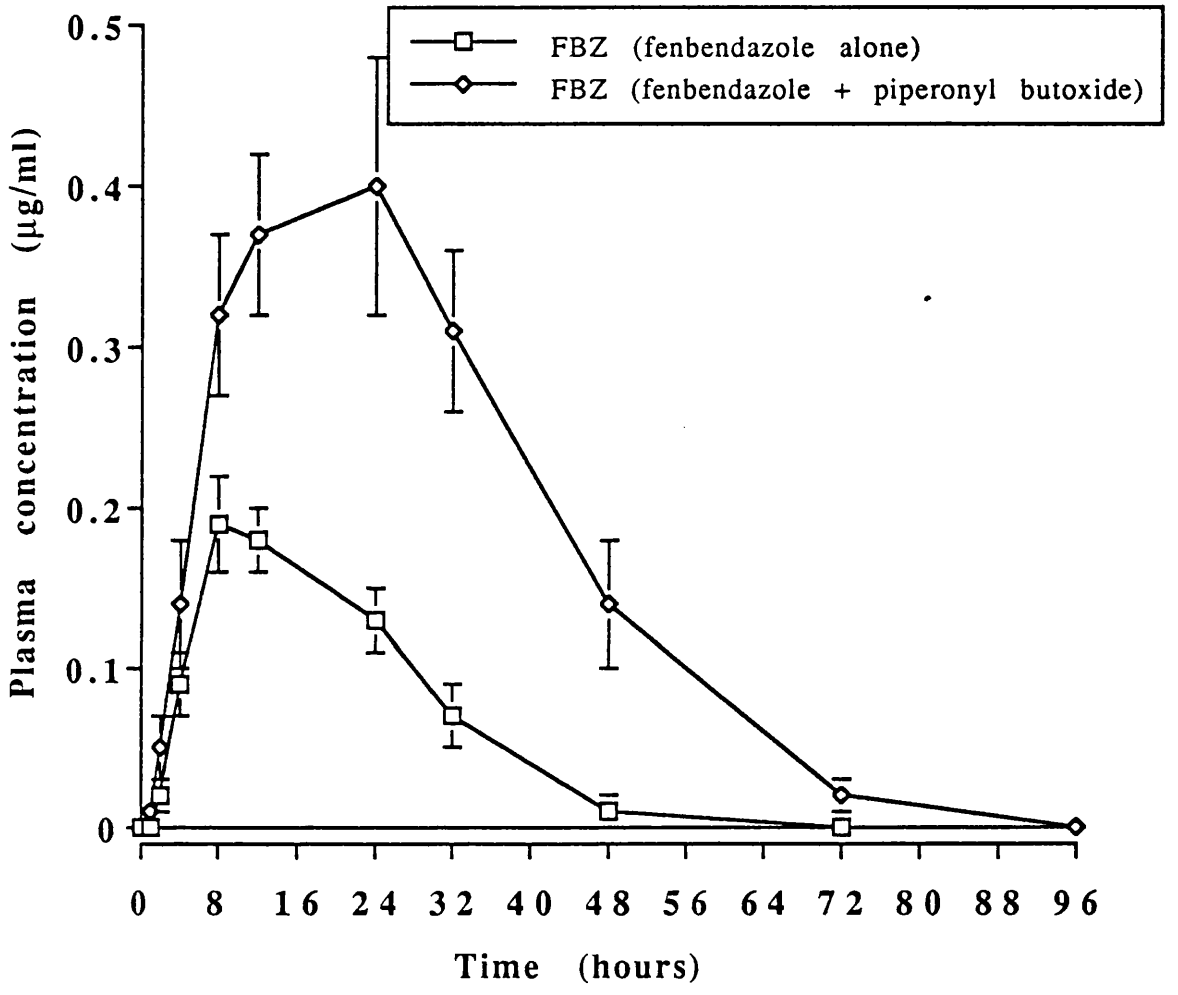

Figure 3-3. Plasma concentrations of fenbendazole (mean ± SEM) following administration of fenbendazole alone (n=6) or with piperonyl butoxide (n=6) in goats.

Table 3-2. Pharmacokinetic parameters of fenbendazole (FBZ) in goats following administration of fenbendazole (7.5 mg/kg) either alone or in combination with piperonyl butoxide (0.5 g/kg).

	Mean ± SEM (n=6)	
	FBZ	
	Fenbendazole alone	Fenbendazole with piperonyl butoxide
AUC_{obs} (μg.h/ml)	4.76 ± 0.65	15.59 ± 2.52*
$AUMC_{obs}$(μg.h^2/ml)	88.83 ± 15.99	422.68 ± 89.74*
MRT (h)	18.42 ± 1.03	25.85 ± 1.85*
Cmax (μg/ml)	0.19 ± 0.02	0.45 ± 0.07
tmax (h)	9.00 ± 0.02	16.33 ± 2.75

* Significantly different from fenbendazole alone ($P < 0.05$)

Table 3-3. Plasma concentrations of fenbendazole sulphoxide (FBSO) (μg/ml) in goats following administration of fenbendazole (7.5 mg/kg) either alone or in combination with piperonyl butoxide (0.5 g/kg).

	Mean ± SEM (n=6)	
Time (hours)	Fenbendazole alone	Fenbendazole with Piperonyl butoxide
0	0.00 ± 0.00	0.00 ± 0.00
0.25	0.00 ± 0.00	0.00 ± 0.00
0.50	0.00 ± 0.00	0.00 ± 0.00
1	0.00 ± 0.00	0.00 ± 0.00
2	0.00 ± 0.00	0.00 ± 0.00
4	0.02 ± 0.01	0.03 ± 0.01
8	0.14 ± 0.02	0.21 ± 0.04
12	0.22 ± 0.03	0.44 ± 0.08
24	0.25 ± 0.03	0.58 ± 0.08
32	0.19 ± 0.03	0.60 ± 0.08
48	0.03 ± 0.01	0.31 ± 0.08
72	0.00 ± 0.00	0.03 ± 0.02
96	0.00 ± 0.00	0.00 ± 0.00

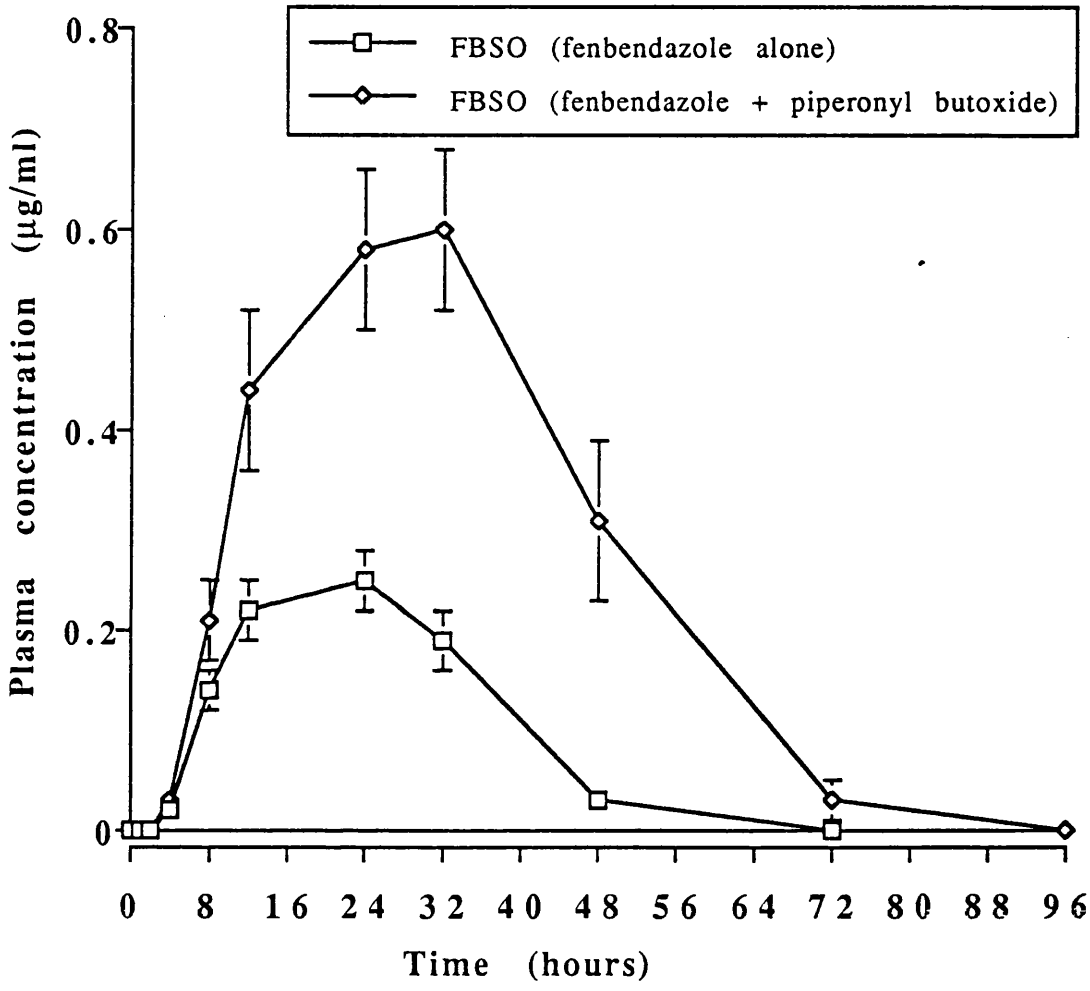

Figure 3-4. Plasma concentrations of fenbendazole sulphoxide (mean ± SEM) following administration of fenbendazole alone (n=6) or with piperonyl butoxide (n=6) in goats

Table 3-4. Pharmacokinetic parameters of fenbendazole sulphoxide (FBSO) (μg/ml) in goats following administration of fenbendazole (7.5 mg/kg) either alone or in combination with piperonyl butoxide (0.5 g/kg).

	Mean ± SEM (n=6)	
	FBSO	
	Fenbendazole alone	Fenbendazole with piperonyl butoxide
AUC_{obs} (μg.h/ml)	7.72 ± 0.99	24.40 ± 3.45*
$AUMC_{obs}$(μg.h^2/ml)	186.44 ± 28.48	772.99 ± 142.76*
MRT (h)	23.95 ± 1.01	30.70 ± 2.02*
Cmax (μg/ml)	0.25 ± 0.03	0.65 ± 0.08*
tmax (h)	23.00 ± 1.00	26.67 ± 1.33

* Significantly different from fenbendazole alone ($P < 0.05$)

Table 3-5. Plasma concentrations of fenbendazole sulphone (FBSO2) (μg/ml) in goats following administration of fenbendazole (7.5 mg/kg) either alone or in combination with piperonyl butoxide (0.5 g/kg).

	Mean ± SEM (n=6)	
Time (hours)	Fenbendazole alone	Fenbendazole with Piperonyl butoxide
0	0.00 ± 0.00	0.00 ± 0.00
0.25	0.00 ± 0.00	0.00 ± 0.00
0.50	0.00 ± 0.00	0.00 ± 0.00
1	0.00 ± 0.00	0.00 ± 0.00
2	0.00 ± 0.00	0.00 ± 0.00
4	0.00 ± 0.00	0.01 ± 0.01
8	0.02 ± 0.00	0.04 ± 0.01
12	0.04 ± 0.01	0.06 ± 0.02
24	0.09 ± 0.01	0.08 ± 0.02
32	0.09 ± 0.01	0.09 ± 0.01
48	0.03 ± 0.01	0.11 ± 0.01
72	0.00 ± 0.00	0.05 ± 0.01
96	0.00 ± 0.00	0.01 ± 0.00

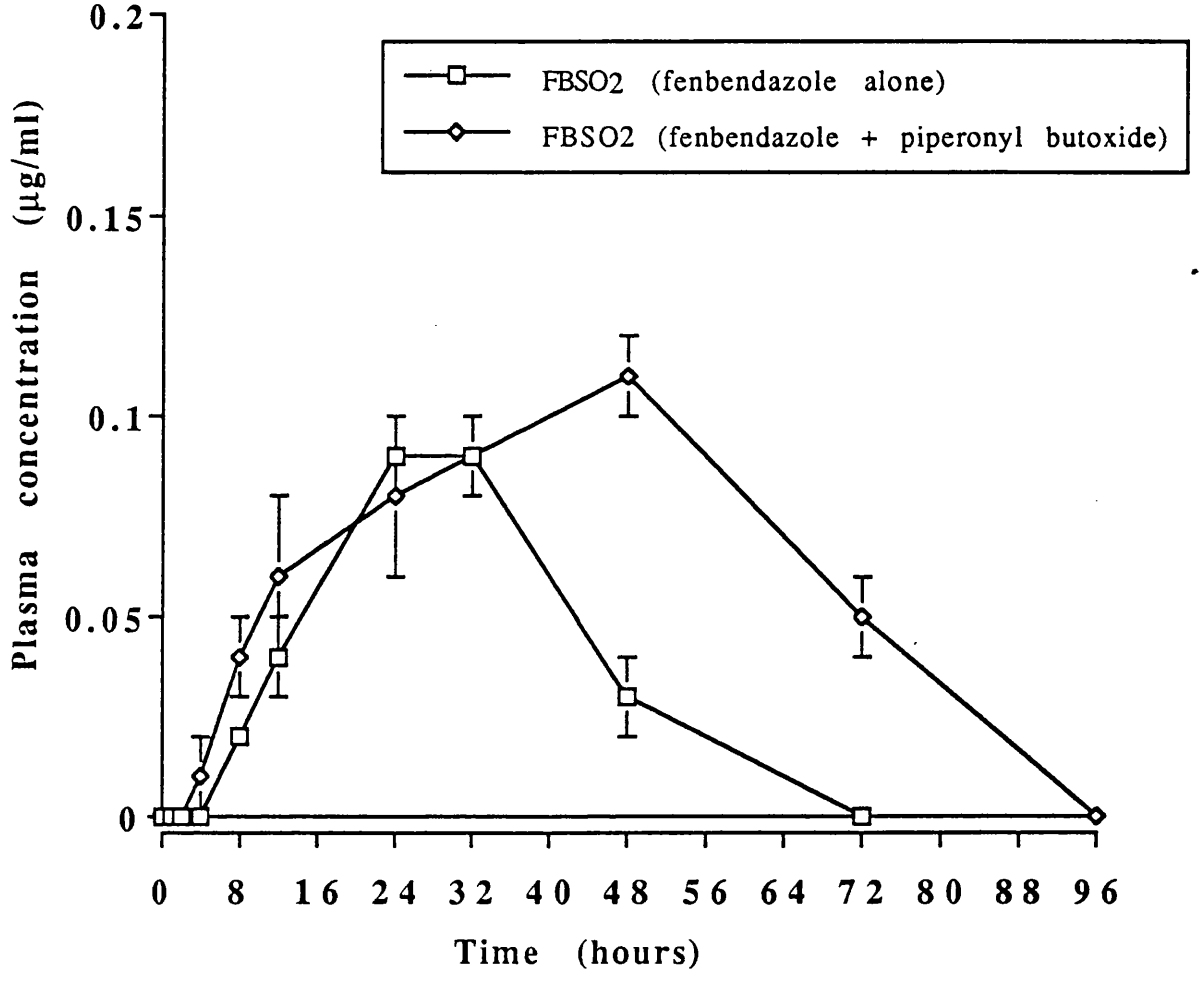

Figure 3-5. Plasma concentrations of fenbendazole sulphone (mean ± SEM) following administration of fenbendazole alone (n=6) or with piperonyl butoxide (n=6) in goats.

Table 3-6. Pharmacokinetic parameters of fenbendazole sulphone (FBSO2) (μg/ml) in goats following administration of fenbendazole (7.5 mg/kg) either alone or in combination with piperonyl butoxide (0.5 g/kg).

	Mean ± SEM (n=6)	
	FBSO2	
	Fenbendazole alone	Fenbendazole with piperonyl butoxide
AUC_{obs} (μg.h/ml)	3.02 ± 0.40	6.03 ± 0.68*
$AUMC_{obs}$(μg.h^2/ml)	104.17 ± 15.93	262.36 ± 29.22*
MRT (h)	34.37 ± 1.52	43.90 ± 3.20*
Cmax (μg/ml)	0.08 ± 0.01	0.12 ± 0.01
tmax (h)	29.33 ± 1.33	34.00 ± 5.82

* Significantly different from fenbendazole alone ($P < 0.05$).

0.08 µg/ml for FBZ, FBSO and FBSO2, respectively; when fenbendazole was given after piperonyl butoxide administration, the Cmax values for each of these metabolites were increased by137%, 160% and 50%, respectively. Areas under the plasma concentrations versus time curves and mean residence times were significantly increased ($P < 0.05$) for FBZ and metabolites when piperonyl butoxide was given prior to fenbendazole administration. The concentrations of albendazole were under the limit of detection when administered alone or in combination with piperonyl butoxide. Albendazole sulphoxide and albendazole sulphone plasma concentrations are shown in Tables 3-7 and 3-9, Appedices B-15, B-17, B-19 and B-21 and Figures 3-6 and 3-7. Large interindividual variations were observed and although a 93% increase in the AUC of ABSO2 occured after pretreatment with piperonyl butoxide, there were no statistically significant changes in the kinetic variables of albendazole metabolites (Tables 3-8 and 3-10, Appendices B-16, B-18, B-20 and B-22).

3.3.2 Albendazole metabolites in sheep

Given alone or in combination with piperonyl butoxide, albendazole was not detected in plasma at any sampling time. Plasma concentrations of the sulphoxide and the sulphone metabolites are reported in Tables 3-11 and 3-13, Appendices B-23, B-25, B-27 and B-29 and plotted in Figures 3-8 and 3-9. The AUC of the sulphoxide metabolite (ABSO) was increased from 25.61 µg.h/ml (albendazole alone) to 45.41 µg.h/ml (albendazole with piperonyl butoxide)(Table 3-12; Appendices B-24 and B-26). The MRT and the tmax of the same metabolite were prolonged by 4.86 and 6.00 hours, respectively, when the combination ABZ-PB was given. Increases in the AUC and MRT of ABSO2 were also observed with the combination (Table3-14; Appendices B-28 and B-30).

3.3.3 Piperonyl butoxide in sheep and goats

Piperonyl butoxide was slowly absorbed following intramuscular administration. It reached plasma levels of 0.26 µg/ml in goats and 0.45 µg/ml in sheep 10 minutes after injection and peak plasma concentrations occured 10 hours (sheep) and 15 hours (goats) after drug administration and were significantly higher in sheep than in goats (Tables 3-15 and 3-16; Appendices B-31, B-32, B-33 and

Table 3-7. Plasma concentrations of albendazole sulphoxide (ABSO) (μg/ml) in goats following administration of albendazole (7.5 mg/kg) either alone or in combination with piperonyl butoxide (0.5 g/kg).

	Mean ± SEM (n=6)	
Time (hours)	albendazole alone	albendazole with Piperonyl butoxide
0	0.00 ± 0.00	0.00 ± 0.00
0.25	0.02 ± 0.01	0.01 ± 0.01
0.5	0.09 ± 0.01	0.10 ± 0.03
1	0.29 ± 0.08	0.26 ± 0.07
2	0.72 ± 0.22	0.74 ± 0.17
4	1.37 ± 0.30	1.58 ± 0.36
8	1.74 ± 0.28	1.54 ± 0.17
12	1.65 ± 0.28	1.33 ± 0.13
24	0.37 ± 0.19	0.69 ± 0.41
32	0.03 ± 0.03	0.34 ± 0.43
48	0.00 ± 0.00	0.09 ± 0.09
72	0.00 ± 0.00	0.01 ± 0.01
96	0.00 ± 0.00	0.00 ± 0.00

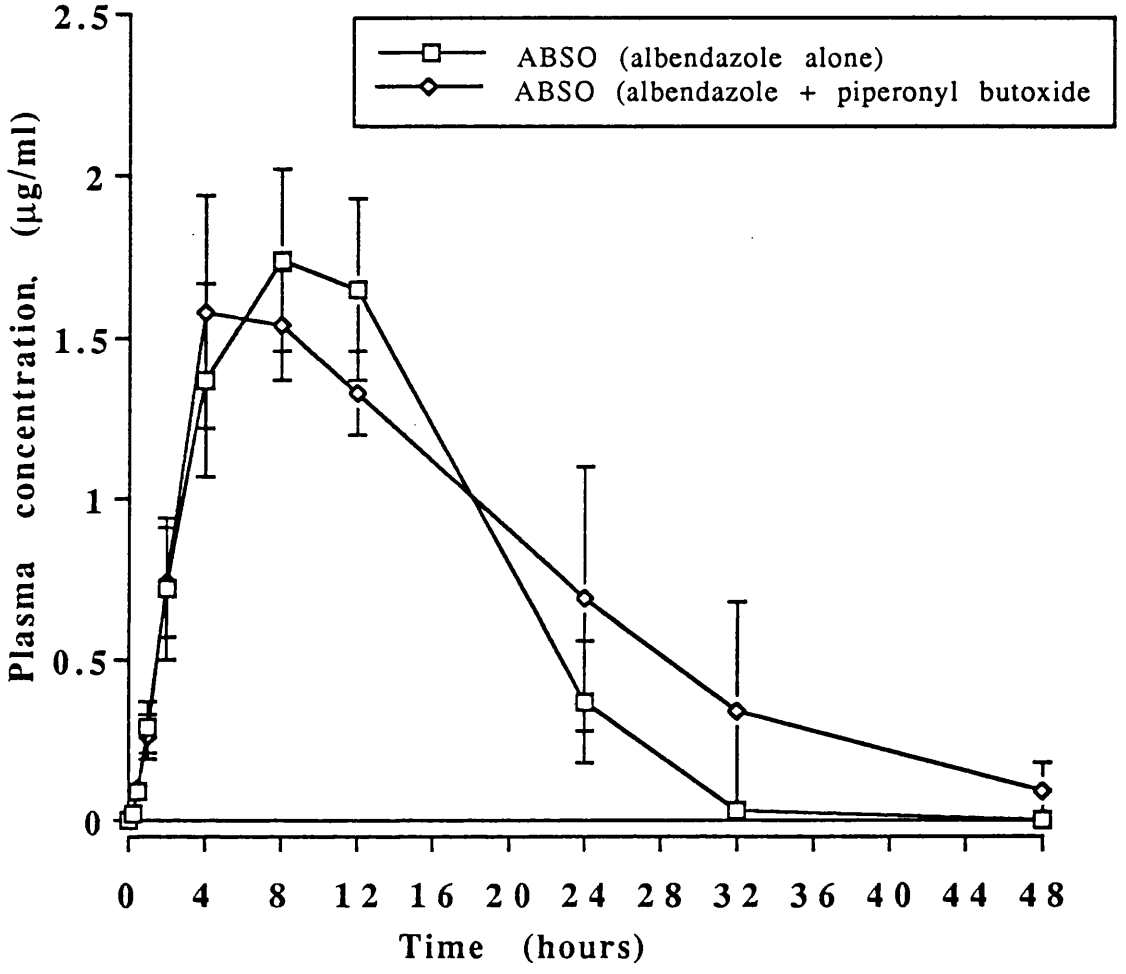

Figure 3-6. Plasma concentrations of albendazole sulphoxide (mean ± SEM) following administration of albendazole alone (n=6) or with piperonyl butoxide (n=6) in goats.

Table 3-8. Pharmacokinetic parameters for albendazole sulphoxide (ABSO) in goats following administration of albendazole at 7.5 mg/kg either alone or in combination with piperonyl butoxide (0.5 g/kg)

	Mean ± SEM (n=6)	
	ABSO	
	albendazole alone	albendazole with piperonyl butoxide
AUC_{obs} (μg.h/ml)	29.63 ± 3.77	35.82 ± 9.13
$AUMC_{obs}$(μg.h^2/m)	333.85 ± 69.78	593.51 ± 324.05
MRT (h)	10.92 ± 1.29	12.94 ± 2.94
Cmax (μg/ml)	2.00 ± 0.27	2.11 ± 0.22
tmax (h)	8.67 ± 1.61	10.00 ± 3.06

Table 3-9. Plasma concentrations of albendazole sulphone (ABSO2) (μg/ml) in goats following administration of albendazole (7.5 mg/kg) either alone or in combination with piperonyl butoxide (0.5 g/kg).

	Mean ± SEM (n=6)	
Time (hours)	albendazole alone	albendazole with Piperonyl butoxide
0	0.00 ± 0.00	0.00 ± 0.00
0.25	0.00 ± 0.00	0.00 ± 0.00
0.50	0.00 ± 0.00	0.00 ± 0.00
1	0.01 ± 0.01	0.00 ± 0.00
2	0.04 ± 0.02	0.04 ± 0.02
4	0.15 ± 0.04	0.20 ± 0.05
8	0.34 ± 0.07	0.49 ± 0.09
12	0.57 ± 0.09	0.72 ± 0.11
24	0.56 ± 0.20	0.95 ± 0.24
32	0.13 ± 0.08	0.32 ± 0.17
48	0.00 ± 0.00	0.22 ± 0.22
72	0.00 ± 0.00	0.00 ± 0.00
96	0.00 ± 0.00	0.00 ± 0.00

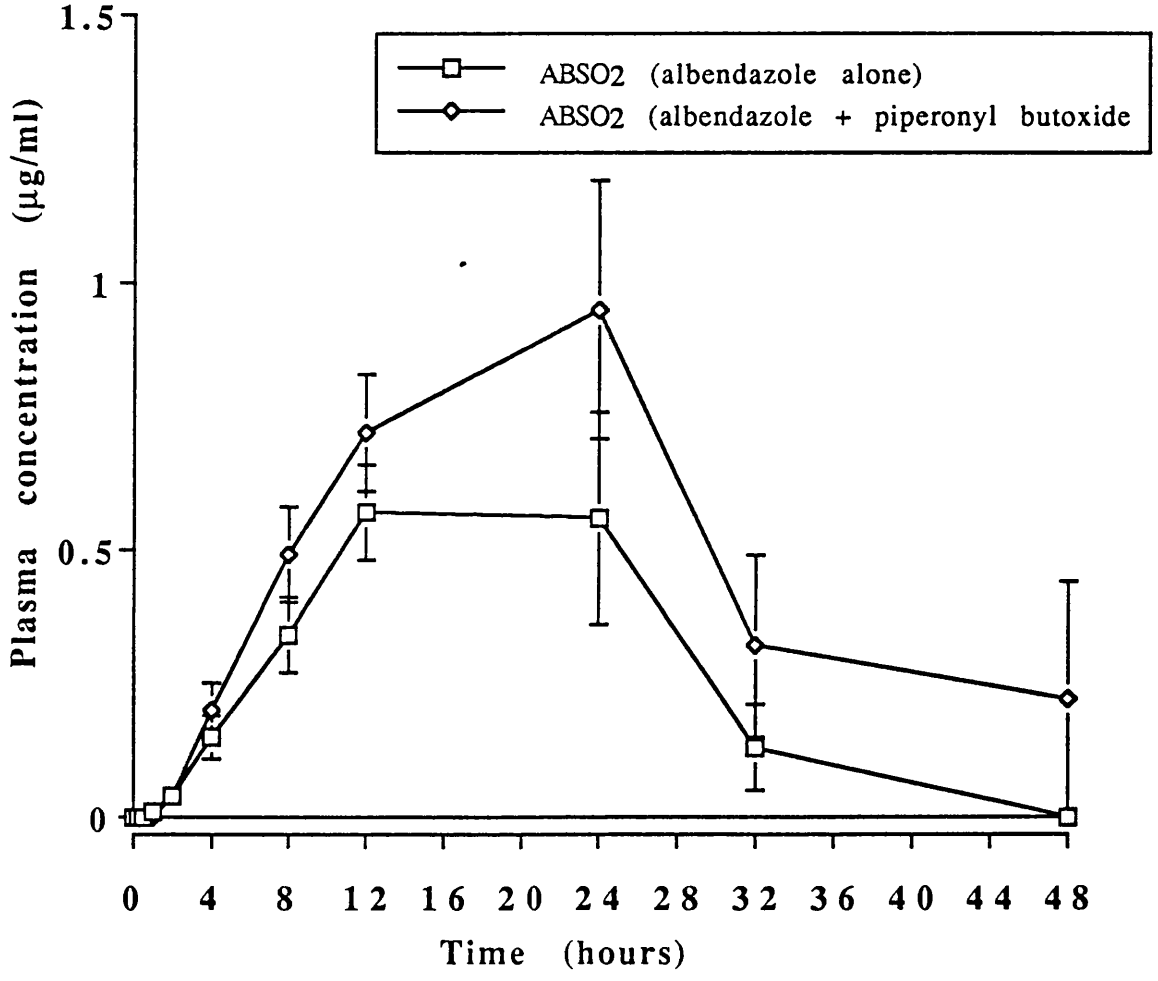

Figure 3-7. Plasma concentrations of albendazole sulphone (mean ± SEM) following administration of albendazole alone (n=6) or with piperonyl butoxide (n=6) in goats.

Table 3-10. Pharmacokinetic parameters for albendazole sulphone (ABSO2) in goats following administration of albendazole at 7.5 mg/kg either alone or in combination with piperonyl butoxide (0.5 g/kg).

	Mean ± SEM (n=6)	
	ABSO2	
	albendazole alone	albendazole with piperonyl butoxide
AUC_{obs} (μg.h/ml)	13.57 ± 2.20	26.17 ± 5.26
$AUMC_{obs}$(μg.h^2/m)	250.77 ± 58.78	651.36 ± 260.12
MRT (h)	16.19 ± 1.81	21.13 ± 3.83
Cmax (μg/ml)	0.78 ± 0.14	1.19 ± 0.14
tmax (h)	18.00 ± 2.68	26.00 ± 4.82

Table 3-11. Plasma concentrations of albendazole sulphoxide (ABSO) (μg/ml) in sheep following administration of albendazole (7.5 mg/kg) either alone or in combination with piperonyl butoxide (0.5 g/kg).

	Mean ± SEM (n=5)	
Time (hours)	albendazole alone	albendazole with Piperonyl butoxide
0	0.00 ± 0.00	0.00 ± 0.00
0.25	0.08 ± 0.02	0.00 ± 0.00
0.50	0.30 ± 0.07	0.08 ± 0.03
1	0.66 ± 0.16	0.25 ± 0.06
2	1.40 ± 0.25	0.59 ± 0.14
4	1.95 ± 0.34	1.10 ± 0.25
8	1.47 ± 0.16	2.43 ± 0.38
12	1.05 ± 0.06	2.20 ± 0.32
24	0.26 ± 0.06	0.94 ± 0.14
32	0.02 ± 0.01	0.35 ± 0.08
48	0.00 ± 0.00	0.00 ± 0.00
72	0.00 ± 0.00	0.00 ± 0.00
96	0.00 ± 0.00	0.00 ± 0.00

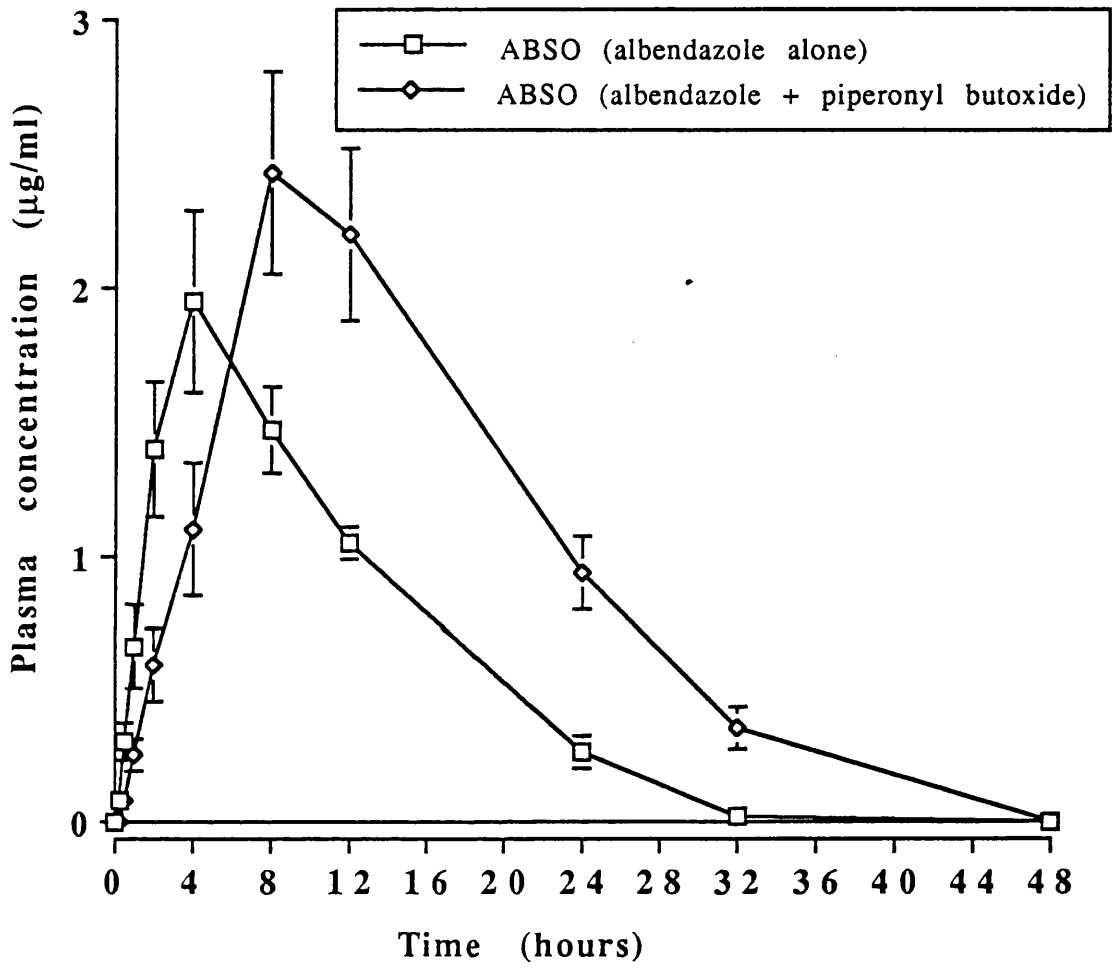

Figure 3-8. Plasma concentrations of albendazole sulphoxide (mean ± SEM) following administration of albendazole alone (n=5) or with piperonyl butoxide (n=5) in sheep.

Table 3-12. Pharmacokinetic parameters of albendazole sulphoxide (ABSO) in sheep following administration of albendazole at 7.5 mg/kg either alone or in combination with piperonyl butoxide

	Mean ± SEM (n=5)	
	ABSO	
	Albendazole alone	Albendazole with piperonyl butoxide
AUC_{obs} (μg.h/ml)	25.61 ± 2.24	45.41 ± 4.29*
$AUMC_{obs}$(μg.h^2/ml)	244.19 ± 21.04	665.57 ± 84.32**
MRT (h)	9.66 ± 0.81	14.52 ± 0.96*
Cmax (μg/ml)	2.01 ± 0.30	2.35 ± 0.29
tmax (h)	4.40 ± 0.98	10.40 ± 0.98**

*(P<0.05); **(P<0.01) Significantly different from albendazole alone.

Table 3-13. Plasma concentrations of albendazole sulphone (ABSO2) (μg/ml) in sheep following administration of albendazole (7.5 mg/kg) either alone or in combination with piperonyl butoxide (0.5 g/kg).

	Mean ± SEM (n=5)	
Time (hours)	albendazole alone	albendazole with Piperonyl butoxide
0	0.00 ± 0.00	0.00 ± 0.00
0.25	0.00 ± 0.00	0.00 ± 0.00
0.50	0.00 ± 0.00	0.00 ± 0.00
1	0.02 ± 0.01	0.00 ± 0.00
2	0.06 ± 0.02	0.04 ± 0.02
4	0.16 ± 0.03	0.14 ± 0.06
8	0.28 ± 0.03	0.37 ± 0.06
12	0.34 ± 0.03	0.61 ± 0.08
24	0.33 ± 0.03	0.77 ± 0.06
32	0.07 ± 0.04	0.45 ± 0.15
48	0.00 ± 0.00	0.11 ± 0.06
72	0.00 ± 0.00	0.00 ± 0.00
96	0.00 ± 0.00	0.00 ± 0.00

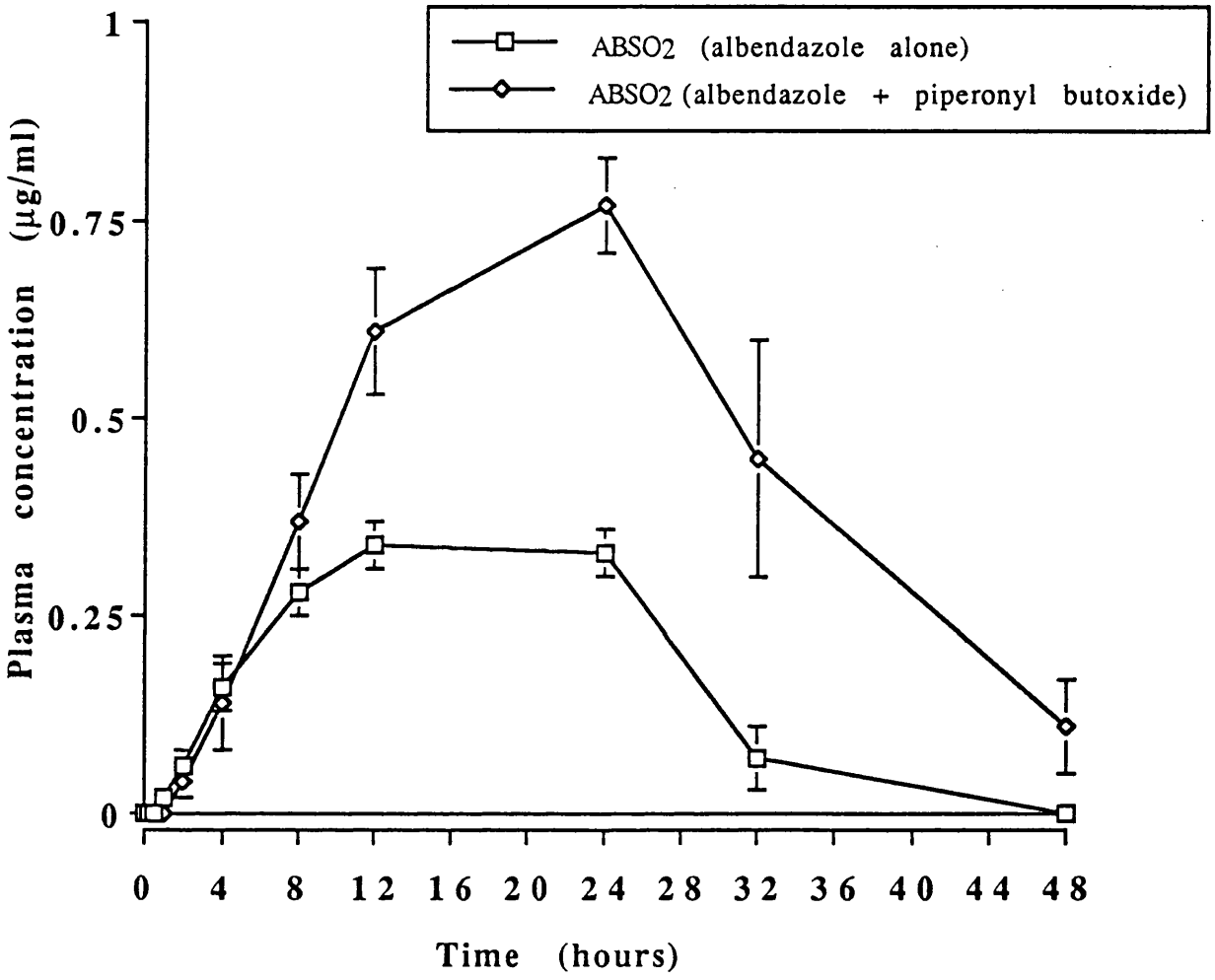

Figure 3-9. Plasma concentrations of albendazole sulphone (mean ± SEM) following administration of albendazole alone (n=5) or with piperonyl butoxide (n=5) in sheep.

Table 3-14. Pharmacokinetic parameters for albendazole sulphone (ABSO2) in sheep following administration of albendazole at 7.5 mg/kg either alone or in combination with piperonyl butoxide.

	Mean ± SEM (n=5)	
	ABSO2	
	albendazole alone	albendazole with piperonyl butoxide
AUC_{obs} (μg.h/ml)	8.22 ± 0.76†	22.32 ± 2.23**
$AUMC_{obs}$(μg.h^2/ml)	133.95 ± 17.86	541.96 ± 99.97*
MRT (h)	16.12 ± 0.74	23.52 ± 2.54*
Cmax (μg/ml)	0.36 ± 0.03††	0.80 ± 0.05**
tmax (h)	14.40 ± 2.40	26.00 ± 2.68**

* ($P<0.05$); ** ($P<0.01$) Significantly different from albendazole alone.
† ($P<0.05$); †† ($P<0.01$) Significantly different from corresponding values in goats.

Table 3-15. Plasma concentrations of piperonyl butoxide (μg/ml) in sheep and goats following intramuscular injection of piperonyl butoxide at 0.5 g/kg bodyweight.

	Mean ± SEM	
Time (h)	Goats (n=6)	Sheep (n=5)
0	0.00 ± 0.00	0.00 ± 0.00
0.167	0.26 ± 0.04	0.45 ± 0.08
0.50	0.38 ± 0.09	0.67 ± 0.10
1	0.36 ± 0.11	1.07 ± 0.26
2	0.59 ± 0.08	1.55 ± 0.39
3	0.68 ± 0.08	2.01 ± 0.44
5	0.87 ± 0.08	2.14 ± 0.41
9	1.11 ± 0.15	2.19 ± 0.26
13	1.32 ± 0.16	2.67 ± 0.41
25	1.06 ± 0.18	2.49 ± 0.39
33	0.92 ± 0.17	1.52 ± 0.28
49	0.83 ± 0.15	0.82 ± 0.12
73	0.70 ± 0.09	0.83 ± 0.13
97	0.63 ± 0.07	0.93 ± 0.27

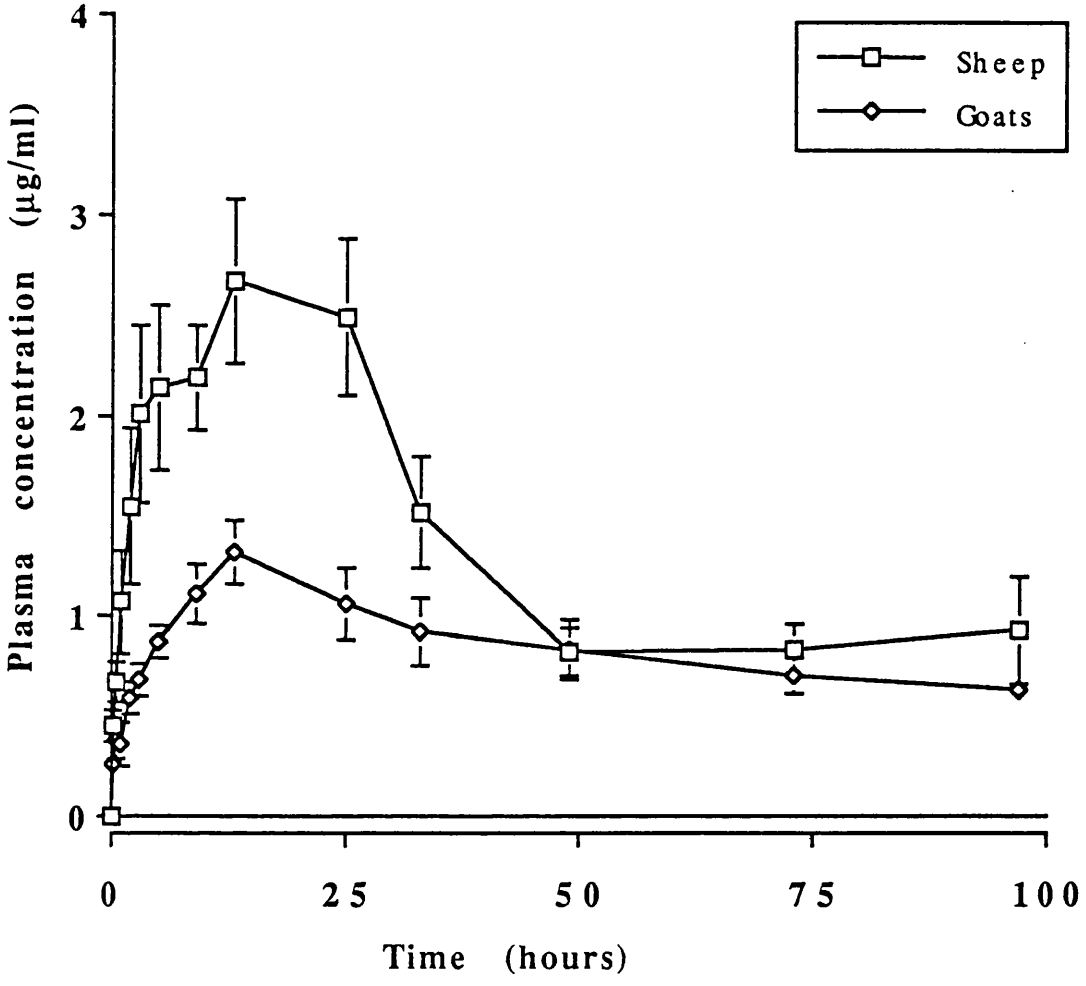

Figure 3-10. Plasma concentrations of piperonyl butoxide (mean ± SEM) following intramuscular administration to sheep (n=5) and goats (n=6).

Table 3-16. Pharmacokinetic parameters of piperonyl butoxide administered intramuscularly to sheep and goats at a dose rate of 0.5 g/kg bodyweight.

	Mean ± SEM	
	Sheep (n=5)	Goats (n=6)
AUC_{obs} (μg.h/ml)	132.96 ± 18.93	82.10 ± 10.99
$AUMC_{obs}$(μg.h^2/ml)	4969.3 ± 835.45	3588.7 ± 454.26
MRT (h)	37.04 ± 1.67	44.01 ± 0.83**
Cmax (μg/ml)	2.92 ± 0.40	1.17 ± 0.20*
tmax (h)	10.20 ± 1.50	15.00 ± 2.00*

* ($P<0.05$); ** ($P<0.01$) Significantly different from sheep.

B-34; Figure 3-10). The AUC of piperonyl butoxide was 62% higher in sheep.

3.4 Discussion

The alteration of liver drug oxidative metabolism by pretreatment with piperonyl butoxide resulted in significant changes in the pharmacokinetic patterns of the two benzimidazole compounds studied. These changes were mainly expressed as increases in the bioavailability of the two metabolites measured and also, in the case of fenbendazole, of the parent compound. Previous studies carried out *in vitro* and *in vivo* have shown that the rate of sulphoxidation and sulphonation of the benzimidazole drugs could be altered by metabolic inhibitors which interfere with the microsomal flavine monooxygenase and, or, cytochrome P450-dependent activities (Fargetton *et al.*, 1986; Galtier *et al.*, 1986a; Lanusse *et al.*, 1991; Murray *et al.*, 1992; Lanusse *et al.*, 1993c).

Piperonyl butoxide is a substrate for cytochrome P450-dependent oxidative metabolism and its biotransformation by this system results in the formation of metabolic intermediates which bind covalently to cytochrome P450 enzymes; the complex thus formed results in an inhibition of the enzymatic activity of the system (Franklin, 1977). In this study, the absence of albendazole parent drug from the plasma of both piperonyl butoxide-pretreated animals and control (albendazole alone) animals supports the suggestion that, in ruminants, albendazole sulphoxidation is far more predominantly catalysed by the flavine monooxygenase than by the cytochrome P450-dependent monooxygenase (Galtier *et al.*, 1986a; Delatour *et al.*, 1994). On the other hand, the increased area under the plasma concentration-time curve, mean residence time and the delayed peak plasma concentration of albendazole sulphoxide observed in sheep when albendazole was given following piperonyl butoxide administration, demonstrates the inhibition of the cytochrome P450-dependent sulphonating reaction (Souhaili-El Amri *et al.*, 1988b) by this metabolic inhibitor. There was also a significant increase in the AUC, MRT and tmax of the sulphone metabolite which was due, at least partly, to a slower rate of sulphone formation. Another reason for the increase in the bioavailability of the sulphone stems from the nature of the metabolism that this compound undergoes. According to Gyurik *et al* (1981), albendazole sulphone is hydroxylated in aromatic or

alkyl positions with possible further conjugation; the hydroxylation could be catalysed by a cytochrome P450-enzyme system which was inhibited in the present study by piperonyl butoxide resulting in increased albendazole sulphone. Infection of lambs with *Fasciola hepatica*, which also decreases hepatic mixed function oxidase activity (Galtier et al., 1983; 1985a; 1986b), has been shown to result in a dramatic increase of albendazole sulphone plasma concentrations (Galtier *et al.*, 1991).

More than 3-fold increases in the AUC of fenbendazole and fenbendazole sulphoxide were observed when fenbendazole was given following metabolic inhibition with piperonyl butoxide. There is evidence from *in vitro* inhibition studies that the sulphoxidation of fenbendazole involves both the flavine-monooxygenase and the cytochrome P450 enzyme systems whereas for the sulphonation and hydroxylation of fenbendazole sulphoxide, evidence only exists to implicate the cytochrome P450-monoxygenase system (Murray *et al.*, 1992). Although, this provides an explanation of the changes caused by piperonyl butoxide to the kinetic disposition of both FBZ and FBSO, there is also a possiblity that the increase in the bioavailability of the parent compound is a consequence of increased sulphoxide concentrations; since distribution of this metabolite into the gastrointestinal tract, its reduction into the sulphide by the gut microflora (Marriner, 1980) followed by reabsorption would indeed increase the systemic availability of FBZ. The AUC and MRT of fenbendazole sulphone were also increased, as a result of a retarded sulphonation and may be also, as a consequence of an inhibition of FBSO2 hydroxylation.

Overall, there was a difference in the extent to which piperonyl butoxide affected the pharmacokinetics of the two benzimidazole drugs in goats. It could be that different isozymes of cytochrome P450 are involved in the metabolic pathways of the two anthelmintics and that these isozymes are differently affected by the metabolic inhibitor used. Isozyme specificity of piperonyl butoxide has been previously shown in rats using different probe drugs for hepatic drug-metabolizing activity (Bachmann, 1989). While the clearance of antipyrine, used as a probe for the cytochrome P450 IIB1 and P450 IIB2 isoforms, was decreased by 50% following pretreatment with piperonyl butoxide, the disposition of quinidine, expressing the activity of the cytochrome P450 IIIA subfamily, was unaffected by this inhibitor. Another

factor may be the relative importance of hydroxylation for the metabolites of the two benzimidazoles studied. Hennessy (1985) has shown that the total (free and conjugated) hydroxylated albendazole sulphoxide accounted for 3% of the total dose of albendazole administered to sheep whereas hydroxylated fenbendazole sulphoxide represented nearly 26% of the dose of fenbendazole given; On the other hand, 3% of the total dose of albendazole was converted to hydroxylated albendazole sulphone when only 0.6% of the total dose of fenbendazole was metabolised into hydroxylated sulphone. The extent of hydroxylation and the possible effect of piperonyl butoxide inhibition on hydroxylating reactions, may help to explain why at the same dosage rate of piperonyl butoxide, different changes in the disposition of albendazole and fenbendazole metabolites were observed.

There was also a difference between the two animal species used, in the effect of PB on albendazole pharmacokinetics. Although the AUC of ABSO2 was doubled in PB-pretreated goats, inhibition did not affect markedly the plasma kinetics of ABSO in this animal species whereas in sheep, the plasma disposition of both metabolites was significantly affected by metabolic inhibition. This interspecies difference could be due to the relatively higher bioavailability of piperonyl butoxide observed in sheep in the present study; the plasma concentrations achieved in the goats, although high enough to alter the metabolism of fenbendazole, might not have been sufficient to cause significant changes in the metabolism of albendazole.

Comparative studies have revealed considerable differences between ruminant species regarding the pharmacokinetics of anthelmintic drugs. Most of these studies emphasised the differences encountered between sheep and goats. It has been reported that thiabendazole was metabolised faster in goats than in sheep (Weir & Bogan, 1985). Lower plasma concentrations of oxfendazole and metabolites were also observed in goats compared to sheep (Bogan *et al.*, 1987; Sangster *et al.*, 1991; Hennessy *et al.*, 1993a). This lower bioavailability may account for the poor efficacy of oxfendazole in goats when administered at the dosage rate recommended for sheep (Kettle *et al.*, 1983). A similar suggestion has been made by Gillham & Obendorf (1985) to explain the poor efficacy of levamisole in goats. These differences extend to

fasciolicide drugs; the AUC of clorsulon after oral adminstration in goats was 60 % of that in sheep (Sundlof *et al.*, 1991; Sundlof & Whitlock, 1992) and the rate of closantel elimination was 2 to 3-times greater in goats than in sheep (Hennessy *et al.*, 1993b). It has been reported by Hennessy *et al.* (1993c) that the AUC of ABSO following administration of albendazole was lower in goats than in sheep, though no major difference in the elimination rate was observed, but the AUC and elimination half-life of ABSO2 were similar between the two species. These authors suggested that the difference in the systemic availability of ABSO was unlikely to be due to a difference in the metabolic rate and that it could be related to a greater sequestration of ABSO in the liver of goats. In the present study, the only difference found between sheep and goats regarding the pharmacokinetics of ABSO following administration of albendazole was the longer tmax (P=0.05) observed in the latter species. This could be due to a slower digesta flow rate in the goat (Hennessy *et al.*, 1993c). Regarding the kinetics of the sulphone metabolite (ABSO2) it is not clear why higher AUC and Cmax were found in goats .

A substantial difference was observed between the albendazole pharmacokinetic values found in the present study and those reported in a previous experiment (see Chapter 2). Because the two experiments were conducted two years apart and four of the goats were used in both experiments, the difference could well be due to age. Some drug-metabolising enzyme activities have indeed been shown to increase with age (Kaddouri *et al.*, 1990; Kawalek and El Said, 1990). However, McKellar *et al* (1993b) did not demonstrate any differences in the pharmacokinetics of netobimin or albendazole metabolites between 3 and 9 month old lambs.

In conclusion, this study shows that piperonyl butoxide has increased significantly the bioavailability of abendazole and fenbendazole metabolites and this could be exploited in increasing the efficacy of benzimidazole drugs.

Chapter 4

Dose titration and efficacy studies with the combination fenbendazole-piperonyl butoxide in sheep

4.1 Introduction

The systemic availability of fenbendazole metabolites has been shown to increase dramatically following pretreatment of goats with piperonyl butoxide (see Chapter 3).

The present study was undertaken in order to determine the effect of the combination in sheep, at various oral dose rates of piperonyl butoxide and to evaluate the efficacy of the drug mixture against benzimidazole-resistant gastric nematodes.

4.2 Materials and Methods

4.2.1 Animals

All animals were maintained indoors under conditions described previously (Section 2.2.1).

4.2.2 Experimental design

4.2.2.1 Oral bioavailability of piperonyl butoxide

Two (02) 1 year-old Scottish Blackface sheep were given piperonyl butoxide orally at a dose rate of 0.5 g/kg bodyweight. Blood samples were taken prior to drug administration and 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 12, 24, 32, 48, 72, 96, 120, 144 and 319 hours thereafter.

4.2.2.2 Dose titration study

Six (06) Scottish Blackface sheep were used in a 6-way crossover design study (Table 4-1) whereby a fixed dose of fenbendazole (Panacur 2.5% at 5 mg/kg bodyweight) was co-administered with 0, 15, 31, 63, 125 and 250 mg/kg bodyweight of piperonyl butoxide. 4-week washout periods were observed between crossovers. Because of the risk of piperonyl butoxide aspiration during oral dosing it was decided for the present study, to administer the drugs by stomach tube. Immediately after drug administration, the tube (10 mm x 2 m, Vet Drug Company plc, Falkirk, UK) was flushed with 20 ml of water. Blood samples were taken and stored as previously described (Section 2.2.2) and were analysed by HPLC for the quantification of FBZ metabolites.

Table 4-1. Piperonyl butoxide dosing schedule adopted during the dose titration study (dose rates are in mg/kg).

	Animal number					
	76	77	78	97	98	99
Crossover 1	0	250	125	63	125	250
Crossover 2	15	0	250	31	63	125
Crossover 3	31	15	0	15	31	63
Crossover 4	63	31	15	0	15	31
Crossover 5	125	63	31	250	0	15
Crossover 6	250	125	63	125	250	0

4.2.2.3 Efficacy trial

This was carried out in comformity with the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines (Powers *et al.*, 1982).

Twenty-four (24) parasite-naive Suffolk cross lambs were allocated into four groups (A, B, C and D) of six animals each. Each lamb received an oral dose of 6,000 infective larvae (L3) of bezimidazole-resistant *Ostertagia circumcincta* and 2,400 (L3) benzimidazole-resistant *Haemonchus contortus* (both parasite strains were purchased from the Moredun Institute, Edinburgh, UK). Twenty-eight days after infection, group B was treated with fenbendazole at the recommended dose rate, group C received piperonyl butoxide at the selected dose rate of 63 mg/kg bodyweight, group D was treated with the combination fenbendazole:piperonyl butoxide (5 mg/kg: 63 mg/kg) and animals in group A were left untreated as controls. Seven days after treatment, faecal samples were taken, all animals were killed and their abomasa collected for parasite counting.

4.2.3 Drug analysis

4.2.3.1 Extraction

The extraction of piperonyl butoxide was identical to that described previously (Section 3.2.4.2).

For the extraction of FBZ metabolites, a solvent extraction using chloroform was performed. To 1 ml of plasma were added 200 µl of ammonium chloride, 0.2 g of sodium chloride and 6 ml of chloroform. After shaking for 10 minutes on a slow rotary mixer, the samples were centrifuged for 20 minutes at 1700 *g*. The supernatant was carefully removed and discarded and 4 ml of the organic phase were transfered to a 10ml thin-walled tube. Samples were evaporated to dryness under nitrogen, reconstituted in 150 µl methanol then processed on the HPLC system.

4.2.3.2 HPLC system

The HPLC conditions for the analysis of piperonyl butoxide were as previously described (Section 3.2.4.3).

A computerised (PC 1000, Spectra-Physics Analytical Inc., UK) HPLC system comprising a gradient pump (model P 4000), a UV-detector (model Spectra-Focus), an autosampler (model AS 3000) and a

controller (model SN 4000), was used for the analysis of FBZ, FBSO and FBSO2. The mobile phase was a mixture acetonitrile-water to which glacial acetic acid (BDH Ltd., Pool, UK) was added (0.5 %, v/v). It was pumped through the column (C18 Nucleosil 5, 10 cm X 4.6 mm, HPLC Technology Ltd., Cheshire, UK) in a linear gradient fashion changing from 35:65 (acetonitrile:water) to 82:18 for 8 minutes, 82:18 to 35:65 for 1 minute and the last ratio was maintained for 3 minutes. The flow rate was 1.5 ml/minute. The detection was at 292 nm. The retention times were 1.60 minutes (FBSO), 2.60 minutes (FBSO2) and 5 minutes (FBZ).

Recovery and precision of the extraction and HPLC detection are reported in Appendix C-1.

4.2.4 Faecal egg numbers and parasite counting

4.2.4.1 Faecal egg counts

These were carried out using the McMaster technique modified by Gordon and Whitlock (1939). Three grams of feces were homogenized in 42 ml of water and the suspension passed through a 250 μ sieve (Endecotts Ltd, London, England). Fifteen ml of the filtrate were collected in a glass tube and centrifuged for 3 minutes at 1500 *g*. The supernatant was discarded and the pellet was resuspended in a saturated salt (NaCl) solution. both chambers of the McMaster slide (Gelman Hawksley Ltd, Harrowden, Northampton) were filled with the suspension (0.15 ml) and the number of eggs counted. The result was multiplied by a factor of 50 to give the number of eggs per gram (epg) of faeces.

4.2.4.2 Worm counting

After opening the abomasum along its greater curvature, the mucosal surface was washed under slow running water and the contents made up to 2 litres. Duplicate samples of 200 ml were taken and 1ml of iodine (45% solution) was added to each sample. In order to count possible immature or arrested larvae, the mucosa was scraped off and incubated for 6 hours with a pepsin-hydrochloric acid mixture at 42ºC. The digest was made up to 2 litres and again duplicate samples of 200 ml were taken and iodine was added to each sample.

After thorough mixing, 2 ml of abomasal content or digesta sample was transfered to a lined petri dish. The sample was decolourised with a sodium thiosulphate solution, the parasites were identified and counted. The procedure was repeated ten times so that 20 ml

of each sample were screened and results for each parasite species multiplied by 100 to give the total number of worms in each abomasal sample.

4.2.5 Pharmacokinetic and statistical analysis

Pharmacokinetic variables were determined as previously described (Section 2.2.4).

Data from the dose titration study were analysed using the Wilcoxon signed rank-test and a $P<0.05$ was considered significant. Data from the efficacy trial were compared using the Mann-Whitney U-test with $P<0.01$ as a level of significance. Geometric means were determined after Log_{10} $(x + 1)$ transformation where x is the worm or faecal egg number:

$$\text{Geometric mean} = \text{Antilog}\ [1/n\ \Sigma\ \text{Log}\ (x_i + 1)]$$

Percentage efficacy was calculated as follows:

[Mean(control) - mean (treated)/mean (control)] x 100

4.3 Results

4.3.1 Piperonyl butoxide

Plasma levels of piperonyl butoxide and the related pharmacokinetic data are displayed on Tables 4-2 and 4-3 respectively. Fifteen minutes after administration, piperonyl butoxide reached a mean plasma concentration of 4.53 µg/ml. The peak concentration was reached at 12 to 24 hours postadministration and the drug was not detectable after 13 days (Figure 4-1).

4.3.2 Fenbendazole metabolites

Mean plasma concentrations (Figures 4-2, 4-3 and 4-4) and mean pharmacokinetic parameters of FBZ metabolites obtained at different dose rates of piperonyl butoxide, are shown in Tables 4-4 to 4-15. The corresponding individual values are shown in Appendices C-2 to C-37. The AUC of fenbendazole, fenbendazole sulphoxide and fenbendazole sulphone increased linearly with increasing doses of piperonyl butoxide ($r^2=0.98$ for FBZ, $r^2=0.93$ for FBSO and $r^2=0.95$ for FBSO2) (Figure 4-5). However, when the

Table 4-2. Plasma concentrations of piperonyl butoxide (μg/ml) in sheep following oral administration at 0.5 g/kg bodyweight.

	Animal number		
Time (hours)	95	96	Mean
0	0.00	0.00	0.00
0.25	6.76	2.29	4.53
0.5	7.22	2.24	4.73
0.75	7.20	2.98	5.09
1	8.28	2.91	5.60
2	9.85	5.10	7.48
3	8.69	6.33	7.51
4	8.80	6.46	7.63
6	8.23	6.05	7.14
8	9.34	8.10	8.72
12	9.72	8.75	9.24
24	11.68	7.34	9.51
32	11.47	8.65	10.06
48	11.67	8.09	9.88
72	2.70	1.45	2.08
96	0.52	0.59	0.56
120	0.24	0.26	0.25
144	0.26	0.13	0.20
319	0.00	0.00	0.00

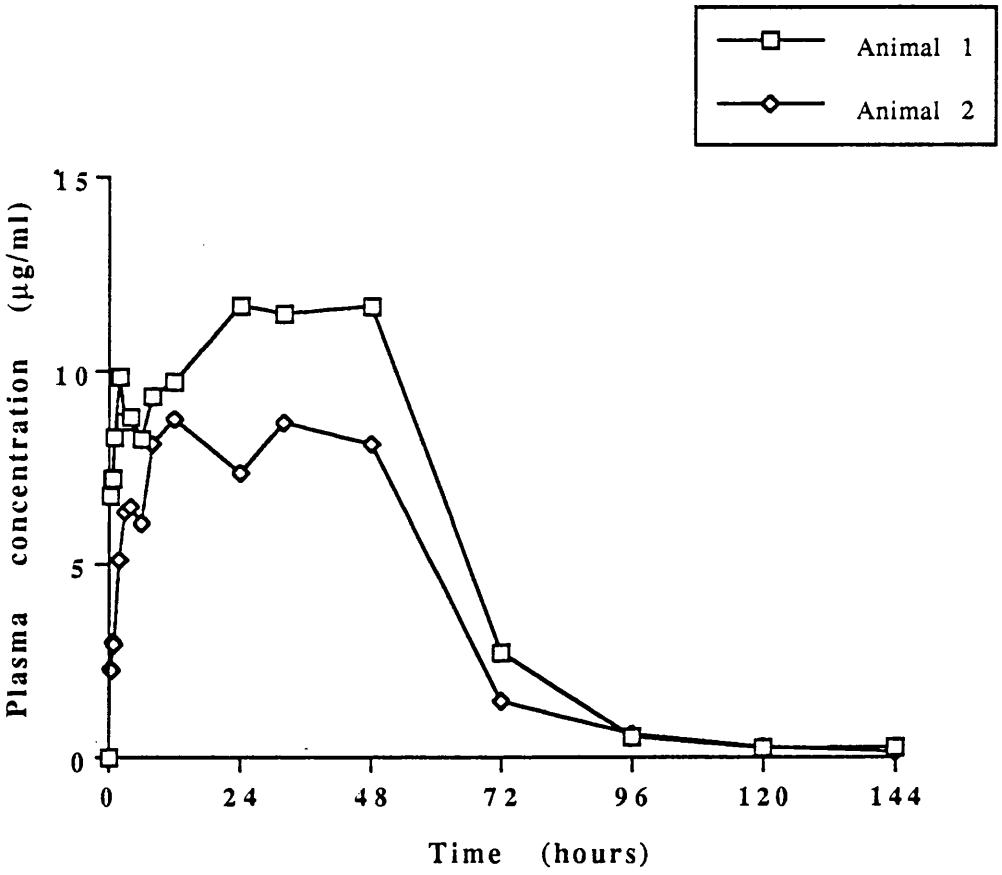

Figure 4-1. Plasma concentrations of piperonyl butoxide administered orally to sheep (n=2) at 0.5 g/kg bodyweight.

Table 4-3. Pharmacokinetic parameters of piperonyl butoxide in sheep following oral administration at 0.5 g/kg bodyweight.

	Animal number		
	Sheep 95	Sheep 96	Mean
AUC_{obs} (μg.h/ml)	761.2	538.4	649.8
$AUMC_{obs}$(μg.h^2/ml)	30053	20503	25278
MRT (h)	39.48	38.08	38.78
Cmax (μg/ml)	11.67	8.75	10.21
tmax (h)	24	12	18

Table 4-4. Plasma concentrations of FBZ metabolites (mean ± SEM) following administration of fenbendazole (5 mg/kg) alone.

	Mean ± SEM (n=6)		
Time (h)	FBZ	FBSO	$FBSO_2$
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.25	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
2	0.08 ± 0.03	0.01 ± 0.01	0.00 ± 0.00
4	0.18 ± 0.03	0.07 ± 0.02	0.00 ± 0.00
8	0.20 ± 0.03	0.18 ± 0.02	0.02 ± 0.00
12	0.19 ± 0.04	0.23 ± 0.03	0.04 ± 0.01
24	0.16 ± 0.02	0.25 ± 0.04	0.09 ± 0.01
32	0.11 ± 0.01	0.22 ± 0.04	0.09 ± 0.01
48	0.06 ± 0.01	0.11 ± 0.02	0.10 ± 0.01
72	0.02 ± 0.00	0.02 ± 0.01	0.06 ± 0.01
96	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.01

Table 4-5. Plasma concentrations of FBZ metabolites (mean ± SEM) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (15 mg/kg).

	Mean ± SEM (n=6)		
Time (h)	FBZ	FBSO	$FBSO_2$
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.25	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1	0.02 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
2	0.05 ± 0.03	0.01 ± 0.01	0.00 ± 0.00
4	0.11 ± 0.01	0.04 ± 0.02	0.01 ± 0.00
8	0.18 ± 0.01	0.14 ± 0.02	0.02 ± 0.00
12	0.17 ± 0.01	0.21 ± 0.02	0.04 ± 0.01
24	0.17 ± 0.01	0.25 ± 0.02	0.08 ± 0.01
32	0.14 ± 0.02	0.24 ± 0.02	0.09 ± 0.01
48	0.09 ± 0.02	0.14 ± 0.02	0.10 ± 0.01
72	0.02 ± 0.00	0.03 ± 0.00	0.07 ± 0.01
96	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.00

Table 4-6. Plasma concentrations of FBZ metabolites (mean ± SEM) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (31 mg/kg).

	Mean ± SEM (n=6)		
Time (h)	FBZ	FBSO	$FBSO_2$
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.25	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.50	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1	0.02 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
2	0.07 ± 0.03	0.01 ± 0.00	0.00 ± 0.00
4	0.20 ± 0.05	0.06 ± 0.02	0.00 ± 0.00
8	0.29 ± 0.06	0.21 ± 0.04	0.02 ± 0.00
12	0.28 ± 0.04	0.30 ± 0.04	0.04 ± 0.01
24	0.27 ± 0.02	0.39 ± 0.05	0.09 ± 0.01
32	0.20 ± 0.02	0.37 ± 0.04	0.12 ± 0.01
48	0.16 ± 0.06	0.23 ± 0.05	0.12 ± 0.02
72	0.03 ± 0.00	0.06 ± 0.01	0.08 ± 0.01
96	0.01 ± 0.00	0.01 ± 0.00	0.05 ± 0.00

Table 4-7. Plasma concentrations of FBZ metabolites (mean ± SEM) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (63 mg/kg).

	Mean ± SEM (n=6)		
Time (h)	FBZ	FBSO	$FBSO_2$
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.25	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.5	0.02 ± 0.01	0.01 ± 0.01	0.00 ± 0.00
1	0.05 ± 0.03	0.02 ± 0.02	0.00 ± 0.00
2	0.12 ± 0.06	0.06 ± 0.05	0.00 ± 0.00
4	0.25 ± 0.04	0.14 ± 0.07	0.00 ± 0.00
8	0.31 ± 0.03	0.27 ± 0.04	0.02 ± 0.00
12	0.26 ± 0.04	0.34 ± 0.04	0.03 ± 0.00
24	0.28 ± 0.05	0.46 ± 0.09	0.08 ± 0.01
32	0.20 ± 0.05	0.42 ± 0.09	0.11 ± 0.01
48	0.11 ± 0.03	0.22 ± 0.05	0.13 ± 0.02
72	0.05 ± 0.01	0.06 ± 0.02	0.09 ± 0.01
96	0.01 ± 0.01	0.01 ± 0.01	0.05 ± 0.01

Table 4-8. Plasma concentrations of FBZ metabolites (mean ± SEM) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (125 mg/kg).

	Mean ± SEM (n=6)		
Time (h)	FBZ	FBSO	$FBSO_2$
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.25	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.5	0.02 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
1	0.07 ± 0.03	0.01 ± 0.00	0.00 ± 0.00
2	0.16 ± 0.05	0.03 ± 0.01	0.00 ± 0.00
4	0.34 ± 0.07	0.13 ± 0.04	0.00 ± 0.00
8	0.47 ± 0.07	0.36 ± 0.07	0.01 ± 0.00
12	0.55 ± 0.10	0.58 ± 0.12	0.03 ± 0.01
24	0.52 ± 0.05	0.79 ± 0.10	0.09 ± 0.02
32	0.39 ± 0.04	0.69 ± 0.07	0.13 ± 0.02
48	0.21 ± 0.02	0.38 ± 0.04	0.17 ± 0.02
72	0.06 ± 0.02	0.11 ± 0.03	0.13 ± 0.01
96	0.02 ± 0.01	0.03 ± 0.01	0.08 ± 0.01

Table 4-9. Plasma concentrations of FBZ metabolites (mean ± SEM) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (250 mg/kg).

	Mean ± SEM (n=6)		
Time (h)	FBZ	FBSO	$FBSO_2$
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.25	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.5	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1	0.04 ± 0.01	0.01 ± 0.00	0.00 ± 0.00
2	0.13 ± 0.04	0.03 ± 0.01	0.00 ± 0.00
4	0.29 ± 0.04	0.11 ± 0.03	0.00 ± 0.00
8	0.38 ± 0.03	0.28 ± 0.05	0.01 ± 0.00
12	0.48 ± 0.05	0.47 ± 0.07	0.02 ± 0.01
24	0.63 ± 0.05	0.74 ± 0.07	0.08 ± 0.02
32	0.62 ± 0.05	0.78 ± 0.08	0.12 ± 0.02
48	0.54 ± 0.04	0.61 ± 0.03	0.18 ± 0.03
72	0.17 ± 0.03	0.24 ± 0.03	0.18 ± 0.02
96	0.05 ± 0.01	0.06 ± 0.01	0.12 ± 0.01

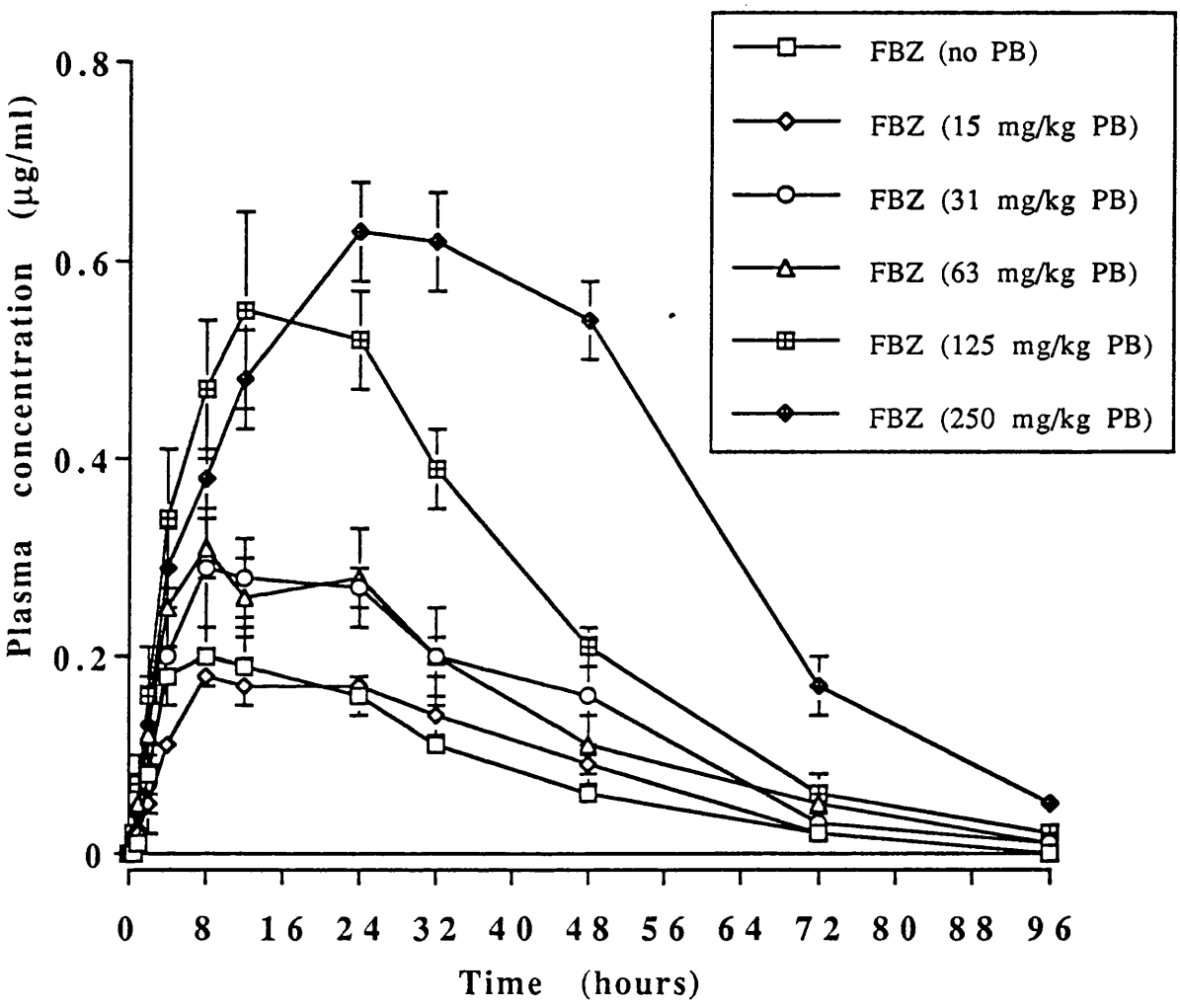

Figure 4-2. Plasma concentrations of fenbendazole (mean ± SEM) following administration of fenbendazole in combination with various dose rates of piperonyl butoxide in sheep (n=6).

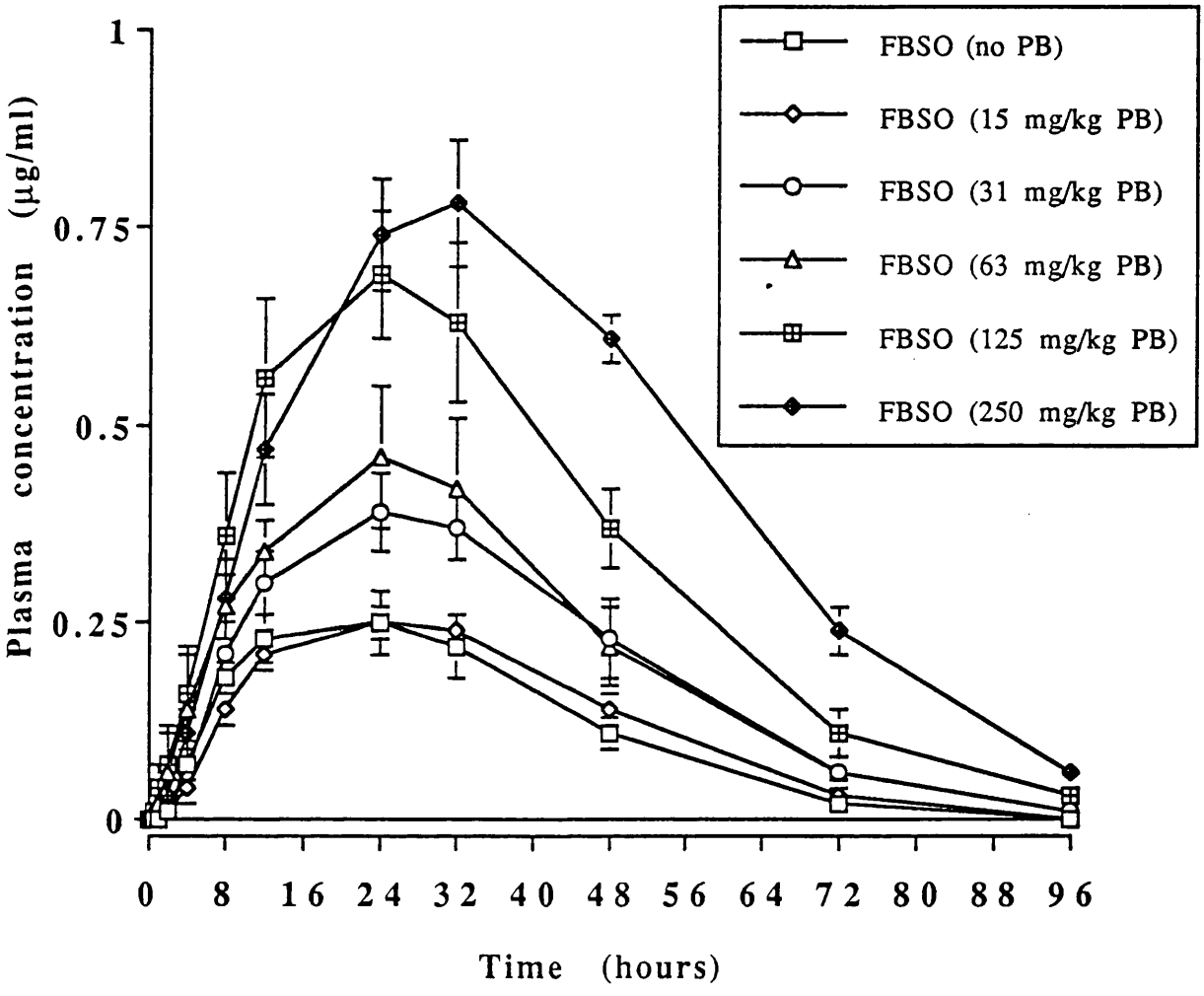

Figure 4-3. Plasma concentrations of fenbendazole sulphoxide (mean ± SEM) following administration of fenbendazole in combination with various dose rates of piperonyl butoxide in sheep (n=6).

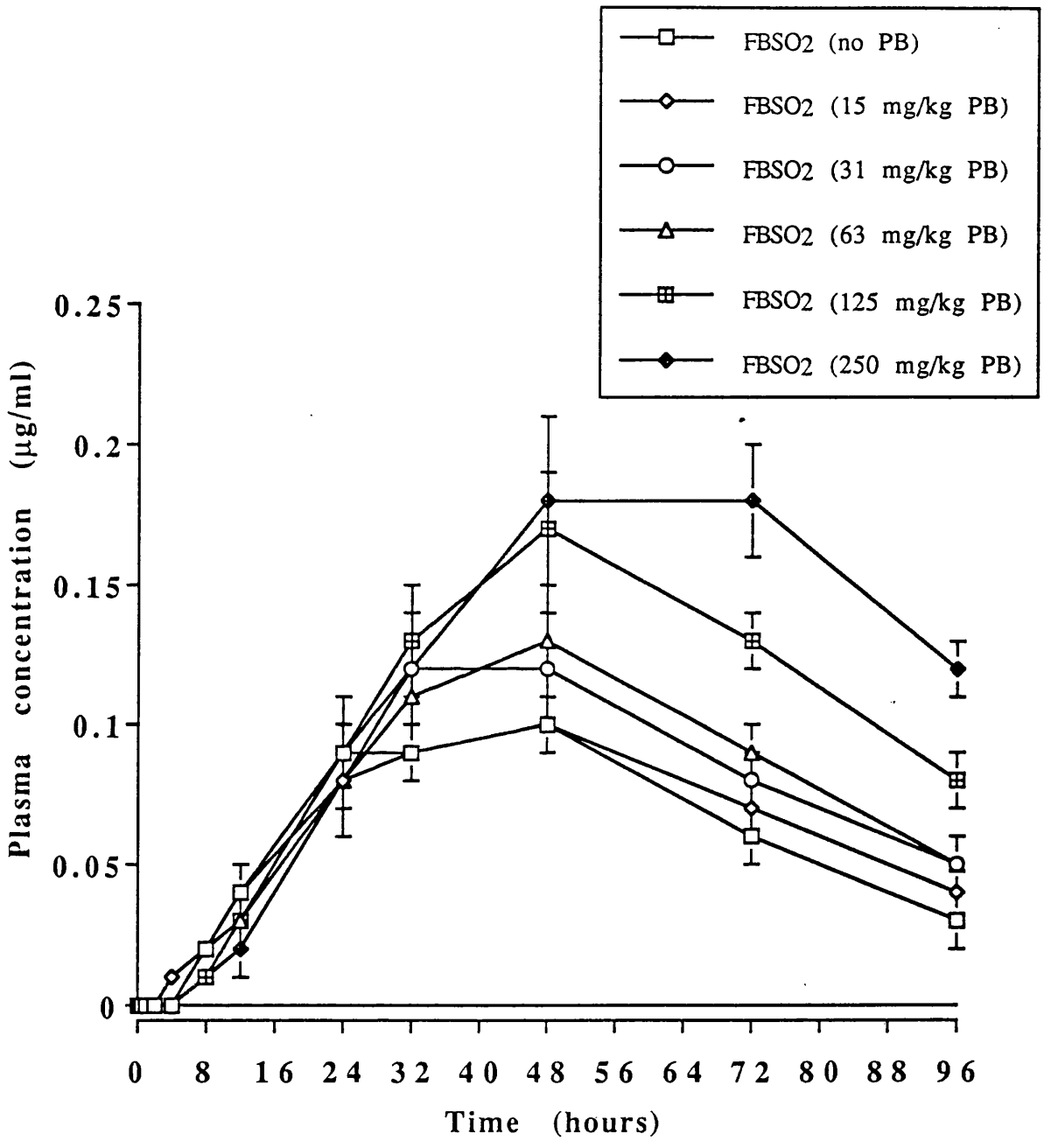

Figure 4-4. Plasma concentrations of fenbendazole sulphone (mean ± SEM) following administration of fenbendazole in combination with various dose rates of piperonyl butoxide in sheep (n=6)

Table 4-10. Pharmacokinetic parameters of FBZ metabolites following administration of fenbendazole (5 mg/kg) alone.

	Mean ± SEM (n=6)		
	FBZ	FBSO	$FBSO_2$
AUC_{obs} (μg.h/ml)	7.54 ± 0.79	10.62 ± 1.59	5.94 ± 0.60
$AUMC_{obs}$(μg.h^2/ml)	194.79 ± 25.05	313.18 ± 55.34	281.25 ± 34.80
MRT (h)	25.52 ± 2.02	28.72 ± 1.61	46.72 ± 1.74
Cmax (μg/ml)	0.24 ± 0.02	0.27 ± 0.03	0.11 ± 0.01
tmax (h)	10.00 ± 3.01	20.67 ± 3.64	34.67 ± 4.46

Table 4-11. Pharmacokinetic parameters of FBZ metabolites following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (15 mg/kg).

	Mean ± SEM (n=6)		
	FBZ	FBSO	$FBSO_2$
AUC_{obs} (μg.h/ml)	8.09 ± 0.63	11.26 ± 0.60	6.29 ± 0.54
$AUMC_{obs}$(μg.h^2/ml)	240.27 ± 29.78	365.98 ± 23.91	310.58 ± 27.03
MRT (h)	29.21 ± 1.36	32.47 ± 1.10*	49.62 ± 2.35
Cmax (μg/ml)	0.20 ± 0.00	0.26 ± 0.02	0.11 ± 0.01
tmax (h)	14.33 ± 4.63	22.33 ± 2.75	46.67 ± 5.63

* Significantly different ($P < 0.05$) from corresponding value in control (see Table 4-10)

Table 4-12. Pharmacokinetic parameters of FBZ metabolites following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (31 mg/kg).

	Mean ± SEM (n=6)		
	FBZ	FBSO	$FBSO_2$
AUC_{obs} (μg.h/ml)	12.91 ± 1.57*	18.07 ± 2.03*	7.55 ± 0.90
$AUMC_{obs}$(μg.h^2/ml)	379.32 ± 61.68*	611.38 ± 73.14*	374.13 ± 42.95*
MRT (h)	29.26 ± 1.92	33.78 ± 0.85*	49.75 ± 0.98
Cmax (μg/ml)	0.35 ± 0.05	0.42 ± 0.05*	0.13 ± 0.02
tmax (h)	20.67 ± 6.23	30.67 ± 3.68	37.33 ± 2.67

* Significantly different ($P< 0.05$) from corresponding value in control (see Table 4-10).

Table 4-13. Pharmacokinetic parameters of FBZ metabolites following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (63 mg/kg).

	Mean ± SEM (n=6)		
	FBZ	FBSO	$FBSO_2$
AUC_{obs} (μg.h/ml)	12.80 ± 1.92*	19.78 ± 3.05*	7.61 ± 0.82
$AUMC_{obs}$(μg.h^2/ml)	372.84 ± 79.14*	636.41 ±131.93	396.13 ± 50.52*
MRT (h)	27.62 ± 2.56	29.38 ± 4.03	51.28 ± 2.14
Cmax (μg/ml)	0.35 ± 0.03*	0.53 ± 0.04*	0.13 ± 0.01
tmax (h)	12.67 ± 3.64	23.33 ± 4.18	42.67 ± 3.96

* Significantly different ($P< 0.05$) from corresponding value in control (see Table 4-10).

Table 4-14. Pharmacokinetic parameters of FBZ metabolites follow administration of fenbendazole (5 mg/kg) in combination with piperc butoxide (125 mg/kg).

	Mean ± SEM (n=6)		
	FBZ	FBSO	$FBSO_2$
AUC_{obs} (μg.h/ml)	23.39 ± 1.59*	33.32 ± 2.68*	9.97 ± 0.77
$AUMC_{obs}$(μg.h^2/ml)	673.61 ± 40.20*	1114.63 ± 82.94*	540.77 ± 39.85*
MRT (h)	29.29 ± 2.14	33.83 ± 2.11*	54.43 ± 1.87*
Cmax (μg/ml)	0.57 ± 0.09*	0.79 ± 0.10*	0.17 ± 0.02*
tmax (h)	20.00 ± 2.53	25.33 ± 1.33	47.33 ± 3.64

* Significantly different ($P< 0.05$) from corresponding value in control (see Table 4-10).

Table 4-15. Pharmacokinetic parameters of FBZ metabolites following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (250 mg/kg).

	Mean ± SEM (n=6)		
	FBZ	FBSO	$FBSO_2$
AUC_{obs} (μg.h/ml)	35.58 ± 1.60*	40.73 ± 2.03*	11.73 ± 1.28
$AUMC_{obs}$(μg.h^2/ml)	312.97 ± 75.81*	1604.93 ± 53.26*	683.80 ± 65.06*
MRT (h)	36.97 ± 1.71*	39.71 ± 1.60*	58.79 ± 1.19*
Cmax (μg/ml)	0.66 ± 0.05*	0.79 ± 0.08*	0.20 ± 0.02
tmax (h)	32.00 ± 3.58*	28.05 ± 6.40	64.00 ± 5.06

* Significantly different ($P < 0.05$) from corresponding value in control (see Table 4-10).

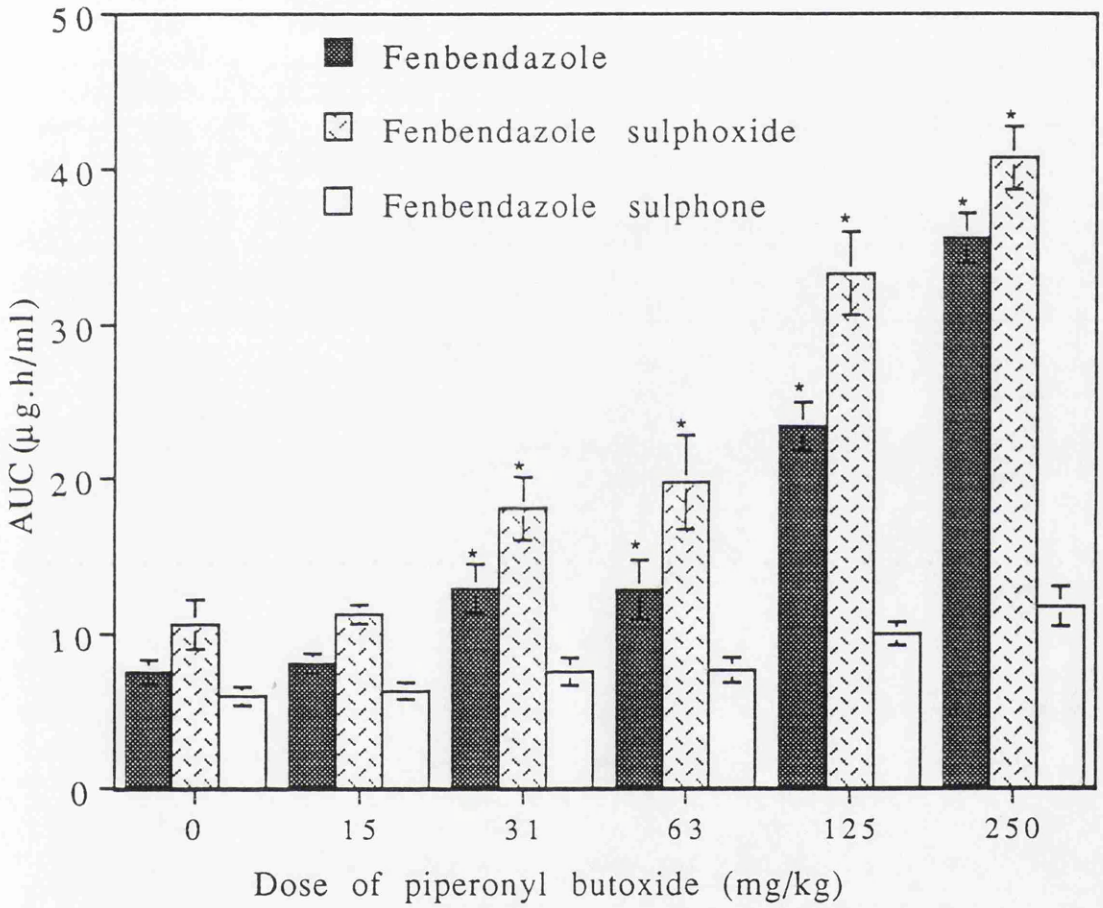

Figure 4-5. Area under the plasma concentration-time curve (AUC) values of fenbendazole metabolites at various dose rates of piperonyl butoxide.

* Significantly different from control values ($P<0.05$)

dose of piperonyl butoxide administered was 250 mg/kg, the AUC of FBZ and FBSO showed a 4-fold increase from the control value, while that of the sulphone metabolite only doubled (figure 4-5) therefore decreasing the ratio AUC (FBSO2)/ AUC (FBZ) from the control value of 0.79 to 0.33 and the ratio AUC(FBSO2)/AUC(FBSO) from 0.56 to 0.29. The mean residence time (MRT) of fenbendazole sulphoxide increased significantly from the control value ($P< 0.05$), with four of the fenbendazole-piperonyl butoxide dose combinations. Mean maximum concentrations (Cmax), particularly those of FBZ and FBSO, also tended to increase with the combination. The time until maximum concentration was also prolonged for all metabolites but statistical significance was only reached with FBZ when the highest dose of piperonyl butoxide was given.

4.3.3 Anthelmintic efficacy of the combination fenbendazole-piperonyl butoxide

The results of the efficacy trial are shown on Tables 4-16, 4-17, 4-18 and Appendix C-38. Piperonyl butoxide, given on its own had no effect on parasitism. While fenbendazole given alone was not effective (less than 8% efficacy) in reducing the number of BZ-resistant *O. circumcincta*, the administration of the combination FBZ:PB showed a 98% efficacy against this parasite strain. The combination was also more effective against BZ-resistant *H. contortus* (99% reduction) than the conventional treatment (85% reduction). Only one animal showed a positive faecal egg count after treatment with the combination.

4.4 Discussion

A sustained plasma level of piperonyl butoxide was obtained following oral administration and concentrations higher than those measured following intramuscular injection of the drug were achieved (see Chapter 3). However, considering the very high dose given, relatively low plasma levels were attained which could be due to poor absorption, first-pass effect or vast tissue distribution. There is no published information on the disposition of piperonyl butoxide in ruminants and in rats oral administration resulted in poor gastrointestinal absorption and rapid excretion in the urine and faeces (Haley, 1978). Although it would be an oversimplification to predict the inhibitory effect of piperonyl butoxide when given orally or intramuscularly simply from the

Table 4-16. Mean worm counts and percentage reductions (*Ostertagia circumcincta*) in different treatment groups.

	Arithmetic mean (± SD)	Geometric mean	% reduction (arithmetic)	%reduction (geometric)
Group A (n=6)	2985 ± 654	2911	-	-
Group B (n=6)	2875 ± 1308	2681	3.7	7.9
Group C (n=6)	3783 ± 441	3760	-	-
Group D (n=6)	450 ± 565*	64.5	84.9	97.8

Group A (control)
Group B (fenbendazole alone)
Group C (piperonyl butoxide alone)
Group D (fenbendazole + piperonyl butoxide)
* Significantly different ($P < 0.01$) from group A, B and C.

Table 4-17. Mean worm counts and percentage reductions (*Haemonchus contortus*) in different treatment groups.

	Arithmetic mean (± SD)	Geometric mean	% reduction (arithmetic)	%reduction (geometric)
Group A (n=6)	345 ± 67	340	-	-
Group B (n=6)	58 ± 29*	51.7	83.0	84.8
Group C (n=6)	320 ± 133	297	-	-
Group D (n=6)	7 ± 8**,†	3.5	98.0	99.0

Group A (control)
Group B (fenbendazole alone)
Group C (piperonyl butoxide alone)
Group D (fenbendazole + piperonyl butoxide)

* Significantly different ($P<0.01$) from group A and C
** Significantly different ($P<0.005$) from group A and C
† Significantly different ($P<0.01$) from group B

Table 4-18. Mean faecal egg counts (epg) and percentage reductions in different treatment groups.

	Arithmetic mean (± SD)	Geometric mean	% reduction (arithmetic)	%reduction (geometric)
Group A (n=6)	492 ± 267	438	-	-
Group B (n=6)	100 ± 84**	27.7	79.7	93.7
Group C (n=6)	483 ± 342	373	-	-
Group D (n=6)	8 ± 20**	1.9	98.4	99.6

Group A (control)
Group B (fenbendazole alone)
Group C (piperonyl butoxide alone)
Group D (fenbendazole + piperonyl butoxide)
** Significantly different ($P<0.005$) from group A and C

plasma disposition of the drug, it is at least evident that, since it is present in the general circulation, the drug has reached the target organ (liver). Since it is the metabolites of piperonyl butoxide that bind to and inhibit microsomal oxidases, measurement of metabolite products in blood but also in liver tissue would give a better estimate of the inhibitory activity, this was however beyond the scope of this thesis.

The pharmacokinetic behaviour of fenbendazole was similar to that described by Marriner & Bogan (1981b) with fenbendazole sulphoxide being the main metabolite detected in plasma. A direct comparison of FBZ disposition in sheep with that described in goats (chapter 3) was not possible since the dose rates used were different; it is of interest, however, to note that with a 33% higher dose given to goats the AUC of FBZ and FBSO were lower than in sheep by 37% and 27%, respectively. Lower bioavailability was also found in goats as compared to sheep when fenbendazole sulphoxide was given (Bogan *et al.*, 1987; Sangster *et al.*, 1991; Hennessy *et al.*, 1993a). The response to metabolic inhibition, expressed in the alteration of FBZ metabolites disposition, increased with increasing doses of piperonyl butoxide. The linear increase pattern observed with the AUC of FBZ, FBSO and FBSO2 resembles that described by Hennessy *et al* (1985) when oxfendazole was co-administered with increasing doses of parbendazole. Parbendazole is a potent inhibitor of the polymerization of mammalian tubulin (Friedman & Platzer, 1978), and it has been suggested that by binding temporarily to liver tubulin, PBZ may reduce the rate of metabolism and excretion of co-administered oxfendazole; there is however evidence that benzimidazoles and their metabolites can act as inhibitors of microsomal cytochrome P450 (Murray *et al.*, 1992). A declining ratio FBSO2/FBSO was observed with the coadministration of increasing doses of piperonyl butoxide indicating a slower rate of sulphonation. The delay in the tmax and the prolonged mean residence times observed also reflect a slower clearance of the benzimidazole metabolites.

The results of the efficacy study give further support to the hypothesis that anthelmintic efficacy parallels the concentration of active metabolites in the bloodstream (Prichard *et al.*, 1978; Hennessy *et al.*, 1985; Kwan *et al.*, 1988). The increased AUC of

both fenbendazole and its active metabolite (FBSO) following concomitant administration of fenbendazole and piperonyl butoxide markedly improved the efficacy of the benzimidazole anthelmintic against BZ-resistant strains of *O. circumcincta*. Indeed, whereas this strain was almost totally refractory to fenbendazole given alone, it was highly susceptible to the combination ($P<0.01$). The strain of *Haemonchus contortus* did not show a high degree of resistance since fenbendazole given alone at the therapeutic dosage rate significantly removed this parasite ($P<0.01$). Nevertheless, the coadministration of piperonyl butoxide with fenbendazole significantly improved the removal of adult *H. contortus* ($P<0.01$). The faecal egg counts were significantly reduced by both fenbendazole and fenbendazole with piperonyl butoxide; the reduction, although higher with the combination was not significantly different ($P=0.062$) from that attributed to the benzimidazole given alone. Taking into account the much higher fecundity of *H. contortus* in comparison to *O. circumcincta*, a higher difference in terms of faecal egg production would have been expected between the group that received fenbendazole alone and the one that received the drug combination, had the level of resistance of the *H. contortus* strain been higher.
Piperonyl butoxide given alone had no effect on worm burden or faecal egg excretion and therefore the interaction between fenbendazole and piperonyl butoxide is not an additive one. Also, the potentiation is unlikely to occur at the parasite level since knowledge so far acquired on drug metabolism in helminths suggests that nematodes, unlike insects are not equipped with a microsomal cytochrome system that would enable them to detoxify xenobiotics (Barrett, 1981). The synergism observed here is most likely due to an improvment of the pharmacokinetic profile of the potentiated drug in the animal host and not in the parasite, as it is the case for the synergistic action of piperonyl butoxide towards pyrethrins (O'Brien, 1969; Haley, 1978).

In conclusion, piperonyl butoxide administered orally with fenbendazole and at much lower doses than in the preliminary studies (chapter 3), significantly increased the systemic availability of the two anthelmintically active moieties (FBZ and FBSO). This was reflected in a high efficacy of the drug combination against benzimidazole-resistant nematodes.

Chapter 5

Metabolism (S-oxidation) of benzimidazole anthelmintics: *in vitro* studies

5.1 Introduction

The metabolism of benzimidazole anthelmintics occurs mainly in the hepatic cells (see Chapter 1). Oxidation of ABZ into ABSO *in vitro* has been demonstrated in rat (Fargetton *et al.*, 1986; Souhaili-El Amri *et al.*, 1988a,b), pig (Souhaili-El Amri *et al.*, 1987), sheep (Galtier *et al.*, 1986a; Lanusse *et al.*, 1993c), cattle (Lanusse *et al.*, 1993b) and human (Rolin *et al.*, 1989) liver microsomes. Initially, it was proposed that sulphoxidation of ABZ was a flavin-monooxygenase (FMO) dependent reaction, while the oxidation of ABSO into ABSO2 (sulphonation) was cytochrome P450-dependent. There is now evidence to suggest that the enzymatic systems involved in the sulphoxidation of ABZ are species-dependent, i.e, both cytochrome P450 and FMO systems act equally in rats and probably in dogs and man, while FMO is the predominant system in ruminants (Delatour *et al.*, 1994). Both systems are also involved in the sulphoxidation of fenbendazole (FBZ) in rats (Murray *et al.*, 1992). There are no reports on the *in vitro* metabolism of triclabendazole (TCBZ).

Hepatic subcellular fractions (microsomes) or intact hepatocytes maintained in culture are useful *in vitro* models for studies on the biotransformation of benzimidazole drugs. *In vitro* models may help understand aspects of BZ pharmacokinetic behaviour (e.g. first-pass effect), and also help assess effects of various factors that can alter their metabolism.

The aim of the present study, was to compare the *in vitro* sulphoxidation and sulphonation of three thiosubstituted benzimidazoles- fenbendazole, triclabendazole and albendazole - and to determine the effect of piperonyl butoxide on the metabolism of each of these benzimidazole drugs, using microsomal preparations and primary culture of hepatocytes isolated from rat livers. The involvement of cytochrome P450 in the biotransformation of triclabendazole was investigated using 1-aminobenzotriazole (1-ABT) as inhibitor of cytochrome P450 (Mugford *et al.*, 1992).

5.2 Materials and methods

5.2.1 Microsomal preparations

5.2.1.1 Chemicals

Nicotinamide adenosine dinucleotide phosphate (NADP), isocitrate dehydrogenase, trisodium isocitrate, Trizma base, Trizma hydrochloride and glycerol were all purchased from Sigma Chemical Co.(St Louis, MO, USA). All other chemicals were obtained from BDH Chemicals Ltd (Poole, UK). Standard drugs were obtained from sources mentioned earlier (Chapter 3) with the exception of triclabendazole metabolites (Ciba Geigy AG, Basel, Switzerland).

5.2.1.2 Isolation

Four (04) female Sprague-Dawley rats weighing 300-400 grams were used for the preparation of liver microsomes. They were killed by decapitation, the abdomen was opened and the liver perfused with ice-cold saline (0.9% NaCl solution) through the portal vein. The liver was excised, drained of excess moisture and weighed. All procedures were carried out at 0-4º C. The organ was collected in 3 volumes of 1.15% KCl and finely chopped before homogenization. This was carried out using a Potter-Elvehjem homogenizer; three to four passages of the teflon pestle were usually sufficient to disrupt the liver tissue. The liver homogenate was centrifuged for 20 minutes at 9000 *g* in a Beckman J2-21 (California., USA) refrigerated centrifuge. This centrifugation allowed the removal of cell debris, nuclei and mitochondria. After removing the floating fat layer with a spatula, the supernatant was decanted in 4 or 6 Beckman Ultra-Clear tubes (California, USA) and centrifuged at 105000 *g* for 75 minutes in a Beckman L8-55 refrigerated ultracentrifuge. The cytosolic fraction (supernatant) was discarded and, using an ultra-turrax (Janke & Kunkel GmbH & Co., Germany) the microsomal pellet was resuspended in 6 ml of 0.1 M Tris-phosphate buffer (pH 7.4) containing 20% (v/v) glycerol. The suspensions were frozen in liquid nitrogen and stored at -80ºC until used for incubation assays . Fifty to one hundred microliters of the microsomal suspension were used for the determination of total protein and cytochrome P450 concentrations. Microsomal protein concentrations were measured using a commercially available kit (Sigma Chemical Co., St Louis, MO, USA) which is based on the

method developed by Lowry *et al.* (1951) and modified by Peterson (1977). Briefly, an alkaline cupric tartrate reagent complexes with the peptide bonds and forms a purple -blue colour when a phenol reagent is added. Bovine serum albumin was used as a standard. Absorbance was read at 750 nm in a Pye-Unicam SP8-400 spectrophotometer (Cambridge, UK). Spectrophotometric determination of cytochrome P450 was carried out according to the method of Omuro and Sato (1964). This method is based on the ability of cytochrome P450 as a haemoprotein to bind to carbon monoxide (CO) and result in a characteristic absorption spectrum at 450 nm (hence the name) when the haem iron is reduced (sodium dithionite is used as a reducing reagent). A molar extinction coefficient of 91 $mM^{-1}cm^{-1}$ was used to convert absorbance units to nmoles of cytochrome P450.

5.2.1.3 Drug incubation

Incubations were conducted aerobically in a shaking water bath at 37º C for a period of 1 hour. Incubation mixtures containing 4 mg of microsomal protein, 50 µM of test drug (fenbendazole, oxfendazole, albendazole and triclabendazole were each dissolved in 5 µl dimethylsulphoxide), an NADPH-generating system consisting of 1mM NADP+, 0.15 M $MgCl_2$, isocitrate dehydrogenase (0.2 Units/ml) and 15 mM trisodium isocitrate, were made up in a final volume of 1 ml 0.1M Tris buffer (pH 7.4) in 10-ml glass test tubes. Piperonyl butoxide was dissolved in 5 µl dimethylsulphoxide (DMSO) and used at a final concentration of 100 µM. Tubes without microsomes were used as controls for possible nonenzymatic drug conversion. Incubations were terminated by putting tubes in boiling water for 2 minutes followed immediately by freezing at -20ºC until analysis. Incubations were conducted in triplicates.

5.2.2 Hepatocyte culture

5.2.2.1 Chemicals

Williams' Medium E (WME), foetal calf serum, penicillin-streptomycin, L-glutamine were purchased from Gibco BRL-Life Technologies Ltd (Renfrewshire, UK). Collagenase type 1A, bovine pancreas insulin, bovine serum albumin and 1-aminobenzotriazole were purchased from Sigma Chemical Co.(St Louis, MO, USA)

5.2.2.2 Isolation of cells

Three (03) female Sprague-Dawley rats were used. They were anaesthetised in an induction chamber using halothane (Fluothane, ICI Pharmaceuticals, Cheshire, England) and oxygen (BOC Limited., Surrey, England). Anaesthesia was induced by turning the vaporiser setting to 3% and the oxygen flowmeter to 1L/minute. The sleeping rat was then removed from the chamber and anaesthesia was maintained with halothane at 1.5-2% delivered through a face mask.

The method used for cell isolation was based on the the two-step procedure described by Seglen (1975) which consists of an initial perfusion with calcium-free buffer in order to remove blood components, followed by perfusion with collagenase buffer which enables the dispersion of liver tissue into single cells. The abdomen was opened and a loose ligature was placed around the portal vein which was then punctured with a 20-gauge cannula (Ecouen, France) and the perfusion immediately started with the calcium-free buffer dispensed by a peristaltic pump at a flow rate of 40 ml/minute.When the liver started blanching (indicating that the cannula was in the vein), the ligature was secured and to avoid build-up of pressure and permit perfusate efflux, the lower vena cava was cut. Buffers used and duration of perfusions are described in Appendix D-1.

5.2.2.3 Culture and drug incubation

Cells were washed three times before seeding; once with the washing buffer and twice with the medium. Washing consisted of resuspending the cells in the buffer or medium (two tubes of 50 ml each) by gently inverting the tubes. After centrifugation at 90 *g* for 3 minutes, the buffer or medium was discarded and replaced and the procedure repeated with new medium. Cells were then counted and their viability assessed by trypan blue exclusion which is based on the ability of intact (viable) cells to exclude dyes such as trypan blue which is taken up by damaged cells and become stained. Cell suspensions of $1x10^6$ viable cells per ml of medium were made up. Cells were then plated in 6-mm plastic dishes (Nunc, Roskilde, Denmark) at a density of $4x10^6$ viable hepatocytes per dish (i.e, 4 ml of the suspension). Composition of the media are reported in Appendix D-2.

After 3-4 hours pre-incubation to allow seeded cells to attach, the attachment medium (with floating dead cells) was removed and replaced with serum-free medium (Appendix D-2). Drugs (fenbendazole, albendazole or triclabendazole) dissolved in DMSO were added. Piperonyl butoxide was added as required. 1-aminobenzotriazole was added to triclabendazole incubation dishes at a final concentration of 10 mM. Cells were incubated in a humidified incubator (Heraeus Instruments GmbH, Hanau, Germany) at 37ºC under an atmosphere of 95% O_2/5% CO_2 (v/v) Incubations were carried out in triplicates. After 24 hours, the samples, i.e., medium together with the cells which were detached with cell scrapers (Nunc, Roskilde, Denmark) were collected in 10-ml plastic tubes and frozen until analysis.

5.2.3 Drug analysis

Drugs and their respective metabolites were analysed according to the methods described in Section 4.2.3. Triclabendazole and its metabolites were extracted using a solid phase extraction method. To 1 ml of sample was added 1ml acetonitrile, the mixture was vortexed for 20 seconds and then centrifuged at 1000 *g* for 15 minutes. The supernatant was mixed with an equal volume of water and then passed through a preconditioned C18 SepPak cartridge (see Section 2.2.3.3). Five millilitres of water followed by 0.4 ml of methanol were passed through the cartridge and the drug was eluted with 3 ml of methanol. HPLC conditions were identical to those described for FBZ (Section 4.2.3).

Because of the poor aqueous solubility of the benzimidazoles, especially of fenbendazole, it was difficult to obtain a solution or even a homogeneous suspension in the incubates; this led to spurious recoveries of parent drug when samples were transfered several times and when an aliquot and not the total sample was analysed. This technical problem was reported by other authors (Marriner, 1980) and we could not overcome it to achieve an accurate estimation of parent compounds in hepatocyte culture medium. This was however possible with microsomal preparations since samples were not transfered many times, total samples were used for drug extraction and each incubation tube was rinsed with 1 ml acetonitrile which was then used for the sample extraction.

5.2.4. Statistical analysis

Extent of conversion and amount of unchanged drug, with and without metabolic inhibition were compared by analysis of variance and a $P<0.05$ was considered significant.

5.3 Results

5.3.1 incubation with hepatic microsomes

Protein and cytochrome P450 contents of the microsomal specimens used for incubations are reported in Appendix D-3.

Incubation of the substrates without microsomes did only result in a slight sulphoxidation (0.4%) for albendazole (Appendix D-4).

In microsomal incubations, all substrates were converted to their respective sulphoxide and sulphone metabolites. The extent of metabolism was however variable between drugs as can be seen from both the disappearance of substrate (Appendix D-5, D-6, D-7, D-8 and Figure 5-1) and the formation of sulphoxide (Appendix D-9, D-13 and D-15) and sulphone (Appendix D-10, D-11, D-14 and D-16) metabolites; while triclabendazole and albendazole were extensively metabolised (97% and 82%, respectively) fenbendazole was only partially converted to its two S-oxide metabolites (16 %). Oxfendazole incubated for 1 hour was also slowly converted to fenbendazole sulphone (13 %).

Effect of inhibition with piperonyl butoxide (PB) on the metabolism of FBZ, FBSO, ABZ and TCBZ are shown in Figures 5-2, 5-3, 5-4 and 5-5, respectively (see also Appendix D-9 to D-16). Sulphoxidation and sulphonation of fenbendazole were reduced by 65% and 67%, respectively, when fenbendazole was incubated with PB. Also, the amount of unchanged fenbendazole was significantly higher ($P<0.01$) (Appendix D-5, Figure 5-1) with metabolic inhibition. The rate of albendazole sulphoxidation was significantly diminished ($P<0.05$) by PB. Albendazole sulphonation, although very slow and variable, was also reduced from 0.14 ± 0.05 to 0.05 ± 0.03 nmol/mg microsomal protein/hour. Unchanged ABZ remaining in the incubation mixture (Figure 5-1) was significantly increased ($P<0.01$) from 8.48 ± 1.91 to 25.25 ± 3.55 nmol/ml when PB was added to the reaction mixture. Triclabendazole sulphoxidation did not change with inhibition; sulphonation was however reduced by 61%. Amount of unchanged TCBZ was 1.24 ± 0.96 nmol/ml when TCBZ was incubated alone and 6.33 ± 3.85 nmol/ml when

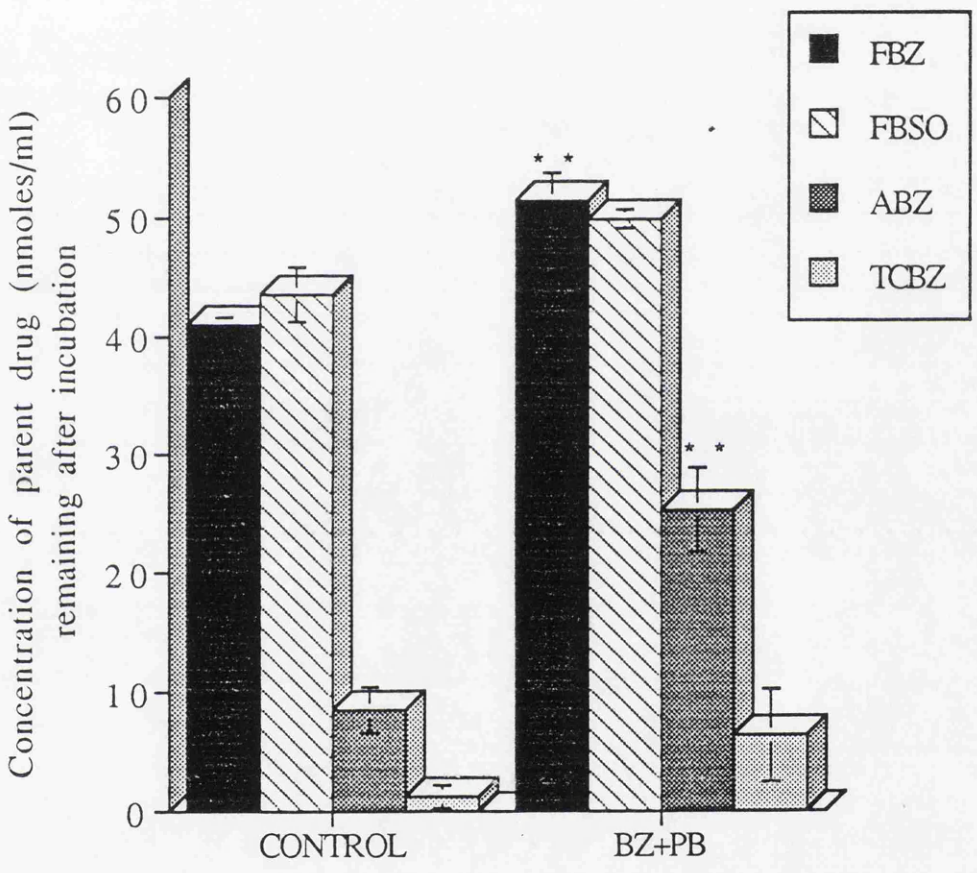

Figure 5-1. Amount of unchanged drug remaining in the microsomal reaction mixture after incubation of BZ (50 μM) with (BZ+PB) and without (control) piperonyl butoxide (n=4). ** Significantly different from control ($P<0.01$)

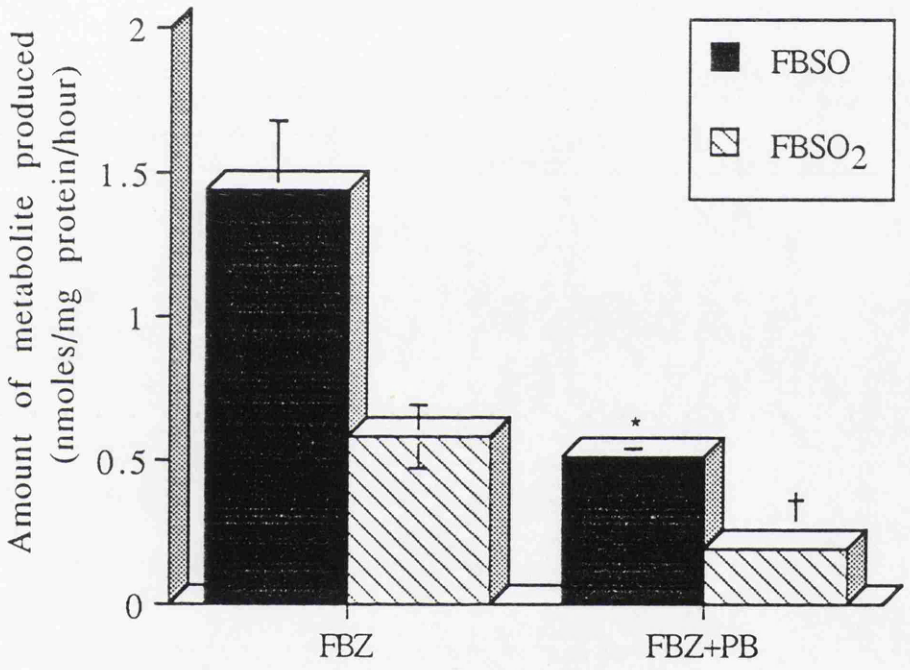

Figure 5-2. Effect of piperonyl butoxide (PB) (100 μM) on microsomal fenbendazole metabolism in rat liver (n=4).
*Significantly different from control (P<0.05)
†Significantly different from control (P<0.001)

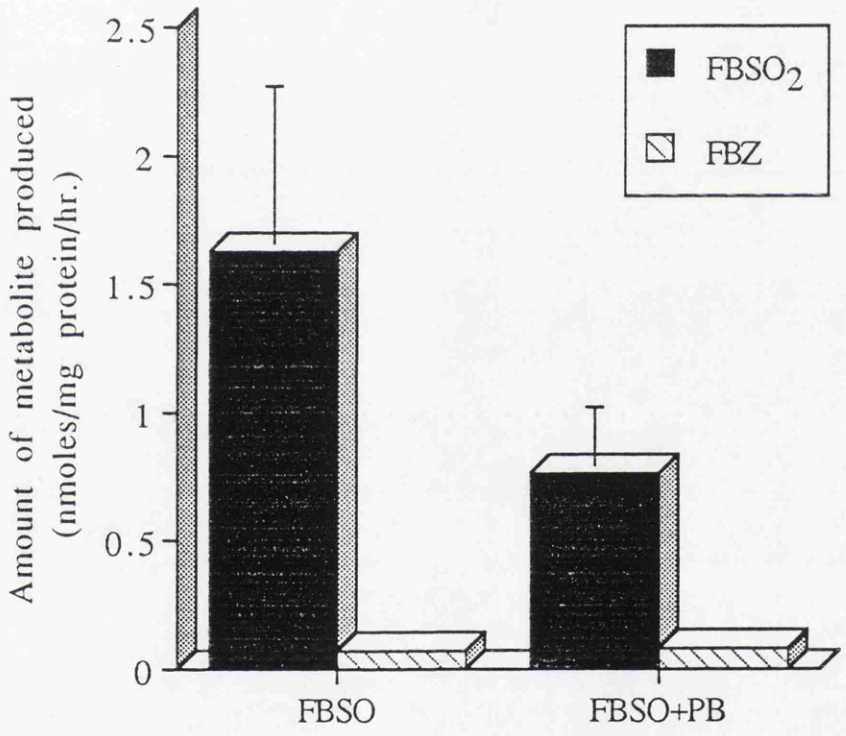

Figure 5-3. Effect of piperonyl butoxide (PB) (100 μM) on microsomal fenbendazole sulphoxide (FBSO) metabolism in rat liver (n=3).

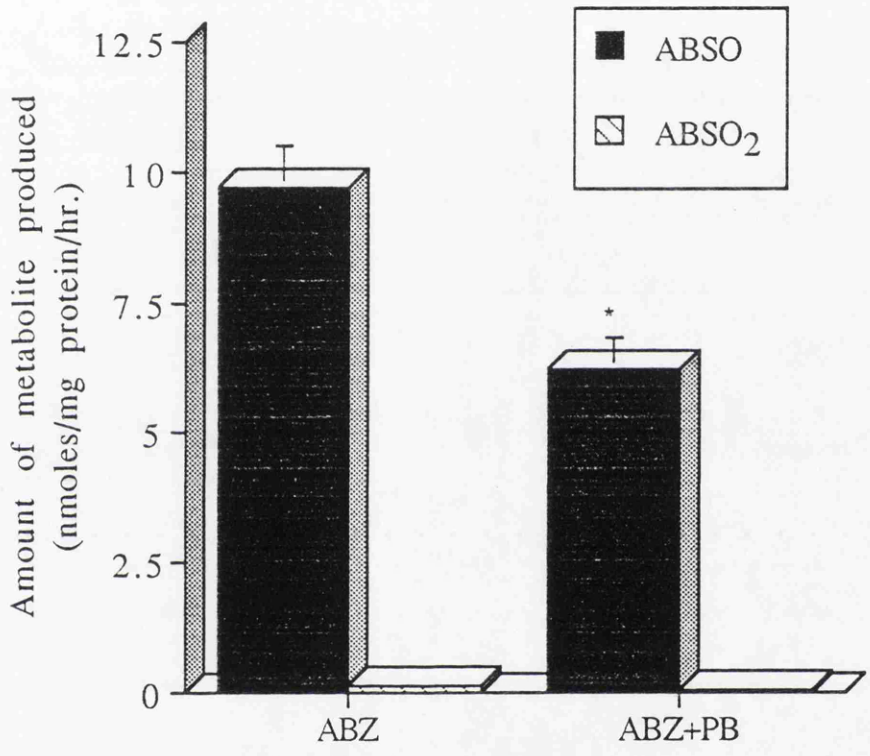

Figure 5-4. Effect of piperonyl butoxide (PB) (100 μM) on microsomal albendazole metabolism in rat liver (n=4).
*Significantly different from control ($P<0.05$)

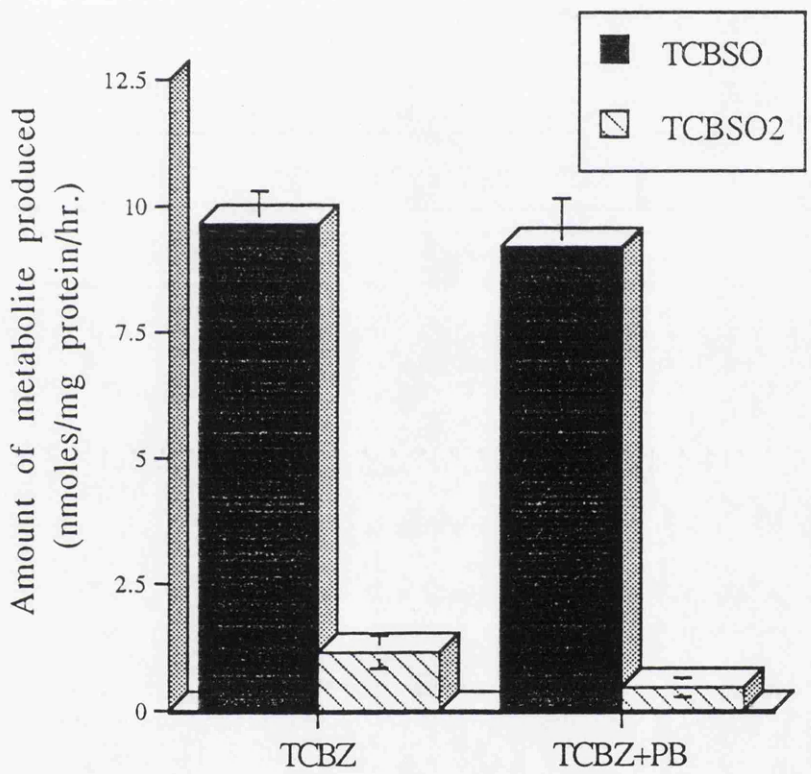

Figure 5-5. Effect of piperonyl butoxide (PB) (100 μM) on triclabendazole (TCBZ) microsomal metabolism in rat liver (n=4).

incubated with PB. Conversion of FBSO into FBSO2 was reduced by 53% when FBSO was incubated with PB. Minimal reduction of FBSO into FBZ occured (Appendix D-12) and this was not affected by inhibition of cytochrome P450 with PB.

5.3.2 Incubation with hepatocytes

Cell viability as assessed by trypan blue exclusion was between 70-85%, and after the pre-incubation period, viable cells were well attached to the plastic dish.

With a mean velocity ranging between 232.8-290.3 pmoles of sulphoxide metabolite formed/10^6 viable hepatocytes /hour (Appendix D-21 and D-23), triclabendazole was the most extensively S-oxidised substrate. Fenbendazole was more slowly sulphoxidised than albendazole and triclabendazole (Appendix D-17). However, among the benzimidazole substrates tested, albendazole demonstrated the lowest rate of sulphonation (Appendix D-20).

Inhibition with 1-aminobenzotriazole (1-ABT) (Figure 5-8) resulted in a highly significant decrease ($P<0.001$) in both sulphoxidation and sulphonation of triclabendazole (Appendix D-21 and D-22).

The effect of piperonyl butoxide is shown in Figures 5-6, 5-7 and 5-9 for fenbendazole, albendazole and triclabendazole, respectively. Sulphoxidation of FBZ, ABZ and TCBZ was reduced by 69%, 64% and 50%, respectively. Inhibition resulted in undetectable levels of albendazole sulphone (Appendix D-20, Figure 5-7). Sulphonation of FBZ and TCBZ were also significantly reduced.by piperonyl butoxide.

5.4 Discussion

In this study, the S-oxidation of three modern benzimidazoles was investigated using two *in vitro* models. In both models, one striking observation was the difference in the speed at which substrates were oxidised, and particularly the difference between fenbendazole and the two other BZ studied (TCBZ and ABZ). It appears clearly from the result of this study that triclabendazole and albendazole are far more rapidly metabolised than fenbendazole. This finding correlates well with several *in vivo* pharmacokinetic studies. Indeed, whereas triclabendazole and albendazole undergo first-pass effect in all species studied and are absent or detected at trace amounts in the systemic circulation

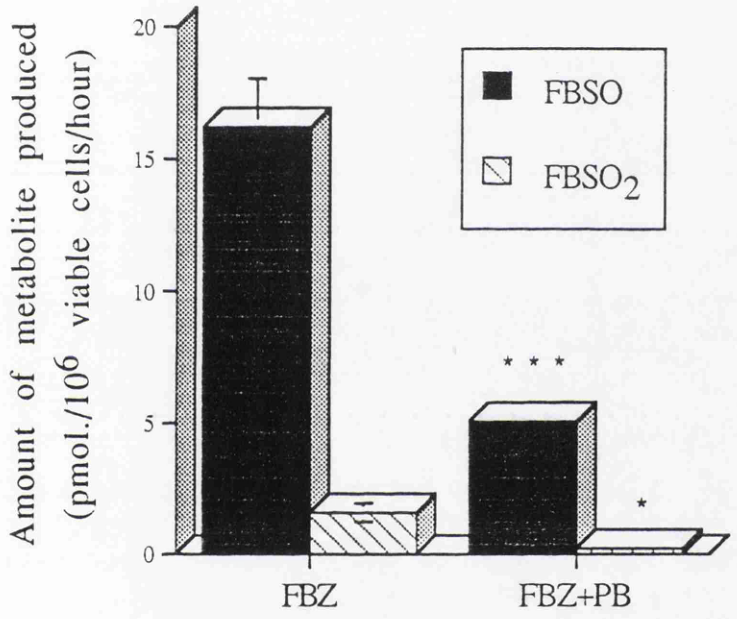

Figure 5-6. Effect of piperonyl butoxide (PB)(100 μM) on fenbendazole metabolism in rat hepatocytes (n=3).
*Significantly different from control (P<0.05)
***Significantly different from control (P<0.005)

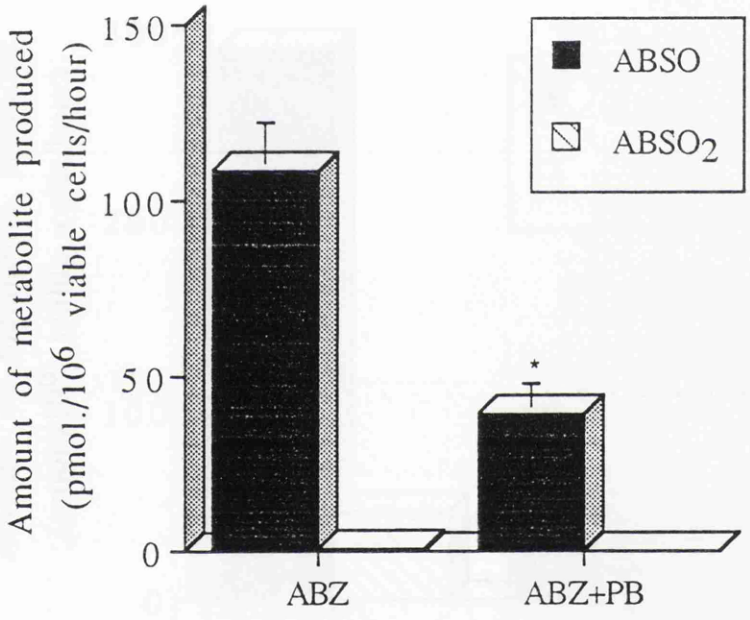

Figure 5-7. Effect of piperonyl butoxide (PB) (100 μM) on albendazole metabolism in rat hepatocytes (n=3).
*Significantly different ($P<0.05$)

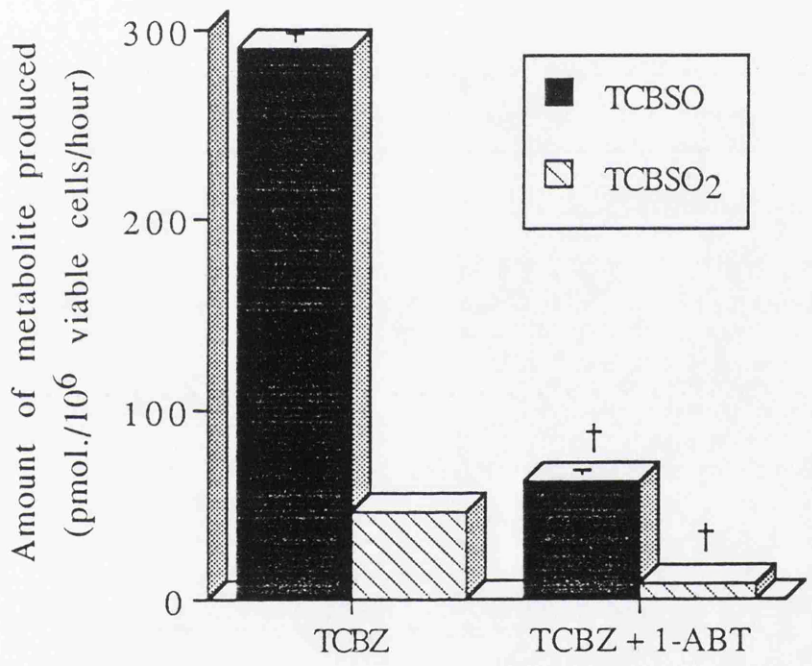

Figure 5-8. Effect of 1-aminobenzotriazole (1-ABT) (10 mM) on triclabendazole metabolism in rat hepatocytes (n=3).
†Significantly different from control ($P<0.001$)

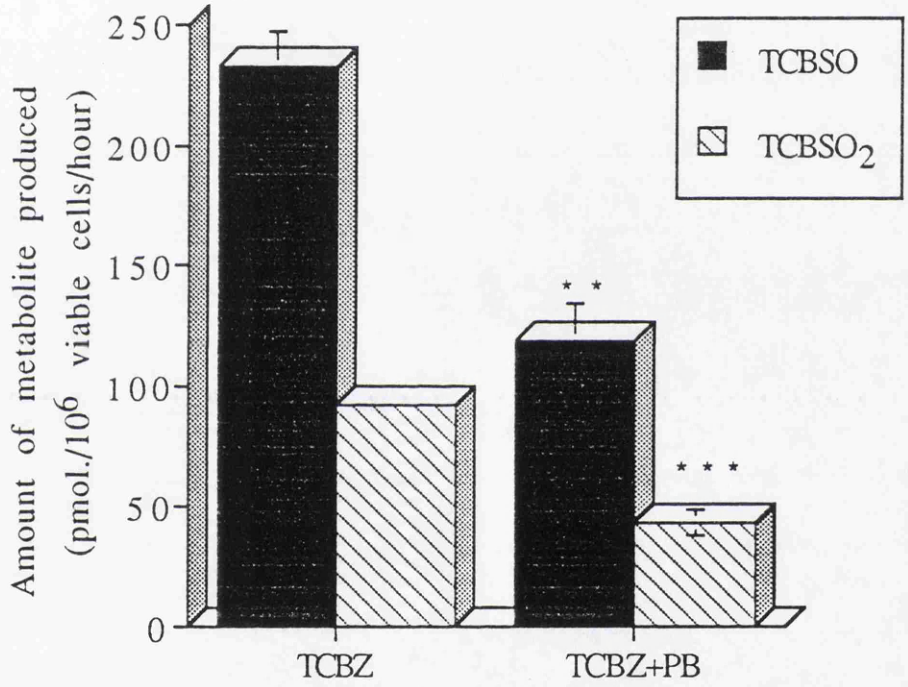

Figure 5-9. Effect of piperonyl butoxide (PB) (100 μM) on triclabendazole metabolism in rat hepatocytes (n=3).
** Significantly different from control (P<0.01)
***Significantly different from control (P<0.005)

after administration (Marriner & Bogan, 1980; Gyurik *et al.*, 1981; Mohammed-Ali *et al.*, 1986; Hennessy *et al.*, 1987; Bogan *et al.*, 1988a; Hennessy *et al.*, 1989; Also see Chapter 2), fenbendazole, is more slowly converted to its sulphoxide metabolite and is detected and persists for several hours postadministration (Marriner & Bogan, 1981b; Ngomuo *et al.*, 1984; Also Chapters 3 and 4). Two factors may influence the rate of sulphoxidation; the hydrophobic character of the molecules and their steric nature. The first feature is now seen as a minor factor in the interaction of benzimidazole derivatives with cytochrome P450 (Little & Ryan., 1982; Murray *et al.*, 1992). The present experiment supports this suggestion since if water solubility was a determinant factor, more extensive metabolism would have been expected for fenbendazole than for albendazole, the former compound being more hydrophobic than the latter. This was not the case in the present experiment. The steric nature of benzimidazole derivatives is more likely to be a crucial factor involved in the accessibility of the P450-heme to the sulphur site (Murray *et al.*, 1992) and the proximity of benzene rings to this site seems to hinder such interactions (Hennessy, 1993b). The sulphur in the fenbendazole molecule is linked to two benzene rings; the phenyl substituent and the benzene ring of the benzothiazolyl nucleus; this location could result in steric hindrance. Albendazole has an aliphatic substituent and the sulphur is therefore only linked to one benzene ring. The methylthio group of triclabendazole is located on position -2 with no benzene group in the vicinity of the sulphur thus making it more 'approachable'. These structural features may explain why triclabendazole is more sulphoxidised than albendazole and the latter more so than fenbendazole.

In both hepatic microsomes and hepatocyte cultures, piperonyl butoxide was shown to reduce substantially the S-oxidation of the drugs studied. Interestingly, the degree of inhibition seemed to be inversely related to the initial (without inhibition) velocity, i.e., fenbendazole more extensively inhibited than albendazole and the latter more so than triclabendazole. In fact triclabendazole sulphoxidation was unaffected by piperonyl butoxide in microsomal preparations. It could be that the formation of PB reactive metabolites which are responsible for the inhibition happens relatively slowly in comparison to the rapid TCBZ sulphoxidation. TCBZ sulphonation, a slower reaction than the sulphoxidation, was

diminished by PB. The increase in the concentration of unchanged TCBZ in microsomal mixtures could have been caused by an inhibited hydroxylation (not measured in this study) of the parent compound. In contrast to the finding with hepatic microsomes, cultured hepatocytes did show a decrease in TCBZ sulphoxidation following incubation with piperonyl butoxide. This could be due to several factors which distinguish intact cells from subcellular fractions including uptake by and distribution within the cells of both drug and inhibitor, NADPH cofactor-supply and the sequential arrangement of both cytosolic and microsomal biotransformations within the cells. Also the presence of bovine serum albumin in the culture medium would be expected to cause protein binding with TCBZ and slow down its uptake and metabolism by the cells. It appears, therefore, that in many respects, primary culture of hepatocytes constitute a more realistic model for the in-vivo situation.

The main limitation encountered in the study of benzimidazole metabolism *in vitro* was the low expression of sulphonation. This meant that a comparison of the impact of inhibition on the two oxidative reactions was not possible. Nonetheless, in order to verify that the decrease in sulphonation with piperonyl butoxide was not a consequence of lower sulphoxide concentrations, inhibition of sulphonation was further investigated by incubating oxfendazole at high concentrations (50 μM) with and without inhibition. This resulted in a reduction of sulphonation which did not reach statistical significance because of the large interindividual variation. However, in all cases the ratio sulphone/sulphoxide was decreased by inhibition which suggest that the sulphonation was independently affected by inhibition.

Inhibition of TCBZ S-oxidation by 1-aminobenzotriazole, confirms the involvement of the cytochrome P450-dependant monooxygenase system in the hepatic biotransformation of this anthelmintic. 1-aminobendzotriazole has been shown to cause a 70% inhibition of P450 at the concentration used (10 mM) with no detectable effect on other metabolizing enzyme systems (Mugford *et al.*, 1992). Also 1-aminobenzotriazole has been shown to inhibit P450 isozymes responsible for catalysing ethoxycoumarin O-deethylation (ECOD) and ethoxyresorufin O-deethylation (EROD), activities characteristic of the P450 1A and 2B subfamilies (Ryan *et*

al., 1979; Guengerich *et al.*, 1982; Dutton and Parkinson, 1989). It could be that S-oxidation of TCBZ is catalysed by isozymes belonging to either or both subfamilies. The P4501A subfamily has been shown to interract with albendazole (Souhaili-El Amri *et al.*, 1988b) and oxfendazole (Gleizes *et al.*, 1991a,b), both are thiosubstituted benzimidazoles. It is therefore possible that this subfamily has an affinity for sulphur-containing benzimidazoles (Gleizes *et al.*, 1991a), including triclabendazole.

In conclusion, a comparative study of BZ-metabolism *in vitro* was carried out and triclabendazole was found to be the most extensively metabolised molecule of the three S-substituted benzimidazoles studied. The inhibition studies with piperonyl butoxide give support to the results already reported *in vivo* (see Chapters 3 and 4) by demonstrating the *in vitro* effect of piperonyl butoxide at the main site of BZ biotransformation. The involvement of cytochrome P450 as an important catalyzing system for TCBZ S-oxidation was confirmed.

Chapter 6

Effect of early treatment with rafoxanide on antipyrine clearance in sheep infected with *Fasciola hepatica*

6.1 Introduction

Infection of lambs with the liver fluke *Fasciola hepatica* has been shown to alter significantly the function of some hepatic drug-metabolising systems (Galtier *et al.* 1986b). Fluke infection causes an impairment of the oxidative drug metabolism activity as a result of a decrease in hepatic cytochrome P450-mediated reactions. Consequently, the plasma clearance of antipyrine (Figure 6-1), a pharmacokinetic marker of the hepatic oxidative activity, has been shown to decrease between 4 and 16 weeks after moderate infection with *Fasciola hepatica* (Tufenkji *et al.*, 1988).

Rafoxanide, 3,5-diiodo-3'-chlor-4-(p-chloro-phenoxy)-salicylanilide, belongs to an important group of flukicidal drugs (figure 6-2) characterised by their high protein binding and long persistence in the plasma (see Chapter 1; section 1.1.1.2). The elimination half-lives of rafoxanide and the related salicylanilides closantel and oxyclozanide are very long and in sheep, they are in the magnitude of 16.6, 14.4 and 6.4 days, respectively (Mohammed-Ali & Bogan, 1987). *In vivo* testing of salicylanilides against early stages of *Fasciola hepatica* has been complicated since small immature larvae are difficult to count and most efficacy trials have determined the number of readily identifiable adult parasites at some stage after treatment. Since salicylanilides have a long residence time in the plasma, and immature flukes reach their adult stage while sustained concentrations of drug are still present in the bloodstream, it has been suggested that the efficacy of these compounds against early stages of *Fasciola hepatica* may have been overestimated and that only mature forms are truly susceptible to this group of flukicides (Mohammed-Ali & Bogan, 1987). However, studies on the efficacy of closantel against immature flukes (Maes *et al.* 1988) showed similar fluke removal when animals infected with 6-week old *Fasciola hepatica* were killed and liver parasites counted one week after treatment (7 weeks post-infection) or 6 weeks after treatment (12 weeks after infection). The same authors have suggested that immature flukes may not have been killed by the drug but only arrested in their development.

The effect of fasciolicide treatment on the restoration of liver metabolic capacity has been reported in one study. Sheep treated with a flukicide benzimidazole , luxabendazole, 8 weeks post-infection had

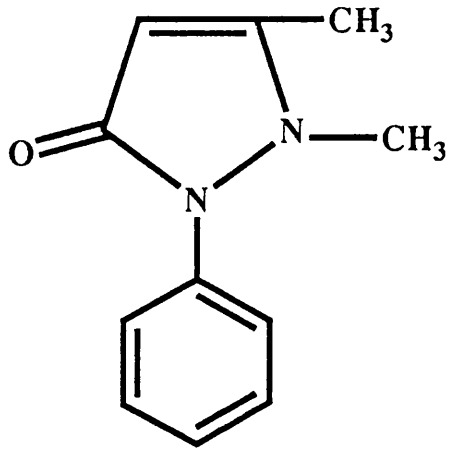

Figure 6-1. Chemical structure of antipyrine

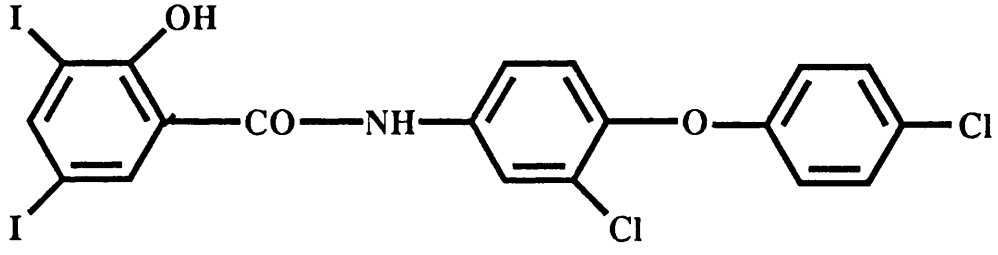

Figure 6-2. Chemical structure of rafoxanide

normal antipyrine elimination half-life 3 weeks after the anthelmintic was given (Burrows *et al.* 1991).

The aim of the present study was to investigate the stage specificity of rafoxanide by measuring antipyrine plasma clearance and relevant biochemical parameters (GLDH and γGT) throughout the course of an experimental liver fluke infection in lambs.

6.2 Materials and Methods

6.2.1 Animals

Eighteen 10-month old Scottish Blackface sheep weighing 26.92±0.77 kg were used in this study. They were parasite-free and kept indoors throughout the entire period of investigation with hay, cereal concentrate (Stewarts, Larbert, UK) and water provided *ad libitum*.

6.2.2 Chemicals

Antipyrine was purchased from Sigma Chemical Co.(St. Louis, MO, USA). Rafoxanide pure compound was donated by Merck Sharp & Dohme development laboratories (Hertfordshire, UK). Rafoxanide (Flukanide 3% W/V, oral drench) for administration to animals was obtained from MSD AGVET (Hertforshire, UK). All organic solvents were purchased from Rathburn Chemicals Ltd. (Walkerburn, UK)

6.2.3 Drug administration and sample collection

Antipyrine was dissolved in sterile water and was administered as a 100 mg/ml solution into the left jugular vein at a dose rate of 25 mg/kg. For each test, blood samples were taken from the contralateral vein prior to and 0.083, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h after the bolus injection of antipyrine. Rafoxanide was administered orally at the recommended dose rate of 7.5 mg/kg. Blood samples for rafoxanide pharmacokinetics were taken prior to and 1, 2, 3, 4, 8, 12, 19, 27, 33, 39, 47, 53, 63, 70 and 76 days after rafoxanide administration. All blood samples were centrifuged, plasma collected and stored at -20 ºC until analysis by HPLC.

6.2.4 Experimental design

Antipyrine was administered to all animals one month before infection at which time they were allocated to three groups A, B and C, of six animals each. Sheep in groups A and B were then each infected with 200 *Fasciola hepatica* metacercariae (MAFF. New Haw, Weybridge, Surrey). Group C remained uninfected. Four weeks after infection,

antipyrine was administered again, and immediately after the 24h-blood sample was collected, sheep in group B and C received an oral dose of rafoxanide. Antipyrine was subsequently administered to the three groups of animals at 6, 8, 10, 12 and 14 weeks post-infection. All animals were killed 15 weeks post-infection and their livers (Figure 6-3) cut into slices for the determination of adult fluke numbers.

6.2.5 Drug analysis

6.2.5.1 Antipyrine

Antipyrine was extracted from alkalinised plasma using a mixture of dichloromethane/pentane as described by Danhof *et al.* (1979). Briefly, to 1 ml of plasma was added 100 µl of sodium hydroxide (NaOH, 2N); after homogenization antipyrine was extracted twice with DCM/pentane (50/50). The organic phase was separated from the plasma by centrifugation at 4000 rpm for 5 minutes; it was then collected and evaporated to dryness at 40ºC. The HPLC. system comprised a solvent delivery pump (Gilson Model 802, Scotlab Instrument sales Ltd, Glasgow, UK) connected to an ODS-Hypersil (5µ) column (16 cm x 5 mm, ID) (Shandon Southern, Cheshire, UK) and a u.v. detector (Model SP 100, Burke Electronics Ltd, Glasgow, UK). The mobile phase was a mixture methanol-water-0.17M perchloric acid (76:24:0.05, v/v/v) running at a flow rate of 1ml/min. The u.v. detector was set at a wavelength of 254 n.m. Recoveries and precision of the method are displayed in Appendix E-1. The limit of analytical detection was below 0.05 µg/ml.

6.2.5.2 Rafoxanide

Plasma samples were precipitated with acetonitrile and rafoxanide was extracted with 4ml of chloroform. After centrifugation, the organic phase was evaporated to dryness at 50ºC, the residue was then reconstituted with methanol and injected on to an ODS-Hypersil (5µl) column (16 cm x 5 mm). The mobile phase was a mixture of methanol-water- 0.17M perchloric acid (95:5:0.05, v/v/v) running at a flow rate of 1ml/min. The u.v. detector (Model SP 8450, Burke Electronics Ltd. Glasgow, UK) was set at a wavelength of 282 n.m. Drug-free plasma fortified with rafoxanide over the concentration range of 0.5-20 µg/ml was included with each assay. Recovery of rafoxanide from plasma to which the drug had been added was evaluated by reference to peak heights resulting from direct injection of rafoxanide standard solutions. The mean recovery obtained was 78.69%. and the inter-assay

coefficients of variation was 5.93% (Appendix E-2). The limit of analytical detection achieved was 0.02 μg/ml.

6.2.6 Biochemistry

Glutamate dehydrogenase (GLDH) activity, gamma-glutamyl transferase (γGT) activity and albumin concentrations were measured in plasma before infection and at 4, 6, 8, 10, 12, 14 weeks after infection with *Fasciola hepatica*. Glutamate dehydrogenase was analysed as recommended by the German Society of Clinical Chemistry (Anonymous, 1972) using test kits purchased from Randox Laboratories Ltd., Crumlin (UK). Gamma-glutamyl transferase activity was measured according to the method described by Szasz (1974) using test kits obtained from Boehringer Mannheim (Meylan, France). Plasma albumin concentrations were measured by a photometric method (Webster, 1977).

6.2.7 Pharmacokinetic and statistical analysis

A multiexponential modelling computer program CSTRIP (Sedman & Wagner, 1976) was used for the pharmacokinetic analysis of antipyrine plasma concentration time profiles obtained from each individual. Using the Akaike information criterion (Yamaoka *et al.*, 1978), plasma concentrations of antipyrine were best fitted to a biexponential equation which can be expressed as follows;

$$C_P = Ae^{-\alpha t} + Be^{-\beta t}$$

Where C_P is the plasma concentration (μg/ml) at time *t*. A and B are the intercepts on the Y-axis of the distribution and elimination slopes, respectively. Alfa and β are the rate constants (expressed in h^{-1}) of distribution and elimination, respectively and e is the base of the natural logarithm. At time zero, the plasma concentration (Cpo) is equal to the sum of the intercepts. The elimination half-life ($t1/2\beta$) was derived from the relationship $t1/2\beta = 0.693/\beta$ The area under the curve (AUC), in μg.h/ml, was calculated as the sum of the ratios of the intercepts and the corresponding first-order rate constants, i.e, $AUC = A/\alpha + B/\beta$. The total body clearance (Clb) expressed in ml/kg.min was deduced from the ratio D/AUC, where D is the dose of antipyrine administered. The antipyrine clearance index which is defined as the ratio of the mean body clearance in the post-infection or post-treatment phase to the mean body clearance in the pre-infection or pre-treatment phase, was also calculated (Bachmann, 1989). The apparent volume of the central compartment (Vc) expressed in ml/kg

was calculated as D/Cpo. The apparent volume of distribution (Vdarea) was calculated from the ratio D/AUC.β. The apparent volume of distribution at steady-state (Vdss) was calculated using transfer rate constants k12 and k21 from the formula (k12+k12/k21).Vc, where 1 and 2 refer to the body compartments. The pharmacokinetic parameters of rafoxanide were determined from observed values as previously described (Section 2.2.4).

All pharmacokinetic data were analysed using non-parametric statistical tests. Comparisons between phases within the same group were performed using a Wilcoxon signed-rank test. A Mann-Whitney U-test was used for between group comparisons. Biochemical parameters were compared by analysis of variance. Values of $P \leq 0.05$ were taken as significant.

6.3 Results

The establishment of flukes was high with more than 50% of administered metacercariae maturing to their adult stage in the infected group which did not receive rafoxanide (Table 6-1 and Appendix E-3 and E-4). The number of adult flukes recovered from the livers of infected-rafoxanide treated sheep indicated an efficacy of 85%. Mean values for plasma protein, albumin, globulin, GGT and GLDH are presented in Table 6-2 (infected untreated), Table 6-3 (infected treated) and Table 6-4 (uninfected treated). Individual values are reported in Appendix E-5 to E-11 (infected untreated), Appendix E-12 to E-18 (infected treated) and Appendix E-19 to E-25 (uninfected treated). Glutamate dehydrogenase activity was increased in both untreated and treated infected sheep between 4 and 12 weeks after fluke infection and two peaks were observed for each group (Figure 6-4). These occured at 4 and 8 weeks post-infection (207 and 219 IU/L respectively) for the untreated group and at 4 and 12 weeks (244 and 102 IU/L respectively) for the rafoxanide-treated group. No changes from the normal range were observed in the unparasitised group. Gamma-glutamyl transferase activity increased in all animals (Figure 6-5), however the greatest rise occurred in the infected untreated group which had maximum activity of 232 IU/L, 10 weeks post-infection. The plasma albumin concentration in all animals prior to infection was 30.5 ± 2.0 g/l (mean ± SEM) with no significant alteration occuring throughout the study, except in the infected untreated group (A). In this group, the albumin concentration fell significantly ($P \leq 0.05$) from a pre-infection value of 31.8 ± 2.6 g/l to 23.7 ± 0.6 g/l at

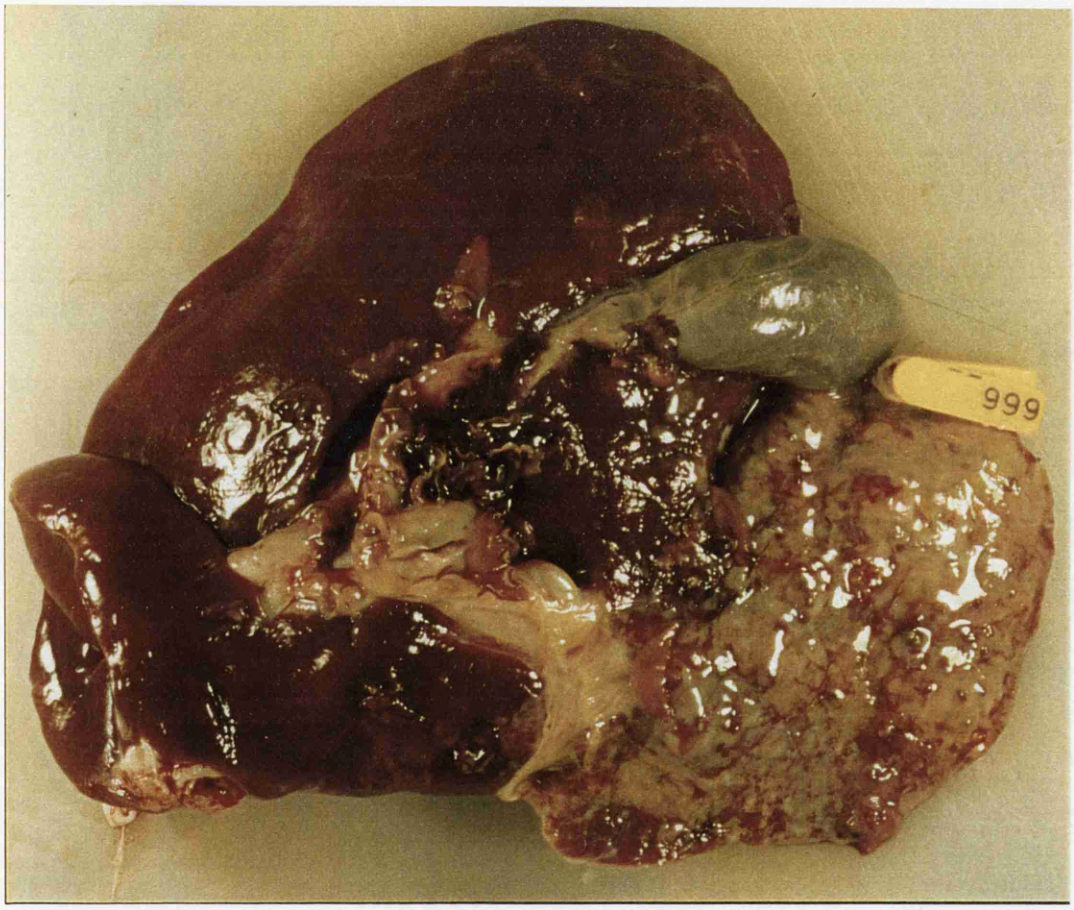

Figure 6-3. Liver lesions caused by *F. hepatica* in one infected untreated sheep.

Table 6-1. Number of adult *Fasciola hepatica* (mean ± SEM) recovered at necropsy in infected control (group A), infected treated (group B) and uninfected treated (group C) sheep.

	Number of flukes (mean ± SEM)	Efficacy (%)
Group A	106.67 ± 11.4	-
Group B	15.67 ± 4.46	85.31
Group C	-	-

Table 6-2. Plasma parameters (mean ± SEM) in group A (infected untreated) before and at different times after *F. hepatica* infection.

	Time postinfection						
	Control	4 weeks PI	6 weeks PI	8 weeks PI	10 weeks PI	12 weeks PI	14 weeks PI
Total protein (g/l)	62.2 ±1.6	70.2 ±1.5	74.3 ±1.1	73.5 ±1.7	76.0 ±1.8	75.3 ±1.9	61.2 ±2.1
Albumin (g/l)	31.8 ±2.6	31.7 ±1.2	31.2 ±0.9	29.7 ±0.8	29.0 ±1.0	27.5 ±0.9	23.7 ±0.6*
Globulin (g/l)	30.3 ±1.7	38.5 ±2.1*	43.2 ±1.8***	43.8 ±2.1***	48.7 ±1.1***	47.8 ±2.1***	37.5 ±2.5*
γGT (U/L)	32.8 ±3.5	56.7 ±3.5***	61.2 ±1.9***	98 ±19**	232 ±13***	170 ±25***	88.3 ±9.2***
GLDH (U/L)	2.2 ±0.4	207 ±44**	158 ±21***	219 ±19***	207 ±34***	108 ±18**	68.2 ±36.5

* Significantly different from control values ($P<0.05$)

** Significantly different from control values ($P<0.01$)

*** Significantly different from control values ($P<0.001$)

PI (postinfection)

Table 6-3. Plasma parameters (mean ± SEM) in group B (infected treated) before and at different times after *F. hepatica* infection.

	Time postinfection						
	control	4 weeks PI	6 weeks PI	8 weeks PI	10 weeks PI	12 weeks PI	14 weeks PI
Total protein (g/l)	66.7 ±3.1	75.2 ±2.8	74.3 ±2.3	68.3 ±1.5	69.0 ±2.1	71.5 ±2.3	68.5 ±2.8
Albumin (g/l)	31.3 ±2.2	32.7 ±0.9	31.7 ±0.4	30.7 ±0.5	32.7 ±0.5	33.0 ±0.6	29.3 ±0.8
Globulin (g/l)	35.3 ±4.0	42.5 ±2.7*	42.7 ±2.1*	37.7 ±1.2	36.3 ±1.9	38.5 ±2.2	39.2 ±3.2
γGT (U/L)	36.2 ±1.2	64.7 ±7.8	55.5 ±5.1	54.8 ±4.0	53.3 ±3.1	85.8 ±7.2	76.2 ±7.4
GLDH (U/L)	4.0 ±1.0	244 ±33***	26.0 ±7.3*	25.3 ±5.6**	42.3 ±8.1**	102 ±28*	5.5 ±1.7

* Significantly different from control values ($P<0.05$)

** Significantly different from control values ($P<0.01$)

*** Significantly different from control values ($P<0.001$)

PI (postinfection)

Treatment with rafoxanide was at 4 weeks postinfection.

Table 6-4. Plasma parameters (mean ± SEM) in group C (uninfected treated) before and at different times after rafoxanide treatment.

	Time posttreatment						
	Control (-8weeks)	Control (TD)	2 weeks PT	4 weeks PT	6 weeks PT	8 weeks PT	10 weeks PT
Total protein (g/l)	66.7 ±2.3	68.7 ±1.9	69.1 ±1.3	67.8 ±1.4	67.0 ±1.8	69.5 ±2.0	67.7 ±1.8
Albumin (g/l)	33.3 ± 1.3	34.2 ±1.0	32.5 ±0.7	31.3 ±0.7	32.5 ±0.9	33.3 ±1.4	30.8 ±0.9
Globulin (g/l)	33.3 ±2.8	34.5 ±1.2	36.7 ±0.9	36.5 ±1.1	34.5 ±1.5	36.2 ±1.7	35.2 ±1.0
γGT (U/l)	28.2 ±2.3	33.2 ±1.6	33.0 ±1.7	40.7 ±1.8**	44.2 ±2.5***	51.2 ±2.8***	51.5 ±3.3***
GLDH (U/l)	3.2 ±1.2	3.8 ±0.8	6.8 ±2.5	2.0 ±0.9	6.3 ±1.7	12.4 ±3.7	5.8 ±1.9

** Significantly different from control values (P<0.01)

*** Significantly different from control values (P<0.001)

PT (Posttreatment)

TD (treatment day; values determined immediately prior to treatment)

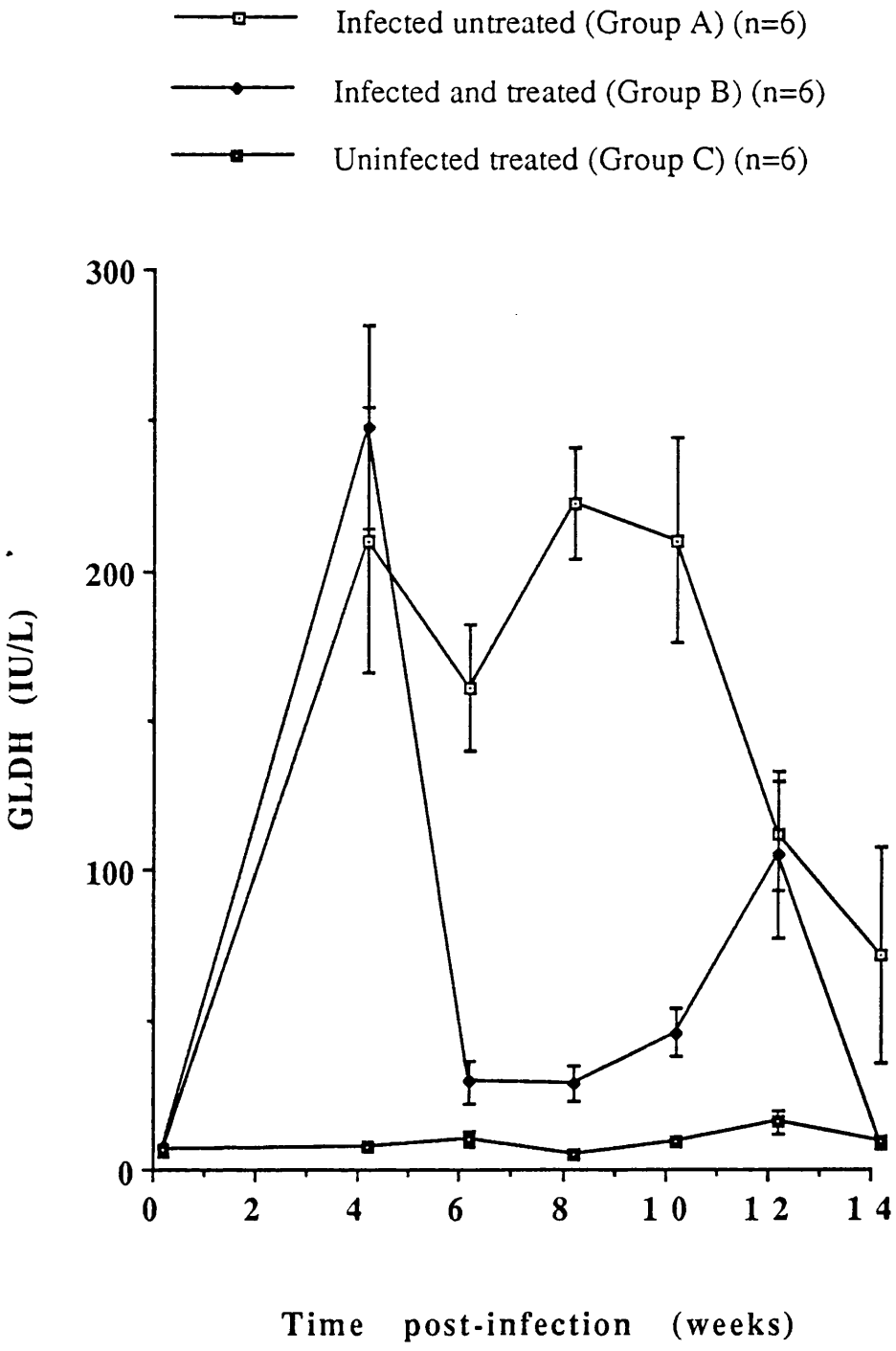

Figure 6-4. Serum glutamate dehydrogenase (GLDH) activity (IU/L) (mean ± SEM) in the three animal groups.

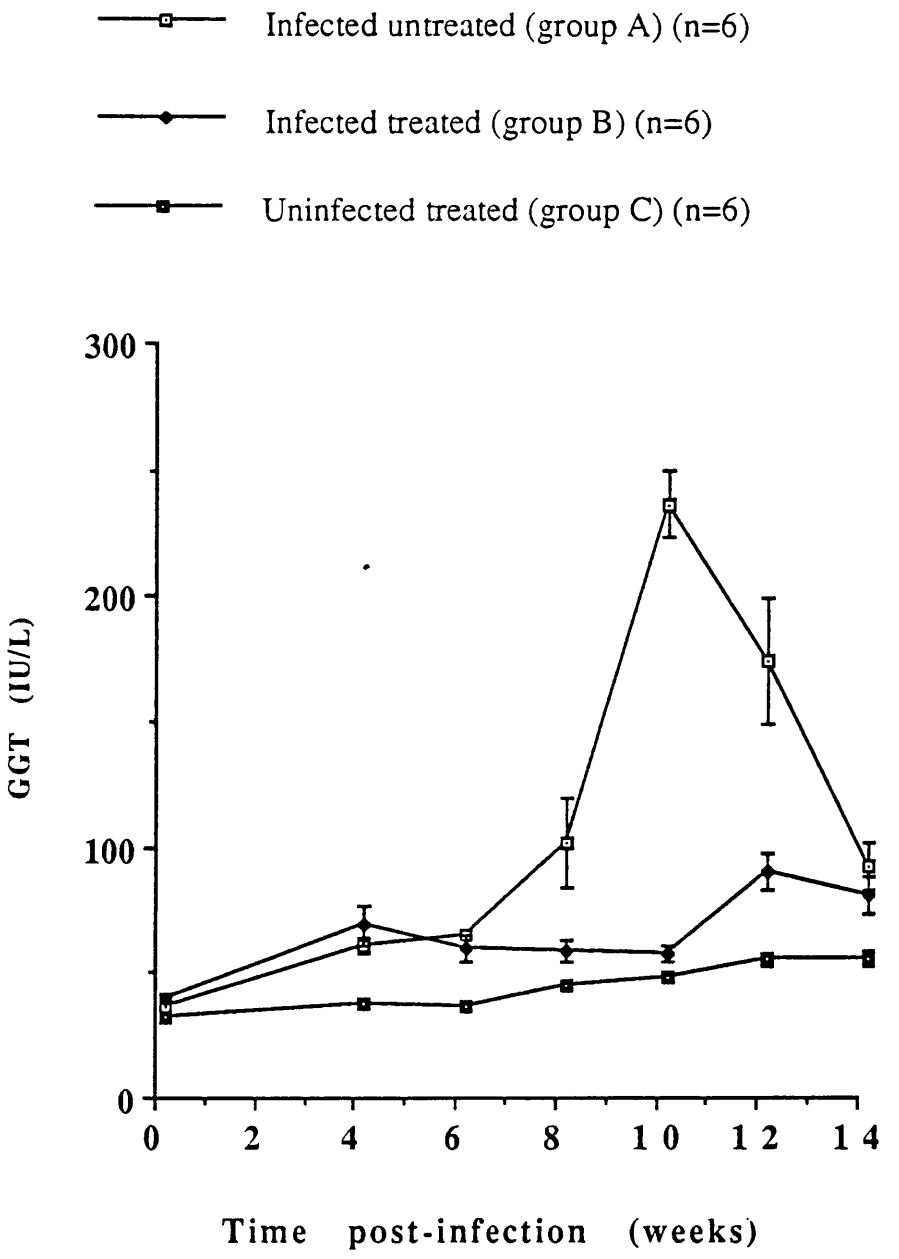

Figure 6-5. Serum gamma-glutamyl transferase (GGT) activity (IU/L) (mean ± SEM) in the three animal groups.

14 weeks post-infection. Plasma globulin concentrations increased significantly in infected untreated sheep and in infected treated sheep before treatment and two weeks after treatment.

Mean plasma concentrations of rafoxanide are presented in Table 6-11 (group B) and Table 6-13 (group C). The corresponding pharmacokinetic variables are reported in Table 6-12 (group B) and Table 6-14 (group C). Individual data are presented in Appendix E-69 to E-72. There were no significant differences in the pharmacokinetic parameters of rafoxanide between infected and uninfected sheep.

Mean plasma concentrations and pharmacokinetic parameters of antipyrine are shown in Table 6-5 and Table 6-6 (group A); Table 6-7 and Table 6-8 (group B) and Table 6-9 and Table 6-10 (group C). Individual values are dispayed in Appendix E-26 to E-39 (group A), Appendix E-40 to E-53 (group B) and Appendix E-54 to E-67 (group C). The decay of plasma antipyrine was best described by a biexponential equation. The semilogarithmic plots of mean plasma concentrations of antipyrine versus time obtained before and at two different occasions after *Fasciola hepatica* infection (group A) and, or, rafoxanide treatment (group B and C) are depicted in Figures 6-6, 6-7 and 6-8, respectively. In parasitised untreated sheep antipyrine body clearance was significantly decreased and the elimination half-life significantly prolonged between 8 and 14 weeks post-infection. In the same group clearance indices were lower than in the uninfected or the infected treated group (Figure 6-9; Appendix E-68). There was little variation in clearance indices in the parasitised untreated sheep from 8 to 14 weeks after infection. In the infected treated animals, antipyrine body clearance was maintained close to the control value until 14 weeks post-infection when it was decreased by 23% as reflected by the reduced clearance index at that stage of infection. Increases in both area under the plasma concentration time curve and biological elimination half-life correlated with the decrease in plasma clearance (Table 6-6 and Table 6-8). There were no significant changes in the volume of distribution in any of the groups. No significant alterations in the plasma kinetics of antipyrine were observed in the uninfected treated group (Table 6-10).

Table 6-5. Plasma concentrations of antipyrine (mean ± SEM) in group A (infected untreated) before and at different times after *F. hepatica* infection.

	Time postinfection						
Time (hours)	Control	4 weeks PI	6 weeks PI	8 weeks PI	10 weeks PI	12 weeks PI	14 weeks PI
0.083	43.51 ± 1.58	47.24 ± 1.95	47.33 ± 2.02	49.85 ± 2.37	44.42 ± 1.67	46.17 ± 2.64	45.29 ± 1.33
0.5	28.50 ± 1.28	30.38 ± 0.61	30.75 ± 1.52	33.68 ± 1.32	31.26 ± 1.72	31.18 ± 1.19	30.12 ± 1.39
1	19.38 ± 1.21	22.99 ± 1.11	21.25 ± 1.31	24.15 ± 1.01	24.19 ± 1.10	25.23 ± 1.23	24.01 ± 0.75
2	10.61 ± 1.34	14.07 ± 0.72	12.65 ± 0.96	15.67 ± 0.73	15.88 ± 1.31	16.57 ± 1.42	15.90 ± 1.70
3	6.66 ± 1.12	9.23 ± 0.65	8.26 ± 1.05	10.75 ± 0.61	11.37 ± 1.57	10.83 ± 0.65	10.82 ± 1.17
4	4.62 ± 0.95	6.09 ± 0.59	5.33 ± 0.80	7.98 ± 0.94	8.36 ± 1.42	7.47 ± 0.36	8.35 ± 1.23
6	1.93 ± 0.54	2.91 ± 0.31	2.31 ± 0.41	3.97 ± 0.60	3.79 ± 0.54	3.78 ± 0.19	3.81 ± 0.54
8	1.11 ± 0.30	1.49 ± 0.17	1.19 ± 0.26	2.21 ± 0.44	2.24 ± 0.53	2.17 ± 0.16	2.65 ± 0.71
12	0.27 ± 0.08	0.39 ± 0.05	0.33 ± 0.07	0.71 ± 0.17	0.67 ± 0.18	0.69 ± 0.06	0.79 ± 0.20
24	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	0.05 ± 0.01	0.01 ± 0.01	0.03 ± 0.02

PI (postinfection)

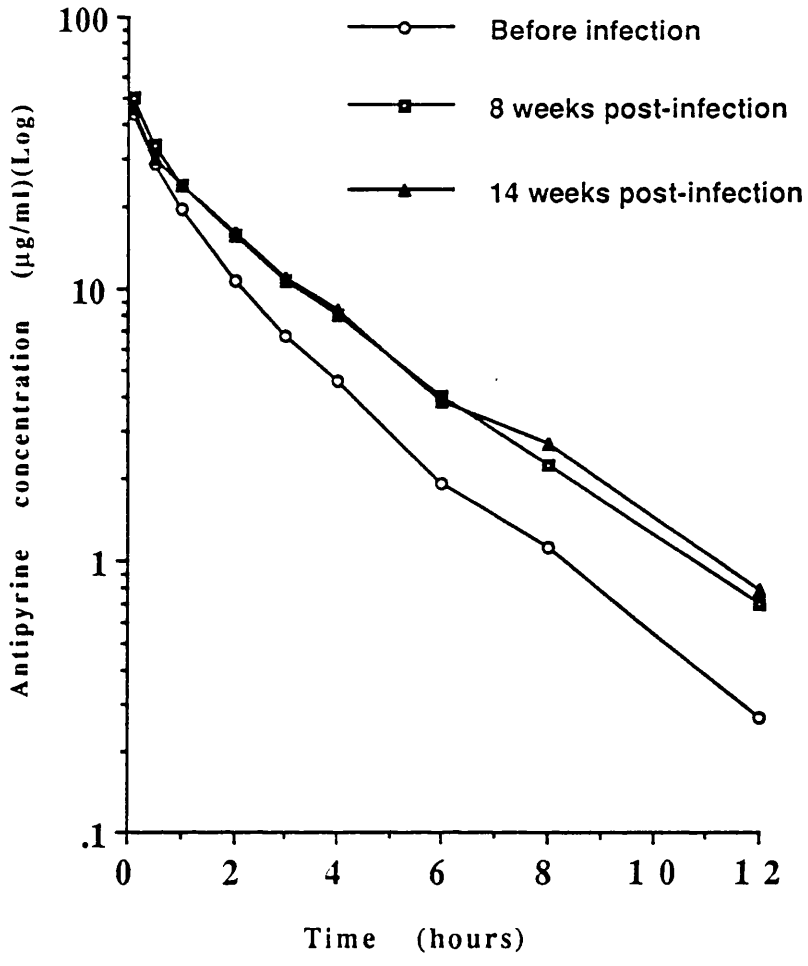

Figure 6-6. Mean plasma concentrations of antipyrine in infected untreated sheep (group A, n=6).

Table 6-6. Pharmacokinetic parameters of antipyrine (mean ± SEM) in group A (infected untreated) before and at different times after *F. hepatica* infection.

	Time postinfection						
	Control	4 weeks PI	6 weeks PI	8 weeks PI	10 weeks PI	12 weeks PI	14 weeks PI
Cpo (μg/ml)	48.58 ±2.14	52.57 ±2.28	53.12 ±2.30	54.48 ±3.21	46.12 ±2.08	50.42 ±3.71	49.66 ±1.72
AUC (μg.h/ml)	70.29 ±7.97	87.21 ±3.78	79.79 ±6.30	102.82 ±7.46*	101.82 ±10.09*†	101.18 ±4.80*	102.84 ±10.47*
t1/2β (h^{-1})¥	1.90	1.91	1.87	2.21*	2.44	2.22*	2.30*
Clb (ml/min.kg)	6.22 ±0.72	4.79 ±0.22	5.34 ±0.50	4.11 ±0.26*	4.23 ±0.40*†	4.13 ±0.21*	4.24 ±0.35*
Vc (ml/kg)	520.1 ±24.8	480.6 ± 22.8	475.4 ± 22.4	458.9 ± 30.7	547.4 ±23.8	512.5 ±46.0	506.4 ±16.8
Vd(area) (ml/kg)	979.6 ±104.4	780.8 ±24.0	874.5 ±62.7	815.2 ± 23.3	903.6 ±71.3	798.8 ±40.9	840.0 ±36.1
Vdss (ml/kg)	763.5 ±20.8	699.1 ±16.8	741.2 ±45.6	690.5 ±21.5	762.5 ±35.1	720.2 ±30.9	758.1 ±27.4

*Significantly different from preinfection ($P \leq 0.05$)

†Significantly different from corresponding values in group C($P \leq 0.05$).

¥Harmonic mean

PI (postinfection)

Table 6-7. Plasma concentrations of antipyrine (mean ± SEM) in group B (infected treated) before and at different times after *F. hepatica* infection.

	Time postinfection						
Time (hours)	Control	4 weeks PI	6 weeks PI	8 weeks PI	10 weeks PI	12 weeks PI	14 weeks PI
0.083	42.81 ± 1.95	44.11 ± 2.43	44.66 ± 1.73	43.83 ± 3.45	43.65 ± 6.42	43.37 ± 0.73	49.37 ± 4.09
0.5	29.21 ± 1.78	28.77 ± 0.91	28.47 ± 1.20	29.62 ± 1.62	28.90 ± 1.89	30.78 ± 1.33	31.29 ± 1.62
1	22.39 ± 1.79	21.38 ± 1.51	21.40 ± 1.54	23.14 ± 1.22	19.40 ± 0.99	21.90 ± 1.78	25.47 ± 1.73
2	13.49 ± 1.30	13.12 ± 1.49	12.76 ± 1.56	13.35 ± 1.08	10.88 ± 1.27	12.60 ± 1.44	16.34 ± 1.14
3	8.91 ± 0.98	8.26 ± 1.27	7.40 ± 1.41	9.30 ± 1.01	7.03 ± 0.84	8.75 ± 1.64	11.63 ± 1.32
4	6.30 ± 0.89	5.78 ± 1.16	5.09 ± 1.14	6.17 ± 0.82	4.90 ± 0.80	5.60 ± 1.14	8.76 ± 1.06
6	3.08 ± 0.53	3.11 ± 1.04	2.51 ± 0.66	2.89 ± 0.62	1.95 ± 0.38	2.75 ± 0.60	4.77 ± 0.69
8	1.70 ± 0.36	1.87 ± 0.74	1.39 ± 0.50	1.42 ± 0.31	0.81 ± 0.18	1.46 ± 0.31	3.11 ± 0.61
12	0.53 ± 0.13	0.57 ± 0.32	0.45 ± 0.17	0.42 ± 0.16	0.27 ± 0.06	0.48 ± 0.12	1.07 ± 0.26
24	0.01 ± 0.01	0.02 ± 0.02	0.02 ± 0.02	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.01	0.04 ± 0.01

PI (postinfection)

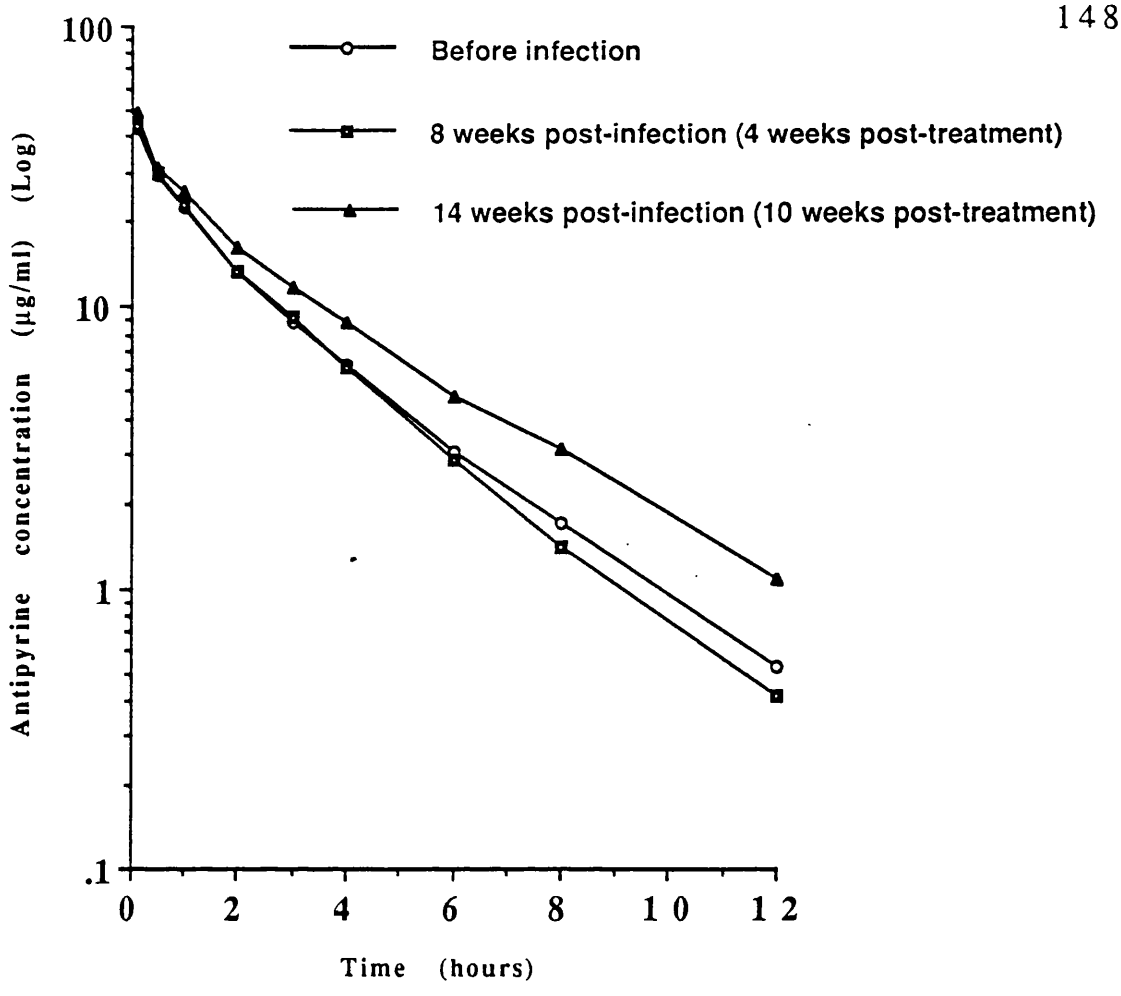

Figure 6-7. Mean plasma concentrations of antipyrine in infected rafoxanide-treated sheep (group B, n=6).

Table 6-8. Pharmacokinetic parameters of antipyrine (mean ± SEM) in group B (infected treated) before and at different times after *F. hepatica* infection.

	Time postinfection						
	Control	4 weeks PI	6 weeks PI	8 weeks PI	10 weeks PI	12 weeks PI	14 weeks PI
Cpo (μg/ml)	47.23 ±2.38	51.40 ±3.07	50.30 ±3.27	47.61 ±4.04	51.58 ±7.21	46.83 ±0.80	54.94 ±5.42
AUC (μg.h/ml)	86.61 ± 8.39	85.07 ±12.4	78.77 ±10.20	85.62 ±8.29	70.74 ±6.98	83.22 ±9.62	112.66 ±11.07*
t1/2β (h^{-1})¥	2.05	1.83	1.93	1.88	1.85	2.13	2.54
Clb (ml/min.kg)	5.09 ±0.60	5.33 ±0.68	5.68 ±0.79	4.99 ±0.41	6.02 ±0.60	5.25 ±0.63	3.90 ±0.38*
Vc (ml/kg)	536.0 ±26.4	494.6 ±27.8	507.3 ±32.0	545.4 ±48.5	523.8 ±60.0	534.7 ±9.2	483.4 ±58.9
Vd(area) (ml/kg)	911.2 ±54.1	840.9 ±34.9	962.7 ±70.6	812.9 ±41.9	1002.2 ±121.5	976.7 ±86.3	860.0 ±72.6
Vdss (ml/kg)	780.5 ±38.4	713.6 ±21.2	765.2 ±29.9	720.1 ±16.9	766.5 ±50.2	755.9 ±25.4	760.3 ±45.3

*Significantly different from preinfection ($P \leq 0.05$)
¥Harmonic mean
PI (postinfection)

Table 6-9. Plasma concentrations of antipyrine (mean ± SEM) in group C (uninfected treated) before and at different times after rafoxanide treatment.

	Time posttreatment						
Time (hours)	8 weeks PreT	1 day PreT	2 weeks PT	4 weeks PT	6 weeks PT	8 weeks PT	10 weeks PT
0.083	44.79 ± 3.37	46.08 ± 2.83	46.26 ± 2.03	53.16 ± 3.44	44.61 ± 1.68	48.06 ± 4.28	52.61 ± 2.36
0.5	27.83 ± 1.47	30.28 ± 1.81	28.66 ± 0.87	29.37 ± 1.12	27.36 ± 2.20	28.81 ± 1.93	28.62 ± 1.62
1	19.62 ± 0.93	21.02 ± 1.24	20.20 ± 0.88	21.98 ± 1.11	18.22 ± 1.52	19.30 ± 1.41	21.82 ± 1.83
2	11.88 ± 0.88	12.56 ± 1.02	11.14 ± 1.26	13.10 ± 0.72	10.19 ± 2.01	10.54 ± 1.87	12.65 ± 1.92
3	7.66 ± 0.82	8.54 ± 0.94	6.50 ± 1.27	8.71 ± 1.06	6.28 ± 1.64	6.32 ± 1.44	7.73 ± 1.92
4	5.02 ± 0.66	5.27 ± 0.79	4.36 ± 1.08	6.06 ± 1.11	4.25 ± 1.47	4.47 ± 1.33	5.58 ± 1.47
6	2.13 ± 0.38	2.42 ±0.54	2.10 ± 0.70	2.78 ± 0.58	2.17 ± 0.94	2.14 ± 0.81	3.10 ± 1.05
8	1.26 ± 0.28	1.26 ± 0.40	1.05 ± 0.40	1.45 ± 0.36	1.04 ± 0.46	1.14 ± 0.48	1.69 ± 0.64
12	0.36 ± 0.10	0.33 ± 0.11	0.30 ± 0.14	0.49 ± 0.21	0.35 ± 0.17	0.30 ± 0.12	0.50 ± 0.20
24	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.02 ± 0.01	0.00 ± 0.00	0.03 ± 0.02

PT (posttreatment)

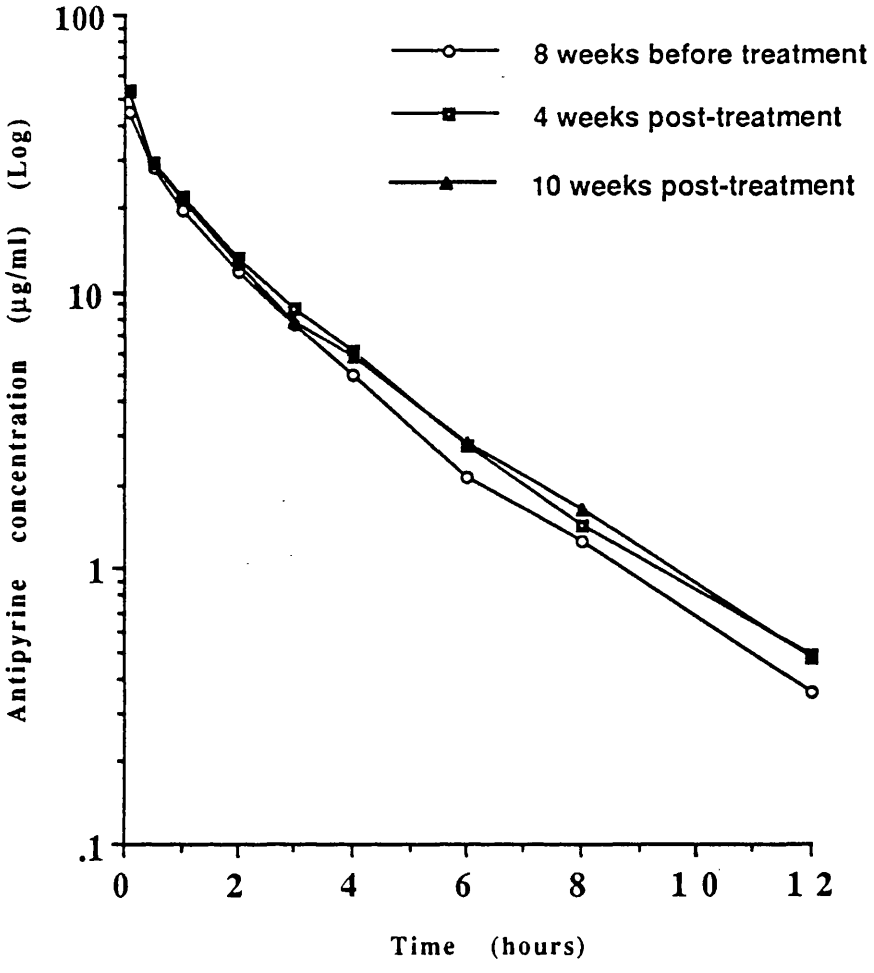

Figure 6-8. Mean plasma concentrations of antipyrine in uninfected treated sheep (group C, n=6).

Table 6-10. Pharmacokinetic parameters of antipyrine (mean±SEM) in group C (uninfected treated) before and at different times after rafoxanide treatment.

	Time postinfection						
	8 weeks PreT	1 day PreT	2 weeks PT	4 weeks PT	6 weeks PT	8 weeks PT	10 weeks PT
Cpo (μg/ml)	50.60 ±4.13	50.81 ±3.31	51.15 ±2.88	61.48 ±4.63	48.60 ±2.51	54.27 ±5.19	61.08 ±3.53
AUC (μg.h/ml)	74.88 ± 6.31	79.61 ±7.9	71.98 ±8.59	85.86 ±8.20	68.80 ±12.11	71.52 ±10.93	84.04 ±13.24
t1/2β (h^{-1})¥	1.82	1.77	1.72	1.87	1.82	1.77	1.94
Clb (ml/min.kg)	5.73 ±0.50	5.41 ±0.48	5.97 ±0.50	5.01 ±0.38	6.74 ±0.90	6.29 ±0.80	5.50 ±0.76
Vc (ml/kg)	507.6 ±33.5	502.3 ±31.9	497.4 ±31.0	419.8 ±35.0	521.5 ±27.7	486.3 ±54.6	429.1 ±31.9
Vd(area) (ml/kg)	902.6 ±33.5	825.5 ±38.3	890.2 ±37.8	812.6 ±12.4	1044.5 ±52.2	963.9 ±71.4	920.4 ±49.9
Vdss (ml/kg)	790.5 ±31.8	719.5 ±32.1	729.8 ±21.5	760.3 ±28.2	773.6 ±10.4	733.7 ±47.4	743.0 ±22.9

¥Harmonic mean

PreT (pretreatment)

PT(posttreatment)

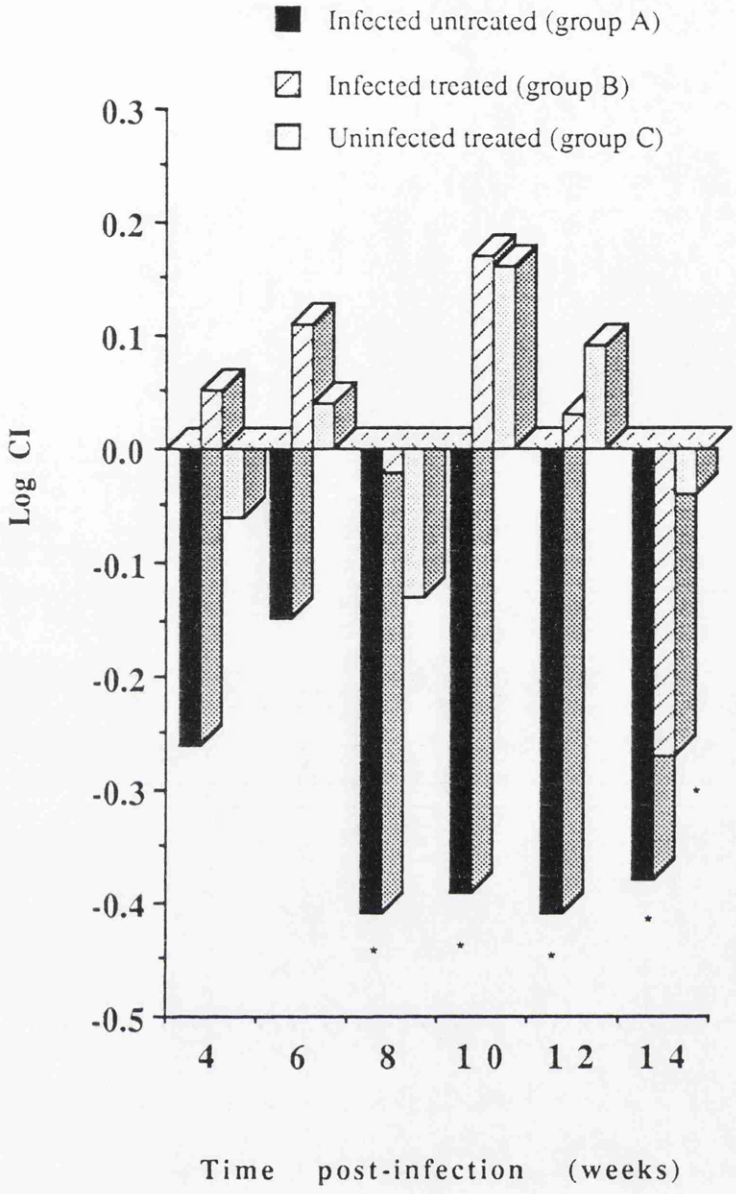

Figure 5-8. Clearance Index (CI) of antipyrine at different stages of infection and/or treatment in group A , B and C.
* Body clearance (Clb) significantly different from the control ($P \leq 0.05$)

CI = mean Clb (post-infection and, or, post-treatment)/mean Clb (control)

Table 6-11. Plasma concentrations of rafoxanide (μg/ml) in infected and uninfected sheep.

	Mean ± SEM (n=6)	
Time (days)	Group B (Infected)	Group C (uninfected)
0	0.00 ± 0.00	0.00 ± 0.00
1	17.67 ± 1.37	18.84 ± 1.35
2	19.31 ± 0.97	20.65 ± 1.45
3	19.48 ± 0.96	20.64 ± 1.32
4	18.55 ± 0.94	18.73 ± 1.03
8	14.64 ± 0.69	14.36 ± 0.91
12	11.80 ± 0.50	11.50 ± 0.85
19	7.44 ± 0.21	7.24 ± 0.74
27	4.17 ± 0.73	4.66 ± 0.60
33	2.95 ± 0.36	3.11 ± 0.60
39	2.01 ± 0.23	1.83 ± 0.46
47	1.09 ± 0.14	1.08 ± 0.31
52	0.78 ± 0.14	0.87 ± 0.27
63	0.44 ± 0.09	0.51 ± 0.16
70	0.29 ± 0.06	0.38 ± 0.12
76	0.18 ± 0.04	0.28 ± 0.09

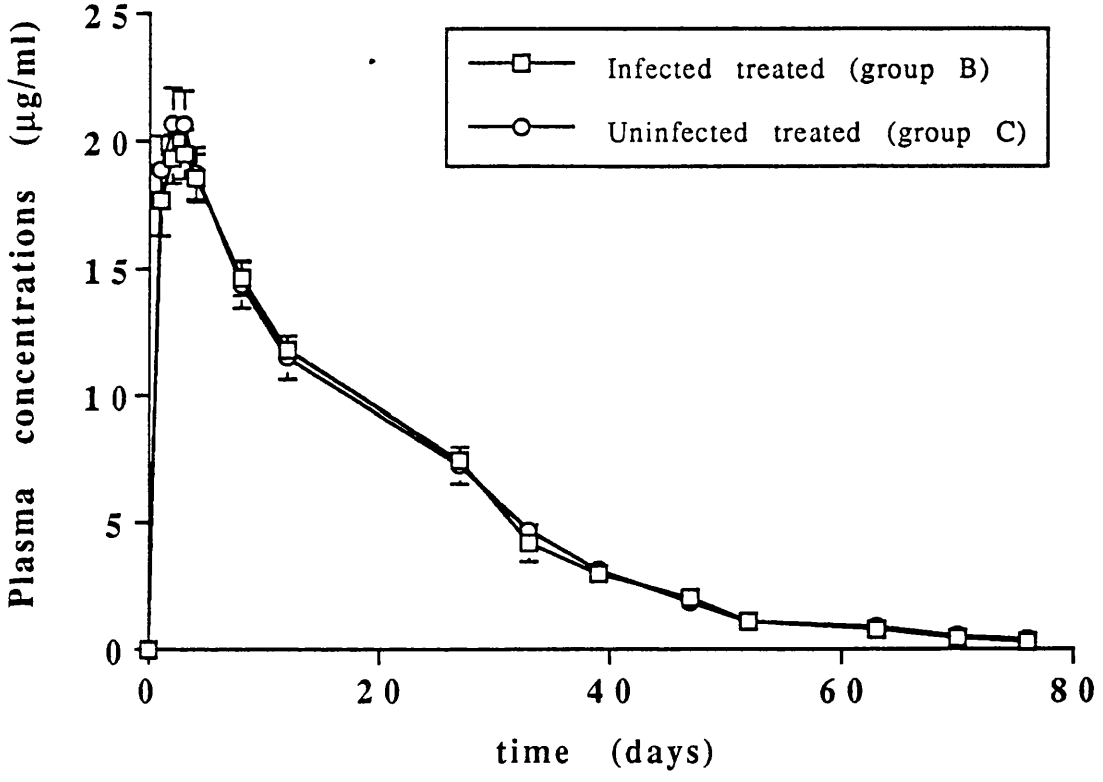

Figure 6-10. Plasma concentrations of rafoxanide (mean ± SEM) in infected (group B, n=6) and uninfected (group C, n=6) sheep.

Table 6-12. Pharmacokinetic parameters of rafoxanide in infected and uninfected sheep.

	Mean ± SEM (n=6)	
	Group B (infected)	Group C (uninfected)
AUC_{obs} (µg.d/ml)	362.76 ± 9.27	367.8 ± 29.4
$AUMC_{obs}$ (µg.d^2/ml)	5607 ± 330	5773 ± 856
MRT (d)	15.45 ± 0.81	15.29 ± 1.22
Cmax (µg/ml)	19.95 ± 0.91	21.77 ± 1.39
tmax (d)	2.67 ± 0.33	2.00 ± 0.37

6.4 Discussion

6.4.1 Suitability of the animal model

Sheep are the host species in which *Fasciola hepatica* infection imposes the greatest economic loss and it is in this species that salicylanilides are most widely used. The use of antipyrine as a pharmacokinetic marker for liver damage caused by *Fasciola hepatica* in sheep has been previously reported (Tufenkji *et al.*, 1988, Burrows *et al.*, 1991). Antipyrine possesses pharmacokinetic characteristics which make it suitable for assessing liver function during the course of various types of hepatic dysfunction (Poulsen and Loft, 1988; St Peter and Awni, 1991). Antipyrine is (1) eliminated principally through biotransformation reactions which take place almost entirely in the liver, metabolic processes involved are catalysed by the hepatic cytochrome P450-enzyme system (which has been shown to be impaired by liver fluke infection); (2) Its volume of distribution equals body water and protein binding is negligible (less than 10%), variations in plasma protein concentrations are therefore unlikely to affect its pharmacokinetic disposition; (3) it is characterised by a low extraction ratio; liver blood flow rate has therefore little bearing on the elimination of the drug. With such pharmacokinetic features, and provided that animals are kept in a stable environment, changes in the clearance of this probe drug reflect liver damage inflicted by the fluke burden. Also the evolution of the disease following flukicidal therapy could be assessed, provided that direct or indirect interaction between the therapeutic agent and the probe drug do not occur.

In the present study, the clearance of antipyrine was significantly decreased in the infected untreated sheep (group A) at 8, 10, 12 and 14 weeks after infection, indicative of liver damage attributable to the parasites, thus demonstrating the applicability of the model. Also, conducting the tests under similar environmental conditions, minimises intraindividual variation in antipyrine disposition (Vessel & Penno, 1983). In the present study, small intraindividual variation were observed (less than 6% for the plasma clearance and the elimination half-life) after two antipyrine tests undertaken 8 weeks apart on the uninfected sheep (group C) before they were dosed with rafoxanide (Table 6-10). This group also served as a control for any interaction between antipyrine and rafoxanide.

6.4.2 Antipyrine pharmacokinetics

The mean plasma clearance and elimination half-life of antipyrine in pre-infection animals is consistent with that reported by Tufenkji *et al.* (1988). Although it has been shown that 4 weeks after infection with *Fasciola hepatica*, the cytochrome P450 activities and the antipyrine clearance start to decrease (Galtier *et al.*, 1983, Galtier *et al.*,1986b, Tufenkji *et al.*, 1988), in the present study it was not until 8 weeks post-infection that the first significant decrease in antipyrine body clearance occurred. This could be related to breed differences of the hosts in their susceptibility to the parasite. Alternatively, differences in the pathogenicity of the strains of parasite used could be the reason, since impairment of host metabolic capacity has been imputed to toxic excretions by the fluke (Galtier *et al.*, 1983, Galtier *et al.*, 1985b, Galtier *et al.*, 1986b). Flukicidal treatment with the salicylanilide drug rafoxanide at 4 weeks post-infection prevented the decrease in the rate of elimination of antipyrine for up to 12 weeks post-infection. This was due to the action of rafoxanide on the parasite rather than to its intrinsic effect on antipyrine metabolism, since rafoxanide did not induce any significant change in the plasma kinetics of antipyrine at any post-treatment stage when given to uninfected sheep. A significant decrease in antipyrine clearance was observed 14 weeks post-infection in group B. This could be due to parasites which survived the treatment depressing the oxidative metabolism of the liver when they matured to the adult stage. Another explanation may be that rafoxanide exerted a transient inhibitive action on parasites development. The inhibited parasites may have resumed development 8 to 10 weeks after treatment, as the plasma concentration of rafoxanide declined.

6.4.3 Biochemical changes

In the infected treated animals, the biochemical findings indicate an early effect of rafoxanide reflected by the fall in GLDH activity 2 weeks after treatment. Surprisingly, however, 6 weeks later GLDH activity rose to values equivalent to those in the parasitised untreated sheep. Gamma-glutamyl dehydrogenase is indicative of liver necrosis induced by migrating immature flukes (Sykes *et al.*, 1980a, Cornelius, 1989), therefore the increased activity of the enzyme manifested later in the course of infection in treated animals supports the hypothesis of a drug-induced retarded development. Studies based on the measurement of fluke size at different stages after treatment with

closantel (Maes *et al.*, 1988, Maes *et al.*, 1990), the sulphonamide clorsulon (Malone *et al.*,1984), or even with the more recent flukicide triclabendazole (Buscher et al., 1987) indicate a stunting effect of the drugs on the parasite. Maes *et al* (1988) postulated that arrested forms eventually resume their development and reach maturity after a delay of at least 10 weeks. The same phenomenon could explain the putative high efficacy (98%) of rafoxanide reported by Armour & Corba (1970) against 4 week-old *Fasciola hepatica* when assessed by counting adult flukes at 10 weeks post-infection rather than at more advanced stages of infection. The peak of γGT activity occurred two weeks after that of GLDH (10 weeks post-infection in the untreated animals) and is in agreement with previous observations which established that the γGT increase was mainly caused by adult flukes reaching the bile ducts (Sykes *et al.*, 1980a, Galtier *et al.*, 1986b). The increase in γGT in uninfected sheep was within the normal range and could be explained by an inductive effect of rafoxanide. It has previously been reported that increased γGT is induced by xenobiotics such as the steroid drug prednisolone (Kramer, 1989). Increased globulinaemia in infected sheep is consistent with previous findings (Sykes *et al.*, 1980b).

6.4.4 Rafoxanide pharmacokinetics

Kinetic parameters of rafoxanide were similar in infected and uninfected sheep. Studying the disposition of radiolabelled rafoxanide in healthy and *Fasciola hepatica*-infected rats, Galtier and collegues. (1985a) made a similar observation. This could be related to the fact that salicylanilides are not extensively metabolised (McKellar & Kinabo, 1991) and therefore their elimination is not compromised by changes in liver metabolism which may be caused by subclinical *Fasciola hepatica* infection. Another factor that could modify the disposition of extensively plasma protein bound drug would be hypoalbuminaemia associated with haematophagic *Fasciola hepatica* . In the present study no changes were observed in plasma protein except in the untreated group (A) where a hypoalbuminaemia was observed 14 weeks after infection. However, this was not clinically significant.

Hepatic drug-metabolism during the course of fascioliasis has been studied using bromosulfophthalein (BSP) as another probe drug, whose clearance has been shown to decrease with fluke infection (Tufenkji *et al.*, 1987). In sheep experimentally infected with *Fasciola hepatica* the elimination half-life and the volume of distribution of

pentobarbital were increased causing a prolonged narcosis (Tufenkji *et al.*, 1991). The macrolide compound, erythromycin, which is extensively metabolised in the liver by N-demethylation also has its elimination impaired during acute and subacute fascioliasis in sheep whereas no changes were observed with oxytetracycline, a less extensively biotransformed antibiotic (Burrows *et al.*, 1991). The plasma kinetics of albendazole metabolites were also altered by subclinical fluke infection in lambs (Galtier *et al.*, 1991) *In vitro* studies on microsomal UDP-glucuronyl transferase activity and microsomal cytochrome P450 activity indicate that the metabolism of the fasciolicide drugs nitroxynil and oxyclozanide could be impaired during fascioliasis (Facino *et al.*, 1984, Facino *et al.*, 1985).

In conclusion, this study gives a further insight in the understanding of salicylanilide action against immature stages of *Fasciola hepatica*. Although rafoxanide acts at an early stage of the parasite as evidenced by the unchanged clearance of antipyrine and the decline of GLDH activity during the pre-patent period of the infection, changes in these parameters occurring later in the course of infection indicate that rafoxanide may not kill immature flukes but temporarily inhibit their development.

Chapter 7

General Discussion

The first study reported in this thesis was set out to generate pharmacokinetic data on three chemically related benzimidazole compounds, in goats. It was found, that albendazole and albendazole sulphoxide, given at the same dose rate of 7.5 mg/kg, were bioequivalent. The albendazole-prodrug netobimin given at 7.5 mg/kg, which is 37% lower than the equimolar dose, resulted in a 45 % lower AUC for the sulphoxide metabolite than that obtained after ABZ administration. Assuming first-order conversion, it could therefore be estimated that the gastrointestinal microflora of the goat is 87% efficient at converting the prodrug into albendazole.

Knowledge of anthelmintic disposition in goats is important given the contribution of this animal species in the development of anthelmintic resistance (Jackson, 1993). Reported studies have shown that a number of anthelmintic drugs are less bioavailable in goats than they are in sheep (Weir & Bogan, 1985; Bogan *et al.*, 1987; Sangster *et al.*, 1991; Sundlof & Whitlock, 1992; Hennessy *et al.*, 1993a,b). Concurrent deworming of sheep and goats is generally based on a dosage regime established in the former species. Nematodes harboured by goats are consequently exposed to sublethal levels of anthelmintic, become resistant and are passed on to sheep.

For these reasons, goats used in the studies described in this thesis were given higher doses of albendazole and fenbendazole than those recommended for sheep. For the latter drug, it is even the manufacturer's recommendation to use the same dose as recommended for sheep. When fenbendazole was given to goats at 7.5 mg/kg, The AUC (FBZ) was 4.76 μg.h/ml, whereas in sheep a lower dose (5 mg/kg) resulted in an AUC (FBZ) of 7.54 μg.h/ml. Taking sheep as the reference species, the relative bioavailability corrected for dose (Shargel & Yu, 1993b) achieved in goats was only 42%. Fenbendazole systemic availability was also found to be lower in goats than in cattle (Short *et al.*, 1987a,b).

Interspecies differences in biovailability were less pronounced with albendazole in our studies. However a recent report suggested a lower AUC of ABSO in goats than in sheep (Hennessy *et al.*, 1993c). This was explained by a higher liver sequestration, rather than metabolism, of the sulphoxide metabolite in goats,

the elimination rate being the same in both species. It would be of interest to compare the hepatic metabolism of albendazole *in vitro* and also to determine liver tissue concentrations of ABSO in both species.

One of the main objectives of this thesis was to explore the link between pharmacokinetic behaviour and clinical efficacy of anthelmintic drugs. Because of the extensive hepatic metabolism which benzimidazole molecules undergo and also because their mode of action is based on the intensity as well as the duration of parasite exposure, BZ's represented an ideal anthelmintic group for pharmacokinetic/pharmacodynamic manipulation through inhibition of hepatic drug-metabolism. In the preliminary studies, a dramatic increase in the bioavailability of benzimidazole active metabolites was observed when the metabolic inhibitor piperonyl butoxide was administered intramuscularly one hour prior to anthelmintic administration. However, piperonyl butoxide failed to increase significantly the plasma ABSO levels in goats, despite a substantial increase in the level of the same metabolite in sheep. This may have been caused by the lower availability of the inhibitor observed in the former species. If this is the case, piperonyl butoxide given orally, could have a more pronounced inhibitory effect as it is more bioavailable by this route. Alternatively, goats could be less responsive to inhibition of albendazole metabolism by piperonyl butoxide in which case, *in vitro* comparative studies would prove valuable in elucidating the difference. In a similar context, Lanusse and Prichard (1992a,b) reported that when netobimin was administered concurrently with the FMO-inihbitor methimazole, a much higher response in terms of sulphoxidase inhibition, was achieved in sheep than in cattle. This observation was later substantiated by a comparative study using liver microsomal preparations from sheep and cattle (Lanusse *et al.*, 1993c).

An attempt was made to study the absolute bioavailability of piperonyl butoxide given at 0.5 g/kg, however, unfortunately, the intravenous administration proved fatal in one animal and the study was aborted. A lower dose and a more appropriate

formulation would probably enable a proper investigation of the bioavailability of this drug. Also piperonyl butoxide is reported to be readily absorbed through the skin. This route could also be investigated in different animal species and compared to other routes of administration in terms of BZ potentiation. Another topic which deserves exploration is the metabolic pathway of piperonyl butoxide and the characterization of the reactive metabolites which cause the metabolic inhibition. It is also important to determine the toxicity of this drug when given orally to ruminants. However, in the rat, orally administered piperonyl butoxide is reported to have a very low toxicity (Haley, 1978).

The dose titration study in sheep showed that significantly higher levels of FBZ and FBSO could be achieved with a dose rate of piperonyl butoxide 16 times lower than the dose administered in the preliminary study. This study also showed that piperonyl butoxide is absorbed and has an inhibitory effect when given orally. This was an important prerequisite for any further consideration concerning the potential benefits of the combination as all BZ, with the exception of netobimin, are limited to oral formulations.

The efficacy trial with the combination fenbendazole-piperonyl butoxide confirmed previous reports that sustained higher plasma levels of active metabolites resulted in enhanced efficacy against BZ-resistant nematodes (Prichard *et al.*, 1978; Hennessy *et al.*, 1985). The BZ-resistant strains of *O. circumcincta* and *H. contortus* proved highly susceptible to the combination. The ratio PB/FBZ used in this trial was 12.6/1; this was because the efficacy trial, for deadline reasons, had to overlap with the analytical phase of the dose titration study and a choice of a dose for piperonyl butoxide had to be made. It can be seen from the end result of the dose titration study that a dose of 31 mg/kg could have been used. Future efficacy assessments in sheep should therefore be tried at a ratio amounting to 6.2/1.

There was no apparent effect of PB given alone, on nematode burden with both strains studied and the potentiating effect is likely to be limited to changes induced in the pharmacokinetic

pattern of the BZ in the host. Notwithstanding this, it would be interesting to look at the effect of the combination against nematodes *in vitro* and to determine wether piperonyl butoxide affects the transcuticular delivery of benzimidazole drugs. Studies in insects have shown that piperonyl butoxide delays the penetration of pyrethrins through cuticles (O'Brien, 1967). Effect of piperonyl butoxide on the absorbtion rate of BZ by the animal host also requires investigation.

Benzimidazoles seem to undergo more extensive metabolism in the equine species than they do in ruminants or in man (Marriner & Bogan, 1985; Bogan *et al.*, 1987). A fast sulphonation of FBZ resulted in no detectable levels of the sulphoxide metabolite in horse's plasma after administration of fenbendazole (Marriner & Bogan, 1985). Studies on the effect of metabolic inhibition with piperonyl butoxide in this species would provide further information on how the benzimidazoles are handled by the equine liver.

A relatively new and unexplored aspect of the pharmacokinetic/pharmacodynamic properties of the benzimidazole drugs is their chiral nature and the enantioselective biotransformation which they undergo (Delatour *et al.*, 1994). Cytochrome P450 Inhibition is likely to change the proportion (+)/(-) which could have an impact on anthelmintic activity since it is generally believed that individual enantiomers of a chiral compound exibit different biological actions. Characterization of the biological action (eudismic ratio) and toxicity of enantiomers is clearily needed. This would provide a direction to follow in terms of metabolic manipulation of the benzimidazoles in different target species.

The use of metabolic inhibition for therapeutic benefit has been reported in man and it has been shown, for example that the cost of cyclosporin therapy can be dramatically lowered by administering the drug with ketoconazole. The interaction was due to the inhibition by ketoconazole of P450IIIA, which is implicated in the metabolism of cyclosporin (First *et al.*, 1989). Also, in relation to helminth therapy, a study on the therapeutic effect of mebendazole against larval echinococcosis in man suggested that higher and more effective mebendazole

concentrations could be achieved by inhibition of the drug metabolizing capacity of the liver rather than by increasing the dose of mebendazole (Witassek & Bircher, 1983). In dogs, McKellar *et al.* (1990, 1993a) have shown that gastrointestinal absorption of fenbendazole follows a zero-order process and increasing the dose does not enhance the bioavailability of this drug; metabolic inhibition may also prove useful in this species.

The *in vitro* S-oxidation of thiosubstituted benzimidazoles revealed dramatic differences in the rates at which these drugs were metabolised. In both microsomal preparations and cultured hepatocytes, triclabendazole was found to be extensively converted into its sulphoxide and sulphone metabolites, more so than albendazole or fenbendazole. Although it is generally believed that TCBZ metabolites achieve much higher plasma levels than ABZ or FBZ metabolites because of their extensive binding to plasma proteins, it is possible that a more efficient S-oxidation of the parent TCBZ may contribute to the high plasma AUC for TCBSO and TCBSO2. It would be interesting to look at the S-oxidation of the three benzimidazoles and their respective sulphoxide metabolites when incubated with and without albumin in the microsomal reaction mixture and monitor the impact of protein binding on the microsomal metabolism of the drugs.

Interspecies differences in the pharmacokinetic pattern of triclabendazole metabolites have been reported (Bogan *et al.*, 1988a; Oukessou *et al.*, 1991; Sanyal, 1994) *in vitro* studies with different species would provide useful data to corroborate the differences observed *in vivo*.

The *in vitro* inhibition of ABZ and FBZ with piperonyl butoxide confirmed the changes observed in the pharmacokinetic behaviour of the two benzimidazoles when coadministered with the inhibitor. The nature of this inhibition was not studied. However, since the substrate concentrations used were similar to the Michaelis constant Km (53.6 μM) reported for ABZ sulphoxidation (Fargetton *et al.*, 1986), it was assumed that saturation of the system was not achieved and therefore both possibilities of competitive and noncompetitive inhibition exist.

It was found that the S-oxidation of TCBZ was affected by piperonyl butoxide and it would be interesting to investigate this effect *in vivo*. 1-aminobenzotriazole also had a dramatic effect on the metabolism of TCBZ confirming the catalytic activity of the cytochrome P450-enzyme system in the oxdative biotransformation of this anthelmintic. Further work is now needed in order to identify the P450 isozymes involved. Combining immunoinhibition and molecular biology techniques would facilitate such characterization. Also the involvement of the flavin-monoxygenase system ought to be considered and investigated using heat-treated microsomes and purified FMO from hog liver microsomes.
It has been shown that *in vitro,* unchanged TCBZ inhibited the motility of immature and adult *F. hepatica* and that this action was slow and necessitated the accumulation of TCBZ within the parasite (Bennett and Kohler, 1987). The use of metabolic inhibitors, such as piperonyl butoxide and 1-aminobenzotriazole may help understand further this effect by inhibiting the conversion of the parent drug into the sulphoxide metabolite.

Hydroxylated metabolites of the benzimidazoles investigated in this thesis, were not measured because of the very low or undetectable levels they achieve in the systemic circulation and it was not within the scope of this project to measure them in bile or in urine where they tend to be the main metabolites. However, given that hydroxylation is likely to be a cytochrome P450 catalyzed reaction (Gibson & Skett, 1994), it would be interesting to examine the effect of inhibitors on this reaction. Moreover, hydroxyfenbendazole (OH.FBZ) has been shown to bind to parasite tubulin (Lacey *et al.*, 1987) and authors have attributed part of the efficacy of fenbendazole and oxfendazole against intestinal parasites, to this metabolite. It would be worth investigating the effect of OH.FBZ administered *per se* on abomasal parasites.

The study described in the last chapter was set out to elucidate whether the action of salicylanilides against juvenile flukes was due to their peak level or to the long lasting residual levels which

are contemporaneous with the adult stage of the fluke. It was concluded that peak plasma levels achieved by rafoxanide had an action against 4-week-old *F. hepatica*; however, liver function tests, namely antipyrine clearance and GLDH activity, suggest that this action is partially a result of a 'stunting' rather than a lethal effect. As drug levels in the blood circulation decline, it is suggested that flukes which have been retarded in their development would, eventually achieve maturity and start laying eggs (Maes *et al.*, 1988).

The antipyrine clearance test proved to be a useful tool in this study since it permitted the assessment of liver damage caused by fluke infection and also, together with GLDH activity measurements, it enabled us to follow the evolution and outcome of flukicidal treatment with rafoxanide.

Although, in the present study fluke infection did not have any effect on the pharmacokinetic behaviour of rafoxanide, a number of studies have shown that drugs which, unlike rafoxanide, are extensively metabolised in the liver, may have their elimination impaired by fascioliasis. However, all studies so far published on hepatic drug metabolism during fluke infection have been limited to the effect of single moderate infections and therefore are not reflective of pasture infections where sheep are exposed to a continuous intake of metacercariae which leads to the cumulative establishment of trematodes of mixed age in the liver. The effect of these natural infections on the metabolic response of parasitised animals to xenobiotics would be of interest. Also, it is worth investigating how liver fluke impairs hepatic drug-metabolising capacity. An initial step would involve *in vitro* drug metabolism studies using excretory-secretory (E-S) products from *F. hepatica.*

Unlike liver fluke infection, infection with *H. contortus* has been shown to increase significantly the body clearance of antipyrine (Kawalek and Fetterer, 1990). It was suggested that *H. contortus* may have an inductive effect on cytochrome P450 mediated metabolism via a generalised increase in hepatic protein synthesis associated with the physiologic response to compensate the loss of plasma proteins and other blood components caused by the infection. A closer examination of the effect of nematodes

on drug metabolism would be useful given that many anthelmintic drugs are extensively biotransformed and their clearance may therefore be altered by nematode infections.

In conclusion, the work carried out throughout this project has mainly led to the development of a drug combination which associates a benzimidazole with a potentiating metabolic inhibitor. Many aspects of this combination are still to be scrutinised. An important one is the safety to both the animal treated and, in relation to food-producing animals, to the consumer. This research program has also involved a comparison of the *in vitro* metabolism of S-substituted benzimidazoles. Finally, a better understanding of the flukicidal action of salicylanilides has been achieved.

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Appendix A

Pharmacokinetics of albendazole, albendazole sulphoxide and netobimin in goats

Appendix A-1. Recovery and coefficients of variation for albendazole metabolites following ether extraction from plasma

Added concentration (µg/ml)	Recovery (%)	Between-assay coefficient of variation (%)
	albendazole	
0.05	101.29 (n=6)	4.97
0.10	90.95 (n=6)	2.79
0.25	93.25 (n=6)	5.43
0.50	96.13 (n=6)	4.68
1.0	98.24 (n=6)	3.76
Mean	95.97 (n=30)	4.33
	albendazole sulphoxide	
0.10	68.69 (n=8)	8.47
0.25	73.55 (n=8)	10.19
0.5	76.68 (n=8)	6.82
1	83.25 (n=8)	6.05
2	83.02 (n=8)	6.78
Mean	77.04 (n=40)	7.66
	albendazole sulphone	
0.10	84.66 (n=8)	4.52
0.25	82.12 (n=8)	3.82
0.50	88.85 (n=8)	6.51
1.0	90.57 (n=7)	4.26
Mean	86.55 (n=31)	4.78

Coefficient of variation = (SD/mean recovery) X 100

Appendix A-2. Recovery and coefficients of variation for netobimin metabolites following solid-phase extraction from plasma

Added concentration (μg/ml)	Recovery (%)	Between-assay coefficient of variation (%)
	netobimin	
0.25	84.43 (n=6)	10.92
0.50	97.67 (n=7)	13.40
1	95.33 (n=8)	9.04
2	92.96 (n=5)	16.22
Mean	92.60 (n=26)	12.40
	albendazole	
0.05	92.46 (n=6)	15.04
0.10	99.53 (n=6)	5.68
0.25	92.65 (n=6)	9.29
0.50	93.15 (n=7)	7.92
1	102.56 (n=7)	5.16
Mean	96.07 (n=32)	8.62
	albendazole sulphoxide	
0.10	82.18 (n=6)	8.17
0.25	85.43 (n=6)	6.23
0.50	77.53 (n=6)	5.10
1	73.81 (n=6)	7.86
2	79.34 (n=6)	7.37
Mean	79.66 (n=30)	6.95
	albendazole sulphone	
0.10	77.20 (n=6)	7.99
0.25	91.48 (n=6)	6.62
0.50	87.39 (n=6)	6.13
1	83.10 (n=6)	7.05
Mean	84.79 (n=24)	6.95

Coefficient of variation = (SD/mean recovery) X 100

Appendix A-3. Plasma concentrations of albendazole sulphoxide (μg/ml) in goats following oral administration of albendazole at 7.5 mg/kg bodyweight.

	Animal number						
Time (h)	G1	G3	G7	G10	G11	G12	G13
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.05	0.00	0.00
0.5	0.09	0.07	0.00	0.11	0.14	0.09	0.00
1	0.23	0.17	0.18	0.25	0.24	0.22	0.08
2	0.62	0.39	0.42	0.60	0.58	0.53	0.27
4	1.08	0.71	0.95	1.32	0.97	0.99	0.59
8	2.15	1.74	2.01	2.47	2.07	2.40	1.24
12	2.09	2.32	2.74	2.58	2.24	2.90	1.73
24	1.36	1.48	1.45	1.47	1.46	2.51	1.03
32	0.54	0.38	0.59	0.51	0.69	1.81	0.15
48	0.00	0.00	0.00	0.00	0.00	0.10	0.00
72	0.00	0.00	0.00	0.00	0.00	0.00	0.00
96	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Appendix A-4. Pharmacokinetic parameters of albendazole sulphoxide (ABSO) in goats following oral administration of albendazole at a dose rate of 7.5 mg/kg bodyweight

	Animal number						
	G1	G3	G7	G10	G11	G12	G13
AUC_{obs} (μg.h/ml)	49.78	47.76	55.16	56.44	33.14	53.11	85.50
$AUMC_{obs}$(μg.h^2/ml)	818.2	789.4	914.7	893.9	518.5	910.1	1758.5
MRT (h)	16.44	16.53	16.58	15.84	15.65	17.14	20.57
Cmax (μg/ml)	2.15	2.32	2.74	2.58	1.73	2.24	2.30
tmax (h)	8.00	12.00	12.00	12.00	12.00	12.00	12.00

Appendix A-5. Plasma concentrations of albendazole sulphone (μg/ml) in goats following oral administration of albendazole at 7.5 mg/kg bodyweight.

	Animal number						
Time (h)	G1	G3	G7	G10	G11	G12	G13
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.00	0.00	0.00	0.02	0.00	0.00	0.00
1	0.00	0.00	0.02	0.02	0.00	0.00	0.00
2	0.03	0.03	0.05	0.04	0.03	0.04	0.00
4	0.04	0.07	0.12	0.11	0.07	0.08	0.05
8	0.21	0.27	0.34	0.33	0.17	0.26	0.20
12	0.34	0.51	0.65	0.55	0.36	0.69	0.40
24	0.68	1.12	1.22	0.97	0.91	0.98	0.88
32	0.77	1.19	1.35	1.02	0.64	1.14	1.20
48	0.02	0.00	0.00	0.02	0.00	0.00	0.79
72	0.00	0.00	0.00	0.00	0.00	0.00	0.00
96	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Appendix A-6. Pharmacokinetic parameters of albendazole sulphone (ABSO2) in goats following oral administration of albendazole at a dose rate of 7.5 mg/kg bodyweight.

	Animal number						
	G1	G3	G7	G10	G11	G12	G13
AUC_{obs} (μg.h/ml)	20.17	30.90	35.41	28.51	20.60	30.36	43.01
$AUMC_{obs}$(μg.h^2/ml)	518.0	784.3	886.1	709.7	505.1	748.7	1470.0
MRT (h)	25.69	25.39	25.02	24.89	24.52	24.66	34.18
Cmax (μg/ml)	0.77	1.19	1.35	1.02	0.91	1.14	1.20
tmax (h)	32.00	32.00	32.00	32.00	24.00	32.00	32.00

Appendix A-7. Plasma concentrations of albendazole sulphoxide (μg/ml) in goats following oral administration of albendazole sulphoxide at 7.5 mg/kg bodyweight.

	Animal number						
Time (h)	G1	G3	G7	G10	G11	G12	G13
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.05	0.04	0.00	0.03	0.05	0.06	0.04
0.5	0.15	0.11	0.14	0.19	0.19	0.19	0.14
1	0.36	0.25	0.42	0.55	0.61	0.49	0.47
2	0.92	0.50	1.00	1.20	1.25	1.07	1.09
4	1.78	0.92	1.81	2.18	2.81	1.77	1.98
8	2.01	1.67	2.47	2.94	2.89	2.69	3.45
12	2.31	1.96	2.68	2.43	3.04	2.88	3.58
24	1.87	0.68	1.49	1.01	1.56	1.87	2.34
32	0.89	0.00	0.62	0.28	0.25	0.81	1.63
48	0.00	0.00	0.00	0.00	0.00	0.00	0.14
72	0.00	0.00	0.00	0.00	0.00	0.00	0.00
96	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Appendix A-8. Pharmacokinetic parameters for albendazole sulphoxide (ABSO) following administration albendazole sulphoxide to goats at a dose rate of 7.5 mg/kg bodyweight.

	Animal number						
	G1	G3	G7	G10	G11	G12	G13
AUC_{obs} (μg.h/ml)	62.96	32.90	60.95	53.49	65.33	69.59	96.19
$AUMC_{obs}$(μg.h^2/ml)	1101.0	417.5	957.0	707.5	892.7	1147.3	1803.4
MRT (h)	17.49	12.69	15.70	13.23	13.66	16.49	18.75
Cmax (μg/ml)	2.31	1.96	2.68	2.94	3.04	2.88	3.58
tmax (h)	12.00	12.00	12.00	8.00	12.00	12.00	12.00

Appendix A-9. Plasma concentrations of albendazole sulphone (ABSO2) (μg/ml) in goats following oral administration of albendazole sulphoxide at 7.5 mg/kg bodyweight.

	Animal number						
Time (h)	G1	G3	G7	G10	G11	G12	G13
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.03	0.05	0.00	0.03
2	0.05	0.05	0.07	0.07	0.10	0.07	0.07
4	0.11	0.12	0.20	0.20	0.30	0.15	0.18
8	0.27	0.31	0.49	0.50	0.47	0.38	0.44
12	0.47	0.59	0.76	0.66	0.70	0.59	0.57
24	1.00	1.29	1.25	0.94	1.18	1.05	0.87
32	1.14	0.24	1.31	0.92	0.89	1.06	1.00
48	0.00	0.00	0.00	0.02	0.00	0.03	0.63
72	0.00	0.00	0.00	0.00	0.00	0.00	0.00
96	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Appendix A-10. Pharmacokinetic parameters of albendazole sulphone (ABSO2) in goats following oral administration of albendazole sulphoxide at a dose rate of 7.5 mg/kg bodyweight.

	Animal number						
	G1	G3	G7	G10	G11	G12	G13
AUC_{obs} (μg.h/ml)	28.93	22.18	36.97	28.83	31.03	30.62	40.24
$AUMC_{obs}$ (μg.h^2/ml)	733.0	469.9	894.3	679.9	710.7	758.7	1267.3
MRT (h)	25.34	21.19	24.19	23.59	22.90	24.78	31.49
Cmax (μg/ml)	1.14	1.29	1.31	0.94	1.18	1.06	1.00
tmax (h)	32.00	24.00	32.00	24.00	24.00	32.00	32.00

Appendix A-11. Plasma concentrations of albendazole sulphoxide (ABSO) (μg/ml) in goats following oral administration of netobimin at 7.5 mg/kg bodyweight.

	Animal number						
Time (h)	G1	G3	G7	G10	G11	G12	G13
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.00	0.00	0.00	0.00	0.03	0.00	0.00
1	0.18	0.11	0.06	0.06	0.12	0.19	0.21
2	0.46	0.47	0.34	0.18	0.53	0.59	0.67
4	1.05	0.63	0.72	0.61	1.09	0.98	1.17
8	1.55	0.95	0.88	1.04	1.65	1.21	1.68
12	1.73	1.00	0.96	1.13	1.76	1.09	1.43
24	0.76	0.27	0.51	1.19	0.90	0.76	0.98
32	0.23	0.00	0.11	0.49	0.18	0.23	0.56
48	0.00	0.00	0.00	0.00	0.00	0.00	0.00
72	0.00	0.00	0.00	0.00	0.00	0.00	0.00
96	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Appendix A-12. Pharmacokinetic parameters of albendazole sulphoxide (ABSO) in goats following oral administration of netobimin at a dose rate of 7.5 mg/kg bodyweight

	Animal number						
	G1	G3	G7	G10	G11	G12	G13
AUC_{obs} (μg.h/ml)	34.37	17.18	20.34	33.14	36.01	27.86	39.35
$AUMC_{obs}$(μg.h^2/ml)	500.5	200.3	294.7	623.5	512.7	427.4	657.5
MRT (h)	14.56	11.66	14.49	18.81	14.49	15.34	16.71
Cmax (μg/ml)	1.73	1.00	0.96	1.19	1.76	1.21	1.68
tmax (h)	12.00	12.00	12.00	24.00	12.00	8.00	8.00

Appendix A-13. Plasma concentrations of albendazole sulphone (ABSO2) (µg/ml) in goats following oral administration of netobimin at 7.5 mg/kg bodyweight.

	Animal number						
Time (h)	G1	G3	G7	G10	G11	G12	G13
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.04	0.00	0.04	0.05	0.00	0.00
4	0.06	0.09	0.09	0.09	0.11	0.07	0.07
8	0.18	0.20	0.20	0.16	0.22	0.20	0.16
12	0.32	0.35	0.40	0.33	0.33	0.27	0.41
24	0.53	0.71	0.73	0.53	0.64	0.61	0.51
32	0.56	0.00	0.63	0.43	0.60	0.57	0.64
48	0.00	0.00	0.00	0.00	0.00	0.00	0.00
72	0.00	0.00	0.00	0.00	0.00	0.00	0.00
96	0.00	0.00	0.00	0.00	0.00	0.00	0.00

ppendix A-14. Pharmacokinetic parameters of albendazole sulphone (ABSO2) goats following oral administration of netobimin at a dose rate of 7.5 mg/kg dyweight

	Animal number						
	G1	G3	G7	G10	G11	G12	G13
AUC_{obs} (µg.h/ml)	15.48	11.03	19.09	14.07	17.53	16.11	16.91
$AUMC_{obs}$(µg.h^2/ml)	379.4	211.6	462.5	330.3	424.2	398.4	413.5
MRT (h)	24.51	19.18	24.23	23.48	24.20	24.73	24.45
Cmax (µg/ml)	0.56	0.71	0.73	0.53	0.64	0.61	0.64
tmax (h)	32.00	24.00	24.00	24.00	24.00	24.00	32.00

Appendix B

Effect of piperonyl butoxide on the pharmacokinetics of albendazole and fenbendazole in sheep and goats

Appendix B-1. Recovery and coefficients of variation for piperonyl butoxide and albendazole metabolites following extraction by precipitation

Added concentration (μg/ml)	Recovery (%)	Between-assay coefficient of variation (%)
	piperonyl butoxide	
0.25	102.88 (n=6)	7.97
0.50	93.59 (n=7)	3.94
1	100.41 (n=7)	6.16
2	99.80 (n=8)	5.41
5	101.97 (n=7)	9.95
Mean	92.60 (n=35)	6.69
	albendazole	
0.10	99.09 (n=6)	4.27
0.50	95.46 (n=6)	3.72
1	93.23 (n=6)	9.06
Mean	95.93 (n=18)	5.68
	albendazole sulphoxide	
0.10	85.13 (n=8)	10.62
0.50	83.78 (n=8)	4.77
1	89.82 (n=6)	6.44
2	93.11 (n=7)	2.31
Mean	87.96 (n=29)	6.04
	albendazole sulphone	
0.10	82.56 (n=7)	6.90
0.50	86.49 (n=7)	5.71
1	91.40 (n=6)	9.49
Mean	84.79 (n=20)	7.38

Coefficient of variation = (SD/mean recovery) X 100

Appendix B-2. Recovery and coefficients of variation for fenbendazole metabolites following extraction by precipitation

Added concentration (µg/ml)	Recovery (%)	Between-assay coefficient of variation (%)
	fenbendazole	
0.10	84.11 (n=4)	9.25
0.25	83.31 (n=5)	8.59
0.50	78.27 (n=5)	11.74
Mean	81.90 (n=14)	9.86
	fenbendazole sulphoxide	
0.10	86.87 (n=5)	10.21
0.25	85.03 (n=6)	11.35
0.50	92.60 (n=5)	9.06
1	90.98 (n=6)	8.10
Mean	88.87 (n=22)	9.68
	fenbendazole sulphone	
0.10	83.06 (n=6)	11.58
0.50	85.98 (n=5)	11.19
1	88.42 (n=5)	9.65
Mean	85.82 (n=16)	10.81

Coefficient of variation = (SD/mean recovery) X 100

Appendix B-3. Plasma concentrations of fenbendazole (FBZ) (μg/ml) in goats following administration of fenbendazole (7.5 mg/kg) alone.

	Animal number					
Time (h)	G1	G3	G7	G10	SW	SB
0	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.02	0.00	0.03	0.02	0.03	0.00
4	0.12	0.04	0.13	0.05	0.13	0.04
8	0.18	0.11	0.25	0.10	0.22	0.25
12	0.18	0.15	0.24	0.09	0.18	0.24
24	0.15	0.10	0.11	0.07	0.14	0.22
32	0.07	0.10	0.07	0.05	0.02	0.13
48	0.00	0.00	0.00	0.00	0.00	0.03
72	0.00	0.00	0.00	0.00	0.00	0.00
96	0.00	0.00	0.00	0.00	0.00	0.00

Appendix B-4. Pharmacokinetic parameters of fenbendazole (FBZ) in goats following administration of fenbendazole (7.5 mg/kg) alone.

	Animal number					
	G1	G3	G7	G10	SB	SW
AUCobs (μg.h/ml)	4.89	3.96	5.30	2.60	7.40	4.40
AUMCobs ($\mu g.h^2/ml$)	87.42	80.80	85.97	48.50	163.04	67.25
MRT (h)	17.88	20.40	16.24	18.65	22.03	15.30
Cmax (μg/ml)	0.18	0.15	0.25	0.10	0.25	0.22
tmax (h)	10	12	8	8	8	8

Appendix B-5. Plasma concentrations of fenbendazole (FBZ) (μg/ml) in goats following administration of fenbendazole (7.5 mg/kg) and piperonyl butoxide (0.5 g/kg).

	Animal number					
Time (h)	G1	G3	G7	G10	SW	SB
0	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.00	0.00	0.00	0.00	0.00	0.00
1	0.02	0.00	0.01	0.00	0.02	0.00
2	0.13	0.01	0.08	0.00	0.02	0.04
4	0.34	0.11	0.16	0.05	0.05	0.13
8	0.50	0.38	0.27	0.34	0.26	0.18
12	0.57	0.30	0.25	0.45	0.38	0.25
24	0.39	0.50	0.19	0.45	0.67	0.20
32	0.34	0.45	0.15	0.42	0.34	0.18
48	0.16	0.10	0.02	0.30	0.20	0.04
72	0.00	0.00	0.00	0.06	0.03	0.00
96	0.00	0.00	0.00	0.00	0.01	0.00

Appendix B-6. Pharmacokinetic parameters of fenbendazole (FBZ) in goats following administration of fenbendazole (7.5 mg/kg) and piperonyl butoxide (0.5 gr/kg).

	Animal number					
	G1	G3	G7	G10	SB	SW
AUC_{obs} (μg.h/ml)	18.97	16.67	7.79	22.09	8.13	19.90
$AUMC_{obs}$ (μg.h^2/ml)	452.97	431.11	157.21	715.64	186.96	592.20
MRT (h)	23.88	25.87	20.19	32.40	23.00	29.77
Cmax (μg/ml)	0.57	0.50	0.28	0.45	0.25	0.67
tmax (h)	12	24	8	18	12	24

Appendix B-7. Plasma concentrations of fenbendazole sulphoxide (FBSO) (μg/ml) in goats following administration of fenbendazole (7.5 mg/kg) alone.

	Animal number					
Time (h)	G1	G3	G7	G10	SW	SB
0	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00
4	0.03	0.00	0.02	0.02	0.03	0.00
8	0.16	0.09	0.18	0.10	0.17	0.14
12	0.27	0.14	0.27	0.13	0.23	0.27
24	0.31	0.20	0.23	0.13	0.29	0.34
32	0.19	0.14	0.13	0.12	0.28	0.29
48	0.00	0.02	0.00	0.03	0.05	0.07
72	0.00	0.00	0.00	0.00	0.00	0.00
96	0.00	0.00	0.00	0.00	0.00	0.00

Appendix B-8. Pharmacokinetic parameters of fenbendazole sulphoxide (FBSO) in goats following administration of fenbendazole (7.5 mg/kg) alone.

	Animal number					
	G1	G3	G7	G10	SB	SW
AUC_{obs} (μg.h/ml)	8.27	5.56	6.80	4.84	11.00	9.87
$AUMC_{obs}$ (μg.h^2/ml)	178.76	137.28	137.04	122.00	290.56	253.00
MRT (h)	21.62	24.69	20.15	25.21	26.41	25.63
Cmax (μg/ml)	0.31	0.20	0.23	0.13	0.34	0.29
tmax (h)	24	24	24	18	24	24

Appendix B-9. Plasma concentrations of fenbendazole sulphoxide (FBSO) (μg/ml) in goats following administration of fenbendazole (7.5 mg/kg) and piperonyl butoxide (0.5 g/kg).

	Animal number					
Time (h)	G1	G3	G7	G10	SW	SB
0	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00
4	0.02	0.02	0.07	0.02	0.01	0.04
8	0.42	0.21	0.22	0.15	0.11	0.16
12	0.70	0.69	0.33	0.40	0.27	0.25
24	0.88	0.45	0.42	0.66	0.69	0.39
32	0.65	0.83	0.38	0.66	0.69	0.36
48	0.34	0.24	0.05	0.48	0.58	0.16
72	0.00	0.00	0.00	0.11	0.09	0.00
96	0.00	0.00	0.00	0.00	0.01	0.00

Appendix B-10. Pharmacokinetic parameters of fenbendazole sulphoxide (FBSO) in goats following administration of fenbendazole (7.5 mg/kg) and piperonyl butoxide (0.5 g/kg).

	Animal number					
	G1	G3	G7	G10	SB	SW
AUC_{obs} (μg.h/ml)	30.74	25.68	13.49	30.62	14.18	31.69
$AUMC_{obs}$ (μg.h^2/ml)	868.08	730.32	334.28	1106.2	415.04	1184.0
MRT (h)	28.24	28.44	24.78	36.13	29.27	37.36
Cmax (μg/ml)	0.88	0.83	0.42	0.66	0.39	0.69
tmax (h)	24	32	24	28	24	28

Appendix B-11. Plasma concentrations of fenbendazole sulphone (FBSO2) (μg/ml) in goats following oral administration of fenbendazole (7.5 mg/kg) alone.

	Animal number					
Time (h)	G1	G3	G7	G10	SW	SB
0	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00	0.00	0.00
8	0.02	0.01	0.02	0.01	0.01	0.01
12	0.04	0.02	0.05	0.01	0.02	0.04
24	0.09	0.07	0.09	0.04	0.05	0.08
32	0.09	0.08	0.07	0.04	0.06	0.12
48	0.03	0.06	0.03	0.03	0.04	0.09
72	0.00	0.01	0.00	0.00	0.01	0.01
96	0.00	0.00	0.00	0.00	0.00	0.00

Appendix B-12. Pharmacokinetic parameters of FBSO2 in goats following administration of fenbendazole(7.5 mg/kg) alone.

	Animal number					
	G1	G3	G7	G10	SB	SW
AUCobs (μg.h/ml)	3.04	3.27	2.80	1.74	2.56	4.70
AUMCobs ($\mu g.h^2/ml$)	92.01	120.80	81.22	63.32	93.61	174.06
MRT (h)	30.24	36.94	28.98	36.45	36.57	37.03
Cmax (μg/ml)	0.09	0.08	0.09	0.04	0.06	0.12
tmax (h)	28	32	24	28	32	32

Appendix B-13. Plasma concentrations of fenbendazole sulphone (FBSO2) (μg/ml) in goats following administration of fenbendazole (7.5 mg/kg) and piperonyl butoxide (0.5 g/kg).

	Animal number					
Time (h)	G1	G3	G7	G10	SW	SB
0	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.02	0.00	0.00	0.00	0.00	0.00
4	0.05	0.00	0.00	0.00	0.00	0.00
8	0.09	0.04	0.04	0.01	0.00	0.05
12	0.12	0.14	0.04	0.01	0.00	0.05
24	0.16	0.06	0.06	0.04	0.05	0.09
32	0.13	0.12	0.08	0.07	0.06	0.10
48	0.13	0.15	0.08	0.11	0.10	0.09
72	0.06	0.02	0.02	0.10	0.07	0.03
96	0.01	0.00	0.00	0.01	0.02	0.01

Appendix B-14. Pharmacokinetic parameters of FBSO2 in goats following administration of fenbendazole (7.5 mg/kg) and piperonyl butoxide (0.5 g/kg).

	Animal number					
	G1	G3	G7	G10	SB	SW
AUC_{obs} (μg.h/ml)	8.86	6.80	4.06	6.13	5.36	4.98
$AUMC_{obs}$ (μg.h^2/ml)	347.81	252.09	155.80	332.87	219.19	266.41
MRT (h)	39.28	37.09	38.36	54.31	40.87	53.47
Cmax (μg/ml)	0.16	0.14	0.08	0.11	0.10	0.10
tmax (h)	24	12	40	48	32	48

Appendix B-15. Plasma concentrations of albendazole sulphoxide (ABSO) (μg/ml) in goats following oral administration of albendazole (7.5 mg/kg) alone.

	Animal number					
Time (h)	G1	G3	G7	G10	SW	SB
0	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.04	0.03	0.00	0.02	0.00
0.5	0.13	0.10	0.07	0.13	0.07	0.05
1	0.45	0.20	0.15	0.62	0.15	0.15
2	1.27	0.30	0.40	1.52	0.34	0.49
4	2.60	0.95	0.83	1.95	0.75	1.14
8	2.51	2.67	1.21	1.47	1.07	1.52
12	1.55	3.00	1.30	1.16	1.61	1.27
24	0.00	0.61	0.05	0.02	1.20	0.31
32	0.00	0.00	0.00	0.00	0.16	0.00
48	0.00	0.00	0.00	0.00	0.02	0.00
72	0.00	0.00	0.00	0.00	0.00	0.00
96	0.00	0.00	0.00	0.00	0.00	0.00

Appendix B-16. Pharmacokinetic parameters of albendazole sulphoxide (ABSO) in goats following administration of albendazole (7.5 mg/kg) alone.

	Animal number					
	G1	G3	G7	G10	SW	SB
AUC_{obs} (μg.h/ml)	32.53	44.28	18.98	24.00	34.38	23.63
$AUMC_{obs}$ (μg.h^2/ml)	264.49	532.31	186.81	191.65	567.59	260.23
MRT (h)	8.13	12.02	9.84	7.98	16.51	11.01
Cmax (μg/ml)	2.60	3.00	1.30	1.95	1.61	1.52
tmax (h)	4	12	12	4	12	8

Appendix B-17. Plasma concentrations of albendazole sulphoxide (ABSO) (μg/ml) in goats following administration of albendazole (7.5 mg/kg) and piperonyl butoxide (0.5 g/kg).

	Animal number					
Time (h)	G1	G3	G7	G10	SW	SB
0	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.02	0.05	0.00	0.00
0.5	0.07	0.06	0.11	0.25	0.05	0.04
1	0.22	0.15	0.40	0.56	0.13	0.12
2	0.68	0.87	0.94	1.37	0.25	0.32
4	1.53	2.00	2.85	1.96	0.51	0.63
8	1.56	1.76	2.08	1.75	0.92	1.19
12	1.28	1.09	1.00	1.19	1.56	1.83
24	0.37	0.21	0.00	0.27	2.68	0.59
32	0.00	0.00	0.00	0.01	2.04	0.00
48	0.00	0.00	0.00	0.00	0.55	0.00
72	0.00	0.00	0.00	0.00	0.04	0.00
96	0.00	0.00	0.00	0.00	0.00	0.00

Appendix B-18. Pharmacokinetic parameters of albendazole sulphoxide (ABSO) in goats following administration of albendazole (7.5 mg/kg) and piperonyl butoxide (0.5 g/kg).

	Animal number					
	G1	G3	G7	G10	SW	SB
AUC_{obs} (μg.h/ml)	25.98	25.30	26.63	27.80	27.78	81.42
$AUMC_{obs}$ (μg.h^2/ml)	282.18	238.09	199.90	266.98	363.98	2209.9
MRT (h)	10.86	9.41	7.51	9.60	13.10	27.14
Cmax (μg/ml)	1.56	1.76	2.85	1.96	1.83	2.68
tmax (h)	8	8	4	4	12	24

Appendix B-19. Plasma concentrations of albendazole sulphone (ABSO2) (μg/ml) in goats following administration of albendazole (7.5 mg/kg) alone .

	Animal number					
Time (h)	G1	G3	G7	G10	SW	SB
0	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.00	0.00	0.00	0.00	0.00	0.00
1	0.02	0.02	0.00	0.03	0.00	0.00
2	0.08	0.04	0.00	0.11	0.01	0.02
4	0.25	0.12	0.12	0.28	0.06	0.07
8	0.49	0.41	0.32	0.54	0.13	0.17
12	0.73	0.83	0.56	0.65	0.26	0.41
24	0.00	1.44	0.31	0.29	0.71	0.58
32	0.00	0.00	0.00	0.00	0.26	0.49
48	0.00	0.00	0.00	0.00	0.00	0.01
72	0.00	0.00	0.00	0.00	0.00	0.00
96	0.00	0.00	0.00	0.00	0.00	0.00

Appendix B-20. Pharmacokinetic parameters of albendazole sulphone (ABSO2) in goats following administration of albendazole (7.5 mg/kg) alone.

	Animal number					
	G1	G3	G7	G10	SW	SB
AUC_{obs} (μg.h/ml)	8.69	23.12	9.22	11.29	13.02	16.08
$AUMC_{obs}$ (μg.h^2/ml)	89.02	439.98	139.84	152.99	300.11	382.66
MRT (h)	10.25	19.03	15.17	13.55	23.06	16.08
Cmax (μg/ml)	0.73	1.44	0.56	0.65	0.71	0.58
tmax (h)	12	24	12	12	24	24

Appendix B-21. Plasma concentrations of albendazole sulphone (ABSO2) (μg/ml) in goats following administration of albendazole (7.5 mg/kg) and piperonyl butoxide (0.5 g/kg).

	Animal number					
Time (h)	G1	G3	G7	G10	SW	SB
0	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.07	0.00	0.08	0.08	0.00	0.00
4	0.19	0.29	0.35	0.24	0.00	0.14
8	0.40	0.68	0.74	0.55	0.09	0.47
12	0.71	0.85	0.87	0.73	0.20	0.97
24	1.19	1.00	0.00	0.97	0.75	1.78
32	0.25	0.13	0.00	0.32	1.13	0.10
48	0.00	0.00	0.00	0.00	1.33	0.00
72	0.00	0.00	0.00	0.00	0.00	0.00
96	0.00	0.00	0.00	0.00	0.00	0.00

Appendix B-22. Pharmacokinetic parameters of albendazole sulphone (ABSO2) in goats following administration of albendazole (7.5 mg/kg) and piperonyl butoxide (0.5 g/kg).

	Animal number					
	G1	G3	G7	G10	SW	SB
AUC_{obs} (μg.h/ml)	22.86	21.95	11.09	22.42	49.62	29.06
$AUMC_{obs}$ (μg.h^2/ml)	465.05	396.76	111.64	446.48	1912.8	575.44
MRT (h)	20.35	18.08	10.07	19.91	38.55	19.80
Cmax (μg/ml)	1.19	1.00	0.87	0.97	1.33	1.78
tmax (h)	24.00	24.00	12.00	24.00	48.00	24.00

Appendix B-23. Plasma concentrations of albendazole sulphoxide (ABSO) (μg/ml) in sheep following administration of albendazole (7.5 mg/kg) alone.

	Animal number				
Time (h)	1	2	3	4	5
0	0.00	0.00	0.00	0.00	0.00
0.25	0.07	0.10	0.10	0.00	0.14
0.5	0.26	0.30	0.39	0.07	0.49
1	0.56	0.53	0.99	0.18	1.06
2	1.70	1.39	1.61	0.43	1.86
4	2.71	2.60	1.82	0.83	1.78
8	1.90	1.74	1.29	1.04	1.36
12	1.19	1.18	1.05	0.94	0.89
24	0.23	0.38	0.10	0.41	0.16
32	0.00	0.03	0.02	0.04	0.00
48	0.00	0.00	0.00	0.00	0.00
72	0.00	0.00	0.00	0.00	0.00
96	0.00	0.00	0.00	0.00	0.00

Appendix B-24. Pharmacokinetic parameters of albendazole sulphoxide (ABSO) in sheep following administration of albendazole alone at 7.5 mg/kg bodyweight.

	Animal number				
	1	2	3	4	5
AUC_{obs} (μg.h/ml)	30.64	30.98	23.59	19.56	23.30
$AUMC_{obs}$ (μg.h^2/ml)	268.33	307.50	201.25	248.68	195.19
MRT (h)	8.76	9.93	8.53	12.72	8.38
Cmax (μg/ml)	2.71	2.60	1.82	1.04	1.86
tmax (h)	4	4	4	8	2

Appendix B-25. Plasma concentrations of albendazole sulphoxide (μg/ml) in sheep following administration of albendazole (7.5 mg/kg) and piperonyl butoxide (0.5 g/kg).

	Animal number				
Time (h)	1	2	3	4	5
0	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00
0.5	0.10	0.08	0.17	0.05	0.00
1	0.28	0.23	0.46	0.21	0.09
2	0.86	0.54	0.94	0.41	0.20
4	1.38	1.04	1.85	0.87	0.37
8	1.78	1.47	2.41	3.52	2.98
12	1.65	1.68	1.80	2.60	3.29
24	0.95	1.05	0.42	1.09	1.21
32	0.35	0.48	0.05	0.40	0.47
48	0.00	0.00	0.00	0.00	0.00
72	0.00	0.00	0.00	0.00	0.00
96	0.00	0.00	0.00	0.00	0.00

Appendix B-26. Pharmacokinetic parameters of albendazole sulphoxide (ABSO) in sheep following administration of albendazole (7.5 mg/kg) and piperonyl butoxide (0.5 g/kg).

	Animal number				
	1	2	3	4	5
AUC_{obs} (μg.h/ml)	39.70	39.71	36.21	53.98	57.46
$AUMC_{obs}$ (μg.h^2/ml)	597.13	658.93	395.32	789.28	887.19
MRT (h)	15.04	16.59	10.92	14.62	15.44
Cmax (μg/ml)	1.78	1.68	2.41	2.60	3.29
tmax (h)	8	12	8	12	12

Appendix B-27. Plasma concentrations of albendazole sulphone (ABSO2) (μg/ml) in sheep following oral administration of albendazole (7.5 mg/kg) alone.

	Animal number				
Time (h)	1	2	3	4	5
0	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00
0.5	0.00	0.00	0.00	0.00	0.00
1	0.00	0.02	0.03	0.00	0.03
2	0.07	0.05	0.09	0.00	0.11
4	0.20	0.16	0.19	0.05	0.22
8	0.35	0.29	0.28	0.16	0.34
12	0.39	0.35	0.35	0.22	0.38
24	0.36	0.44	0.31	0.25	0.29
32	0.01	0.12	0.01	0.23	0.00
48	0.00	0.00	0.00	0.00	0.00
72	0.00	0.00	0.00	0.00	0.00
96	0.00	0.00	0.00	0.00	0.00

Appendix B-28. Pharmacokinetic parameters of albendazole sulphone (ABSO2) in sheep following administration of albendazole (7.5 mg/kg) alone.

	Animal number				
	1	2	3	4	5
AUC_{obs} (μg.h/ml)	8.95	10.37	7.87	5.74	8.15
$AUMC_{obs}$ (μg.h^2/ml)	141.49	196.65	123.37	88.31	119.95
MRT (h)	15.82	18.96	15.68	15.40	14.72
Cmax (μg/ml)	0.39	0.44	0.35	0.25	0.38
tmax (h)	12.00	24.00	12.00	12.00	12.00

Appendix B-29. Plasma concentrations of albendazole sulphone (ABSO2) (μg/ml) in sheep following administration of albendazole (7.5 mg/kg) and piperonyl butoxide (0.5 g/kg).

	Number number				
Time (h)	1	2	3	4	5
0	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00
0.5	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.00
2	0.10	0.00	0.07	0.02	0.00
4	0.35	0.05	0.21	0.08	0.00
8	0.39	0.23	0.57	0.29	0.35
12	0.68	0.30	0.80	0.60	0.69
24	0.68	0.59	0.81	0.83	0.96
32	0.08	0.69	0.49	0.84	0.17
48	0.00	0.33	0.05	0.19	0.00
72	0.00	0.00	0.00	0.00	0.00
96	0.00	0.00	0.00	0.00	0.00

Appendix B-30. Pharmacokinetic parameters of albendazole sulphone (ABSO2) following administration of albendazole (7.5 mg/kg) and piperonyl butoxide (0.5 g/kg).

	Animal number				
	1	2	3	4	5
AUC_{obs} (μg.h/ml)	15.96	24.25	24.40	28.41	18.56
$AUMC_{obs}$ (μg.h^2/ml)	276.18	760.12	528.33	772.06	373.12
MRT (h)	17.30	31.35	21.66	27.18	20.10
Cmax (μg/ml)	0.68	0.69	0.81	0.84	0.96
tmax (h)	18	32	24	32	24

Appendix B-31. Plasma concentrations of piperonyl butoxide (µg/ml) in goats following intramuscular injection of piperonyl butoxide at 0.5 g/kg bodyweight.

	Animal number					
Time (h)	G1	G3	G7	G10	SW	SB
0	0.00	0.00	0.00	0.00	0.00	0.00
0.167	0.17	ND	0.19	ND	0.37	0.29
0.5	0.23	ND	0.29	ND	0.64	0.37
1	0.30	0.17	0.37	0.00	0.67	0.64
2	0.38	0.68	0.46	0.46	0.88	0.68
3	0.50	0.61	0.49	0.62	1.01	0.84
5	0.81	0.86	0.59	0.82	1.14	0.98
9	1.02	1.06	0.49	1.32	1.54	1.21
13	1.46	1.23	0.59	1.66	1.65	1.33
25	1.03	0.89	0.58	1.25	1.82	0.81
33	0.77	0.76	0.47	0.94	1.73	0.86
49	0.72	0.53	0.52	0.81	1.52	0.86
73	0.63	0.58	0.36	0.86	0.97	0.79
97	0.55	0.65	0.61	0.86	0.76	0.36

Appendix B-32. Pharmacokinetic parameters for piperonyl butoxide administered intramuscularly to goats at a dose rate of 0.5 g/kg.

	Animal number					
	G1	G3	G7	G10	SB	SW
AUC_{obs} (µg.h/ml)	75.11	68.75	47.79	93.42	79.74	127.80
$AUMC_{obs}$ (µg.h^2/ml)	3194.2	3001.1	2272.8	4225.5	3376.9	5461.6
MRT (h)	42.52	43.65	47.55	45.23	42.35	42.75
Cmax (µg/ml)	1.46	1.23	0.59	1.66	1.33	1.82
tmax (h)	13	13	13	13	13	25

Appendix B-33. Plasma concentrations of piperonyl butoxide (μg/ml) in sheep following intramuscular injection of piperonyl butoxide at 0.5 g/kg bodyweight.

	Animal number				
Time (h)	1	2	3	4	5
0	0.00	0.00	0.00	0.00	0.00
0.167	0.37	0.31	0.40	0.40	0.78
0.5	0.51	0.50	0.57	0.72	1.05
1	0.62	0.50	1.01	1.28	1.94
2	0.83	0.87	1.18	1.97	2.87
3	1.43	1.21	1.53	2.30	3.59
5	1.36	1.86	1.53	2.35	3.62
9	1.83	2.48	1.80	3.09	1.75
13	2.19	3.89	1.80	2.08	3.41
25	1.89	3.31	1.58	2.12	3.55
33	1.18	2.14	0.72	1.38	2.18
49	0.64	1.18	0.56	0.71	0.99
73	0.82	1.25	0.50	0.68	0.93
97	0.85	1.97	0.47	0.68	0.68

Appendix B-34. Pharmacokinetic parameters for piperonyl butoxide administered intramuscularly to sheep at a dose rate of 0.5 g/kg

	Animal number				
	1	2	3	4	5
AUC_{obs} (μg.h/ml)	108.40	185.90	84.05	119.12	167.31
$AUMC_{obs}$ (μg.h^2/ml)	4315.0	7839.7	2935.4	4123.6	5632.8
MRT (h)	39.80	42.17	34.92	34.62	33.67
Cmax (μg/ml)	2.19	3.89	1.80	3.08	3.62
tmax (h)	13	13	11	9	5

Appendix C

Dose titration and efficacy studies with the combination fenbendazole-piperonyl butoxide in sheep

Appendix C-1. Recovery and coefficients of variation for fenbendazole metabolites following chloroform extraction from plasma

Added concentration (µg/ml)	Recovery (%)	Between-assay coefficient of variation (%)
	fenbendazole	
0.05	96.66 (n=6)	10.80
0.10	93.45 (n=6)	8.19
0.25	93.63 (n=6)	11.09
0.50	84.26 (n=6)	1.95
1	84.43 (n=6)	8.12
Mean	90.49 (n=30)	8.03
	fenbendazole sulphoxide	
0.05	85.35 (n=7)	7.70
0.10	92.78 (n=7)	4.10
0.25	92.84 (n=7)	4.90
0.50	89.24 (n=7)	5.20
1	86.24 (n=7)	4.29
Mean	89.29 (n=35)	5.24
	fenbendazole sulphone	
0.05	87.03 (n=7)	7.36
0.10	91.69 (n=7)	7.86
0.25	87.29 (n=7)	7.00
0.50	93.59 (n=7)	5.18
1	86.58 (n=7)	3.34
Mean	89.24 (n=30)	6.15

Coefficient of variation = (SD/mean recovery) X 100

Appendix C-2. Plasma concentrations of fenbendazole (FBZ) (μg/ml) following administration of fenbendazole (5mg/kg) alone.

	Animal number					
Time (h)	76	77	78	97	98	99
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.50	0.00	0.00	0.00	0.00	0.00	0.00
1	0.01	0.00	0.00	0.00	0.03	0.03
2	0.07	0.19	0.04	0.04	0.13	0.03
4	0.15	0.29	0.14	0.10	0.27	0.11
8	0.21	0.13	0.31	0.14	0.26	0.17
12	0.20	0.08	0.31	0.10	0.25	0.17
24	0.20	0.09	0.22	0.18	0.16	0.13
32	0.13	0.06	0.15	0.07	0.13	0.12
48	0.06	0.02	0.07	0.09	0.05	0.09
72	0.01	0.00	0.02	0.02	0.01	0.03
96	0.00	0.00	0.00	0.00	0.00	0.00

Appendix C-3. Pharmacokinetic parameters of fenbendazole (FBZ) (μg/ml) following administration of fenbendazole (5mg/kg) alone.

	Animal number					
	76	77	78	97	98	99
AUCobs (μg.h/ml)	8.00	4.34	10.08	6.64	8.47	7.70
AUMCobs (μg.h^2/ml)	200.70	79.73	253.60	206.40	189.60	238.70
MRT (h)	25.08	18.39	25.16	31.09	22.39	31.01
Cmax (μg/ml)	0.21	0.29	0.31	0.18	0.27	0.17
tmax (h)	8	4	10	24	4	10

Appendix C-4. Plasma concentrations of fenbendazole (FBZ) (μg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (15 mg/kg).

	Animal number					
Time (h)	76	77	78	97	98	99
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.50	0.00	0.02	0.00	0.00	0.00	0.00
1	0.00	0.09	0.00	0.00	0.01	0.00
2	0.01	0.19	0.02	0.02	0.03	0.05
4	0.06	0.16	0.10	0.09	0.11	0.13
8	0.14	0.15	0.20	0.16	0.21	0.19
12	0.16	0.13	0.20	0.19	0.18	0.15
24	0.18	0.17	0.21	0.16	0.16	0.13
32	0.20	0.09	0.18	0.12	0.11	0.11
48	0.16	0.06	0.10	0.07	0.06	0.09
72	0.02	0.01	0.04	0.02	0.01	0.02
96	0.00	0.00	0.02	0.00	0.00	0.00

Appendix C-5. Pharmacokinetic parameters of fenbendazole (FBZ) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (15 mg/kg).

	Animal number					
	76	77	78	97	98	99
AUCobs (μg.h/ml)	9.92	6.70	10.19	7.38	7.02	7.33
AUMCobs ($\mu g.h^2/ml$)	328.80	170.10	334.50	210.70	180.90	216.60
MRT (h)	33.16	25.39	32.83	28.56	25.77	29.57
Cmax (μg/ml)	0.20	0.19	0.21	0.19	0.21	0.19
tmax (h)	32	2	24	12	8	8

Appendix C-6. Plasma concentrations of fenbendazole (FBZ) (μg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (31 mg/kg).

	Animal number					
Time (h)	76	77	78	97	98	99
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.50	0.00	0.00	0.00	0.00	0.00	0.01
1	0.01	0.01	0.02	0.00	0.05	0.02
2	0.03	0.05	0.09	0.02	0.19	0.06
4	0.11	0.11	0.27	0.10	0.40	0.18
8	0.23	0.15	0.48	0.18	0.46	0.21
12	0.27	0.12	0.39	0.26	0.39	0.23
24	0.25	0.16	0.32	0.27	0.32	0.27
32	0.16	0.13	0.22	0.23	0.21	0.25
48	0.12	0.07	0.13	0.43	0.11	0.07
72	0.02	0.03	0.03	0.04	0.01	0.03
96	0.00	0.01	0.01	0.00	0.00	0.01

Appendix C-7. Pharmacokinetic parameters of fenbendazole (FBZ) (μg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (31 mg/kg).

	Animal number					
	76	77	78	97	98	99
AUCobs (μg.h/ml)	10.76	7.37	15.28	18.15	14.64	11.27
AUMCobs ($\mu g.h^2/ml$)	305.90	236.70	405.80	667.02	338.00	322.50
MRT (h)	28.42	32.10	26.56	36.75	23.08	28.62
Cmax (μg/ml)	0.27	0.16	0.48	0.43	0.46	0.27
tmax (h)	12	24	8	48	8	24

Appendix C-8. Plasma concentrations of fenbendazole (μg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (63 mg/kg).

	Animal number					
Time (h)	76	77	78	97	98	99
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.01	0.00	0.00	0.00	0.00
0.50	0.00	0.06	0.01	0.01	0.00	0.02
1	0.01	0.19	0.02	0.03	0.01	0.02
2	0.05	0.39	0.04	0.10	0.03	0.09
4	0.21	0.41	0.25	0.23	0.13	0.25
8	0.21	0.33	0.38	0.35	0.27	0.33
12	0.18	0.13	0.36	0.26	0.37	0.28
24	0.22	0.04	0.37	0.34	0.40	0.29
32	0.17	0.02	0.32	0.19	0.31	0.16
48	0.13	0.01	0.18	0.07	0.18	0.09
72	0.07	0.03	0.10	0.01	0.04	0.02
96	0.00	0.00	0.05	0.00	0.00	0.00

Appendix C-9. Pharmacokinetic parameters of fenbendazole (μg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (63 mg/kg).

	Animal number					
	76	77	78	97	98	99
AUCobs (μg.h/ml)	11.51	5.90	19.37	11.67	16.76	11.57
AUMCobs ($\mu g.h^2/ml$)	390.52	108.00	664.70	278.30	502.80	292.70
MRT (h)	33.92	18.30	34.32	23.85	29.99	25.31
Cmax (μg/ml)	0.22	0.41	0.38	0.35	0.40	0.33
tmax (h)	24	4	8	8	24	8

Appendix C-10. Plasma concentrations of fenbendazole (FBZ) (μg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (125 mg/kg).

	Animal number					
Time (h)	76	77	78	97	98	99
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.02	0.00
0.50	0.00	0.05	0.01	0.00	0.03	0.00
1	0.01	0.21	0.04	0.02	0.07	0.05
2	0.05	0.31	0.08	0.08	0.31	0.11
4	0.16	0.45	0.31	0.26	0.64	0.22
8	0.28	0.65	0.49	0.42	0.68	0.31
12	0.29	0.94	0.58	0.37	0.70	0.40
24	0.32	0.53	0.61	0.42	0.63	0.63
32	0.26	0.33	0.43	0.34	0.56	0.44
48	0.23	0.13	0.18	0.25	0.20	0.29
72	0.08	0.03	0.01	0.13	0.05	0.05
96	0.02	0.02	0.01	0.04	0.01	0.00

Appendix C-11. Pharmacokinetic parameters of fenbendazole (FBZ) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (125 mg/kg).

	Animal number					
	76	77	78	97	98	99
AUCobs (μg.h/ml)	17.08	24.93	22.90	22.44	29.11	23.88
AUMCobs (μg.h^2/ml)	597.60	571.10	588.47	794.40	753.80	736.30
MRT (h)	34.98	22.91	25.69	35.41	25.89	30.83
Cmax (μg/ml)	0.32	0.94	0.42	0.42	0.70	0.63
tmax (h)	24	12	24	24	12	24

Appendix C-12. Plasma concentrations of fenbendazole (μg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (250 mg/kg).

	Animal number					
Time (h)	76	77	78	97	98	99
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.50	0.01	0.02	0.01	0.01	0.01	0.01
1	0.02	0.11	0.04	0.02	0.02	0.03
2	0.06	0.29	0.20	0.09	0.06	0.10
4	0.20	0.42	0.34	0.25	0.18	0.35
8	0.37	0.40	0.49	0.34	0.28	0.42
12	0.38	0.68	0.52	0.36	0.37	0.58
24	0.46	0.76	0.72	0.49	0.61	0.76
32	0.49	0.67	0.58	0.54	0.60	0.82
48	0.48	0.38	0.58	0.51	0.63	0.64
72	0.21	0.06	0.17	0.19	0.26	0.12
96	0.07	0.01	0.06	0.06	0.05	0.04

Appendix C-13. Pharmacokinetic parameters of fenbendazole following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (250 mg/kg).

	Animal number					
	76	77	78	97	98	99
AUCobs (μg.h/ml)	31.19	33.63	38.03	32.00	37.47	41.15
AUMCobs ($\mu g.h^2/ml$)	1253.8	1003.7	1385.5	1260.7	1535.3	1438.8
MRT (h)	40.20	29.85	36.43	39.39	40.98	34.97
Cmax (μg/ml)	0.49	0.76	0.72	0.54	0.63	0.82
tmax (h)	32	24	24	32	48	32

Table C-14. Plasma concentrations of fenbendazole sulphoxide (FBSO) (μg/ml) following administration of fenbendazole (5mg/kg) alone.

	Animal number					
Time (h)	76	77	78	97	98	99
0	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.50	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.01	0.04	0.01	0.00	0.02	0.00
4	0.07	0.15	0.05	0.02	0.12	0.02
8	0.18	0.18	0.21	0.12	0.27	0.11
12	0.29	0.16	0.30	0.14	0.33	0.18
24	0.35	0.12	0.32	0.23	0.30	0.20
32	0.36	0.09	0.28	0.17	0.23	0.16
48	0.19	0.03	0.13	0.09	0.11	0.12
72	0.03	0.00	0.04	0.02	0.02	0.00
96	0.00	0.00	0.01	0.00	0.00	0.00

Table C-15. Pharmacokinetic parameters of fenbendazole sulphoxide (FBSO) (μg/ml) following administration of fenbendazole (5mg/kg) alone.

	Animal number					
	76	77	78	97	98	99
AUCobs (μg.h/ml)	15.61	5.39	13.64	8.28	12.55	8.26
AUMCobs (μg.h^2/ml)	491.00	115.20	425.91	258.96	342.30	245.70
MRT (h)	31.46	21.37	31.21	31.28	27.27	29.74
Cmax (μg/ml)	0.36	0.18	0.32	0.23	0.33	0.20
tmax (h)	32	8	24	24	12	24

Appendix C-16. Plasma concentrations of fenbendazole sulphoxide (FBSO) (µg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (15 mg/kg).

	Animal number					
Time (h)	76	77	78	97	98	99
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.50	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.01	0.00	0.00	0.00	0.00
2	0.00	0.05	0.00	0.00	0.00	0.01
4	0.02	0.13	0.01	0.02	0.03	0.05
8	0.10	0.19	0.10	0.11	0.14	0.18
12	0.15	0.28	0.17	0.19	0.22	0.22
24	0.26	0.33	0.23	0.26	0.22	0.21
32	0.32	0.24	0.22	0.24	0.20	0.21
48	0.20	0.14	0.13	0.13	0.08	0.15
72	0.03	0.02	0.04	0.03	0.02	0.05
96	0.00	0.00	0.01	0.00	0.00	0.00

Appendix C-17. Pharmacokinetic parameters of fenbendazole sulphoxide (FBSO) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (15 mg/kg).

	Animal number					
	76	77	78	97	98	99
AUCobs (µg.h/ml)	12.82	12.93	10.41	10.82	9.09	11.47
AUMCobs (µg.h^2/ml)	447.00	375.00	364.80	353.20	266.90	389.00
MRT (h)	34.86	29.00	35.04	32.64	29.36	33.92
Cmax (µg/ml)	0.32	0.33	0.23	0.26	0.22	0.22
tmax (h)	32	24	24	24	18	12

Appendix C-18. Plasma concentrations of fenbendazole sulphoxide (FBSO) (μg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (31 mg/kg).

	Animal number					
Time (h)	76	77	78	97	98	99
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.50	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.01	0.01	0.01	0.00	0.02	0.01
4	0.05	0.05	0.07	0.02	0.13	0.04
8	0.19	0.13	0.32	0.13	0.33	0.17
12	0.34	0.17	0.40	0.21	0.43	0.27
24	0.48	0.20	0.51	0.38	0.51	0.28
32	0.48	0.20	0.40	0.39	0.43	0.32
48	0.24	0.11	0.21	0.49	0.17	0.18
72	0.05	0.04	0.08	0.05	0.11	0.04
96	0.01	0.01	0.01	0.00	0.01	0.00

Appendix C-19. Pharmacokinetic parameters of fenbendazole sulphoxide (FBSO) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (31 mg/kg).

	Animal number					
	76	77	78	97	98	99
AUC_{obs} (μg.h/ml)	20.33	9.73	20.85	21.74	21.60	14.18
$AUMC_{obs}$ (μg.h^2/ml)	667.19	332.20	676.90	822.30	705.80	463.90
MRT (h)	32.83	34.15	32.47	37.83	32.68	32.72
Cmax (μg/ml)	0.48	0.20	0.51	0.49	0.51	0.32
tmax (h)	28	28	24	48	24	32

Appendix C-20. Plasma concentrations of fenbendazole sulphoxide (FBSO) (μg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (63 mg/kg).

	Animal number					
Time (h)	76	77	78	97	98	99
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.01	0.00	0.00	0.00	0.00
0.50	0.00	0.03	0.00	0.00	0.00	0.00
1	0.00	0.13	0.00	0.00	0.00	0.00
2	0.01	0.30	0.01	0.02	0.00	0.00
4	0.11	0.47	0.05	0.10	0.04	0.09
8	0.21	0.44	0.23	0.25	0.20	0.28
12	0.29	0.19	0.32	0.44	0.38	0.39
24	0.39	0.08	0.45	0.64	0.68	0.50
32	0.40	0.02	0.51	0.57	0.65	0.37
48	0.24	0.00	0.31	0.25	0.33	0.18
72	0.07	0.00	0.14	0.03	0.08	0.02
96	0.01	0.00	0.05	0.01	0.01	0.00

Appendix C-21. Pharmacokinetic parameters of fenbendazole sulphoxide (FBSO) (μg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (63 mg/kg).

	Animal number					
	76	77	78	97	98	99
AUCobs (μg.h/ml)	18.81	6.29	24.43	23.93	27.20	18.03
AUMCobs (μg.h^2/ml)	646.00	65.84	939.70	727.30	922.70	516.90
MRT (h)	34.35	10.47	38.47	30.39	33.92	28.67
Cmax (μg/ml)	0.40	0.47	0.51	0.64	0.68	0.50
tmax (h)	32	4	32	24	24	24

Appendix C-22. Plasma concentrations of fenbendazole sulphoxide(FBSO) (μg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (125 mg/kg).

	Animal number					
Time (h)	76	77	78	97	98	99
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.01	0.00
0.50	0.00	0.05	0.00	0.00	0.01	0.00
1	0.00	0.21	0.00	0.00	0.01	0.01
2	0.01	0.31	0.01	0.01	0.04	0.01
4	0.06	0.45	0.09	0.06	0.23	0.06
8	0.25	0.65	0.30	0.23	0.57	0.18
12	0.39	0.94	0.49	0.38	0.81	0.34
24	0.57	0.53	0.78	0.50	0.98	0.77
32	0.54	0.33	0.74	0.47	0.99	0.72
48	0.42	0.13	0.38	0.39	0.47	0.41
72	0.17	0.03	0.02	0.19	0.18	0.09
96	0.04	0.02	0.03	0.04	0.02	0.01

Appendix C-23. Pharmacokinetic parameters of fenbendazole sulphoxide (FBSO) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (125 mg/kg).

	Animal number					
	76	77	78	97	98	99
AUCobs (μg.h/ml)	29.45	36.69	30.53	27.64	45.16	30.46
AUMCobs (μg.h^2/ml)	1133.4	936.9	963.00	1093.9	1499.5	1061.1
MRT (h)	38.48	25.35	31.55	39.58	33.20	34.83
Cmax (μg/ml)	0.57	1.15	0.78	0.50	0.99	0.77
tmax (h)	24	24	24	24	32	24

Appendix C-24. Plasma concentrations of fenbendazole sulphoxide (FBSO) (μg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (250 mg/kg).

	Animal number					
Time (h)	76	77	78	97	98	99
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.50	0.00	0.00	0.00	0.00	0.01	0.00
1	0.00	0.01	0.00	0.00	0.02	0.01
2	0.01	0.05	0.01	0.01	0.06	0.01
4	0.07	0.21	0.03	0.07	0.18	0.09
8	0.28	0.48	0.14	0.21	0.28	0.31
12	0.44	0.75	0.32	0.31	0.37	0.60
24	0.67	0.94	0.69	0.57	0.61	0.97
32	0.75	0.96	0.65	0.63	0.60	1.08
48	0.61	0.58	0.55	0.57	0.63	0.73
72	0.33	0.14	0.23	0.33	0.26	0.17
96	0.11	0.02	0.07	0.07	0.05	0.02

Appendix C-25. Pharmacokinetic parameters of fenbendazole sulphoxide (FBSO) (μg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (250 mg/kg).

	Animal number					
	76	77	78	97	98	99
AUCobs (μg.h/ml)	42.01	44.75	35.29	36.97	37.47	47.91
AUMCobs ($\mu g.h^2/ml$)	1783.4	1506.0	1456.9	1614.2	1535.3	1733.8
MRT (h)	42.46	33.65	41.29	43.67	40.98	36.19
Cmax (μg/ml)	0.75	0.96	0.69	0.63	0.63	1.08
tmax (h)	32	32	24	32	48	32

Appendix C-26. Plasma concentrations of fenbendazole sulphone (FBSO2) (μg/ml) following administration of fenbendazole (5mg/kg) alone.

	Animal number					
Time (h)	76	77	78	97	98	99
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.50	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00
4	0.00	0.01	0.00	0.00	0.01	0.00
8	0.01	0.03	0.02	0.02	0.03	0.01
12	0.03	0.04	0.05	0.03	0.06	0.03
24	0.07	0.05	0.10	0.10	0.11	0.08
32	0.12	0.06	0.12	0.07	0.10	0.07
48	0.14	0.05	0.10	0.09	0.09	0.10
72	0.08	0.03	0.07	0.05	0.04	0.07
96	0.04	0.00	0.04	0.03	0.02	0.04

Appendix C-27. Pharmacokinetic parameters of fenbendazole sulphone (FBSO2) (μg/ml) following administration of fenbendazole (5mg/kg) alone.

	Animal number					
	76	77	78	97	98	99
AUCobs (μg.h/ml)	7.62	3.41	7.08	5.52	5.93	6.08
AUMCobs (μg.h^2/ml)	384.80	139.80	338.56	261.80	250.20	312.32
MRT (h)	50.50	41.00	47.82	47.42	42.19	51.37
Cmax (μg/ml)	0.14	0.06	0.12	0.10	0.11	0.10
tmax (h)	48	32	32	24	24	48

Appendix C-28. Plasma concentrations of fenbendazole sulphone (FBSO2) (µg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (15 mg/kg).

	Animal number					
Time (h)	76	77	78	97	98	99
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.50	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00
4	0.00	0.01	0.00	0.00	0.01	0.01
8	0.01	0.02	0.01	0.01	0.02	0.03
12	0.03	0.05	0.03	0.01	0.05	0.06
24	0.07	0.09	0.06	0.03	0.09	0.11
32	0.11	0.08	0.08	0.04	0.10	0.13
48	0.16	0.10	0.08	0.08	0.07	0.13
72	0.08	0.04	0.05	0.10	0.04	0.08
96	0.04	0.04	0.04	0.05	0.03	0.02

Appendix C-29. Pharmacokinetic parameters of fenbendazole sulphone (FBSO2) (µg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (15 mg/kg).

	Animal number					
	76	77	78	97	98	99
AUCobs (µg.h/ml)	7.90	5.81	5.12	5.50	5.33	8.05
AUMCobs (µg.h^2/ml)	400.20	269.10	257.60	331.00	236.40	369.20
MRT (h)	50.65	46.31	50.31	60.19	44.36	45.87
Cmax (µg/ml)	0.16	0.10	0.08	0.10	0.10	0.13
tmax (h)	48	48	40	72	32	40

Appendix C-30. Plasma concentrations of fenbendazole sulphone (FBSO2) (μg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (31 mg/kg).

	Animal number					
Time (h)	76	77	78	97	98	99
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.50	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00	0.01	0.01
8	0.02	0.01	0.02	0.01	0.03	0.03
12	0.05	0.03	0.03	0.02	0.05	0.06
24	0.13	0.06	0.08	0.06	0.12	0.11
32	0.17	0.08	0.10	0.09	0.13	0.14
48	0.16	0.08	0.09	0.09	0.10	0.19
72	0.09	0.06	0.08	0.06	0.06	0.11
96	0.06	0.05	0.05	0.03	0.04	0.06

Appendix C-31. Pharmacokinetic parameters of fenbendazole sulphone (FBSO2) (μg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (31 mg/kg).

	Animal number					
	76	77	78	97	98	99
AUCobs (μg.h/ml)	9.90	5.48	6.64	5.48	7.23	10.57
AUMCobs (μg.h^2/ml)	480.20	286.40	343.40	275.80	330.40	528.60
MRT (h)	48.50	52.26	51.71	50.34	45.69	50.01
Cmax (μg/ml)	0.17	0.08	0.10	0.09	0.13	0.19
tmax (h)	32	40	32	40	32	48

Appendix C-32. Plasma concentrations of fenbendazole sulphone (FBSO2) (µg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (63 mg/kg).

	Animal number					
Time (h)	76	77	78	97	98	99
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.50	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00
4	0.00	0.01	0.00	0.00	0.00	0.00
8	0.02	0.03	0.01	0.01	0.01	0.03
12	0.03	0.04	0.01	0.03	0.02	0.04
24	0.08	0.08	0.04	0.07	0.07	0.12
32	0.10	0.07	0.09	0.12	0.12	0.13
48	0.14	0.05	0.11	0.14	0.18	0.13
72	0.11	0.03	0.10	0.08	0.14	0.09
96	0.06	0.02	0.07	0.04	0.06	0.04

Appendix C-33. Pharmacokinetic parameters of fenbendazole sulphone (FBSO2) (µg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (63 mg/kg).

	Animal number					
	76	77	78	97	98	99
AUCobs (µg.h/ml)	8.48	4.07	7.04	7.62	10.02	8.44
AUMCobs (µg.h^2/ml)	454.70	173.90	404.50	384.80	549.00	409.90
MRT (h)	53.62	42.72	57.45	50.50	54.79	48.57
Cmax (µg/ml)	0.14	0.08	0.11	0.14	0.18	0.13
tmax (h)	48	24	48	48	48	40

Appendix C-34. Plasma concentrations of fenbendazole sulphone (FBSO2) (μg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (125 mg/kg).

	Animal number					
Time (h)	76	77	78	97	98	99
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.50	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00
4	0.00	0.01	0.00	0.00	0.00	0.00
8	0.01	0.02	0.01	0.01	0.02	0.01
12	0.02	0.05	0.02	0.02	0.04	0.02
24	0.07	0.16	0.05	0.05	0.11	0.08
32	0.10	0.18	0.08	0.08	0.19	0.12
48	0.15	0.15	0.13	0.16	0.24	0.18
72	0.14	0.08	0.08	0.16	0.15	0.15
96	0.09	0.05	0.09	0.08	0.08	0.07

Appendix C-35. Pharmacokinetic parameters of fenbendazole sulphone (FBSO2) (μg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (125 mg/kg).

	Animal number					
	76	77	78	97	98	99
AUCobs (μg.h/ml)	9.54	9.75	7.26	9.66	13.14	10.48
AUMCobs ($\mu g.h^2/ml$)	547.00	452.90	411.70	567.20	685.60	580.20
MRT (h)	57.34	46.26	56.71	58.72	52.18	55.36
Cmax (μg/ml)	0.15	0.18	0.13	0.16	0.24	0.18
tmax (h)	48	32	48	60	48	48

Appendix C-36. Plasma concentrations of fenbendazole sulphone (FBSO2) (μg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (250 mg/kg).

	Animal number					
Time (h)	76	77	78	97	98	99
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00	0.00	0.00
0.50	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00	0.00	0.00
8	0.01	0.03	0.01	0.01	0.01	0.01
12	0.02	0.06	0.01	0.01	0.01	0.03
24	0.06	0.18	0.05	0.05	0.06	0.10
32	0.10	0.18	0.07	0.09	0.10	0.15
48	0.26	0.21	0.09	0.13	0.14	0.23
72	0.16	0.20	0.11	0.19	0.18	0.24
96	0.12	0.13	0.09	0.12	0.12	0.13

Appendix C-37. Pharmacokinetic parameters of fenbendazole sulphone (FBSO2) (μg/ml) following administration of fenbendazole (5 mg/kg) in combination with piperonyl butoxide (250 mg/kg).

	Animal number					
	76	77	78	97	98	99
AUCobs (μg.h/ml)	12.48	15.12	6.98	10.30	10.48	15.00
AUMCobs ($\mu g.h^2/ml$)	719.40	816.00	420.30	639.20	637.80	870.10
MRT (h)	57.64	53.97	60.22	62.06	60.85	58.01
Cmax (μg/ml)	0.26	0.21	0.11	0.19	0.18	0.24
tmax (h)	48	48	72	72	72	72

Appendix C-38. Individual values for adult worm and faecal egg counts obtained during the efficacy trial.

	Animal number	Faecal egg counts (epg)*	*Haemonchus contortus* (adult worms)†	*Ostertagia circumcincta* (adult worms)
Group A	X6860	900	450	3350
	X1007	300	300	1750
	X1070	250	290	2850
	X1069	350	290	3200
	X1126	400	410	3610
	X1114	750	330	3150
Group B	X1093	100	40	2600
	X1141	0	20	5400
	X1129	200	80	1750
	X1000	150	60	2100
	X 987	0	100	3000
	X1039	150	50	2400
Group C	X1032	600	290	3700
	X1113	250	260	4250
	X1018	700	250	3000
	X1014	100	520	3800
	X1133	1000	440	4150
	X 999	250	160	3800
Group D	X1065	0	0	0
	X1098	0	0	0
	X1175	0	10	200
	X1010	50	0	400
	X1024	0	10	600
	X1035	0	20	1500

* Seven days posttreatment.

† No larval stages were found in the abomasal digests of any of the sheep.

Appendix D

Metabolism (S-oxidation) of benzimidazole anthelmintics: *in vitro* studies

Appendix D-1 Buffer composition

Calcium-free buffer:

NaCl	8.30 g/l
KCl	0.50 g/l
Hepes	2.40 g/l

Adjust pH to 7.4 by adding 5.5 ml of 1M NaOH.(1 litre of buffer).

Collagenase buffer:

NaCl	3.90 g/l
KCl	0.50 g/l
Hepes	24.0 g/l
$CaCl_2.2H_20$	0.70 g/l
Collagenase	0.50 g/l

Adjust pH to 7.6 by adding 66 ml of 1M NaOH (1 litre of buffer).

N.B. Collagenase to be added just before perfusion.

Washing buffer:

NaCl	8.30 g/l
KCl	0.50 g/l
Hepes	2.40 g/l
$CaCl_2.2H_20$	0.18 g/l

Adjust pH to 7.4 by adding 5.5 ml of 1M NaOH (1 litre of buffer)

Perfusion time:

Calcium-free buffer	12 minutes
Collagenase buffer	20 minutes

Appendix D-2 Culture media

Attachment medium

To 100 ml EWM add;

1 ml L-glutamine (i.e, 2mM)

1 ml (10000 IU-10000 µg) Penicillin-streptomycin

5 ml Foetal calf serum

1 µM insulin

Replacement medium

To 100 ml EWM add;

1 ml L-glutamine (2mM)

1 ml of (10000 IU-10000 µg) Penicillin- streptomycin

1.33 ml of 7.5% (W/V) Bovine serum albumin

Appendix D-3 Microsomal protein and cytochrome P450 in rat liver (n=4)

	1	2	3	4	Mean ± SEM
Microsomal protein (mg/g of liver)	21.09	14.52	15.30	14.70	16.40 ± 1.57
Cytochrome P450 (nmol/mg microsomal protein)	0.45	0.52	0.47	0.67	0.53 ± 0.05

Appendix D-4 Amount of drug and metabolites recovered (nmoles/ml) after one hour incubation without microsomes.

	Sulphide	Sulphoxide	Sulphone
FBZ (n=3)	51.72	0.00	0.00
FBSO (n=3)	0.00	47.54	0.00
ABZ (n=3)	49.13	0.21	0.00
TCBZ (n=3)	48.71	0.00	0.00

Appendix D-5. Amount of fenbendazole (FBZ) remaining in the microsomal reaction mixture after one hour incubation.

	FBZ (nmoles/ml) (n=4)				
Inhibitor added	1	2	3	4	Mean ± SEM
None	40.92	39.11	42.22	41.75	41.00 ± 0.68
PB	50.37	45.59	56.08	53.64	51.42 ± 2.27**

**Significantly different from the control value (P<0.01)

Appendix D-6 Amount of fenbendazole sulphoxide (FBSO) remaining in the microsomal reaction mixture after one hour incubation.

	FBSO (nmoles/ml) (n=3)			
Inhibitor added	1	2	3	Mean ± SEM
None	44.48	47.02	39.28	43.59 ± 2.28
PB	49.56	48.80	51.33	49.90 ± 0.75

Appendix D-7 Amount of albendazole (ABZ) remaining in the microsomal reaction mixture after one hour incubation.

	ABZ (nmoles/ml) (n=4)				
Inhibitor added	1	2	3	4	Mean ± SEM
None	12.33	10.67	7.20	3.73	8.48 ± 1.91
PB	20.39	18.47	33.74	28.38	25.25 ± 3.55**

**Significantly different from the control value (P<0.01)

Appendix D-8 Amount of triclabendazole (TCBZ) remaining in the microsomal reaction mixture after one hour incubation.

	TCBZ (nmoles/ml) (n=4)				
Inhibitor added	1	2	3	4	Mean ± SEM
None	0.00	4.11	0.28	0.56	1.24 ± 0.96
PB	0.89	17.04	6.84	0.56	6.33 ± 3.85

Appendix D-9. Effect of piperonyl butoxide (PB) on microsomal sulphoxidation of fenbendazole in rat liver.

	FBSO formed (nmoles/mg protein/hour) (n=4)				
Inhibitor added	1	2	3	4	Mean ± SEM
None	1.99	1.06	1.01	1.70	1.44 ± 0.24
PB	0.76	0.32	0.33	0.63	0.51 ± 0.11*

*Significantly different from the control value ($P<0.05$)

Appendix D-10. Effect of piperonyl butoxide (PB) on microsomal sulphonation of fenbendazole in rat liver.

	$FBSO_2$ formed (nmoles/mg protein/hour) (n=4)				
Inhibitor added	1	2	3	4	Mean ± SEM
None	0.65	0.63	0.54	0.51	0.58 ± 0.03
PB	0.21	0.24	0.15	0.17	0.19 ± 0.02†

† Signficantly different from the control value ($P<0.001$)

Appendix D-11. Effect of piperonyl butoxide (PB) on microsomal sulphonation of fenbendazole sulphoxide (FBSO) in rat liver.

	FBSO2 formed (nmol/mg protein/hour) (n=3)			
Inhibitor added	1	2	3	Mean ± SEM
None	2.16	0.35	2.38	1.63 ± 0.64
PB	1.15	0.27	0.87	0.76 ± 0.26

Appendix D-12. Effect of piperonyl butoxide (PB) on microsomal reduction of fenbendazole sulphoxide (FBSO) in rat liver.

	FBZ formed (nmol/mg protein/hour) (n=3)			
Inhibitor added	1	2	3	Mean ± SEM
None	0.08	0.06	0.07	0.07 ± 0.01
PB	0.08	0.10	0.07	0.08 ± 0.01

Appendix D-13. Effect of piperonyl butoxide (PB) on microsomal sulphoxidation of albendazole in rat liver.

	ABSO formed (nmoles/mg protein/hour) (n=4)				
Inhibitor added	1	2	3	4	Mean ± SEM
None	10.17	7.30	10.63	10.74	9.71 ± 0.81
PB	7.52	4.88	5.55	7.02	6.24 ± 0.62*

*Significantly different from the control value ($P<0.05$)

Appendix D-14 Effect of piperonyl butoxide (PB) on microsomal sulphonation of albendazole in rat liver.

	$ABSO_2$ formed (nmoles/mg protein/hour) (n=4)				
Inhibitor added	1	2	3	4	Mean ± SEM
None	0.13	0.05	0.08	0.29	0.14 ± 0.05
PB	0.10	0.00	0.00	0.10	0.05 ± 0.03

Appendix D-15. Effect of piperonyl butoxide (PB) on microsomal sulphoxidation of triclabendazole in rat liver.

	TCBSO formed (nmoles/mg protein/hour) (n=4)				
Inhibitor added	1	2	3	4	Mean ± SEM
None	7.87	9.72	11.08	9.90	9.64 ± 0.66
PB	9.55	6.46	9.82	10.92	9.19 ± 0.96

Appendix D-16. Effect of piperonyl butoxide (PB) on microsomal sulphonation of triclabendazole in rat liver.

	$TCBSO_2$ formed (nmoles/mg protein/hour) (n=4)				
Inhibitor added	1	2	3	4	Mean ± SEM
None	0.68	0.70	1.13	2.03	1.14 ± 0.32
PB	0.56	0.11	0.21	0.93	0.45 ± 0.19

Appendix D-17. Effect of piperonyl butoxide (PB) on sulphoxidation of fenbendazole by rat hepatocytes

	FBSO formed (pmoles/10^6 viable cells/hour) (n=3)			
Inhibitor added	1	2	3	Mean ± SEM
None	12.60	18.50	17.50	16.20 ± 1.82
PB	5.61	4.95	4.62	5.06 ± 0.29***

***Significantly different from the control value ($P<0.005$)

Appendix D-18. Effect of piperonyl butoxide (PB) on sulphonation of fenbendazole by rat hepatocytes

	$FBSO_2$ formed (pmoles/10^6 viable cells/hour) (n=3)			
Inhibitor added	1	2	3	Mean ± SEM
None	0.94	2.20	1.57	1.75 ± 0.36
PB	0.31	0.00	0.31	0.21 ± 0.10*

*Significantly different from the control value ($P<0.05$)

D-19. Effect of piperonyl butoxide (PB) on sulphoxidation of albendazole by rat hepatocytes

	ABSO formed (pmoles/10^6 viable cells/hour) (n=3)			
Inhibitor added	1	2	3	Mean ± SEM
None	85.54	107.39	132.94	108.62 ± 13.70
PB	31.11	56.66	29.99	39.25 ± 8.71*

* Significantly different from the control value ($P<0.05$).

D-20. Effect of piperonyl butoxide (PB) on sulphonation of albendazole by rat hepatocytes

	$ABSO_2$ formed (pmoles/10^6 viable cells/hour) (n=3)			
Inhibitor added	1	2	3	Mean ± SEM
None	0.70	0.70	0.70	0.70 ± 0.00
PB	0.00	0.00	0.00	0.00 ± 0.00*

* Significantly different from the control value ($P<0.05$)

Appendix D-21. Effect of 1-aminobenzotriazole (1-ABT) on sulphoxidation of triclabendazole by rat hepatocytes

	TCBSO formed (pmoles/10^6 viable cells/hour) (n=3)			
Inhibitor added	1	2	3	Mean ± SEM
None	295.10	301.30	274.40	290.30 ± 8.13
1-ABT	72.10	51.02	63.77	62.30 ± 6.13†

†Significantly different from the control value ($P<0.001$)

Appendix D-22. Effect of 1-aminobenzotriazole (1-ABT) on sulphonation of triclabendazole by rat hepatocytes

	$TCBSO_2$ formed (pmoles/10^6 viable cells/hour) (n=3)			
Inhibitor added	1	2	3	Mean ± SEM
None	46.57	44.41	47.87	46.28 ± 1.01
1-ABT	9.57	7.18	7.98	8.24 ± 0.70†

†Significantly different from the control value ($P<0.001$)

Appendix D-23. Effect of piperonyl butoxide (PB) on sulphoxidation of triclabendazole by rat hepatocytes

	TCBSO formed (pmoles/10^6 viable cells/hour) (n=3)			
Inhibitor added	1	2	3	Mean ± SEM
None	241.20	204.6	252.60	232.80 ± 14.48
PB	148.60	97.87	109.50	118.66 ± 15.34**

**Significantly different from the control value ($P<0.01$)

Appendix D-24. Effect of piperonyl butoxide (PB) on sulphonation of triclabendazole by rat hepatocytes

	$TCBSO_2$ formed (pmoles/10^6 viable cells/hour) (n=3)			
Inhibitor added	1	2	3	Mean ± SEM
None	85.90	94.41	96.00	92.10 ± 3.14
PB	53.72	38.56	36.43	42.90 ± 5.44***

***Significantly different from the control value ($P<0.005$)

Appendix E

Effect of early treatment with rafoxanide on antipyrine clearance in sheep infected with *Fasciola hepatica*

Appendix E-1. Recovery and coefficients of variation for antipyrine extracted from plasma.

Added concentration (µg/ml)	Recovery (%)	Between-assay coefficient of variation (%)
0.5	80.53 (n=4)	6.07
2	81.56 (n=4)	1.37
5	77.59 (n=4)	5.59
10	90.77 (n=4)	2.94
20	89.47 (n=4)	5.35
40	92.73 (n=4)	6.50
Mean	85.44 (n=24)	4.64

Coefficient of variation = (SD/mean recovery) X 100

Appendix E-2. Recovery and coefficients of variation for rafoxanide extracted from plasma.

Added concentration (µg/ml)	Recovery (%)	Between-assay coefficient of variation (%)
0.5	77.10 (n=5)	3.41
1	79.14 (n=5)	8.39
5	78.19 (n=5)	7.43
10	78.68 (n=5)	5.73
20	80.34 (n=5)	3.98
Mean	78.69 (n=25)	5.93

Coefficient of variation = (SD/mean recovery) X 100

Appendix E-3. Number and size of flukes recovered at necropsy in group A (infected untreated)

	Animal number					
	785	790	792	800	999	1000
number of fluke	107	109	107	156	88	73
size of fluke	>12 mm	>12 mm	>12 mm	>12 mm	>12 mm	>12 mm

Appendix E-4. Number and size of flukes recovered at necropsy in group B (infected treated)

	Animal number					
	786	788	789	797	798	799
number of fluke	4	15	20	9	35	11
size of fluke	>12 mm	>12 mm	>12 mm	>12 mm	>12 mm	>12 mm

Appendix E-5. Plasma parameters in group A (before *F. hepatica* infection)

	Animal number					
	785	790	792	800	999	1000
Total protein (g/l)	66	58	63	66	57	63
Albumin (g/l)	35	32	34	35	19	36
Globulin (g/l)	31	26	29	31	38	27
γGT(U/L)	35	20	26	38	44	34
GLDH (U/L)	1.0	2.3	1.8	ND	ND	3.5

Appendix E-6. Plasma parameters in group A (4 weeks after *F. hepatica* infection)

	Animal number					
	785	790	792	800	999	1000
Total protein (g/l)	68	66	74	75	71	67
Albumin (g/l)	32	33	32	33	26	34
Globulin (g/l)	36	33	42	42	45	33
γGT(U/L)	65	46	67	60	53	49
GLDH (U/L)	356	276	53	249	168	137

Appendix E-7. Plasma parameters in group A (6 weeks after *F. hepatica* infection)

	Animal number					
	785	790	792	800	999	1000
Total protein (g/l)	72	71	77	76	77	73
Albumin (g/l)	32	32	28	33	29	33
Globulin (g/l)	40	39	49	43	48	40
γGT(U/L)	59	54	60	61	67	66
GLDH (U/L)	149	100	133	209	232	124

Appendix E-8. Plasma parameters in group A (8 weeks after *F. hepatica* infection)

	Animal number					
	785	790	792	800	999	1000
Total protein (g/l)	70	67	76	74	78	76
Albumin (g/l)	30	30	28	31	27	32
Globulin (g/l)	40	37	48	43	51	44
γGT(U/L)	73	78	83	79	84	188
GLDH (U/L)	184	224	176	194	237	298

Appendix E-9. Plasma parameters in group A (10 weeks after *F. hepatica* infection)

	Animal number					
	785	790	792	800	999	1000
Total protein (g/l)	79	69	74	78	81	75
Albumin (g/l)	32	28	25	30	29	30
Globulin (g/l)	47	51	49	48	52	45
γGT (U/L)	229	227	284	245	188	221
GLDH (U/L)	305	157	224	234	254	69

Appendix E-10. Plasma parameters in group A (12 weeks after *F. hepatica* infection)

	Animal number					
	785	790	792	800	999	1000
Total protein (g/l)	75	68	79	76	81	73
Albumin (g/l)	27	26	24	29	29	30
Globulin (g/l)	48	42	55	47	52	43
γGT(U/L)	260	90	185	208	162	114
GLDH (U/L)	154	31	132	113	137	85

Appendix E-11. Plasma parameters in group A (14 weeks after *F. hepatica* infection)

	Animal number					
	785	790	792	800	999	1000
Total protein (g/l)	58	55	68	64	65	57
Albumin (g/l)	24	24	22	25	22	25
Globulin (g/l)	34	31	46	39	43	32
γGT(U/L)	103	56	119	98	79	75
GLDH (U/L)	14	12	245	42	25	72

Appendix E-12. Plasma parameters in group B (before *F. hepatica* infection)

	Animal number					
	786	788	789	797	798	799
Total protein (g/l)	56	72	69	77	64	62
Albumin (g/l)	30	21	34	34	35	34
Globulin (g/l)	26	51	35	43	29	28
γGT (U/L)	38	34	32	40	36	37
GLDH (U/L)	4.50	ND	7.10	0.40	3.40	4.70

Appendix E-13. Plasma parameters in group B (4 weeks after *F. hepatica* infection)

	Animal number					
	786	788	789	797	798	799
Total protein (g/l)	65	75	77	80	84	70
Albumin (g/l)	30	33	32	36	31	34
Globulin (g/l)	35	42	45	44	53	36
γGT (U/L)	75	45	84	53	86	45
GLDH (U/L)	321	118	339	202	222	265

Appendix E-14 Plasma parameters in group B (6 weeks after *F. hepatica* infection)

	Animal number					
	786	788	789	797	798	799
Total protein (g/l)	67	78	74	76	82	69
Albumin (g/l)	30	32	31	33	32	32
Globulin (g/l)	37	46	43	43	50	37
γGT (U/L)	69	39	55	50	71	49
GLDH (U/L)	59	13	29	9	28	18

Appendix E-15. Plasma parameters in group B (8 weeks after *F. hepatica* infection)

	Animal number					
	786	788	789	797	798	799
Total protein (g/l)	64	69	70	73	70	64
Albumin (g/l)	30	31	30	33	30	30
Globulin (g/l)	34	38	40	40	40	34
γGT (U/L)	69	44	63	54	54	45
GLDH (U/L)	31	16	48	9	28	20

Appendix E-16. Plasma parameters in group B (10 weeks after *F. hepatica* infection)

	Animal number					
	786	788	789	797	798	799
Total protein (g/l)	63	72	74	72	71	62
Albumin (g/l)	32	32	33	35	32	32
Globulin (g/l)	31	40	41	37	39	30
γGT (U/L)	63	49	60	53	53	42
GLDH (U/L)	65	19	64	22	40	44

Appendix E-17. Plasma parameters in group B (12 weeks after *F. hepatica* infection)

	Animal number					
	786	788	789	797	798	799
Total protein (g/l)	64	71	76	74	78	66
Albumin (g/l)	32	32	32	36	33	33
Globulin (g/l)	32	39	44	38	45	33
γGT (U/L)	95	66	109	71	100	74
GLDH (U/L)	81	34	214	37	139	108

Appendix E-18. Plasma parameters in group B (14 weeks after *F. hepatica* infection)

	Animal number					
	786	788	789	797	798	799
Total protein (g/l)	59	68	75	72	75	62
Albumin (g/l)	30	29	26	32	29	30
Globulin (g/l)	29	39	49	40	46	32
γGT (U/L)	104	70	51	74	88	70
GLDH (U/L)	8.40	1.40	6.40	1.10	12.00	3.50

Appendix E-19. Plasma parameters in group C (8 weeks before rafoxanide treatment)

	Animal number					
	787	791	793	794	795	796
Total protein (g/l)	66	67	59	76	69	63
Albumin (g/l)	31	34	30	31	36	38
Globulin (g/l)	35	33	29	45	33	25
γGT (U/L)	32	34	21	32	28	22
GLDH (U/L)	2.10	0.90	2.40	ND	2.20	8.60

Appendix E-20. Plasma parameters in group C (immediately before rafoxanide treatment)

	Animal number					
	787	791	793	794	795	796
Total protein (g/l)	65	70	63	76	70	68
Albumin (g/l)	31	35	31	36	36	36
Globulin (g/l)	34	35	32	40	34	32
γGT (U/L)	32	35	27	37	31	37
GLDH (U/L)	3.60	1.80	4.10	2.10	3.70	7.60

Appendix E-21. Plasma parameters in group C (2 weeks after rafoxanide treatment)

	Animal number					
	787	791	793	794	795	796
Total protein (g/l)	66	72	66	74	68	69
Albumin (g/l)	30	35	31	33	33	33
Globulin (g/l)	36	37	35	41	35	36
γGT (U/L)	30	32	29	40	31	36
GLDH (U/L)	4.90	2.60	2.80	5.40	19.20	6.10

Appendix E-22. Plasma parameters in group C (4 weeks after rafoxanide treatment)

	Animal number					
	787	791	793	794	795	796
Total protein (g/l)	64	70	64	72	67	70
Albumin (g/l)	30	34	29	31	32	32
Globulin (g/l)	34	36	35	41	35	38
γGT (U/L)	38	39	41	49	36	41
GLDH (U/L)	4.00	0.20	2.00	0.70	5.00	0.00

Appendix E-23. Plasma parameters in group C (6 weeks after rafoxanide treatment)

	Animal number					
	787	791	793	794	795	796
Total protein (g/l)	62	67	64	75	67	67
Albumin (g/l)	31	34	32	35	34	29
Globulin (g/l)	31	33	32	40	33	38
γGT (U/L)	42	43	40	56	40	44
GLDH (U/L)	3.80	4.10	13.40	8.00	6.70	1.50

Appendix E-24. Plasma parameters in group C (8 weeks after rafoxanide treatment)

	Animal number					
	787	791	793	794	795	796
Total protein (g/l)	63	70	66	77	69	72
Albumin (g/l)	30	36	32	37	36	29
Globulin (g/l)	33	34	34	40	33	43
γGT (U/L)	43	50	48	62	48	56
GLDH (U/L)	ND	3.90	19.50	14.60	22.10	1.90

Appendix E-25. Plasma parameters in group C (10 weeks after rafoxanide treatment)

	Animal number					
	787	791	793	794	795	796
Total protein (g/l)	62	67	65	73	66	73
Albumin (g/l)	28	32	31	33	33	28
Globulin (g/l)	34	35	34	40	33	35
γGT (U/L)	48	46	47	67	47	54
GLDH (U/L)	3.20	ND	9.00	4.3	11.90	0.60

Appendix E-26. Plasma concentrations of antipyrine in group A (before *F. hepatica* infection)

Time (hours)	Animal number					
	785	790	792	800	999	1000
0.083	44.33	42.05	45.66	44.02	48.20	36.77
0.5	31.37	30.85	29.18	24.07	30.45	25.11
1	20.74	20.79	21.34	14.92	22.10	16.39
2	12.52	10.52	11.69	7.14	15.20	6.58
3	8.35	7.21	6.46	3.53	10.70	3.73
4	6.06	4.84	4.42	2.03	8.14	2.22
6	2.76	1.40	2.07	0.63	4.02	0.68
8	1.48	1.12	1.09	0.30	2.29	0.36
12	0.32	0.23	0.27	0.06	0.59	0.13
24	0.00	0.00	0.00	0.00	0.00	0.00

Appendix E-27. Pharmacokinetic parameters of antipyrine in group A (before *F. hepatica* infection)

	Animal number					
	785	790	792	800	999	1000
Cpo (μg/ml)	50.23	45.80	50.42	50.15	55.10	39.75
AUC (μg.h/ml)	81.77	71.60	73.37	47.86	98.44	48.69
t1/2β (h^{-1})	1.91	1.82	1.78	1.76	2.15	2.04
Clb (ml/min.kg)	5.05	5.65	5.55	8.42	4.25	8.37
Vc (ml/kg)	497.70	545.90	495.80	498.50	453.70	629.00
Vd(area)(ml/kg)	836.20	888.80	865.30	1019.7	790.40	1477.0
Vdss (ml/kg)	735.40	727.00	745.20	787.70	728.80	857.00

Appendix E-28. Plasma concentrations of antipyrine in group A (4 weeks after *F. hepatica* infection)

Time (hours)	Animal number					
	785	790	792	800	999	100
0.083	48.45	52.22	45.82	50.84	38.69	47.39
0.5	32.19	33..73	30.11	30.28	28.13	31.17
1	20.36	28.26	22.01	22.91	22.36	22.03
2	11.00	15.61	13.31	14.05	15.74	14.68
3	6.57	10.20	9.21	8.45	11.20	9.72
4	3.85	6.20	6.81	5.27	8.13	6.27
6	1.71	2.83	3.54	2.55	3.85	2.98
8	0.69	1.69	1.56	1.42	1.75	1.80
12	0.20	0.45	0.57	0.30	0.47	0.36
24	0.00	0.00	0.00	0.00	0.00	0.00

Appendix E-29. Pharmacokinetic parameters of antipyrine in group A (4 weeks after *F. hepatica* infection)

	Animal number					
	785	790	792	800	999	1000
Cpo (μg/ml)	53.65	58.00	50.51	57.90	42.93	52.40
AUC (μg.h/ml)	70.69	97.12	89.04	83.95	92.76	88.86
t1/2β (h^{-1})	1.73	1.89	2.10	1.80	2.11	1.89
Clb (ml/min.kg)	5.78	4.30	4.62	4.93	4.47	4.63
Vc (ml/kg)	466.00	431.00	495.00	431.80	582.40	477.10
Vd(area)(ml/kg)	861.80	703.00	838.60	768.90	756.4	755.90
Vdss (ml/kg)	680.40	639.60	753.10	691.40	738.30	691.90

Appendix E-30. Plasma concentrations of antipyrine in group A (6 weeks after *F. hepatica* infection)

	Animal number					
Time (hours)	785	790	792	800	999	1000
0.083	51.77	39.32	44.74	50.17	46.03	51.96
0.5	32.94	24.30	34.56	33.23	29.66	29.78
1	25.81	16.20	21.92	22.69	19.66	21.24
2	14.08	8.21	13.05	12.56	15.00	12.97
3	7.94	4.16	11.05	7.25	10.91	8.24
4	5.21	2.56	7.92	4.08	7.05	5.15
6	2.07	0.99	3.87	1.65	2.98	2.27
8	0.98	0.44	2.04	0.80	1.88	0.97
12	0.25	0.11	0.54	0.24	0.54	0.29
24	0.00	0.00	0.00	0.00	0.08	0.00

Appendix E-31. Pharmacokinetic parameters of antipyrine in group A (6 weeks after *F. hepatica* infection)

	Animal number					
	785	790	792	800	999	1000
Cpo (μg/ml)	57.46	43.56	53.80	53.73	50.61	59.57
AUC (μg.h/ml)	84.29	51.98	95.89	76.39	90.94	79.25
t1/2β (h^{-1})	1.66	1.55	2.12	1.76	2.84	1.76
Clb (ml/min.kg)	4.93	7.72	4.36	5.32	4.46	5.22
Vc (ml/kg)	435.10	573.90	464.40	465.30	494.00	419.70
Vd(area)(ml/kg)	708.80	1034.5	801.80	810.60	1096.5	794.80
Vdss (ml/kg)	631.30	840.60	723.40	642.80	910.10	699.10

Appendix E-32. Plasma concentrations of antipyrine in group A (8 weeks after *F. hepatica* infection)

	Animal number					
Time (hours)	785	790	792	800	999	1000
0.083	40.70	50.55	49.50	57.61	46.90	53.83
0.5	29.67	29.67	36.30	35.67	33.98	36.76
1	20.63	22.71	23.45	27.23	26.58	24.30
2	14.09	14.01	15.34	15.34	16.38	18.85
3	9.09	10.07	10.46	9.79	11.98	13.09
4	6.74	6.91	6.39	6.62	8.93	12.31
6	2.72	2.92	3.79	2.85	5.29	6.23
8	1.43	1.65	1.57	1.68	2.77	4.18
12	0.41	0.47	0.53	0.42	0.94	1.46
24	0.00	0.00	0.00	0.00	0.00	0.08

Appendix E-33. Pharmacokinetic parameters of antipyrine in group A (8 weeks after *F. hepatica* infection)

	Animal number					
	785	790	792	800	999	1000
Cpo (μg/ml)	43.63	59.94	50.88	64.55	49.09	58.81
AUC (μg.h/ml)	83.97	90.29	96.60	98.09	114.00	134.00
t1/2β (h^{-1})	1.92	2.11	2.10	1.87	2.83	2.79
Clb (ml/min.kg)	4.86	4.57	4.28	4.21	3.64	3.09
Vc (ml/kg)	573.00	417.10	491.40	387.30	509.30	425.10
Vd(area)(ml/kg)	810.40	839.00	784.30	682.50	892.90	747.20
Vdss (ml/kg)	742.00	714.00	665.80	606.60	746.50	670.40

Appendix E-34. Plasma concentrations of antipyrine in group A (10 weeks after *F. hepatica* infection)

	Animal number					
Time (hours)	785	790	792	800	999	1000
0.083	45.30	39.88	50.08	40.40	42.96	47.92
0.5	32.77	26.54	35.67	27.08	36.13	29.38
1	27.03	23.93	24.00	21.23	27.57	21.39
2	20.22	13.12	15.95	14.20	19.24	12.54
3	12.54	6.72	15.85	9.36	15.47	8.28
4	9.99	3.68	12.52	6.95	11.40	5.64
6	4.16	2.48	5.74	3.54	4.56	2.24
8	2.51	0.93	4.50	1.72	2.56	1.22
12	0.88	0.19	1.42	0.52	0.70	0.30
24	0.08	0.00	0.09	0.04	0.06	0.04

Appendix E-35. Pharmacokinetic parameters of antipyrine in group A (10 weeks after *F. hepatica* infection)

	Animal number					
	785	790	792	800	999	1000
Cpo (μg/ml)	46.13	40.72	54.09	41.47	44.60	49.71
AUC (μg.h/ml)	117.43	73.16	132.38	87.49	120.80	79.68
t1/2β (h^{-1})	2.81	1.64	2.85	2.61	2.64	2.65
Clb (ml/min.kg)	3.55	5.41	3.12	4.72	3.43	5.12
Vc (ml/kg)	542.0	613.90	462.20	602.80	560.60	502.90
Vd(area)(ml/kg)	861.7	765.90	769.40	1065.2	784.40	1175.0
Vdss (ml/kg)	738.2	717.60	702.90	890.60	678.20	847.60

Appendix E-36. Plasma concentrations of antipyrine in group A (12 weeks after *F. hepatica* infection)

	Animal number					
Time (hours)	785	790	792	800	999	1000
0.083	48.83	49.16	33.74	52.42	46.44	46.44
0.5	33.12	30.87	27.69	30.43	29.11	35.86
1	25.15	24.12	23.17	25.41	22.57	30.96
2	15.45	15.33	15.90	15.77	13.52	23.45
3	12.53	11.06	11.25	9.46	8.44	12.23
4	8.47	7.52	7.17	7.56	5.94	8.13
6	4.17	3.77	3.83	3.82	2.89	4.21
8	2.57	2.07	2.37	2.17	1.43	2.39
12	0.85	0.55	0.71	0.75	0.45	0.81
24	0.04	0.00	0.03	0.00	0.00	0.00

Appendix E-37. Pharmacokinetic parameters of antipyrine in group A (12 weeks after *F. hepatica* infection)

	Animal number					
	785	790	792	800	999	1000
Cpo (μg/ml)	53.29	55.70	34.39	60.53	51.98	46.65
AUC (μg.h/ml)	108.10	98.43	95.74	100.78	84.61	119.40
t1/2β (h^{-1})	2.51	2.05	2.54	2.21	1.97	2.15
Clb (ml/min.kg)	3.81	4.23	4.27	4.17	4.91	3.41
Vc (ml/kg)	469.10	448.80	727.00	413.00	481.00	535.90
Vd(area)(ml/kg)	828.60	749.20	939.20	800.10	837.20	638.50
Vdss (ml/kg)	738.70	697.00	816.30	727.90	753.40	588.10

Appendix E-38. Plasma concentrations of antipyrine in group A (14 weeks after *F. hepatica* infection)

	Animal number					
Time (hours)	785	790	792	800	999	1000
0.083	44.64	42.80	42.39	50.46	43.42	48.02
0.5	29.76	25.07	27.48	31.29	33.57	33.54
1	24.18	22.62	22.81	23.21	27.58	23.67
2	15.55	13.16	14.94	13.69	24.21	13.85
3	10.37	7.92	10.53	9.91	16.33	9.83
4	8.54	5.39	8.04	6.58	14.04	7.50
6	3.39	2.36	4.39	3.00	6.16	3.56
8	2.09	1.27	3.01	1.78	5.98	1.78
12	0.64	0.33	0.86	0.50	1.72	0.68
24	0.04	0.00	0.04	0.00	0.11	0.00

Appendix E-39. Pharmacokinetic parameters of antipyrine in group A (14 weeks after *F. hepatica* infection)

	Animal number					
	785	790	792	800	999	1000
Cpo (μg/ml)	45.14	50.34	47.34	56.61	46.62	51.89
AUC (μg.h/ml)	98.34	77.82	101.11	91.21	152.43	96.10
t1/2β (h^{-1})	2.57	1.84	2.55	2.02	2.86	2.25
Clb (ml/min.kg)	4.20	5.43	4.15	4.52	2.80	4.31
Vc (ml/kg)	553.80	496.60	528.10	441.60	536.30	481.80
Vd(area)(ml/kg)	933.80	863.20	918.00	791.20	693.80	840.10
Vdss (ml/kg)	787.80	806.30	851.30	708.30	677.90	717.00

Appendix E-40. Plasma concentrations of antipyrine in group B (before *F. hepatica* infection)

Time (hours)	Animal number					
	786	788	789	797	798	799
0.083	47.49	39.37	38.11	43.81	49.26	38.83
0.5	32.03	27.00	23.90	25.51	31.87	34.97
1	29.32	19.96	17.53	19.04	24.17	24.30
2	15.07	13.53	8.40	11.19	16.80	15.93
3	10.59	9.73	4.94	6.97	10.62	10.62
4	7.71	7.60	2.67	4.59	7.95	7.29
6	4.11	3.99	1.05	1.83	3.92	3.59
8	1.85	2.74	0.36	0.98	2.10	2.16
12	0.62	1.00	0.08	0.25	0.56	0.66
24	0.00	0.00	0.00	0.00	0.00	0.05

Appendix E-41. Pharmacokinetic parameters of antipyrine in group B (before *F. hepatica* infection)

	Animal number					
	786	788	789	797	798	799
Cpo (μg/ml)	51.71	42.30	42.25	50.06	55.20	41.86
AUC (μg.h/ml)	102.65	94.52	53.31	68.86	101.10	99.19
t1/2β (h^{-1})	2.18	2.69	1.42	1.77	2.04	2.94
Clb (ml/min.kg)	4.13	4.43	7.69	6.02	4.12	4.16
Vc (ml/kg)	483.50	591.00	591.70	499.40	452.90	597.30
Vd(area)(ml/kg)	780.30	1031.9	947.10	923.60	728.20	1056.3
Vdss (ml/kg)	675.90	920.90	822.00	818.40	678.90	766.90

Appendix E-42. Plasma concentrations of antipyrine in group B (4 weeks after *F. hepatica* infection)

Time (hours)	Animal number					
	786	788	789	797	798	799
0.083	35.79	46.65	43.96	45.30	39.86	53.12
0.5	26.24	31.47	26.15	30.58	29.63	28.52
1	16.71	21.05	17.53	22.44	24.65	25.88
2	9.92	12.15	8.24	16.25	14.68	17.47
3	6.74	6.39	4.08	10.68	9.09	12.59
4	4.61	3.29	2.33	8.43	6.64	9.38
6	1.88	1.24	0.58	4.09	3.29	7.56
8	0.92	0.71	0.26	2.33	1.73	5.25
12	0.18	0.16	0.06	0.49	0.39	2.12
24	0.00	0.00	0.00	0.00	0.00	0.14

Appendix E-43. Pharmacokinetic parameters of antipyrine in group B (4 weeks after *F. hepatica* infection)

	Animal number					
	786	788	789	797	798	799
Cpo (μg/ml)	51.71	50.62	49.10	50.38	41.72	64.87
AUC (μg.h/ml)	63.65	69.43	52.64	99.08	89.11	136.48
t1/2β (h^{-1})	1.73	1.57	1.34	2.02	1.98	3.17
Clb (ml/min.kg)	6.50	5.75	7.76	4.20	4.67	3.12
Vc (ml/kg)	483.50	493.90	509.20	496.20	599.20	385.40
Vd(area)(ml/kg)	971.20	783.00	897.80	736.10	799.40	857.60
Vdss (ml/kg)	675.90	657.40	724.20	698.80	718.80	806.20

Appendix E-44. Plasma concentrations of antipyrine in group B (6 weeks after *F. hepatica* infection)

	Animal number					
Time (hours)	786	788	789	797	798	799
0.083	47.67	41.57	45.57	43.55	38.91	50.69
0.5	30.75	28.15	23.42	27.50	29.26	31.76
1	23.03	19.97	15.82	21.45	20.83	27.30
2	14.14	10.58	7.07	13.81	12.44	18.52
3	8.24	4.46	2.85	8.27	7.85	12.73
4	5.32	3.45	1.27	5.89	4.97	9.64
6	2.44	1.49	0.42	2.63	2.81	5.26
8	1.40	0.56	0.16	1.31	1.20	3.71
12	0.39	0.18	0.03	0.46	0.44	1.20
24	0.00	0.00	0.00	0.00	0.05	0.09

Appendix E-45. Pharmacokinetic parameters of antipyrine in group B (6 weeks after *F. hepatica* infection)

	Animal number					
	786	788	789	797	798	799
Cpo (μg/ml)	52.70	43.82	52.66	48.73	40.56	63.32
AUC (μg.h/ml)	83.99	62.97	45.59	81.67	78.04	120.38
t1/2β (h^{-1})	1.90	1.75	1.23	1.97	2.94	2.92
Clb (ml/min.kg)	4.91	6.31	9.18	5.12	5.08	3.47
Vc (ml/kg)	474.40	570.50	474.70	513.00	616.30	394.80
Vd(area)(ml/kg)	806.40	954.40	976.10	874.00	1292.6	872.60
Vdss (ml/kg)	714.40	738.10	742.30	793.50	900.20	702.60

Appendix E-46. Plasma concentrations of antipyrine in group B (8 weeks after *F. hepatica* infection)

	Animal number					
Time (hours)	786	788	789	797	798	799
0.083	46.64	48.24	35.13	44.50	32.98	55.51
0.5	29.05	34.16	27.37	25.54	26.76	34.86
1	19.36	24.79	20.47	22.83	23.78	27.59
2	9.24	13.27	11.72	14.06	15.06	16.77
3	6.24	12.13	6.92	9.53	8.95	12.02
4	4.35	7.23	4.21	6.08	5.55	9.57
6	1.85	3.36	1.70	2.61	2.10	5.72
8	0.99	2.06	0.75	1.11	0.93	2.65
12	0.26	0.58	0.17	0.23	0.15	1.13
24	0.00	0.00	0.00	0.00	0.00	0.07

Appendix E-47. Pharmacokinetic parameters of antipyrine in group B (8 weeks after *F. hepatica* infection)

	Animal number					
	786	788	789	797	798	799
Cpo (μg/ml)	50.36	51.63	37.36	49.28	35.14	61.88
AUC (μg.h/ml)	69.02	98.22	67.29	81.32	77.50	120.38
t1/2β (h^{-1})	1.92	2.15	1.69	1.68	1.52	2.73
Clb (ml/min.kg)	5.89	4.20	6.05	4.94	5.41	3.45
Vc (ml/kg)	496.40	484.20	669.20	507.30	711.50	404.00
Vd(area)(ml/kg)	973.90	780.60	885.40	717.20	706.50	813.80
Vdss (ml/kg)	764.60	680.50	765.30	665.60	723.30	721.50

Appendix E-48. Plasma concentrations of antipyrine in group B (10 weeks after *F. hepatica* infection)

	Animal number					
Time (hours)	786	788	789	797	798	799
0.083	31.03	42.87	43.40	26.82	46.10	71.70
0.5	30.17	29.13	24.94	23.50	28.98	36.65
1	17.14	20.37	15.81	19.76	21.40	21.91
2	10.05	8.73	6.52	11.56	13.33	15.06
3	7.24	6.86	3.45	7.01	7.88	9.76
4	4.93	4.35	1.69	5.07	5.61	7.75
6	1.70	1.76	0.50	1.96	2.42	3.36
8	0.62	0.74	0.22	0.63	1.25	1.42
12	0.23	0.45	0.05	0.24	0.35	0.28
24	0.00	0.00	0.00	0.00	0.00	0.00

Appendix E-49. Pharmacokinetic parameters of antipyrine in group B (10 weeks after *F. hepatica* infection)

	Animal number					
	786	788	789	797	798	799
Cpo (μg/ml)	33.72	45.51	48.73	44.60	51.46	85.44
AUC (μg.h/ml)	65.03	69.14	47.00	65.17	80.08	98.01
t1/2β (h^{-1})	2.80	2.17	1.34	1.78	1.86	1.75
Clb (ml/min.kg)	6.28	5.74	8.51	6.28	5.16	4.15
Vc (ml/kg)	741.50	549.30	513.00	560.60	485.80	292.60
Vd(area)(ml/kg)	1519.20	1080.50	986.40	964.80	833.40	628.70
Vdss (ml/kg)	867.30	797.90	747.90	892.50	745.30	547.80

Appendix E-50. Plasma concentrations of antipyrine in group B (12 weeks after *F. hepatica* infection)

	Animal number					
Time (hours)	786	788	789	797	798	799
0.083	41.03	45.38	42.33	42.67	45.53	43.29
0.5	29.64	32.41	26.37	29.51	30.68	36.08
1	21.37	22.69	15.96	19.91	22.17	29.31
2	9.45	13.83	9.02	11.43	13.24	18.64
3	6.03	8.66	3.70	9.64	8.84	15.63
4	3.36	6.55	1.91	5.72	6.08	9.98
6	1.45	2.97	0.63	3.60	3.15	4.71
8	0.80	1.95	0.25	1.80	1.84	2.14
12	0.28	0.52	0.08	0.47	0.55	0.95
24	0.00	0.00	0.00	0.00	0.03	0.0.5

Appendix E-51. Pharmacokinetic parameters of antipyrine in group B (12 weeks after *F. hepatica* infection)

	Animal number					
	786	788	789	797	798	799
Cpo (μg/ml)	44.22	49.21	46.65	47.86	48.13	44.88
AUC (μg.h/ml)	66.34	89.72	51.22	83.48	87.90	120.65
t1/2β (h^{-1})	2.54	2.17	1.41	2.16	2.53	2.57
Clb (ml/min.kg)	6.19	4.60	7.78	4.96	4.64	3.33
Vc (ml/kg)	565.40	508.00	535.90	522.40	519.40	557.00
Vd(area)(ml/kg)	1363.1	861.60	951.00	926.40	1018.3	739.90
Vdss (ml/kg)	787.50	735.20	737.20	811.30	814.20	650.20

Appendix E-52. Plasma concentrations of antipyrine in group B (14 weeks after *F. hepatica* infection)

	Animal number					
Time (hours)	786	788	789	797	798	799
0.083	56.31	32.56	44.12	58.27	57.19	47.75
0.5	32.96	26.69	29.23	28.23	37.40	33.22
1	29.00	22.44	21.43	22.23	31.89	25.84
2	19.14	15.54	13.39	13.03	19.34	17.58
3	15.42	9.55	8.97	7.78	14.02	14.04
4	11.09	7.78	6.40	5.35	11.18	10.74
6	5.71	4.35	3.17	2.54	6.04	6.82
8	4.23	2.67	1.55	1.32	3.94	4.95
12	1.84	0.82	0.40	0.44	1.19	1.75
24	0.00	0.04	0.00	0.05	0.05	0.09

Appendix E-53. Pharmacokinetic parameters of antipyrine in group B (14 weeks after *F. hepatica* infection)

	Animal number					
	786	788	789	797	798	799
Cpo (μg/ml)	66.09	33.30	48.68	68.59	61.87	51.10
AUC (μg.h/ml)	138.76	96.22	84.93	83.59	136.98	135.45
t1/2β (h^{-1})	2.79	2.63	1.94	2.67	2.57	2.90
Clb (ml/min.kg)	3.05	4.25	4.85	5.04	3.08	3.13
Vc (ml/kg)	378.30	750.80	513.60	364.50	404.10	489.20
Vd(area)(ml/kg)	738.00	970.80	815.00	1164.9	686.50	784.80
Vdss (ml/kg)	693.00	863.50	746.00	914.70	613.40	731.10

Appendix E-54. Plasma concentrations of antipyrine in group C (8 weeks before treatment)

Time (hours)	Animal number					
	787	791	793	794	795	796
0.083	39.46	61.00	38.96	45.24	41.54	42.53
0.5	30.39	33.65	25.43	27.93	24.67	24.90
1	19.61	23.40	17.53	20.52	17.05	19.60
2	13.17	14.57	11.17	12.31	8.22	11.83
3	10.34	9.08	6.48	7.86	4.57	7.63
4	7.21	5.85	4.40	5.59	2.41	4.65
6	3.37	2.81	1.59	2.12	0.73	2.15
8	2.44	1.49	0.95	1.22	0.38	1.08
12	0.78	0.48	0.24	0.31	0.07	0.26
24	0.00	0.00	0.00	0.00	0.00	0.00

Appendix E-55. Pharmacokinetic parameters of antipyrine in group C (8 weeks before treatment)

	Animal number					
	787	791	793	794	795	796
Cpo (μg/ml)	44.01	70.32	42.85	50.81	46.55	49.07
AUC (μg.h/ml)	91.60	92.19	65.23	76.68	52.42	71.14
t1/2β (h^{-1})	2.47	1.97	1.78	1.83	1.40	1.78
Clb (ml/min.kg)	4.60	4.47	6.27	5.39	7.80	5.85
Vc (ml/kg)	568.00	355.50	583.40	492.00	537.00	509.50
Vd(area)(ml/kg)	984.00	762.80	963.70	854.90	946.80	903.10
Vdss (ml/kg)	869.50	652.20	847.10	764.70	785.80	823.70

Appendix E-56. Plasma concentrations of antipyrine in group C (1 day prior to treatment)

Time (hours)	Animal number					
	787	791	793	794	795	796
0.083	50.15	57.04	40.13	44.75	37.92	46.47
0.5	33.60	35.05	22.82	31.76	27.66	30.78
1	25.47	21.56	16.22	21.11	19.75	22.01
2	16.80	11.82	9.68	11.90	11.17	13.98
3	12.73	7.56	6.87	7.91	6.62	9.52
4	8.48	4.96	3.42	4.52	3.69	6.54
6	4.75	2.22	1.60	1.70	1.19	3.08
8	3.16	1.30	0.61	0.85	0.45	1.17
12	0.83	0.36	0.16	0.21	0.09	0.32
24	0.00	0.00	0.00	0.00	0.00	0.00

Appendix E-57. Pharmacokinetic parameters of antipyrine in group C (1 day prior to treatment)

	Animal number					
	787	791	793	794	795	796
Cpo (μg/ml)	55.37	63.84	46.29	47.53	40.45	51.40
AUC (μg.h/ml)	112.96	82.31	59.08	74.06	63.27	85.97
t1/2β (h^{-1})	2.30	2.01	1.63	1.74	1.40	1.80
Clb (ml/min.kg)	3.68	4.98	6.99	5.55	6.42	4.84
Vc (ml/kg)	451.50	391.60	540.10	526.00	618.10	486.40
Vd(area)(ml/kg)	730.50	865.60	986.70	838.50	775.50	756.30
Vdss (ml/kg)	677.80	668.20	877.20	688.30	713.10	692.10

Appendix E-58. Plasma concentrations of antipyrine in group C (2 weeks after treatment)

Time (hours)	Animal number					
	787	791	793	794	795	796
0.083	45.15	51.24	46.84	45.05	51.38	37.91
0.5	32.20	29.51	27.22	28.76	28.29	25.96
1	23.67	20.65	19.51	18.21	17.88	21.28
2	17.12	10.16	9.81	9.27	8.83	11.67
3	12.56	5.11	5.26	4.86	4.26	6.97
4	9.50	3.18	3.38	2.78	2.55	4.74
6	5.45	1.29	1.31	1.21	0.92	2.42
8	2.93	0.61	0.59	0.51	0.33	1.31
12	0.98	0.16	0.11	0.15	0.08	0.33
24	0.00	0.00	0.00	0.00	0.00	0.00

Appendix E-59. Pharmacokinetic parameters of antipyrine in group C (2 weeks after treatment)

	Animal number					
	787	791	793	794	795	796
Cpo (μg/ml)	48.97	57.82	53.04	49.18	58.51	39.40
AUC (μg.h/ml)	113.51	65.55	62.40	59.80	57.60	73.02
t1/2β (h^{-1})	2.40	1.60	1.51	1.70	1.42	2.07
Clb (ml/min.kg)	3.66	6.22	6.56	6.74	7.03	5.63
Vc (ml/kg)	510.50	432.40	471.30	508.30	427.30	634.50
Vd(area)(ml/kg)	762.00	863.90	856.40	993.10	860.40	1005.2
Vdss (ml/kg)	715.30	696.00	729.20	734.60	676.00	827.90

Appendix E-60. Plasma concentrations of antipyrine in group C (4 weeks after treatment)

	Animal number					
Time (hours)	787	791	793	794	795	796
0.083	56.90	55.40	61.21	44.91	59.90	40.63
0.5	32.94	25.88	29.21	28.69	32.16	27.32
1	26.81	20.68	19.25	20.48	23.39	21.28
2	15.43	13.26	13.29	10.81	11.33	14.45
3	13.31	7.10	7.97	6.46	7.28	10.15
4	10.81	3.78	5.69	4.31	4.21	7.55
6	4.91	1.54	2.30	1.78	1.93	4.24
8	2.97	0.84	1.27	0.72	0.93	1.96
12	1.53	0.17	0.35	0.19	0.20	0.50
24	0.08	0.00	0.00	0.00	0.00	0.00

Appendix E-61. Pharmacokinetic parameters of antipyrine in group C (4 weeks after treatment)

	Animal number					
	787	791	793	794	795	796
Cpo (μg/ml)	65.26	69.48	74.05	49.60	64.95	45.53
AUC (μg.h/ml)	123.61	72.04	81.73	68.96	77.46	91.34
t1/2β (h^{-1})	2.83	1.59	1.89	1.64	1.65	2.06
Clb (ml/min.kg)	3.40	5.86	5.06	5.90	5.28	4.57
Vc (ml/kg)	383.10	359.80	337.60	504.00	384.90	549.10
Vd(area)(ml/kg)	832.80	807.90	826.20	839.00	755.30	814.10
Vdss (ml/kg)	748.40	713.30	703.10	728.40	890.60	777.70

E-62. Plasma concentrations of antipyrine in group C (6 weeks after treatment)

	Animal number					
Time (hours)	787	791	793	794	795	796
0.083	48.45	45.29	37.26	42.96	48.06	45.65
0.5	38.21	23.73	25.22	24.89	25.83	26.30
1	19.89	20.39	17.74	17.05	11.77	22.50
2	16.29	6.85	7.07	7.05	7.13	16.77
3	10.82	4.26	3.29	3.52	3.78	12.01
4	8.46	2.28	1.94	1.53	1.96	9.30
6	4.77	0.99	0.49	0.64	0.65	5.47
8	2.73	0.50	0.26	0.27	0.29	2.18
12	0.94	0.09	0.04	0.07	0.10	0.84
24	0.08	0.00	0.00	0.00	0.00	0.03

E-63. Pharmacokinetic parameters of antipyrine in group C (6 weeks after treatment)

	Animal number					
	787	791	793	794	795	796
Cpo (μg/ml)	46.81	45.93	39.60	48.16	54.70	56.41
AUC (μg.h/ml)	108.20	54.45	48.08	48.77	47.62	105.67
t1/2β (h^{-1})	2.96	1.72	1.63	1.42	1.55	2.40
Clb (ml/min.kg)	3.85	7.60	8.53	8.32	8.11	4.03
Vc (ml/kg)	534.10	544.30	631.30	519.10	457.00	443.20
Vd(area)(ml/kg)	986.40	1129.9	1204.2	1021.0	1088.6	837.10
			0		0	
Vdss (ml/kg)	807.80	760.50	739.70	773.60	761.50	798.70

Appendix E-64. Plasma concentrations of antipyrine in group C (8 weeks after treatment)

	Animal number					
Time (hours)	787	791	793	794	795	796
0.083	32.44	57.32	57.23	43.18	41.65	56.51
0.5	29.71	30.06	30.79	22.59	24.18	35.50
1	23.29	17.01	18.01	16.54	16.87	24.09
2	15.60	9.20	6.87	8.13	6.43	17.00
3	9.83	5.99	2.73	4.00	3.95	11.40
4	7.79	3.76	1.71	2.43	1.88	9.23
6	3.96	1.32	0.50	1.09	0.69	5.27
8	2.00	0.74	0.22	0.48	0.26	3.16
12	0.66	0.21	0.05	0.12	0.07	0.69
24	0.04	0.00	0.00	0.00	0.00	0.00

Appendix E-65. Pharmacokinetic parameters of antipyrine in group C (8 weeks after treatment)

	Animal number					
	787	791	793	794	795	796
Cpo (μg/ml)	34.59	66.07	65.02	50.10	46.37	63.47
AUC (μg.h/ml)	94.50	65.99	53.91	52.22	48.44	114.05
t1/2β (h^{-1})	2.86	1.74	1.33	1.59	1.57	2.23
Clb (ml/min.kg)	4.41	6.04	7.33	7.93	8.42	3.62
Vc (ml/kg)	722.80	378.40	384.50	499.00	539.30	393.90
Vd(area)(ml/kg)	1094.4	910.20	844.90	1091.3	1143.5	698.90
Vdss (ml/kg)	838.50	699.60	582.20	882.70	762.00	637.00

Appendix E-66. Plasma concentrations of antipyrine in group C (10 weeks after treatment)

	Animal number					
Time (hours)	787	791	793	794	795	796
0.083	45.74	48.99	61.78	52.13	52.53	55.43
0.5	27.37	32.47	25.97	24.55	26.78	34.59
1	26.26	23.47	18.50	15.23	20.90	26.57
2	16.08	13.51	9.12	7.61	9.75	19.83
3	10.31	9.62	3.58	4.00	3.82	15.04
4	7.12	6.40	2.56	2.76	2.91	11.73
6	4.91	3.19	0.96	1.14	1.04	7.27
8	2.53	1.86	0.44	0.53	0.46	4.34
12	0.75	0.62	0.13	0.09	0.08	1.30
24	0.05	0.06	0.00	0.00	0.00	0.08

Appendix E-67. Pharmacokinetic parameters of antipyrine in group C (10 weeks after treatment)

	Animal number					
	787	791	793	794	795	796
Cpo (μg/ml)	52.69	51.55	75.48	62.05	61.61	63.12
AUC (μg.h/ml)	102.56	92.84	59.62	54.00	61.06	138.38
t1/2β (h^{-1})	2.76	2.84	1.57	1.54	1.42	2.74
Clb (ml/min.kg)	4.13	4.34	7.06	7.47	6.89	3.03
Vc (ml/kg)	474.5	485.00	331.20	402.90	405.80	396.10
Vd(area)(ml/kg)	931.60	1066.1	959.80	995.90	847.80	718.70
Vdss (ml/kg)	854.50	812.10	711.60	775.60	697.60	673.10

Appendix E-68. Mean clearance indices in different animal groups

	Clearance Index
4 weeks	
Group A	0.77
Group B	1.06
Group C	0.95
6 weeks	
Group A	0.85
Group B	1.14
Group C	1.05
8 weeks	
Group A	0.66
Group B	1.00
Group C	0.88
10 weeks	
Group A	0.68
Group B	1.20
Group C	1.18
12 weeks	
Group A	0.66
Group B	1.06
Group C	1.10
14 weeks	
Group A	0.68
Group B	0.78
Group C	0.96

Appendix E-69. Plasma concentrations of rafoxanide in group B (infected)

	Animal number					
Time (days)	786	788	789	797	798	799
0	0.00	0.00	0.00	0.00	0.00	0.00
1	17.96	22.78	14.72	13.94	20.11	16.51
2	20.53	22.86	19.76	16.08	19.23	17.41
3	19.93	22.15	20.53	15.10	19.82	19.34
4	20.41	19.36	20.76	14.40	18.07	18.29
8	17.39	14.44	14.39	12.24	14.13	15.27
12	13.16	11.58	11.81	9.72	11.60	12.95
19	7.84	8.18	6.80	7.01	7.36	7.43
27	1.24	5.81	5.79	5.09	3.11	3.97
33	2.07	4.13	2.91	3.89	2.21	2.48
39	1.24	2.48	1.96	2.53	2.43	1.43
47	0.99	1.22	1.31	1.57	0.71	0.73
52	0.72	0.99	0.67	1.37	0.52	0.43
63	0.38	0.46	0.48	0.82	0.29	0.19
70	0.24	0.28	0.28	0.57	0.21	0.15
76	0.18	0.16	0.25	0.33	0.13	0.05

Appendix E-70. Pharmacokinetic parameters of rafoxanide group B (infected)

	Animal number					
	786	788	789	797	798	799
AUC $_{obs}$(μg.d/ml)	357.68	404.09	373.00	346.03	345.03	350.71
AUMC$_{obs}$(μg.d^2/ml)	4816	6523	5873	6539	4977	4915
MRT (d)	13.46	16.14	15.75	18.89	14.42	14.02
Cmax (μg/ml)	20.53	22.86	20.76	16.08	20.11	19.34
tmax (d)	2.00	2.00	4.00	2.00	1.00	3.00

Appendix E-69. Plasma concentrations of rafoxanide in group B (infected)

	Animal number					
Time (days)	786	788	789	797	798	799
0	0.00	0.00	0.00	0.00	0.00	0.00
1	17.96	22.78	14.72	13.94	20.11	16.51
2	20.53	22.86	19.76	16.08	19.23	17.41
3	19.93	22.15	20.53	15.10	19.82	19.34
4	20.41	19.36	20.76	14.40	18.07	18.29
8	17.39	14.44	14.39	12.24	14.13	15.27
12	13.16	11.58	11.81	9.72	11.60	12.95
19	7.84	8.18	6.80	7.01	7.36	7.43
27	1.24	5.81	5.79	5.09	3.11	3.97
33	2.07	4.13	2.91	3.89	2.21	2.48
39	1.24	2.48	1.96	2.53	2.43	1.43
47	0.99	1.22	1.31	1.57	0.71	0.73
52	0.72	0.99	0.67	1.37	0.52	0.43
63	0.38	0.46	0.48	0.82	0.29	0.19
70	0.24	0.28	0.28	0.57	0.21	0.15
76	0.18	0.16	0.25	0.33	0.13	0.05

Appendix E-70. Pharmacokinetic parameters of rafoxanide group B (infected)

	Animal number					
	786	788	789	797	798	799
AUC $_{obs}$(μg.d/ml)	357.68	404.09	373.00	346.03	345.03	350.71
AUMC$_{obs}$(μg.d^2/ml)	4816	6523	5873	6539	4977	4915
MRT (d)	13.46	16.14	15.75	18.89	14.42	14.02
Cmax (μg/ml)	20.53	22.86	20.76	16.08	20.11	19.34
tmax (d)	2.00	2.00	4.00	2.00	1.00	3.00

Appendix E-71. Plasma concentrations of rafoxanide in group C (uninfected)

	Animal number					
Time (days)	787	791	793	794	795	796
0	0.00	0.00	0.00	0.00	0.00	0.00
1	17.83	17.15	24.58	17.65	20.60	15.20
2	17.59	20.32	27.41	19.16	18.27	21.16
3	17.59	22.32	24.87	18.25	17.54	23.28
4	16.72	21.38	22.04	17.12	16.10	19.00
8	14.27	17.11	16.11	13.11	10.85	14.72
12	12.79	13.62	11.52	10.36	7.96	12.76
19	9.59	8.00	7.77	6.22	4.28	7.60
27	6.52	4.89	5.10	3.69	2.33	5.41
33	5.52	3.53	2.29	2.17	1.43	3.71
39	3.94	1.72	1.17	1.36	0.71	2.11
47	2.44	0.89	0.70	0.76	0.28	1.42
52	1.98	0.85	0.40	0.58	0.18	1.20
63	1.12	0.40	0.37	0.29	0.07	0.82
70	0.79	0.32	0.26	0.21	0.04	0.66
76	0.54	0.23	0.14	0.16	0.03	0.55

Appendix E-72. Pharmacokinetic parameters of rafoxanide group C (uninfected)

	Animal number					
	787	791	793	794	795	796
AUC $_{obs}$(μg.d/ml)	449.04	403.00	389.00	316.00	250.00	400.00
AUMC$_{obs}$(μg.d^2/ml)	9028	6068	5234	4540	2896	6870
MRT (d)	20.11	15.06	13.46	14.35	11.58	17.16
Cmax (μg/ml)	17.83	22.32	27.41	19.16	20.60	23.28
tmax (d)	1.00	3.00	2.00	2.00	1.00	3.00