THE *in vitro* AND *in vivo* STUDY OF THE PHARMACOKINETICS AND METABOLISM OF ANTHELMINTICS IN *equidae*

A thesis submitted for the degree of

Master of Veterinary Medicine

by

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To my mother, Ruby and my late father, Bertram.

Contents

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Declaration

I, Kaampwe Mayovu Muzandu, do hereby declare that the work presented in this thesis is original, was carried out by me, unless otherwise stated, and has not been presented for an award of a degree in any other University.

Signature:

Date:

Summary

Fenbendazole is a thio-substituted benzimidazole used in the treatment of helminths of domestic animals. It has poor water solubility and is rapidly biotransformed and eliminated from the animals' body. These features are common to the 'newer' benzimidazoles and pose a serious constraint on their efficacy, especially in the equine species with short gut transit time. Anthelmintic activity of the benzimidazoles is related to the period of time the parasite is exposed to effective concentrations of the active compounds (i.e. the parent sulphide and the sulphoxide metabolite), consequently residence time of the active compound is important.

The effect of administering fenbendazole with food , and/or in combination with the metabolic inhibitor, piperonyl butoxide, was investigated. Six adult horses were administered 10 mg/kg fenbendazole with or without food, and with or without piperonyl butoxide, 63 mg/kg, in a series of experiments.

Feeding was shown to affect the disposition kinetics of fenbendazole and it's metabolites. The mean residence time of fenbendazole was only very slightly reduced but that of the sulphoxide was reduced by 67%, and the inactive metabolite (sulphone) was prolonged in the blood when the parent compound was administered with food. Feeding was also found to delay the time to reach C_{max} ,and reduce (by 50.4%) the total area under the plasma concentration-time curve of the active metabolites ($AUC_{sulphide}$ + $AUC_{sulphoxide}$). In respect of these findings, feeding reduces the relative bioavailability of the active metabolites and horses would be better administered fenbendazole on an empty stomach.

The methylenedioxyphenyl (MDP) compound, piperonyl butoxide, was used in the metabolism studies in the horse. When fenbendazole was co-administered with piperonyl butoxide the relative bioavailability of the active metabolites was enhanced. This was attributed to the piperonyl butoxide-mediated metabolic inhibition of the cytochrome P450 enzyme-

catalyzed sulphoxidation and sulphonation reactions resulting in increased amounts of the active compounds. In comparison to the unfed animals not receiving piperonyl butoxide, the piperonyl butoxide-treated unfed animals showed increases in the AUC, AUMC and C_{max} values of 10, 8 and 7 times, respectively, for the sulphide, fenbendazole, whereas the same parameters for the sulphoxide were reduced. In the piperonyl butoxide-treated fed horses, these parameters were even higher, compared to the same parameters in the unfed animals not receiving piperonyl butoxide, and this applied to the sulphoxide values as well. The mean residence times were increased in all cases except for the sulphoxide metabolite in the unfed horses. Piperonyl butoxide markedly improved the effectiveness of fenbendazole whether administered with food or without. *In vitro* metabolism studies using equine liver microsomes were also conducted. Piperonyl butoxide reduced the extent of metabolism of fenbendazole *in vitro* by 38.4% when a fenbendazole: piperonyl butoxide ratio of 1: 5 was used. The rate of sulphoxidation was drastically reduced (by 90%) with the co-administration of the inhibitor but there was little effect on the rate of sulphonation, and the sulphonation: sulphoxidation ratio was increased. The results of the *in vitro* studies supported the *in vivo* findings.

Abbreviations

ABSO	albendazole sulphoxide
$ABSO_2$	albendazole sulphone
ABZ	albendazole
AUC	area under the curve
AUC_{obs}	area under the curve for observed values
AUFS	absorbance units full scale
AUMC	area under the first moment curve
$AUMC_{obs}$	area under the first moment curve for observed values
BZ	benzimidazole
CBZ	cambendazole
C_{max}	Peak plasma concentration
Cp0	plasma concentration at time zero
CV	coefficient of variance
e.g.	for example
epg	eggs per gram
et al.	and others
FAD	flavin adenine dinucleotide
FBZ	fenbendazole
FBZSO	fenbendazole sulphoxide
FLBZ	flubendazole
$FBZSO_2$	fenbendazole sulphone
FMO	flavin-containing monooxygenase
FMN	flavin mononucleotide
g	gram
g	gravity, 10^{-11} N.m/s^2
GI	gastrointestinal
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	litre
M	molar
ml	millilitre
mmol	millimole
MRT	mean residence time
mw	molecular weight
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
OFZ	oxfendazole
OH-FBZ	hydroxyfenbendazole
OH-FBZSO	hydroxyfenbendazole sulphoxide
PB	piperonyl butoxide
PBZ	parbendazole
PI	post infection
PT	post treatment
S-oxidation	sulphoxidation or sulphonation
SD	standard deviation
SEM	standard error of the mean
$t_{1/2\beta}$	elimination half-life
TBZ	thiabendazole
TCBZ	triclabendazole
t_{max}	time to peak plasma concentration
uv	ultraviolet

Vc	volume of the central compartment
Vd_{area}	apparent volume of distribution
Vd_{ss}	volume of distribution at steady state
w/v	weight/volume
v/v	volume/volume
µg	microgram
µl	microlitre
µM	micromolar
°C	degrees centigrade

Chapter 1
Review of literature

1.1 Introduction

The discovery and development of novel chemicals for use against helminth parasites is becoming increasingly more difficult. The cost of research and drug development is very high due to strict regulations on potential toxicity. Lengthy mandatory trials are required to determine likely teratogenic or mutagenic effects and diverse studies on non-target invertebrate and vertebrate species must be undertaken. It has been estimated that the discovery, research and development of a novel pharmaceutical product intended for use in food-producing animals may cost $US 100-200 million and takes about 12 years (Hennessy, 1997). For drugs meant for use in food animals, the additional costs of extensive studies to determine when residues are at acceptable levels have to be considered. For anthelmintic drugs nematode resistance constitutes another major problem. Reports of anthelmintic resistance are increasing worldwide. Helminths have become resistant to various anthelmintics, including the recent endectocides, such as ivermectin and moxidectin and to the salicylanilide, closantel. The problem seems inevitable and the life expectancy of any new anthelmintic compound is limited and unpredictable.

The problems and costs associated with the discovery and development of new anthelmintic drugs have prompted investigations into alternative methods of treatment and management of helminthiosis. These include means of achieving more effective use of currently available anthelmintics and non-chemical control strategies, such as grazing management and biological, immunological and genetic methods.

1.2 Anthelmintics in horses.

Equine anthelmintic drugs can be allocated chemically into seven distinct classes (Table 1.1) with each class affecting a different range of parasites and having a different pharmacological behaviour.

Desirable attributes of an ideal equine anthelmintic include: high efficacy, broad spectrum of activity, low host toxicity and high safety index, easy to administer, environmentally safe, relatively inexpensive and unlikely to select for drug resistance (Herd, 1992). An anthelmintic with all these attributes has yet to be found.

The broad spectrum anthelmintic drugs are commonly classified into three classes: Benzimidazoles and pro-benzimidazoles; Imidazothiazoles and tetrahydropyrimidines; and avermectins and milbemycins. Specific examples of representative drugs are shown in Table 1.1. The other drugs have a narrow spectrum of activity except for some of the organophosphates.

1.2.1 Mode of action

Anthelmintics used in horses affect parasites either by altering metabolic processes necessary for survival of the parasites or by causing a disturbance in muscular activity that results in paralysis (DiPietro and Todd, 1989).

The benzimidazoles and probenzimidazoles affect energy production by inhibiting fumarate reductase and glucose uptake, and binding to tubulin. Binding of tubulin blocks polymerization into microtubules thereby damaging the integrity and transport functions of the absorptive cells within the parasite. Drugs causing a disturbance in neuromuscular activity do so by inhibiting the breakdown of excitatory neurotransmitters,

Table 1.1 Chemical classification of anthelmintics used in the horse and related compounds

Class	Route of administration	Representative drugs
Simple heterocyclics		Phenothiazine, Piperazine
Probenzimidazoles*		Febantel, Thiophanate
Benzimidazoles		
(Thiazoles)	P.O.	Thiabendazole (TBZ)
(Carbamates)	P.O. (I.R.)	Albendazole (ABZ)*
		Fenbendazole (FBZ)
		Mebendazole (MBZ)
		Oxfendazole (OXF)
		Oxibendazole (OXI)
		Tioxidazole*
Macrocyclic lactones		
Avermectins	P.O., i.m./s.c., T	Ivermectin, Abamectin*
		Doramectin*
Milbemycins		Moxidectin, Milbemycin D
		Milbemycin oxime*
Imidazothiazole	P.O.,s.c.,T	Levamisole*
Tetrahydropyrimidines	P.O.	Pyrantel, Morantel*
Organophosphates	P.O.	Dichlorvos, Haloxon, Trichlorphon

*Not licenced for horses

mimicking the inhibitory neurotransmitter or by mimicking the action of the excitatory neurotransmitter (Rew, 1978) or by stimulating glutamate gated chloride ion channels (McKellar and Benchaoui, 1996). The acetylcholinesterase inhibitors include organophosphates (e.g. dichlorvos, haloxon, trichlorphon). Cholinergic agonists (ganglionic) include quaternary ammonium salts (biphenium) imidazoles (levamisole), pyridines (methyrdine) and pyrimidines (pyrantel). Muscle hypopolarisers include piperazines (piperazine). GABA agonists include the macrocyclic lactones (e.g. ivermectin) which are now thought to exert their nematocidal activity by stimulating glutamate gated chloride ion channels.

1.2.2 Anthelmintic resistance

The development of drug resistance is a major drawback in chemotherapeutics worldwide. Anthelmintic drug resistance is the heritable ability of some nematode parasites to survive treatment with anthelmintic drugs at the recommended therapeutic dose levels (Taylor and Hunt, 1989). Side resistance is resistance to different compounds within a class of anthelmintics and multiple resistance is resistance to more than one class of anthelmintic (Prichard *et al.*, 1980). Reversion is a return to susceptibility to an anthelmintic drug by an originally resistant parasite strain (Prichard *et al.*, 1980).

The first report of anthelmintic drug resistance in horses was in North America in 1965 (Drudge and Lyons, 1965) in which strains of equine strongyles became resistant to thiabendazole barely four years after its discovery. Anthelmintic resistance is now recognised throughout the world in small strongyles to various classes of anthelmintics including benzimidazoles (McKellar and Scott, 1990) and pyrantel (Conder and

Campbell, 1995). The majority of reports on anthelmintic resistance involve small strongyles (cyathostomes). Resistance to large strongyles has not been clearly demonstrated.

Development of anthelmintic resistance

As Geerts, Coles and Gryseels (1997) state, the key issue in the development of resistance is the contribution that helminths surviving therapy make to the next generation. These survivors are the most resistant component of the population and carry resistance genes which they pass to their offspring (Sangster, 1996). Over several generations the number of survivors of therapy increase as the number of genes conferring resistance accumulate (Sangster, 1996). A number of factors contribute to the selection of resistance including frequent dosing, underdosing, mass therapy and prolonged use of the same class of anthelmintic.

Selection for resistance appears to occur in three phases (Prichard, 1990a). An initial anthelmintic susceptibility phase occurs whereby there are few drug resistant parasites in the population. This is followed by the development of a phase in which heterozygous resistant individuals predominate and finally, with continued selection pressure, a resistant phase develops which is predominated by homozygous resistant individuals.

Control strategies that involve the slow rotation of anthelmintics rely upon the possibility of reversion to susceptibility occurring in the intervening years between treatments with a single drug (Jackson, 1993). This reversion is most likely to occur during the heterozygous-resistant phase of development (Scott *et al.*, 1991). Unfortunately, a number of field studies on reversion provided little or no evidence of reversion in the

absence of chemotherapy (Uhlinger and Johnstone, 1984; Martin, 1987; Borgsteede and Duyn, 1989).

Detection of anthelmintic resistance

Sound methods of detecting anthelmintic resistance in the field are yet to be developed. The existing assays for identifying anthelmintic resistance early in the developmental process are not efficient for field use. Comprehensive reviews on a variety of assays to monitor resistance have been published (Presidente, 1985; Johansen, 1989; Taylor and Hunt, 1989; Coles, 1990).These assays can be grouped into *in vitro* and *in vivo* techniques (Taylor and Hunt, 1989).

The *in vivo* techniques are suitable for all chemical groups of anthelmintics. The *in vivo* techniques include the faecal egg count reduction test (FECRT) and the controlled efficacy test (CET). Though it has its own limitations, FECRT is probably the best technique for use in the field; it is the simplest and does not require highly skilled labour to carry out. However, it has poor sensitivity and is only capable of detecting high levels of resistance (Conder and Campbell, 1995). The controlled efficacy test is the definitive test for resistance but is very costly in terms of animals and labour (Johansen, 1989).

Most of the *in vitro* tests are best applied for research in the laboratory, they are rapid, sensitive and economical to use. The *in vitro* assays include those based on anthelmintic effects on normal physiological processes such as development, growth, and/or movement of parasitic stages include egg hatch, larval development, larval paralysis, motility and larval migration assays (Conder and Campbell, 1995). Biochemical based assays include the colorimetric assay of acetylcholinesterases (Sutherland *et al.*, 1988; 1989) tubulin binding and polymerization assays (Lacey, 1985; Lacey and Prichard, 1986; Lacey and Snowdon, 1988). Genetic

assays are available with benzimidazoles where resistant populations have been identified using cloned β-tubulin probes (Roos *et al.*, 1990). Of the *in vitro* assays mentioned, only the egg hatch and the larval development assays are suitable for field survey investigations.

Control of Resistance

Since the relative flow of genes through generations is crucial for the development of drug resistance, several steps can be taken to delay and even avoid the problem. (Savioli *et al.*, 1997). Evaluation of anthelmintic efficacy is an important procedure in the detection of drug resistance development in helminths. At least 20% of horses should be tested for the presence of nematode ova 7 to 14 days after drug therapy (Ewert *et al.*, 1991). Each and every horse sharing a grazing area should be subjected to the same anthelmintic protocol and new horses should be treated before being released onto the pasture. A new horse with drug-resistant worms can introduce those worms to the rest of the horses. Frequent treatment intervals predispose to the fast development of drug resistance and therefore these should be minimized to reduce the selection pressure for resistant parasites. The optimum treatment interval will vary according to the following: the number, age and population densities of the target horses; pasture management factors (e.g. manure-handling methods and micro-environment considerations); current level of parasitic infectivity in horses and in the grazing environment; and climatic and seasonal variations that determine the survival of the free-living stages (Uhlinger, 1991).

Another control strategy to delay anthelmintic resistance is the use of a single class of anthelmintics annually to prevent multiple resistance and to rotate classes annually to limit passage of resistant genes (Conder and Campbell, 1995). The concept of rotation relies on the possibility that parasites revert to susceptibility after prolonged withdrawal of one class of

drug, and subsequent reintroduction following administration of a different class (Jackson, 1993). However, a number of field studies conducted have provided little evidence of reversion in the absence of chemotherapy (Jackson, 1993).

Small strongyles that have developed resistance to benzimidazoles may be treated by: using piperazine, or the non-benzimidazole drugs ivermectin, dichlorvos or pyrantel pamoate. The benzimidazole, oxibendazole, has been shown to be effective against small strongyles that have developed resistance to other benzimidazoles (thiabendazole, mebendazole, cambendazole, fenbendazole and oxfendazole), but even this drug shows reduced efficacy after repeated treatments. The co-administration of oxfendazole and parbendazole in sheep resulted in potentiation of the former by the latter by increasing systemic availability of the oxfendazole and thus efficacy (Hennessy *et al.*, 1985).

Animals need to be administered an effective dose of a highly efficacious drug so that heterozygous resistant parasites (with genes which confer partial resistance) are effectively removed along with the susceptible individuals (Conder and Campbell, 1995).

Mathematical models are being developed for investigating the effectiveness of different control strategies but these are faced with various constraints such as limited understanding of the genetics of resistance and insufficient data on the epidemiology of helminthiosis.

1.2.3 Control of endoparasites

Horses, ponies, donkeys and other equidae are hosts to a variety internal parasites. Equine parasites in decreasing order of importance are: large strongyles, small strongyles, ascarids, bots, pinworms, and other parasites

of minor importance including strongyloides, stomach worms, tapeworms, eye worms, and filaroids (Herd, 1992).

It is unfeasible, if not impossible, to eradicate parasitism. Control of helminthiosis is aimed at reducing the worm burden to levels that do not impair health, production and performance. The importance of parasitic disease varies widely with geographic area and management systems. Anthelmintic chemotherapy and chemoprophylaxis are vital in the fight against internal parasites. However, there is need to combine chemotherapy with other control strategies, namely effective managemental practices. This approach, referred to as integrated control, makes optimal use of available methods of control in a flexible way, adapted to local circumstances and to economic reality. Control intervention must be carried out at strategic periods in the parasitic life cycle. Epidemiological information is of vital importance in the design of effective control strategies to prevent the seasonal build-up of pasture contamination and infectivity. Effective management practices may include grazing management, use of optimal stocking rates, appropriate housing or confinement, good sanitation and adequate nutrition.

Considerable research is being carried out on alternative means of control, such as immunological, biological, ecological and genetic methods.

Immunological control

There are very few vaccines used against parasites. For the helminth parasites, only one vaccine is used commercially and this is the one for the lungworm of cattle *Dictyocaulus viviparus* (Jarett *et al.*, 1959). A similar vaccine was developed in India in 1971 (Dhar and Sharma, 1981) for use against the lungworm, *Dictyocaulus filaria*, of sheep. A commercial vaccine against the canine hookworm *Ancylostoma caninum* was

produced (Jarett *et al.*, 1959; Miller 1971, 1978) but was short lived on the animal market due to certain constraints such as short shelf-life, high cost of production and failure to completely remove *Ancylostoma caninum* eggs and faeces even though the dogs did not develop pathological hookworm disease. It was withdrawn in 1975.

Current strategies for vaccine development are grouped into two broad categories, namely exploitation of excretory/secretory (E/S) antigens and 'hidden antigens' (Newton, 1995). Excretory/secretory antigens or products are substances such as enzymes and metabolites that are released from the oral and genital openings, excretory pores and secretory glands of nematodes. 'Hidden ' or concealed antigens are antigens located in the parasite gut. Antibody responses to these hidden antigens are lethal to the parasite when it invades and feeds on host fluids that contain induced antibodies to its internal tissues, such as gut cells (Soulsby, 1994).

The market for equine vaccines is generally small compared to that for the food and other companion animals. The cost of developing, testing and licensing a vaccine are the same regardless of the size of the market (Powell, 1992) and the pharmaceutical industry is hesitant to invest in the development of new equine vaccines, but would rather use technologies developed for vaccines in other species and adapt them to similar products in the horse (Powell, 1992). At present no practical vaccines against helminth parasites of the horse have been made. A horse harbors a mixed infection of parasites and it will be a Herculean task to produce a multivalent vaccine that will be effective against all the 10 major species of cyathostomes, for instance. Conventional vaccines are normally based on whole dead organisms or attenuated live preparations. With the recent advances in recombinant DNA technology, novel vaccines are being produced that are based on surface and extracellular components of

parasites, called protective antigens. Recombinant DNA technology has overcome the previously, apparently unsolvable, problem of how to produce sufficient antigen to make a practical vaccine for helminths. It involves the identification of the effective immunizing epitope (or antigenic protein required for the immune response); the isolation (or synthesis) of the gene coding for it; incorporation of the gene into a vector e.g. a plasmid; transfer of the vector into an appropriate recipient (host) e.g. *Escherichia coli* or yeast; selection and cloning of the host cells carrying the gene; expression of the gene; extraction of the protein; and scaling up production.

Genetic control

The immune response to endoparasites varies between different breeds, different populations and different individuals. Individuals harboring few helminths are genetically 'high' responders and those with heavy burdens are 'low' responders (Soulsby, 1994). Selective breeding for genetically immune hosts to endoparasites will in the long run convert a population to a high responder category and this will be a means of reducing the reliance upon chemotherapy (Rothwell *et al.*, 1991). However, selection for breeding in horses is dependent on other phenotypic characteristics such as performance, appearance and temperament and it may be impossible to combine appropriate conventional characteristics with those of parasitic resistance.

Biological control

Biological control involves the use of predators of parasites to control parasitism. Fungi seem to be the most promising of the potential biological control agents (fungi, bacteria, viruses and protozoa).

In ruminants, a number a successful experiments have been performed in attempts to control the free-living larvae of trichostrongyles by means of

nematode-trapping fungi (Grønvøld *et al.*, 1993a, b, c; Waller and Larsen, 1996, Waller *et al.*, 1994; Wolstrup *et* al, 1994, 1996; Larsen *et al.*, 1992; 1995; Nansen *et al.*, 1995). Recent studies performed by Larsen *et al.*, (1995a, b; 1996) in horses have also demonstrated the potential of the fungus *Duddingtonia flagrans* for controlling free-living stages of horse parasitic nematodes.

1.2.4 Environmental impact of anthelmintic usage

This is an area that is increasingly receiving more attention, particularly from regulatory authorities. Residues or metabolites of phenothiazine, dichlorvos, coumaphos, ruelene and piperazine excreted in bovine faeces had deleterious effects on dung beetle in Texas (Blume *et al.*, 1976). Phenothiazine is known to have harmful effects via excreted metabolites on pasture growth, clover content, nitrification rates and animal production (Southcott, 1980).

Decomposing animal faeces are beneficial to the ecosystem in that they return valuable nutrients to the soil. The dung fauna- fungi, yeast, bacteria, nematodes, insects and earthworms- play an important role in the decomposition process (Spratt, 1997). Insects, including dung beetles, also help aerate the pat, improve the humus content, enhance water percolation and recycle soil nutrients. The effect of ivermectin on the pasture ecosystem has been receiving special attention due to its potent insecticidal activity. Furthermore, it is mainly excreted in faeces mostly unchanged and in bile regardless of the species or route of administration (reviewed by McKellar and Benchaoui, 1996). Ivermectin has adverse effects on many dung degrading beetles and on dipteran flies.

1.3 Benzimidazoles in horses

The first benzimidazole to be introduced was thiabendazole (TBZ) in 1961 (Brown *et al.*, 1961).Since then a number of benzimidazoles with improved efficacy and spectra of activity have been developed. They include mebendazole (MBZ), cambendazole (CBZ), fenbendazole (FBZ), oxfendazole (OXF), oxibendazole (OXI), and luxabendazole (LBZ).

Features common to the benzimidazole drugs are broad-spectrum of anthelmintic activity, low mammalian toxicity and relatively low dosage requirements.

1.3.1 Spectra of activity

All the benzimidazoles (and also the pro- benzimidazoles) used in equines are effective against *Parascaris equorum*, *Oxyuris equi*, adult cyathostomes and adult large strongyles (see Table 1.2). Thiabendazole needs to be given at a higher dosage for *P. equorum*. Thiabendazole, oxibendazole and fenbendazole are effective against *Strongloides westerii* when given at a higher dose. Fenbendazole (at a high dosage) and oxfendazole are effective against migrating cyathostome larvae and large strongyles' larvae. Fenbendazole is effective against *Dictyocaulus arnfieldi* and oxfendazole is effective against *Trichostrongylus axei.* Nevertheless, both drugs are likely be effective against both nematodes but this has not yet been tested (McKellar, personal communications). Mebendazole is effective against both of these parasites. The benzimidazoles are not effective against bots.

1.3.2 Safety, Toxicity and Teratogenicity.

The benzimidazole and pro-benzimidazole anthelmintics have high therapeutic indices. Their low water solubility could account for this low toxicity. The safety index (ratio of maximum tolerated dose to the recommended dose) ranges from 10 to 100 (Herd, 1992). The main toxic effect of benzimidazoles is their teratogenic effect which varies with benzimidazole structure and with target-animal species (Delatour and Parish, 1986). Sheep are the most sensitive of the domestic animals to the teratogenic effects of the benzimidazoles. Differences in the kinetics and drug metabolism in various species may account for the differences in the sensitivity to the teratogenic effects of the benzimidazoles (Delatour and Parish, 1986).

1.3.3 Mode of Action

Benzimidazoles exert their primary action by binding to the cytoskeletal protein, tubulin, and inhibiting the formation of the microtubule matrix which is essential to the normal functioning of all eukaryotic cells (Lacey and Gill, 1994). These anthelmintics are, however, more toxic to helminth cells than mammalian cells. This selective toxicity may be due to differences in the structure of the microtubules in the cells (Reviewed by McKellar and Scott, 1990). Mammalian cells have 13 protofilaments in microtubules whereas nematode cells have protofilaments varying between 11 and 15 (Chalfie and Thompson, 1979; Davis and Gull, 1983). One study revealed that there are differences in the affinities of benzimidazoles for host and parasite tubulin (Friedman and Platzer, 1980) and assumed that these differences could account for the benzimidazoles' selective toxicity. However, a subsequent study failed to support this assumption. The differential pharmacokinetics of the benzimidazoles may also account for the selective toxicity (Köhler and Bachmann, 1981).

1.3.4 Formulation and administration

The benzimidazoles are available in a variety of formulations including powders, pellets, suspensions, pastes and boluses. Most anthelmintics are formulated as oral dosage forms. The formulation of the drug dosage form largely determines the route of administration, which may in turn influence the clinical efficacy of the drug. Physicochemical properties that affect absorption (e.g. lipid solubility and degree of ionization) of the drug, stability of the drug in the gastrointestinal tract and drug release from the dosage form may influence absorption of drugs from oral formulations. The relatively poor water solubility of the benzimidazole anthelmintics precludes their use parenterally.

1.3.5 Pharmacokinetics of benzimidazoles

Pharmacokinetics is the study of the time course of drug and metabolite concentrations in animals and how these are affected by absorption, distribution, biotransformation and elimination processes. A knowledge of pharmacokinetics is essential in pharmaceutical drug research and development and in the optimal clinical use of drug formulations. Bioavailability (sometimes referred to as systemic availability) is defined as the rate and extent to which a drug enters the systemic circulation unchanged following administration by any route (Baggot, 1992). The route of administration of the dosage form affects the rate and the extent of absorption of a drug and may thereby influence the intensity and duration of the pharmacological effect. Maximum bioavailability is attained when a drug is directly administered into the vascular system or when it is administered by an extravascular route and is completely absorbed from that site of administration into the blood stream.

The active benzimidazoles are thought to have a similar mode of action (Coles, 1977) and to be relatively equipotent. The different efficacies of the benzimidazoles *in vivo* have been attributed to different pharmacokinetics in the host (Bogan, 1983), and *in vitro* to different solubilities and consequent absorption by the parasite from culture medium (Scott, 1988), respectively. The benzimidazole anthelmintics are only sparingly soluble in water (Table 1.3). The newer benzimidazoles e.g. albendazole, oxfendazole and fenbendazole have more limited water solubility. Small differences on the solubilities of the anthelmintic drugs may have a significant influence in the absorption and hence on their resultant clinical efficacy. Thiabendazole and cambendazole are the most water soluble of the benzimidazoles. They are extensively dissolved in aqueous ruminal fluid and rapidly absorbed in ruminants (McKellar and Scott, 1990), having time to maximum concentration (t_{max}) values of as little as 4h in cattle (Tocco *et al.*, 1965; Prichard, 1986). The less soluble benzimidazole drugs remain in the plasma for long periods, and since an equilibrium presumably exists between plasma and the gastrointestinal tract, the duration of exposure of the parasites to effective concentrations of drugs is extended.

The absorption of drugs administered orally may be affected by drug release from the dosage form, the stability of the drug in the gastrointestinal tract and the physicochemical properties that govern absorption (e.g. degree of ionization and lipid solubility) of the drug. The absorption of a drug following oral administration involves the transfer of the drug from within the gastrointestinal tract into the plasma of the portal vein, from which it reaches the systemic circulation via the liver. Drugs

Table 1.2 Dose, safety, method of administration, and efficacy of certain benzimidazoles[1] used in horses.

				Mean percentage of efficacy[2]				
Drug	Dose (mg/kg)	Safety index[3]	Method of administration[4]	*P.equorum*	*S. edentatus*	*S. vulgaris*	Small strongyles	*O. equi*
FBZ	5 to 10[5]	200	S,F,O	95	97	97	97	97
MBZ	8.8	45	S,F,O	97	80	97	87	97
OXF	10	10	S,F,O	95	97	97	97	97
OXI	10 to 15	60	S,O	95	97	97	97	97
TBZ	44 to 88[5]	27	S,F,O	42	97	97	95	95

[1] Ewert, *et al.*(1991).

[2] Applies to susceptible parasites only. Resistant populations may be encountered especially for small strongyles.

[3] Dose at which toxic signs first occur . Toxic level is equal to safety index times therapeutic dose.

[4] S = stomach tube, F =feed, O =orally (as paste or drench).

[5] Higher doses are required for *P.equorum*.

Table 1.3. The solubility (μg/ml) of benzimidazoles in phosphate buffer at different pH values (Ngumuo, 1983)

	pH 7.4	pH 6.0	pH 2.2
Thiabendazole	45.01	>24.67	>480.78
Albendazole	0.85	>0.48	>26.58
Fenbendazole	0.05	>0.07	>1.60
Oxfendazole	5.97	>3.01	>44.12
Parbendazole	0.20	>0.27	>27.07

passing through the liver undergo some degree of hepatic metabolism called the 'first pass effect'. The first pass effect in horses is quite significant owing to the high rate of activity of the hepatic microsomal enzymes in this species. Generally, for the benzimidazoles, absorption is rapid with C_{max} values occurring within 2-7 hours after administration for TBZ, FLBZ, or MBZ and 6 to 30 hours for ABZ, FBZ, OXF, OXI, PBZ or TPT. Marked species differences in the kinetics of the benzimidazoles exist. The benzimidazoles have lower bioavailability in monogastric animals (horses and dogs) than in the ruminant species. This is attributed to the rapid gut transit times in monogastrics. In one study carried out, the horse was found to have a gut transit time of 8.47 hours (Dougherty, 1992). In ruminants, the gut transit time may range from 30 to 80 hours depending on the digestibility of the food, since highly digestible food has shorter retention time (McDonald *et al.*, 1995; Warner, 1981). In a study done in horses, oxfendazole and fenbendazole were shown to have area under the plasma concentration curve (AUC) values of 3.17 and 2.32

μg.h/ml respectively after administration of 10mg/kg of oxfendazole (Marriner and Bogan, 1985). After administration of fenbendazole at the same dose rate, lower plasma concentrations of oxfendazole and fenbendazole were obtained, with oxfendazole being below the limit of detection (<0.04μg/ml) and fenbendazole with an AUC of 1.77 μg.h/ml (Marriner and Bogan, 1985). In contrast to other species (sheep, cattle, man), the horse has high sulphone metabolite relative to the sulphoxide and this may be due to the relatively rapid rate of conversion of the sulphoxide to the sulphone (Marriner and Bogan, 1985). Anthelmintic activity is related to the duration of parasite exposure to effective concentrations of the active compound (Baggot and McKellar, 1994), and consequently the pharmacokinetics of FBZ and OXF in horses make these drugs less effective in horses than ruminants if administered at the same dose rates.

Protein binding influences the disposition (distribution and elimination) of a drug. Most drugs are bound mainly to plasma albumin but some are bound to other blood constituents such as alpha$_1$-acid glycoprotein, lipoproteins and erythrocytes. The extent of binding varies with different species and is reversible. Protein binding also affects the intensity of the pharmacological effect. Only free, unbound drug is pharmacologically active and the bound drug is inert. Binding is a function of the concentration of the free drug. With increasing drug concentration and as the plasma protein binding sites become saturated, the amount of free drug increases slowly. This increase is rapid once saturation of the plasma proteins is attained. Multiple drug therapy can result in competition for plasma protein binding sites. One drug can displace the other from a binding site and this may result in toxic effects of the displaced drug as its free drug concentration rises. The pharmacological effect of a drug may also be enhanced in some disease states where the level of plasma albumin or the drug binding capacity of albumin is reduced. Extensive

protein binding can decrease the amount of free drug that passes through the liver, thus reducing the rate of biotransformation and elimination. The rate of elimination is also slowed down in the kidneys as less free drug is removed from the serum by glomerular filtration. Most benzimidazole drugs have a plasma protein binding of less than 50% and a relatively high volume of distribution (Lanusse and Prichard, 1993a).However, the halogenated benzimidazoles, triclabendazole (Mohammed-Ali *et al.*,1987; Hennessy *et al.*, 1987) and lobendazole (Steel and Duwel, 1987) have been shown to have a strong binding to plasma proteins, which may contribute to their relatively long elimination rates and low volumes of distribution.

1.3.6 Effect of parasitism on the pharmacokinetics and metabolism of benzimidazoles

In ruminants, parasitic infection of the gastrointestinal tract has been shown to reduce the bioavailability of benzimidazoles.The bioavailability of fenbendazole and its metabolites in sheep experimentally infected with *Ostertagia circumcincta* and treated with fenbendazole was lower than that observed in uninfected sheep treated with the same dose of fenbendazole (Marriner *et al.*, 1985). Similarly, the bioavailability of oxfendazole was reduced by 25% in goats infected with the same nematode compared to uninfected goats (Bogan *et al.*, 1987). Parasitism of the gastrointestinal tract has been shown to cause elevated abomasal pH (Mostofa and McKellar, 1989; McKellar *et al.*, 1990) and a constant stimulus for gastrin secretion leading to hypergastrinaemia and pronounced hyperplastic changes in the abomasal mucosal mass (Anderson *et al.*, 1988). These changes may affect absorption and ionic-trap-mediated distribution of the different benzimidazole metabolites thereby, probably, altering the pharmacokinetic behaviour and expected efficacy of the anthelmintic drug. Furthermore, these changes may induce

the development of side effects of the anthelmintic therapy. During bovine ostertagiasis, Murray (1969) reported increased permeability of the gastric mucosa to macromolecules. Gastrointestinal parasitism alters gut transit times which may also affect the pharmacokinetic behaviour of the benzimidazole anthelmintics. Elevated gastric pH causes reduced solubility of fenbendazole which would contribute to decreased bioavailability. Debackere and co-workers (1993) reported a decreased bioavailability of the benzimidazole prodrug febantel in lambs afflicted with ostertagiasis, as well as in those infected with *Trichostrongylus colubriformis*. The *T. colubriformis* infection caused reduced gut motility and mucosal villous atrophy which could affect drug absorption. Altered absorption accounts for the changes in bioavailability of the anthelmintics. During parasitism there may also be altered metabolism which could affect the pharmacokinetics of the anthelmintics. However, this is more likely to be as a result of liver damage due to liver fluke infection as the liver is the principal site of drug metabolism. It has been shown that the enzymatic activity of the hepatic microsomal cytochrome P-450-dependent monooxygenase system is depressed in rats (Tekwani *et al.*, 1988) and sheep (Galtier *et al.*, 1986) that have been infected with *Fasciola hepatica* which could lead to altered drug metabolism and clearance (Tufenkji *et al.*,1988). Parasitism may cause extensive liver damage leading to severe hypoalbuminaemia and this could affect the disposition of drugs which are extensively bound (>90%) to plasma proteins.

1.4 Metabolism of Benzimidazoles

Common to all the benzimidazole anthelmintics is the central structure, 1,2-diaminobenzene (Fig 1.1). They differ from each other only at the 2 and 5 substituent positions of the central structure. The benzimidazole compounds can be grouped as follows (Lanusse and Prichard, 1993b). Benzimidazole thiazolyl derivatives include thiabendazole (TBZ) and

cambendazole (CBZ). Benzimidazole methylcarbamates have a substitution on position 5- of the benzene ring. They include parbendazole (PBZ), mebendazole(MBZ), flubendazole (FLBZ), ciclobendazole (CIBZ), oxibendazole (OBZ), luxabendazole (LBZ), albendazole (ABZ), ricobendazole (albendazole sulphoxide, ABZSO), fenbendazole (FBZ) and oxfendazole (OFZ). Halogenated benzimidazole thiols include triclabendazole (TCBZ) and probenzimidazoles include thiophanate (TPT), febantel (FBT) and netobimin (NTB).

The benzimidazole drugs are extensively metabolised *in vivo* with the parent drug being short-lived and metabolic products predominating in plasma and other tissues of the host (Delatour and Parish, 1986; Lanusse, and Prichard, 1993b). Mebendazole is an exception as it is poorly metabolised and is excreted unchanged mostly in faeces with only5-10% excreted in urine. Xenobiotic metabolism occurs primarily in the liver. However, extrahepatic tissues are also involved including blood plasma, kidneys and the gastrointestinal tract. Biotransformation usually results in metabolites that are more polar and less lipid soluble than the parent drug with reduced or no activity (Baggot, 1974). These reactions are usually biphasic. Phase I (non-synthetic) reactions are oxidation, reductions and hydrolysis and a number of enzyme complexes are involved of which the cytochrome P450 mediated mixed function oxidase (M.F.O.) system is the major one. Another important enzyme is the flavin-containing monooxygenase (FMO) system, which plays a major role in the oxidative metabolism of drugs and xenobiotics. Functional groups such as hydroxy, carboxy, amino and sulphydryl groups are introduced into organic substrates. The MFO comprises cytochrome P450, NADPH-cytochrome P450 reductase and lipid.

Cytochrome P450 is a haemoprotein with iron protoporphyrin IX as the prosthetic group, and derives its name from the fact that the pigment

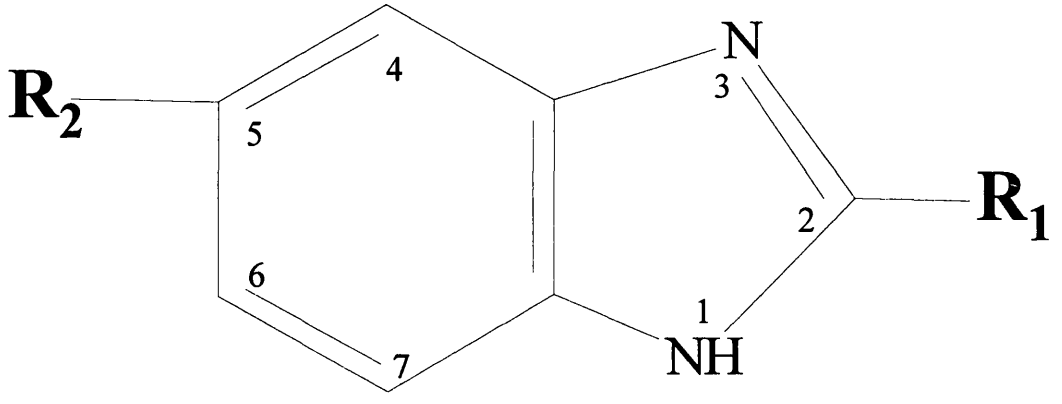

Fig. 1.1 Chemical structure of the benzimidazole nucleus with the primary substituent positions shown.

cytochrome exhibits a spectral absorbance maximum at 450nm when reduced and complexed with carbon monoxide. A common reaction catalysed by cytochrome P450 involves the incorporation of an oxygen atom, derived from "activated" molecular oxygen, into a generally lipophilic substrate. The necessary electrons to derive the reaction (a) are donated by NAD(P)H and cytochrome B_5, and are catalysed by NADPH-cytochrome P450 reductase and cytochrome B_5 reductase, respectively (Porter and Coon, 1991).

$$NADPH + H^+ + O_2 + RH \xrightarrow{\text{cytochrome P450}} NADP^+ H_2O + ROH \quad \text{.....(a),}$$

$$NADPH + H^+ + RH \xrightarrow[\text{P450 reductase}]{\text{NADPH-cytochrome}} NADP^+ H_2O + ROH \quad \text{....(b), where}$$

RH is the oxidisable drug substrate and ROH is the hydroxylated metabolite. The reductases and cytochrome P450 are found in the smooth endoplasmic reticulum and in mitochondria.

Cytochrome P450 exists in multiple forms or isoenzymes that are classified, based on genes coding for specific P450 protein sequences (Nerbert *et al.*, 1991), into a superfamily comprising families and sub-families. A gene family comprises a cytochrome P450 sequence with usually less than 40% resemblance to that from any other family. Cytochrome P450 sequences within a sub-family in mammals show more than 55% resemblance (Witkamp, 1992). An arabic number represents the family, and this is followed by a letter denoting the sub-family, and an arabic number denoting a specific gene within a sub-family.

NADPH-cytochrome P450 reductase is a flavin-containing enzyme (flavoprotein containing both FMN and FAD prosthetic groups) and is responsible for transferring reducing equivalents from NADPH $+H^+$ to cytochrome P450 (see equation b).

The lipid component of the MFO system may be required for substrate binding, facilitation of electron transfer or providing a 'template' for the interaction of cytochrome P450 and NADPH-cytochrome P450 reductase molecules. However, its precise mode of action is yet to be confirmed (Gibson and Skett, 1994).

The FMO enzyme is found in many tissues but highest concentrations occur in the microsomal fraction of the liver. It uses either NADH or NADPH as a source of reducing equivalents in the oxygenation of many nucleophilic organic nitrogen and sulphur compounds (Gibson and Skett, 1994), such as phenothiazines and sulphide benzimidazoles.

Phase II (synthetic, conjugation) reactions entail addition of sulphate groups, glucuronides, acetylation or methylation.

The most common reactions of the benzimidazoles occur at position 5- of the benzene ring. Phase I reactions, such as aliphatic and aromatic hydroxylations, N-, S- and O- dealkylations, N- and S-oxidations and reductive reactions, occur at this position. Another common reaction is the hydrolytic decarboxylation of the carbamate group at position 2- to form aminoderivatives (Gottschall *et al.*, 1990). Metabolism of the benzene nucleus itself is rare e.g. 5-hydroxylation of thiabendazole, and N-methylation.

Liver microsomal sulphoxidation is a common metabolic route for the commercially available thioether and sulphide benzimidazoles (ABZ, FBZ and TCBZ) (Fig. 1.2). and probenzimidazoles (FBT and NTB). They are extensively and rapidly metabolised to their sulphoxide and sulphone derivatives. Sulphoxidation is a reversible process (Marriner and Bogan, 1980; 1981a, b; 1985; Mohammed Ali *et al.*, 1987; Gyurik *et al.*, 1981) resulting in an equilibrium between sulphide and sulphoxide, although the process favours metabolism towards the sulphoxide. Sulphonation follows sulphoxidation and is an irreversible and slower oxidative step resulting in a sulphone metabolite (Averkin *et al.*, 1975). Two microsomal enzymatic systems are involved in sulphoxidation and sulphonation. However, FMO is the predominant system in ruminants, whereas in monogastrics both systems are equally involved (Delatour *et al.*, 1994).

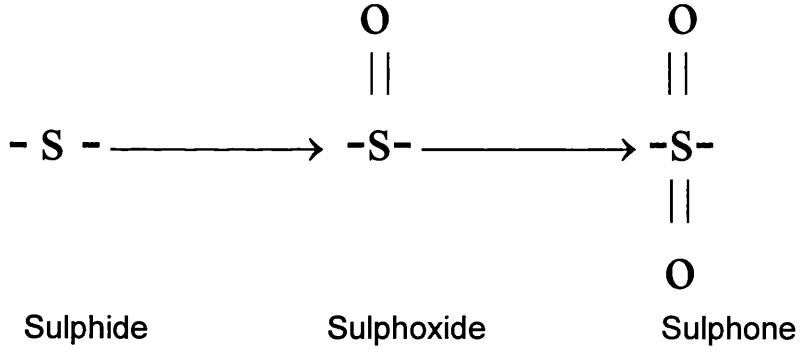

Fig. 1.2 Principal route of metabolism of the sulphide benzimidazoles (e.g. albendazole, fenbendazole and oxfendazole).

Another important metabolic route of the sulphide benzimidazoles is aromatic ring *p*-hydroxylation that leads to the formation of hydroxylated metabolites. These metabolites are excreted directly into the bile as free or conjugated metabolites and are therefore only detected to a limited extent, if at all, in the blood (Short *et al.,* 1987a, b; 1988; Hennessy *et al.*, 1993).

Chirality

Using chiral HPLC Delatour *et al.* (1990a, b; 1991a, b) have demonstrated the presence of both enantiomers of benzimidazole sulphoxides in the plasma of various species following administration of prochiral sulphide parent molecule. Interspecies stereoselective differences for the benzimidazole sulphoxide exist and could be explained by different relative enzyme contributions. Both the FMO and the MFO systems act equally in rats, and probably in other monogastrics (man, dog, horse), while the FMO system is predominant in ruminants. FMO is product selective in favour of production of the (+)-sulphoxide while MFO specifically uses the (-)-sulphoxide as the substrate for sulphonation. The potency ratios (or eudismic ratios) of the enantiomeric pair are yet to be determined, and the importance of enantioselective pharmacokinetics of benzimidazole sulphoxide antipodes on their anti-parasitic effect is still unclear (Landoni *et al.*, 1997).

Aim of the study

The aim of this study was to examine the pharmacokinetic behaviour of the benzimidazole anthelmintic, fenbendazole, and to extend its systemic availability and thus hopefully increase its efficacy in the horse. The effect of food and/or the metabolic inhibitor, piperonyl butoxide, on co-administration with fenbendazole on the bioavailability of the active metabolites (fenbendazole and fenbendazole sulphoxide) was investigated in the horse.

Chapter 2

Effect of food on the pharmacokinetics of fenbendazole with or without piperonyl butoxide in horses

2.1.1 Introduction

Fenbendazole is a broad spectrum sulphide benzimidazole anthelmintic used in dogs, cats, horses, sheep, goats, cattle and birds. Various attempts have been made to improve the efficacy of benzimidazole derivatives. In this study, the effect of administering fenbendazole with food was investigated on the relative bioavailability of the active metabolites in the horse. The time of feeding relative to oral drug administration has been shown to affect the bioavailability and absorption pattern of some drugs in horses. Feeding in horses was shown to decrease the mean plasma concentration and systemic availability of trimethoprim, but did not affect sulphadiazine absorption (Bogan *et al.*, 1984). Absorption for many drugs follows first-order kinetics such that an increased amount of drug is absorbed with increasing dose. An exception is that of absorption of fenbendazole in the dog as it was shown by McKellar *et al.* (1993) that the AUCs of parent fenbendazole and its sulphoxide metabolite do not significantly increase as the dose is increased from 10mg/kg up to 80mg/kg. The factors involved in the effect of food on drug absorption include an increase in stomach pH followed by degradation of pH-susceptible drugs (Boutin-Pannetier, *et al.*, 1986), formation of chelates with calcium and iron, drug binding to macromolecules and irregularities in propulsion or gastrointestinal pH (pH partition hypothesis (Baggot, 1977)) in the presence of food.

One study carried out in dogs (McKellar *et al.*, 1993) showed a significant increase in the systemic bioavailability of fenbendazole following its administration with food. In ruminants the systemic availability of fenbendazole is known to decrease when administered into the abomasum (Marriner and Bogan, 1981a). The rumen is thought of as a drug reservoir and it slows down the passage of digesta and extends the gut transit time of the drug. For an anthelmintic drug to be effective, it needs to achieve effective concentrations at the site of action. The different benzimidazoles are considered to have a similar mode of action and variations in their efficacy and spectrum of activity are associated with differences in pharmacokinetic behaviour. The efficacy and spectrum of activity will be determined by the concentration of the anthelmintic to which the parasite is exposed and the duration the parasite is exposed to toxic concentrations of the drug. Unfortunately, these concentrations are reduced rapidly in the horse due to the high activity of the hepatic microsomal associated metabolic pathways. Thus the 'first pass' effect may significantly decrease the systemic availability of orally administered drugs that are extensively metabolised. Fenbendazole is short-lived in plasma being extensively metabolised to its sulphoxide, sulphone and hydroxylated derivatives (Fig 2-1). The drugs that are active against helminths are the parent compound itself (FBZ) and the sulphoxide (SO) metabolite.

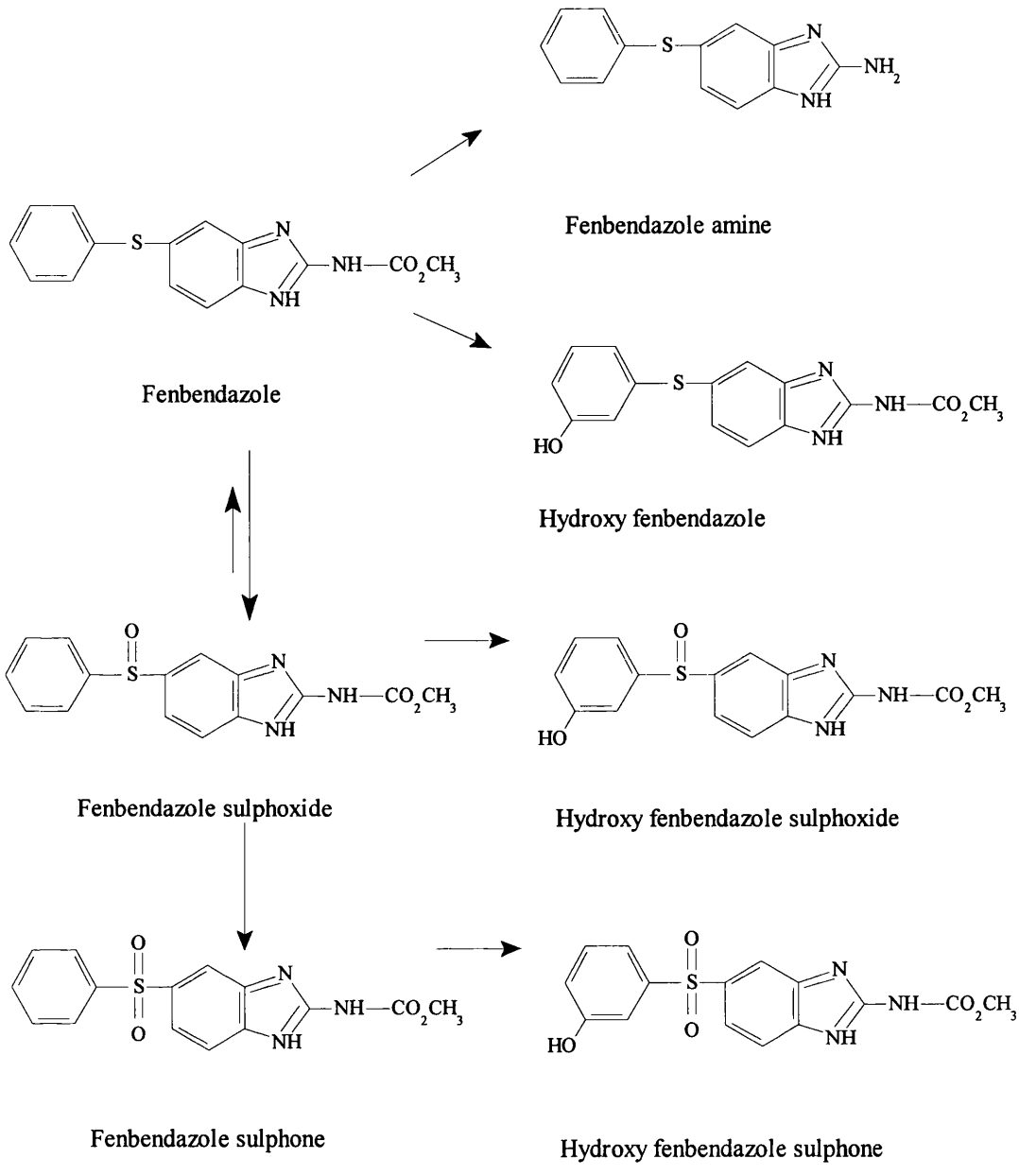

Figure 2-1. Major metabolic pathways fenbendazole

Studies carried out in goats and sheep showed that coadministration of piperonyl butoxide (Fig 2-2) with fenbendazole results in inhibited metabolism of fenbendazole and thus potentiated anthelmintic action associated with the less oxidised moieties (Benchaoui and McKellar, 1996). Piperonyl butoxide inhibits cytochrome P450- dependent oxidative metabolism by forming metabolic intermediates which bind covalently to the cytochrome P450 enzymes to form a complex with inhibitory effects on the MFO system (Franklin, 1977).

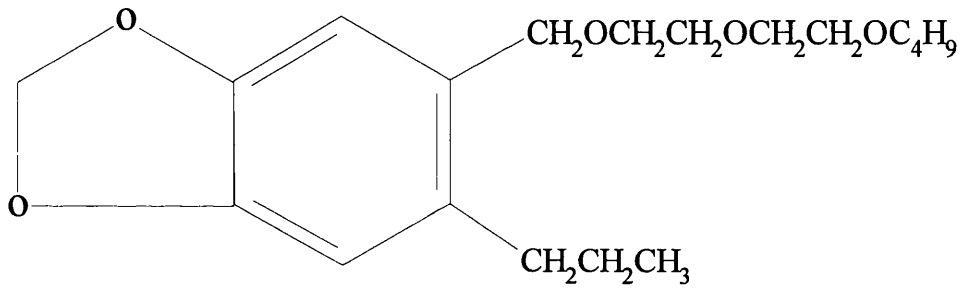

Figure 2-2. Structure of piperonyl butoxide

2.1.2 Aim

The aim of the present study was to determine the effect that the presence or absence of food has on the absorption of fenbendazole and whether the metabolic inhibitor, piperonyl butoxide, could be used to alter the biotransformation of fenbendazole in the horse, thus increasing the relative bioavailability of it's active metabolites.

2.2 Materials and methods

2.2.1 Animals

Six (06) ponies weighing 94 to 216.1 kg were used.

2.2.2 Experimental design

2.2.2.1 Experiment 1

The six ponies were not fed for 12 hours before the administration of the drugs. They were allocated into two groups of three animals each and were administered drugs in a two-way crossover design protocol. In the first phase one group received fenbendazole alone while the second group received both fenbendazole and piperonyl butoxide. In the second phase each group received the alternative treatment so that at the end of this experiment both treatments had been given sequentially to each pony used. A 4-week washout period was allowed between the experimental phases.

2.2.2.2 Experiment 2

The second experiment was carried out three months after the completion of the first experiment. The six ponies were not fed for 12 hours before the administration of drugs. However, they were each given half a bucket of coarse mix (cereal concentrates) one hour before drug administration and the other half just before drug administration. They were allocated into two

groups of three animals each and were administered drugs in a two-way crossover design protocol as in the first experiment.

2.2.3 Drug administration and sampling procedure

Fenbendazole (Panacur, 2.5%, Hoechst Ltd.) was given at a fixed dose rate of 10mg/kg bodyweight administered orally by stomach tube. Piperonyl butoxide (90%) (Aldrich Chemicals) was administered by stomach tube at a fixed dose rate of 63mg/kg.

Blood samples were taken before drug administration and at 1, 2, 3, 4, 6, 8, 12, 24, 32, 48, 72, and 96 hours thereafter. Samples were centrifuged to separate plasma which was then stored at -20 °C and analysed for the measurement of FBZ and its metabolites concentrations.

2.2.4 Drug analysis

Plasma samples were analysed by High Performance Liquid Chromatography following liquid phase extraction.

2.2.4.1 Standard preparation.

Stock solutions (100μg/ml) of pure standard fenbendazole, fenbendazole sulphoxide and fenbendazole sulphone (Hoechst Ltd., Frankfurt/Main, Germany) and albendazole (Sigma Chemical co, UK) were prepared using methanol (Rathburn Chemicals Ltd., UK) as a solvent. These were diluted

to give 10 , 5 , 2.5 , 1 and 0.5 μg/ml standard solutions for calibration and for adding to drug-free plasma samples to determine the recovery.

2.2.4.2 Extraction

Liquid phase extraction was the procedure used for fenbendazole (FBZ) and albendazole (ABZ). The method was adopted from that described by Marriner and Bogan (1980). To 1 ml plasma sample contained in a 10 ml-ground glass tube was added 200 μl of ammonium hydroxide (NH_4OH , 0.1N , pH 10) and 0.4 grams sodium chloride. This was vortexed for 30 seconds and 6 ml of chloroform were added, the sample tubes were stoppered and shaken on a slow rotary mixer for 10 minutes. After centrifugation at 1700g, 4 ml of the organic phase were transferred to a thin-walled 10-ml conical glass tube and evaporated to dryness at 50°C under nitrogen. The residue was reconstituted with 200μl of 35% acetonitrile, placed in an ultrasonic water bath for 1 minute and finally processed on the HPLC system.

2.2.4.3 HPLC system

This comprised a solvent delivery pump (Spectra Physics SP4000) connected to a Capital HPLC nucleosil C18, 5μl column (15cm x 4.6mm) and a UV detector (Model SP 8450 Spectra Physics SP Focus, Burke Electronics Ltd., Glasgow, UK). The autosampler was a Spectra Physics SP AS 3000, the recorder a PC 1000 and the controller was a Model SN 4000.

The mobile phase was a mixture of acetonitrile-water to which glacial acetic acid (BDH Ltd., Pool, UK) (0.5% , v/v) was added. The solvent was pumped through the column in a linear gradient profile changing from 35:65 (acetonitrile: water) to 55:45 in 8 minutes , 55:45 to 35:65 in 0.10 minute and the former ratio was maintained for 4.9 minutes. The flow rate was 1.5ml/minute. The detector wavelength was at 292nm and the retention times for FBZSO, $FBZSO_2$, FBZ and ABZ were 2.6, 4.1, 7.7 and 5.6 minutes respectively.

2.2.4.4 Recovery and precision

Recovery of the compounds from plasma to which the drugs had been added was evaluated by reference to peak areas resulting from direct injection of standard solutions. The precision of the extraction 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 coefficients of interassay variation are reported in Appendix A-1 and Limits of detection were 0.01 μg/ml for FBZSO , $FBZSO_2$, FBZ and ABZ.

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.

2.2.5 Pharmacokinetic and statistical analysis

The individual data from each animal were analysed using non-compartmental model analysis with extravascular input (PC NONLIN version 4.0) (Metzler & Weiner, 1992).

The trapezoidal rule was used to calculate the area under the plasma concentration time curve (AUC_{OBS}) and the area under the first moment curve ($AUMC_{OBS}$) from observed values. The mean residence time (MRT) is the ratio $AUMC_{OBS}/AUC_{OBS}$.

Comparison of pharmacokinetic parameters for each metabolite obtained when animals were administered drugs after feeding or without feeding were made using Wilcoxon sign rank test. A Mann-Whitney *U* test was used to determine the effect of administering fenbendazole with the metabolic inhibitor, piperonyl butoxide. Differences were considered significant when $P < 0.05$.

2.3 Results

Plasma concentrations of fenbendazole (Table 2-1 and 2-7, Appendices A-2, A-4, A-6 and A-8), fenbendazole sulphoxide (Table 2-3 and 2-9, Appendices A-10, A-12, A-14 and A-16) and fenbendazole sulphone (Table 2-5 and 2-11, Appendices A-18, A-20, A-22 and A-24) are plotted in Figs 2-1 and 2-4 (FBZ), 2-2 and 2-5 (FBZSO), and 2-3 and 2-6 ($FBZSO_2$).

Due to the large interindividual variations observed, there were no significant differences in any of the pharmacokinetic parameters of the metabolites obtained between administration of the drug without feeding and after feeding. Administration of fenbendazole with food resulted in an increased area under the plasma concentration time curve (37.5%) of the parent moiety, but the area under the first moment curve was reduced by 28.7%. Feeding produced higher mean maximum plasma concentrations of fenbendazole even though these were delayed by over 3 hours. For the sulphoxide metabolite, the AUC and the AUMC were much lower after feeding, and the MRT, C_{max}, and t_{max} were decreased. The mean maximum plasma concentrations of 0.02μg/ml for FBZ and FBZSO, and 0.04μg/ml for $FBZSO_2$ attained were increased by 100 and 125% for the sulphide and sulphone but reduced by 50% in case of the sulphoxide following feeding. The AUC of fenbendazole parent drug was increased by 37.5% after feeding but the AUMC was reduced by 28.7 %. Feeding resulted in an elevation of the sulphone AUC by 13.9% but a reduction of the AUMC of 62.2%. However, the total AUC for the sulphide and the sulphoxide (active compounds) in unfed horses (1.19μg.h/ml) was twice as high as that in fed horses (0.59μg.h/ml).

Administration of fenbendazole with piperonyl butoxide without feeding produced significant differences in most of the pharmacokinetic

Table 2-1. Plasma concentrations of fenbendazole (FBZ) (μg/ml) following administration of fenbendazole (10mg/kg) alone either with or without food in horses.

	Mean ± SEM	
Time (h)	FBZ without food (n=6)	FBZ with food (n=6)
0	0.002 ± 0.0017	0.000 ± 0.0000
1	0.011 ± 0.0062	0.020 ± 0.0045
2	0.016 ± 0.0063	0.027 ± 0.0057
3	0.014 ± 0.0072	0.026 ± 0.0107
4	0.011 ± 0.0066	0.025 ± 0.0085
6	0.012 ± 0.0050	0.020 ± 0.0057
8	0.008 ± 0.0051	0.023 ± 0.0056
12	0.009 ± 0.0042	0.017 ± 0.0064
24	0.010 ± 0.0032	0.016 ± 0.0052
32	0.003 ± 0.0017	0.000 ± 0.0000
48	0.000 ± 0.0000	0.004 ± 0.0045
72	0.000 ± 0.0000	0.000 ± 0.0004
96	0.006 ± 0.0048	0.000 ± 0.0005

Figure 2-1. Plasma concentrations of fenbendazole (FBZ) (μg/ml) (mean±SEM) following administration of fenbendazole (10mg/kg) alone (n=6) either with or without food in horses.

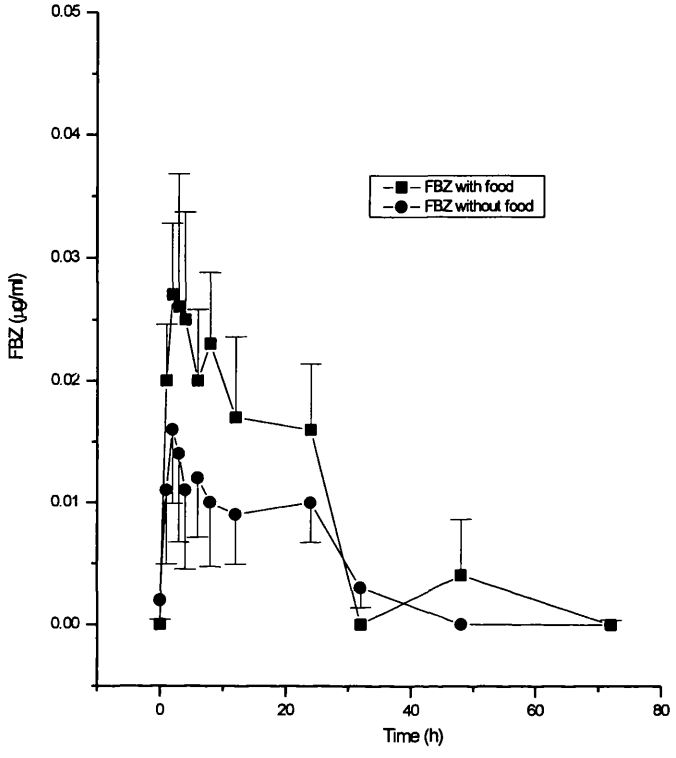

Table 2-2. Pharmacokinetic parameters of fenbendazole (FBZ) following administration of fenbendazole (10mg/kg) alone either with or without food in horses.

	Mean ± SEM	
	FBZ without food (n=6)	FBZ with food (n=6)
AUC_{OBS} (μg.h/ml)	0.32 ± 0.107	0.44 ± 0.099
$AUMC_{OBS}$ (μg.h^2/ml)	6.72 ± 1.644	4.79 ± 1.144
MRT (h)	11.68 ± 3.288	11.40 ± 1.633
C_{max} (μg/ml)	0.02 ± 0.006	0.04 ± 0.010
t_{max} (h)	3.00 ± 1.826	6.50 ± 3.519

Table 2-3. Plasma concentrations of fenbendazole sulphoxide (FBZSO) (μg/ml) following administration of fenbendazole (10mg/kg) alone either with or without food in horses.

	Mean ± SEM	
Time (h)	FBZSO without food (n=6)	FBZSO with food (n=6)
0	0.000 ± 0.0000	0.001 ± 0.0013
1	0.000 ± 0.0000	0.003 ± 0.0014
2	0.000 ± 0.0000	0.007 ± 0.0013
3	0.009 ± 0.0063	0.007 ± 0.0026
4	0.010 ± 0.0065	0.010 ± 0.0021
6	0.009 ± 0.0061	0.008 ± 0.0037
8	0.018 ± 0.0082	0.010 ± 0.0035
12	0.029 ± 0.0099	0.009 ± 0.0043
24	0.033 ± 0.0106	0.001 ± 0.0007
32	0.035 ± 0.0118	0.002 ± 0.0020
48	0.035 ± 0.0114	0.001 ± 0.0010
72	0.004 ± 0.0031	0.000 ± 0.0004
96	0.000 ± 0.0000	0.003 ± 0.0026

Figure 2-2. Plasma concentrations of fenbendazole sulphoxide (FBZSO) (μg/ml) (mean±SEM) following administration of fenbendazole (10mg/kg) alone (n=6) either with or without food in horses.

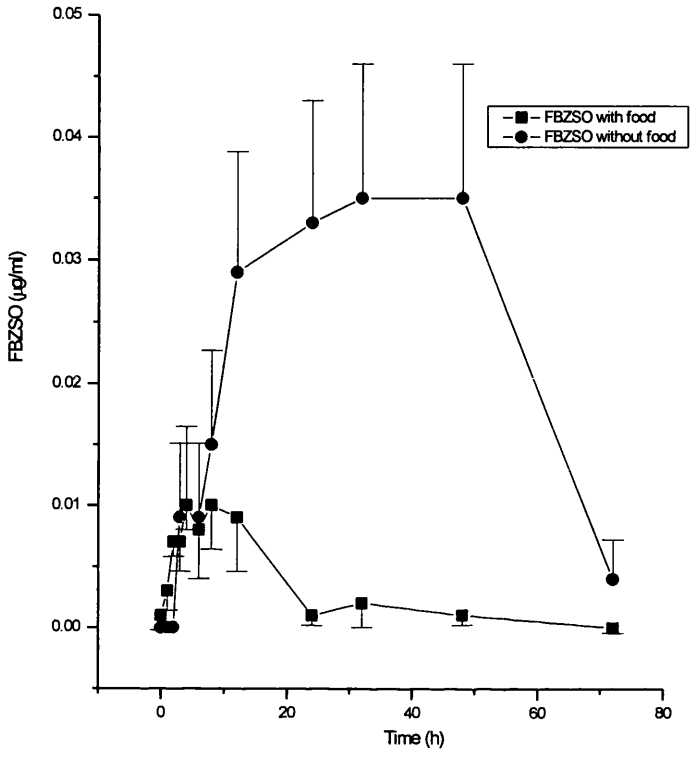

Table 2-4. Pharmacokinetic parameters of fenbendazole sulphoxide (FBZSO) following administration of fenbendazole (10mg/kg) alone either with or without food in horses.

	Mean ± SEM	
	FBZSO without food (n=6)	FBZSO with food (n=6)
AUC_{OBS} (μg.h/ml)	1.87 ± 0.599	0.15 ± 0.059
$AUMC_{OBS}$ (μg.h^2/ml)	64.69 ± 21.494	1.38 ± 0.565
MRT (h)	22.94 ± 7.352	7.53 ± 1.279
C_{max} (μg/ml)	0.03 ± 0.0132	0.01 ± 0.004
t_{max} (h)	22.67 ± 7.843	3.67 ± 0.954

Table 2-5. Plasma concentrations of fenbendazole sulphone ($FBZSO_2$) (μg/ml) following administration of fenbendazole (10mg/kg) alone either with or without food in horses.

	Mean ± SEM	
Time (h)	$FBZSO_2$ without food (n=6)	$FBZSO_2$ with food (n=6)
0	0.000 ± 0.0000	0.004 ± 0.0025
1	0.003 ± 0.0018	0.0023 ± 0.0055
2	0.023 ± 0.0112	0.038 ± 0.0079
3	0.030 ± 0.0160	0.042 ± 0.0095
4	0.030 ± 0.0160	0.062 ± 0.0175
6	0.030 ± 0.0165	0.052 ± 0.0155
8	0.029 ± 0.0166	0.057 ± 0.0125
12	0.021 ± 0.0077	0.047 ± 0.0090
24	0.018 ± 0.0056	0.027 ± 0.0032
32	0.015 ± 0.0059	0.016 ± 0.0037
48	0.011 ± 0.0045	0.020 ± 0.0084
72	0.008 ± 0.0052	0.004 ± 0.0013
96	0.004 ± 0.0033	0.0009 ± 0.0036

Figure 2-3. Plasma concentrations of fenbendazole sulphone ($FBZSO_2$) (μg/ml) (mean±SEM) following administration of fenbendazole (10mg/kg) alone (n=6) either with or without food in horses.

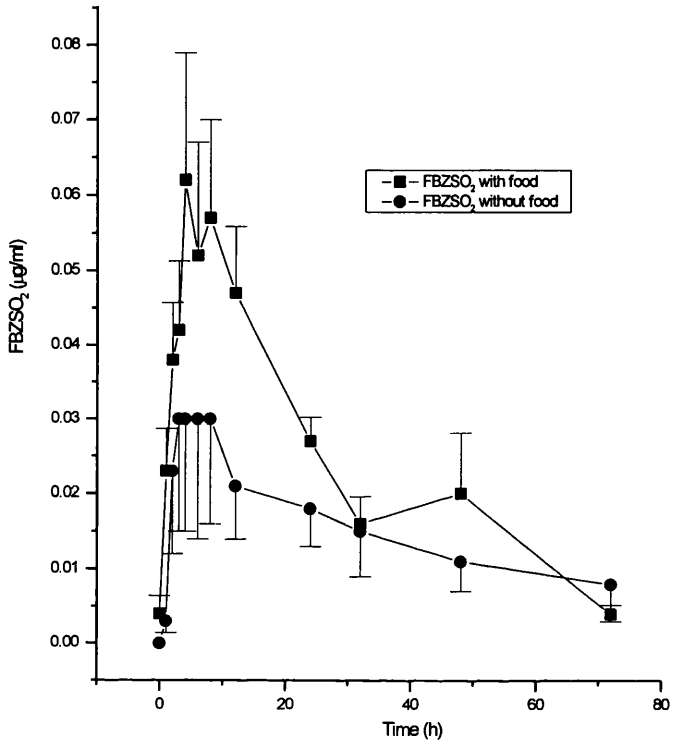

Table 2-6. Pharmacokinetic parameters of fenbendazole sulphone ($FBZSO_2$) following administration of fenbendazole (10mg/kg) alone either with or without food in horses.

	Mean ± SEM	
	$FBZSO_2$ without food (n=6)	$FBZSO_2$ with food (n=6)
AUC_{OBS} (μg.h/ml)	1.29 ± 0.493	1.47 ± 0.186
$AUMC_{OBS}$ (μg.h^2/ml)	44.50 ± 22.145	27.25 ± 3.658
MRT (h)	30.86 ± 5.999	18.77 ± 1.698
C_{max} (μg/ml)	0.04 ± 0.016	0.09 ± 0.015
t_{max} (h)	15.50 ± 7.329	4.83 ± 0.980

Table 2-7. Plasma concentrations of fenbendazole (FBZ) (μg/ml) following administration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) either with or without food in horses.

	Mean ± SEM	
Time (h)	FBZ and PB without food (n=6)	FBZ and PB with food (n=5)
0	0.002 ± 0.0023	0.001 ± 0.0009
1	0.028 ± 0.0090	0.083 ± 0.0265
2	0.054 ± 0.0174	0.118 ± 0.0364
3	0.068 ± 0.0238	0.137 ± 0.0443
4	0.068 ± 0.0216	0.260 ± 0.0771
6	0.083 ± 0.0273	0.180 ± 0.0610
8	0.096 ± 0.0199	0.355 ± 0.1316
12	0.135 ± 0.0163	0.278 ± 0.1189
24	0.133 ± 0.0245	0.157 ± 0.0553
32	0.032 ± 0.0076	0.005 ± 0.0036
48	0.000 ± 0.0000	0.035 ± 0.0251
72	0.000 ± 0.0000	0.000 ± 0.0000
96	0.000 ± 0.0000	0.000 ± 0.0000

Figure 2-4. Plasma concentrations of fenbendazole (FBZ) (μg/ml) (mean±SEM) following administration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) either with or without food in horses.

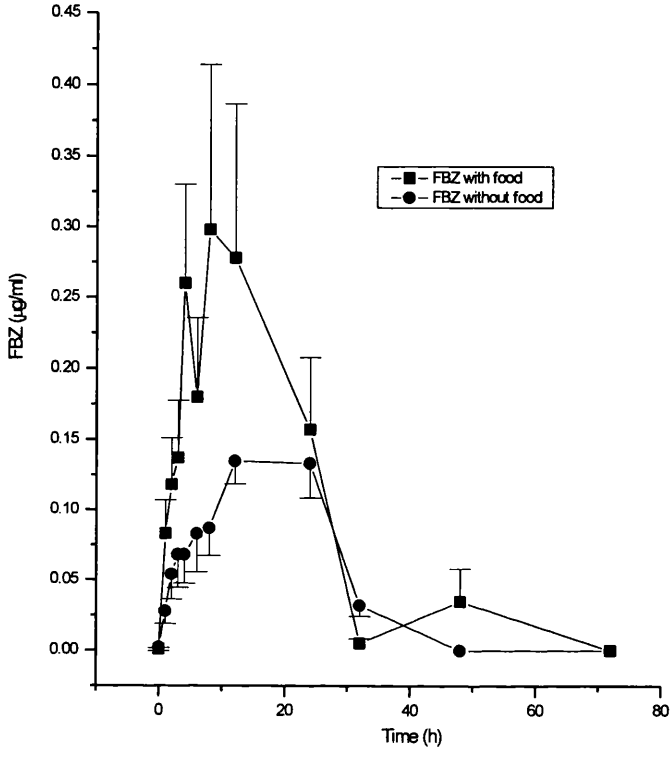

Table 2-8. Pharmacokinetic parameters of fenbendazole (FBZ) following administration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) either with or without food in horses.

	Mean ± SEM	
	FBZ and PB without food (n=6)	FBZ and PB with food (n=5)
AUC_{OBS} (μg.h/ml)	3.51 ± 0.401	5.81 ± 1.961
$AUMC_{OBS}$ (μg.h^2/ml)	61.79 ± 8.453	76.72 ± 25.094
MRT (h)	17.51 ± 1.450	12.63 ± 1.965
C_{max} (μg/ml)	0.16 ± 0.015	0.37 ± 0.104
t_{max} (h)	17.00 ± 3.256	13.60 ± 4.308

Table 2-9. Plasma concentrations of fenbendazole sulphoxide (FBZSO) (μg/ml) following administration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) either with or without food in horses.

	Mean ± SEM	
Time (h)	FBZSO and PB without food (n=6)	FBZSO and PB with food (n=5)
0	0.000 ± 0.0000	0.001 ± 0.0006
1	0.000 ± 0.0000	0.014 ± 0.0082
2	0.000 ± 0.0000	0.021 ± 0.0126
3	0.004 ± 0.0024	0.031 ± 0.0188
4	0.006 ± 0.0029	0.065 ± 0.0359
6	0.010 ± 0.0053	0.052 ± 0.0265
8	0.018 ± 0.0072	0.116 ± 0.0583
12	0.027 ± 0.0069	0.117 ± 0.0717
24	0.030 ± 0.0073	0.060 ± 0.0242
32	0.010 ± 0.0077	0.001 ± 0.0006
48	0.005 ± 0.0032	0.010 ± 0.0059
72	0.000 ± 0.0000	0.000 ± 0.0000
96	0.000 ± 0.0000	0.000 ± 0.0000

Figure 2-5. Plasma concentrations of fenbendazole sulphoxide (FBZSO) (μg/ml) (mean±SEM) following administration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) either with or without food in horses.

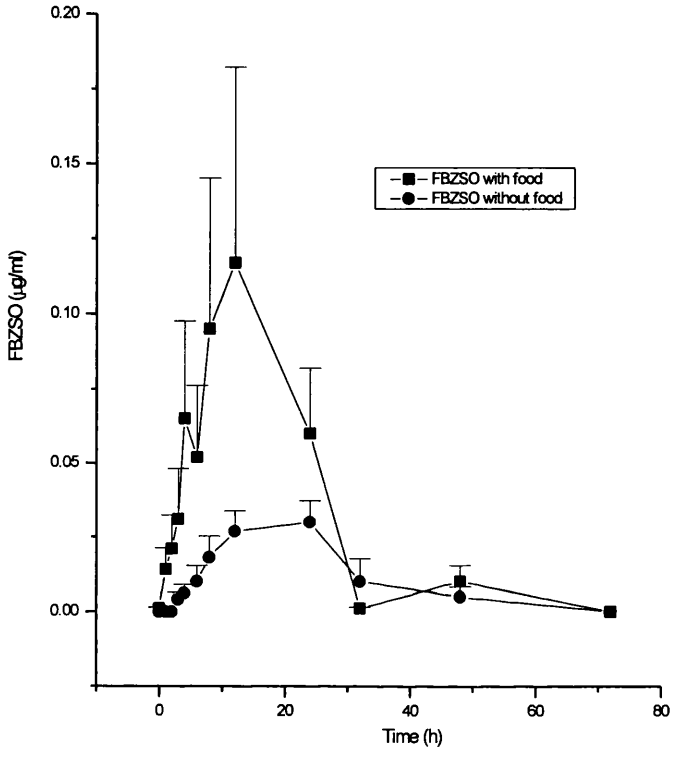

Table 2-10. Pharmacokinetic parameters of fenbendazole sulphoxide (FBZSO) following administration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) either with or without food in horses.

	Mean ± SEM	
	FBZSO and PB without food (n=6)	FBZSO and PB with food (n=5)
AUC_{OBS} (μg.h/ml)	0.82 ± 0.2178	2.08 ± 0.992
$AUMC_{OBS}$ (μg.h^2/ml)	19.15 ± 6.5593	28.97 ± 12.970
MRT (h)	21.05 ± 2.5287	13.56 ± 2.437
C_{max} (μg/ml)	0.04 ± 0.0038	0.13 ± 0.067
t_{max} (h)	19.00 ± 3.2558	14.40 ± 4.118

Table 2-11. Plasma concentrations of fenbendazole sulphone ($FBZSO_2$) (μg/ml) following administration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) either with or without food in horses.

	Mean ± SEM	
Time (h)	$FBZSO_2$ and PB without food (n=6)	$FBZSO_2$ and PB with food (n=5)
0	0.000 ± 0.0000	0.002 ± 0.0024
1	0.000 ± 0.0002	0.002 ± 0.0021
2	0.002 ± 0.0018	0.003 ± 0.0019
3	0.000 ± 0.0000	0.003 ±0.0019
4	0.003 ± 0.0030	0.006 ± 0.0040
6	0.000 ± 0.0000	0.009 ± 0.0025
8	0.010 ± 0.0059	0.016 ± 0.0034
12	0.012 ± 0.0074	0.033 ± 0.0073
24	0.046 ± 0.0119	0.102 ± 0.0341
32	0.035 ± 0.0145	0.015 ± 0.0061
48	0.006 ± 0.0031	0.071 ± 0.0340
72	0.007 ± 0.0045	0.002 ± 0.0012
96	0.002 ± 0.0023	0.002 ± 0.0014

Figure 2-6. Plasma concentrations of fenbendazole sulphone ($FBZSO_2$) (μg/ml) (mean±SEM) following administration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) either with or without food in horses.

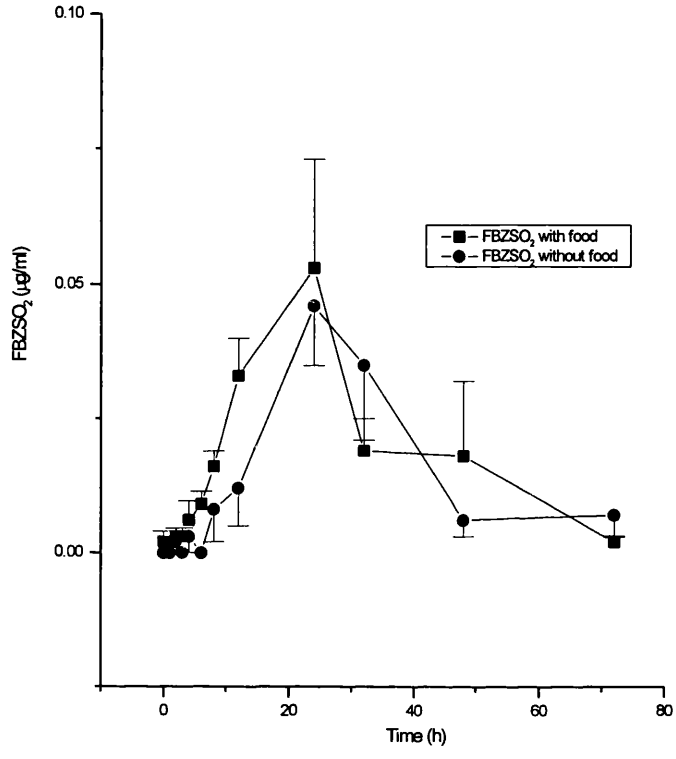

Table 2-12. Pharmacokinetic parameters of fenbendazole sulphone ($FBZSO_2$) following administration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) either with or without food in horses.

	Mean ± SEM	
	$FBZSO_2$ and PB without food (n=6)	$FBZSO_2$ and PB with food (n=5)
AUC_{OBS} (μg.h/ml)	1.29 ± 0.273	1.44 ± 0.328
$AUMC_{OBS}$ (μg.h^2/ml)	45.26 ± 13.192	30.33 ± 7.950
MRT (h)	28.10 ± 7.106	20.19 ± 1.718
C_{max} (μg/ml)	0.05 ± 0.013	0.11 ± 0.028
t_{max} (h)	20.00 ± 5.367	21.60 ± 2.400

parameters of the active metabolites ($P<0.05$). There was no statistical evidence of any difference in those of the sulphone metabolite except for the t_{max}. AUC, AUMC and C_{max} values for fenbendazole were significantly increased following administration of fenbendazole and piperonyl butoxide after feeding. For the sulphoxide and sulphone metabolites only the t_{max} (and also C_{max} for sulphoxide) values were significantly increased following administration of piperonyl butoxide. The inhibitor increased the AUC, AUMC and C_{max} values for the parent drug 10 , 8 and 7 times, respectively, but decreased the same parameters for fenbendazole sulphoxide by 56.1, 70.4 and 50% respectively, in the unfed horses. It only had a slight effect on AUC and AUMC values of the sulphone metabolite (0.0 and 1.7% increment only respectively). In the fed horses , the fenbendazole AUC, AUMC and C_{max} were increased by 12.2, 15 and 8.25 times, respectively, with piperonyl butoxide pre-treatment. The MRT was also slightly increased (10.8%). In contrast to the situation in the unfed horses, the sulphoxide pharmacokinetic parameters in the fed horses were markedly increased by approximately 13, 20, and 12 times for the AUC, AUMC and C_{max} values, respectively, with piperonyl butoxide pre-treatment. The MRT was increased by 80% in this case. In unfed horses, the total AUC for the sulphide and the sulphoxide metabolite registered a more than three-fold increase after administration of piperonyl butoxide. In fed horses, the increase was even greater (more than 13 times).

2.4 Discussion

Fenbendazole was extensively metabolised to the sulphoxide and the sulphone metabolites in the horse with low amounts of the parent drug measured in the plasma and higher amounts of the metabolites. The amounts of the sulphoxide metabolite were found to be very low in relation to the sulphone in the fed horses, and this supports the work of Marriner and Bogan, (1985). This is attributed to the high rate of sulphonation in this species. Feeding was shown to affect the kinetics of the benzimidazole anthelmintics in the horse. The mean maximum plasma concentration of fenbendazole was increased by 100% after feeding but the time taken to reach C_{max} was more than doubled. The persistence of fenbendazole in the plasma was only slightly affected (reduced by 17 minutes). An interesting finding in the unfed horses was the higher amounts (as indicated by the areas under the curve) of the sulphoxide as compared to the sulphone, whereas in the fed horses the situation was the opposite. It may be that feeding slows the rate of absorption of fenbendazole, and that most of the fenbendazole that is absorbed is converted to the sulphoxide and then immediately converted to the sulphone. In unfed horses, fenbendazole could be absorbed at a faster rate such that there is more substrate for sulphoxidation per unit time and this reaction is occurring more rapidly than sulphonation. Hence, the higher sulphoxide amounts in unfed horses. The increased time to C_{max} of fenbendazole suggests a reduced rate of absorption with feeding and the shorter MRT of the sulphoxide compared to the sulphone is probably due

to a faster elimination (e.g. conversion to sulphone), Hence the higher sulphone amounts in fed horses. The sulphoxide MRT in unfed animals was also shorter (by 7.92 hours) than for the sulphone but not as much as that observed in fed horses. Thus feeding would reduce the overall amount of active drugs and increase the persistence of the inactive sulphone in the blood.

Fenbendazole might be extensively bound to food, and this would inevitably delay the absorption rate. However, it's MRT was not much affected. The double peaks observed in the drug plasma concentration-time profiles (see Figs 2-1 and 2-4) suggest an uneven pattern of absorption. Some of the administered drug may have been absorbed directly from the intestine but the bound drug may have been absorbed at a later stage in the colon and /or caecum as it was being released by fermentative digestion. The slightly decreased mean residence time of fenbendazole with feeding does not, however, support this suggestion. Double peaks could also arise from the uneven absorption of drug along the different segments of the GI tract, and from drugs that undergo gasteroenteric recycling.

Piperonyl butoxide produced striking effects on the pharmacokinetics of fenbendazole when administered with it. The plasma concentration of fenbendazole was increased dramatically with metabolic inhibition as evidenced by the higher amount of drug in the body (high AUC and AUMC

values) and higher mean maximum plasma concentrations. An even higher plasma concentration was achieved when the horses were administered drug with food. Higher t_{max} values were required to reach the increased concentrations and in case of fenbendazole this was at 17 hours post treatment, an increase of 14 hours compared to that without pre-treatment with piperonyl butoxide. The sulphoxide AUC and AUMC values were reduced with inhibition in the unfed horses but the AUC was higher in fed animals pre-treated with the inhibitor. The lower sulphoxide areas under the curve were probably due to inhibition of the cytochrome P-450 enzyme system thereby maintaining higher plasma sulphide amounts. The high sulphone metabolite concentrations observed with piperonyl butoxide pre-treatment were not expected. It would seem logical to assume that the lower plasma concentration of the sulphoxide would leave little substrate for sulphonation. In addition, an inhibition of sulphonation was also expected.

In conclusion, feeding in horses reduces the relative plasma concentration of the active moieties of fenbendazole and it is anticipated that this would reflect reduced efficacy of the drug. Fenbendazole should therefore be administered without food in horses. The coadministration of the *S*-oxidation-impairing piperonyl butoxide markedly improved the plasma concentration of fenbendazole and this possible method of improving its anthelmintic efficacy deserves further investigation.

Chapter 3

The *in vitro* metabolism of benzimidazole anthelmintics

3.1 Introduction

Metabolism of xenobiotics occurs mainly in the liver but also occurs in a number of other tissues (e.g. gastrointestinal tract, kidneys). Hepatic subcellular fractions are useful *in vitro* models for the study of benzimidazole compounds and their biotransformation pathways under strictly controlled conditions. Furthermore, *in vitro* studies are less costly compared to *in vivo* studies that require animal purchase, care and maintenance (Van't Klooster, 1992). In addition, the number of experimental animals used in xenobiotic biotransformation studies and toxicity studies may be minimised through the use of *in vitro* experiments.

In the present study, the *in vitro* inhibition of metabolism of fenbendazole in equine liver was investigated and the effect of piperonyl butoxide, a cytochrome P450 inhibitor, was determined. A similar *in vitro* study was undertaken by Benchaoui and McKellar, (1996), using rat liver microsomes. Evidence from *in vitro* studies indicates that the sulphoxidation of fenbendazole involves both the flavin monooxygenase and the cytochrome P450 systems (Benchaoui and McKellar, 1996). *In vitro* studies done using rat liver microsomes showed that fenbendazole metabolites, particularly the sulphone and hydroxylated metabolites, but not the sulphoxide metabolite, also acted to inhibit cytochrome P450 enzyme activity (Murray *et al.*, 1992). Oxfendazole (which is the same as fenbendazole sulphoxide) has been shown to induce cytochrome P450, more specifically the isoenzyme P450 IA2 and it's corresponding

monooxygenases, in rabbit liver microsomes (Gleïzes *et al.*, 1991a) and rabbit liver hepatocytes (Gleïzes *et al.*, 1991b).

3.2 Materials and methods

3.2.1 Liver microsome preparations

3.2.1.1 Chemicals

The chemicals used for the NADPH-generating cofactor solution were as follows: Trizma hydrochloride, Trizma base, isocitrate dehydrogenase, nicotinamide adenine dinucleotide phosphate (NADP), niacinamide (or nicotinamide) and trisodium isocitrate, all obtained from Sigma Chemical Ltd. (UK) ; and $MgCl_2$ from BDH Chemicals Ltd. (Poole, UK).

The standard compounds used were albendazole and oxfendazole from Sigma Chemicals Ltd. (UK), fenbendazole, fenbendazole sulphoxide fenbendazole sulphone from Hoechst Ltd. (Frankfurt/ Main, Germany) and 90% piperonyl butoxide (Aldrich Chemicals Ltd., UK). Solvents used in this experiment were methanol (Rathburn Chemicals Ltd., UK) and varying proportions of acetonitrile (Rathburn Chemicals Ltd., UK) with water and acetic acid (BDH Chemicals Ltd., Poole, UK) and dimethylsulphoxide (DMSO) (BDH Chemicals Ltd., Poole, UK), which was used to dissolve standard drugs to be added to the incubation mixture.

Chemicals used for the isolation of microsomes include 0.9% sodium chloride and 1.15% potassium chloride from BDH Chemicals Ltd., Poole, UK, and Tris buffer in 20% glycerol (Sigma Chemical Ltd., UK).

The materials used included the Beckman J2-21 refrigerated centrifuge and the Beckman L8-70 refrigerated ultracentrifuge (California, USA), an Ultra-turrax (Janke & Kunkel GmbH & Co., Germany), and Potter-Elvehjem homogeniser.

Microsomal protein was measured using the Coomassie® protein Assay Reagent (Pierce Chemical Co, Rochford, UK) with Bovine Serum Albumin (BSA) standard.

3.2.1.2 Isolation

The liver microsomes were prepared from livers obtained from six (6) different horses (A, B, C, D, E, and F). The different weights of the liver portions used are shown in Appendix B-1. Shortly after a horse was killed, the liver was removed, and perfused with ice cold saline (0.9%, NaCl solution) through the hepatic veins. The liver portion was then drained of excess moisture and weighed. All procedures were performed at 0-4 ^{o}C. The liver tissue was placed in 300 ml of 1.15% KCl solution and finely chopped before homogenisation using a Potter-Elvehjem homogeniser. Several passages of the teflon pestle were necessary to disrupt the tissue. The liver homogenate was centrifuged for 20 minutes at about 9000*g* in a Beckman J2-21 refrigerated centrifuge to clean the tissue (removal of cell debris, nuclei and mitochondria). The floating fat layer was removed with a pasteur pipette, and the supernatant decanted in 6 Beckman Ultra-Clear tubes (California, USA). The tubes were centrifuged at 10500*g* for 75 minutes in a Beckman L8-70 refrigerated ultracentrifuge. After discarding

the cytosolic fraction (supernatant), the microsomal pellet was resuspended in 60ml of 0.1M Tris-phosphate buffer (pH 7.4) containing 20% (v/v) glycerol using an Ultra-turrax. The microsomal suspensions (final volumes shown in Appendix B-2) were then stored at -70 °C. until used for incubation assays. Diluted (x500) microsomal suspensions were used in the determination of total protein. The protein content was determined using the Micro-assay procedure of Coomassie® Protein Assay Reagent. The assay is based on the absorbance shift from 465 to 595nm that occurs when Coomassie Brilliant Blue G-250 binds to proteins in an acidic solution. A standard curve using BSA was run with each assay. This is a strongly recommended procedure as the colour response is non-linear over a wide range of protein concentration. A review of the protein content measurement procedure is given by Davies (1988). A determination of cytochrome P450 concentrations was not done.

3.2.1.3 Drug incubation

Incubations were carried out in a shaking water bath at 37 °C for a period of 2 hours, after which they were stopped by placing tubes with the reaction mixture in boiling water for 2 minutes. This was immediately followed by freezing at -20 °C until analysis. Ten-ml glass test tubes were used. One assay of an incubation mixture contained 4mg of microsomal protein suspension, 5μl of test drug (fenbendazole, or fenbendazole sulphoxide) alone or with 5μl of piperonyl butoxide (PB), and 1.0ml of the co-factor solution. The precise amounts are shown in Appendix B-1 and

B-2. Incubations were done in triplicate. Tubes without microsomes were used as controls for possible nonenzymatic drug conversion. Piperonyl butoxide was used at a constant concentration of 50μM. Fenbendazole and fenbendazole sulphoxide concentrations used were 5, 10, 25 and 50μM.

3.3 Drug analysis

3.3.1 Extraction

Fenbendazole and it's respective metabolites were analysed in a similar fashion to the procedure outlined in section 2.2.4. The drug extraction method involved the use of total samples of the incubation mixture and each incubation tube was rinsed with 1.0ml acetonitrile, which was also included for the extraction.

3.3.2 HPLC system

This is as described in section 2.2.4.3 except for the following modifications. The column used was a Genesis nucleosil C18, 4μl, column(15cm X 4.6cm) (Jones Chromatography).

The mobile phase was pumped through the column in a linear gradient profile changing from 40:60 (acetonitrile: water) to 50:50 in 4 minutes, to 70:30 in 8 minutes and this was maintained for up to 12 minutes running time. A wash of the column with 99.5% acetonitrile and 0.5% acetic acid for 7.90 minutes was incorporated between each run, after which the

mobile phase composition returned to 40:40 at 20.10 minutes run time. The column was allowed 10 minutes to equilibrate. Thus each run was quite long (30 minutes) because of the wash, which was necessary to avoid too many irrelevant dirt peaks from being eluted. The retention times were approximately 10.10, 3.78 and 5.83 minutes for fenbendazole, sulphoxide and sulphone respectively, and 7.78 minutes for the albendazole standard.

3.4 Statistical analysis

The extent of conversion and the amount of unchanged drug, with and without metabolic inhibition were compared by analysis of variance. Results were considered significant when $P<0.05$.

3.5. Results

The composition of the co-factor solution needed to generate NADPH during incubation of the (FBZ, FBZSO) with hepatic microsomes is given in Appendix B-1. The microsomal protein content of the different equine livers used is given in appendices B-2, B-3 and B-4.

Incubation done without microsomes resulted in slight sulphonation of the FBZSO (Oxfendazole) parent drug (1.4%) (see Appendix B-5)

Incubation of the benzimidazole drugs resulted in formation of their sulphone and sulphoxide metabolites (for FBZ) and sulphone and sulphide metabolites (for FBZSO). Hydroxylated metabolites were not measured in this study.

The extent of metabolism of fenbendazole was 11, 30.7, 3.56 and 25.7% for the 5, 10, 25 and 50μM substrate concentrations used (Appendix B-7, Figs 3.1, 3.2, 3.3 and 3.4) respectively. Upon addition of the cytochrome P450 inhibitor, piperonyl butoxide (PB), the extent of conversion of fenbendazole was reduced by 29.6, 38.4, 22.9 and 16.9% for 5 and 10 and 25 and 50μm substrate concentrations respectively. The extent of metabolism of FBZSO was also reduced by over 20% on co-administration of PB at 10 and 25μm concentrations (Appendix B-6). Despite these apparently large differences between metabolism of drug with and without PB, no statistical significance could be inferred due to the large interindividual variations. This was also the case for studies on sulphonation and sulphoxidation.

The effect of PB on sulphonation for FBZ was variable for the different concentrations and the different livers used (Appendix B-11, Figs 3.5, 3.6, 3.7 and 3.8) with lower substrate concentrations (5 and 10μm) resulting in increased sulphone formation and higher concentrations (25 and 50μm) resulting in reduced production. The oxfendazole substrate yielded a reduced amount of sulphone of generally more than 70% following addition of PB (Appendix B-8, Figs 3.9, 3.10, 3.11 and 3.12.). It was also observed that decreasing the substrate-PB ratio increased the extent of sulphonation of FBZSO, with maximum sulphonation achieved at the ratio of 1:5 (see Appendix. B-8 and B-11)

Reduction of FBZSO (oxfendazole) substrate to sulphide was also decreased (by over 90%) upon addition of PB (Appendix B-9). The amount of FBZ formed after FBZSO metabolism was generally greater than the amount of $FBZSO_2$ formed (i.e. reduction > sulphonation). However following addition of PB, the two metabolites were formed in approximately equal ratios.

Piperonyl butoxide drastically lowered (by more than 90%) the sulphoxidation of FBZ (Appendix B-10, Figs 3.5, 3.6, 3.7 and 3.8). In the absence of the inhibitor, the extent of sulphonation was less than the extent of sulphoxidation, but the two processes produced more or less equal amounts of metabolite following addition of inhibitor, thereby increasing the sulphonation: sulphoxidation ratio.

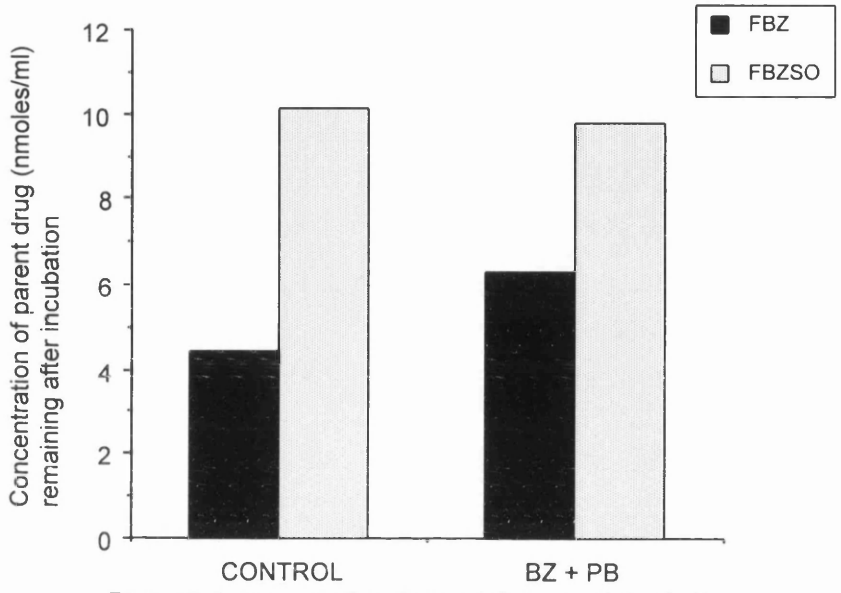

Figure 3-1. Amount of unchanged drug remaining in the microsomal reaction mixture after incubation of BZ (5μM) with (BZ + PB) and without (control) piperonyl butoxide (n=4).

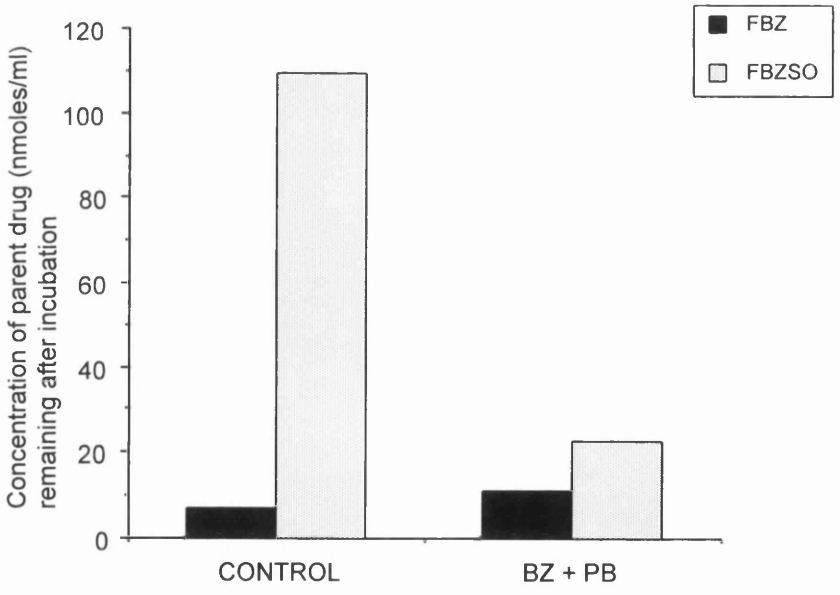

Figure 3-2. Amount of unchanged drug remaining in the microsomal reaction mixture after incubation of BZ (10μM) with (BZ + PB) and without (control) piperonyl butoxide (n=4).

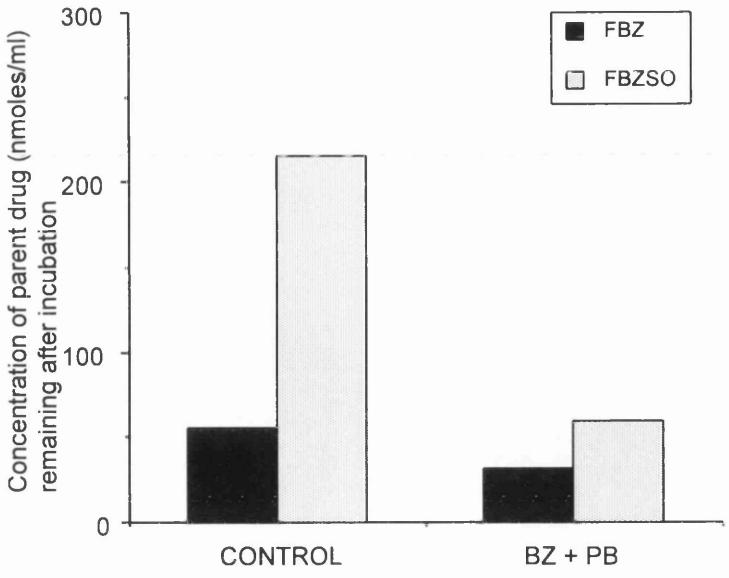

Figure 3.3 Amount of unchanged drug remaining in the microsomal reaction mixture after incubation of BZ (25μM) with (BZ + PB) and without (control) piperonyl butoxide (n=4).

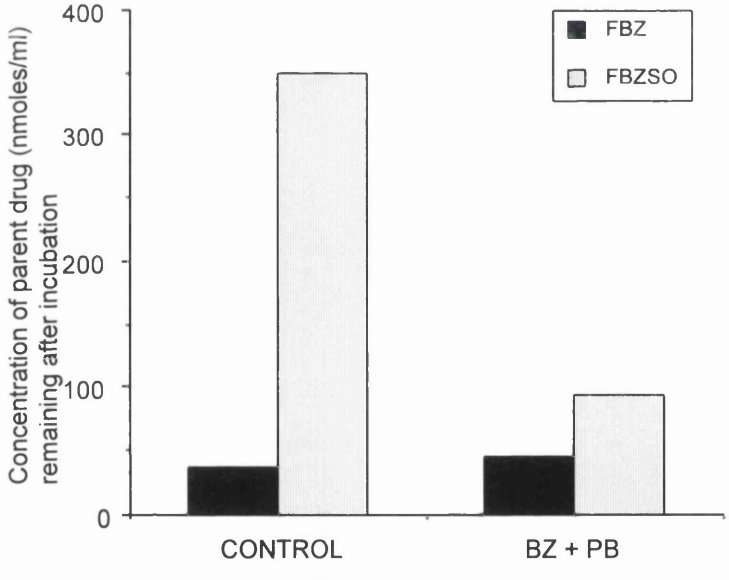

Figure 3.4 Amount of unchanged drug remaining in the microsomal reaction mixture after incubation of BZ (50μM) with (BZ + PB) and without (control) piperonyl butoxide (n=4).

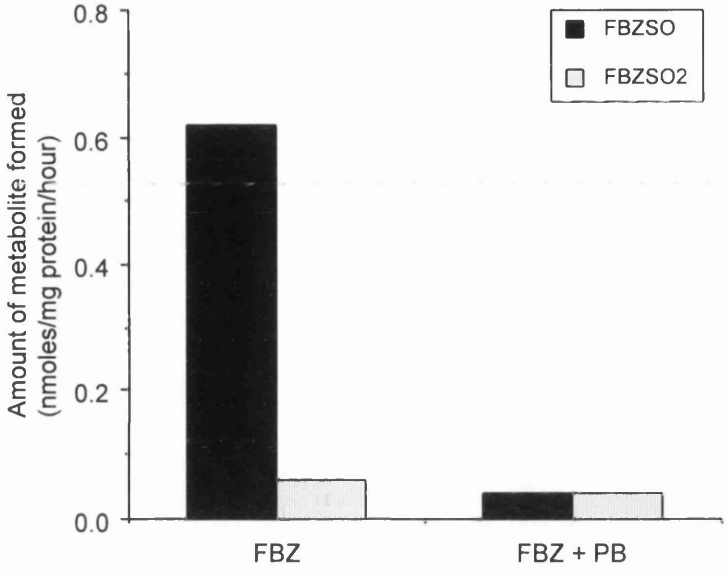

Figure 3.5 Effect of piperonyl butoxide (PB) (50μM) on microsomal fenbendazole (5μM) metabolism in equine liver

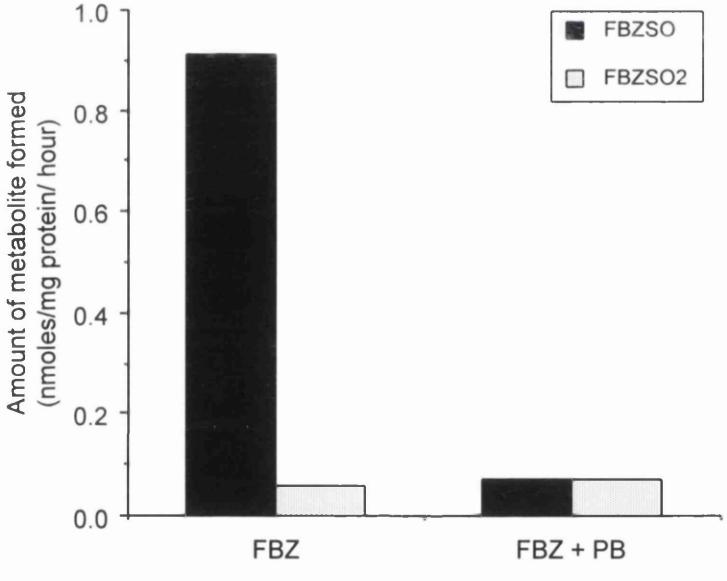

Figure 3.6 Effect of PB (50μM) on microsomal fenbendazole (10μM) metabolism in equine liver (n=4).

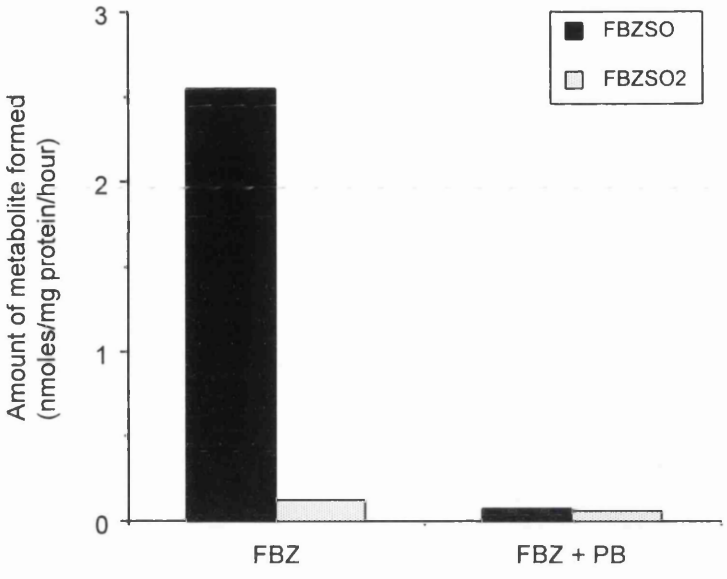

Figure 3.7 Effect of PB (50μM) on microsomal fenbendazole (25μM) metabolism in equine liver (n=4).

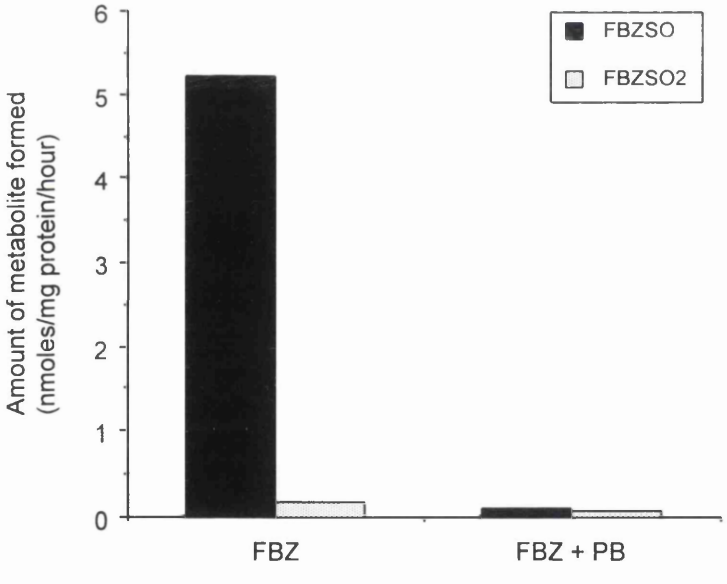

Figure 3.8 Effect of PB (50μM) on microsomal fenbendazole (50μM) metabolism in equine liver (n=4).

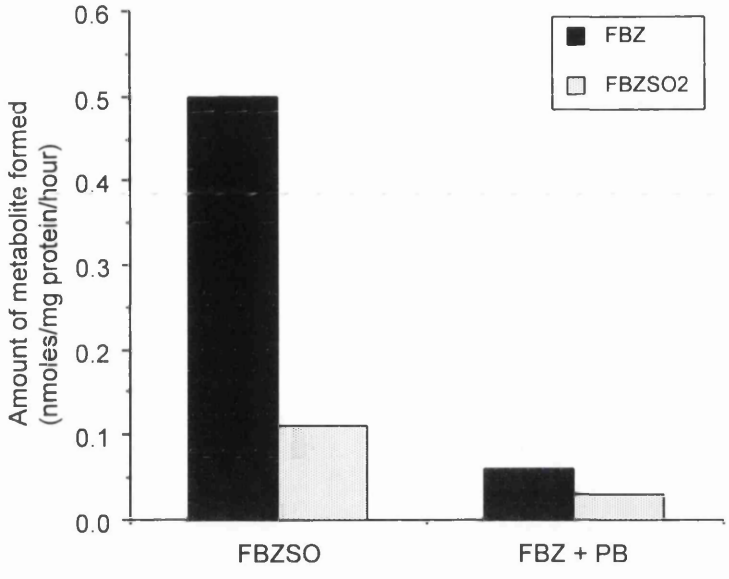

Figure 3.9 Effect of PB (50μM) on microsomal fenbendazole sulphoxide (5μM) metabolism in equine liver (n=3).

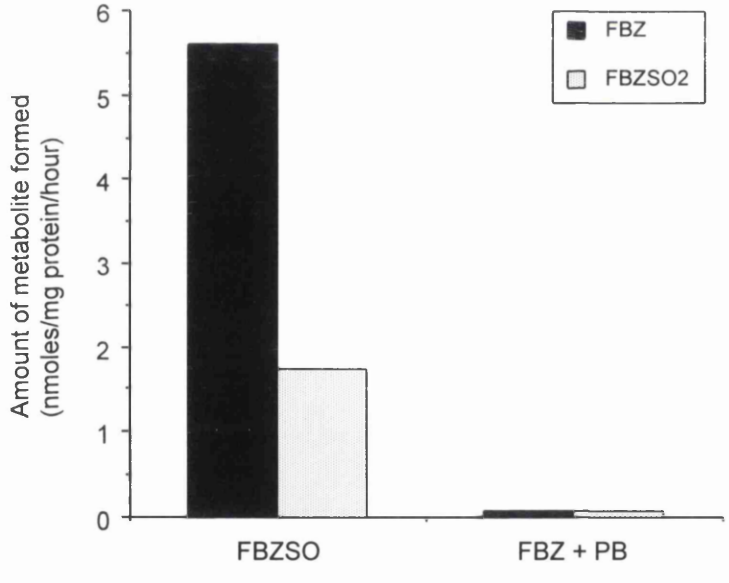

Figure 3.10 Effect of PB (50μM) on microsomal fenbendazole sulphoxide (10μM) metabolism in equine liver (n=4).

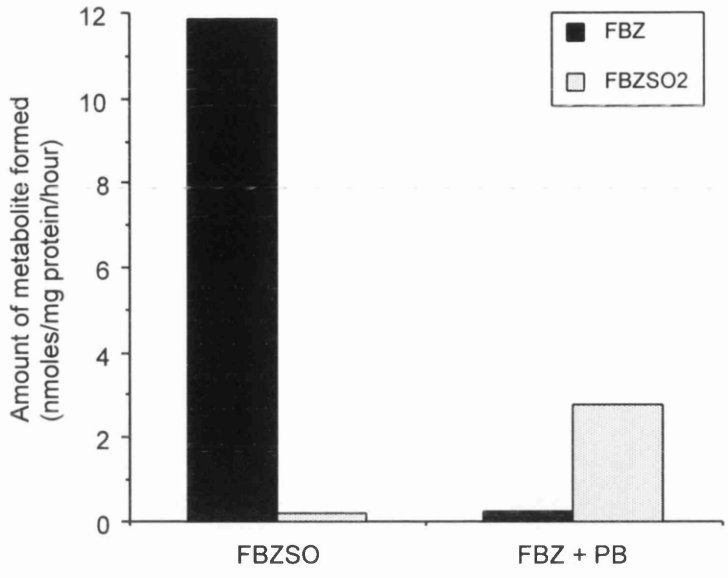

Figure 3.11 Effect of PB (50μM) on microsomal fenbendazole sulphoxide (25μM) metabolism in equine liver (n=4).

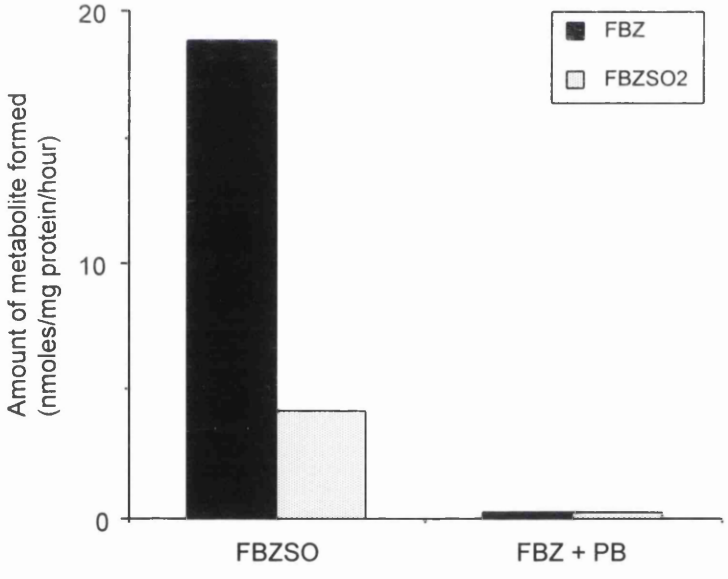

Figure 3.12 Effect of PB (50μM) on microsomal fenbendazole sulphoxide (50μM) metabolism in equine liver (n=4).

3.6 Discussion

Fenbendazole is known to be extensively metabolised *in vivo* to it's sulphoxide, sulphone and hydroxylated metabolites (Short *et al.*,1988). *In vivo* studies conducted with fenbendazole do not quite achieve the extensive metabolism observed in the *in vivo* studies in which only a small fraction of the parent compound remains in the blood. Studies done using rat hepatic microsomes yielded only 16% conversion of fenbendazole into metabolites (Benchaoui and McKellar, 1996). In the present study, the metabolism of fenbendazole was found to be variable with different substrate concentrations and individuals; substrate: PB ratios of 1:5 and 1:1 produced the highest degree of metabolism (30.7 and 25.7%, respectively). The present *in vitro* study was complicated by the large interindividual variations in results. Microsomal protein concentrations (mg) per gram of liver were below the usual 20mg/g liver (Gibson and Skett, 1994) found with other species and this could have affected the extent and rate of metabolism of the parent drugs. The large interindividual variations could be due to one, or several of, the many factors that may affect drug metabolism. For instance, the liver is a heterogenous organ, with differences in drug metabolism between the centrilobular and periportal areas (Gibson and Skett, 1994), and the section from which the sample is extracted may be of relevance in drug metabolism studies. In addition, there was inadequate knowledge about

the clinical history (e.g. age, whether diseased or not, and previous drug treatment) of the horses used in the present study.

The cytochrome P450 inhibitor, piperonyl butoxide, decreased the extent of metabolism of fenbendazole. Sulphoxidation was observed to have been drastically reduced with the addition of the inhibitor and this may explain the lower amounts of sulphone formed at least with the higher sulphide substrate concentrations. It is not clear why the lower concentrations lead to increased expression of sulphonation as the sulphone: sulphoxide ratio in all cases following addition of PB was found to have increased, indicating that PB affected both sulphoxidation and sulphonation. The average sulphone to sulphoxide ratio was 0.045 before and 1.275 after addition of PB. Incubation of hepatic microsomes with fenbendazole sulphoxide revealed a reduction in sulphonation following co-administration with PB indicating that the lower sulphone metabolite concentrations produced were not a result of lower sulphoxide concentrations.

In the present *in vitro* study, piperonyl butoxide reduced the extent of metabolism of fenbendazole by up to 38.4% and that of oxfendazole by over 20%. Even though, the results of *in vitro* cannot be adequately extrapolated to the results of *in vivo* studies, similar changes in metabolic inhibition would be anticipated. The *in vivo* studies reported in chapter 2

revealed much greater changes in the metabolic inhibition by piperonyl butoxide than the changes observed with the *in vitro* studies.

In comparison to the FMO inhibitor, methimazole (MTZ), greater metabolic inhibition *in vivo* appears to have been achieved with piperonyl butoxide. *In vivo* studies carried out in sheep by Lanusse *et al.* (1995) with MTZ showed less metabolic inhibition of fenbendazole parent drug relative to the *in vivo* studies in horses reported in chapter 2. The FBZ parent drug AUC value was only increased by 113% with MTZ as compared to the ten-fold increase with PB. The ratio of the AUC values (FBZ: FBZSO) was slightly increased (from 0.12 to 0.16) with MTZ administration in sheep as compared to a much larger increase (from 0.17 to 4.28) with piperonyl butoxide in horses. However, the fact that different species were used needs to be considered since wide differences in the rate of xenobiotic metabolism exist between horses and sheep. Horses have a more rapid rate of metabolism of some drugs than sheep. In addition to the species differences, a direct comparison is difficult to make due to the different dosage regimens administered- 5mg/kg FBZ and 3mg/kg MTZ in sheep and 10mg/kg FBZ and 63mg/kg PB in horses. It may be that the greater metabolic inhibition observed with PB in horses than that observed with MTZ in sheep was due, or at least partially, to the higher proportion of the inhibitor relative to the benzimidazole. Methimazole had little effect on the sulphoxide metabolite pharmacokinetics, whereas PB caused a significant decrease in the FBZSO AUC and C_{max} values in the unfed horses and an

increase of the same parameters in the fed horses. Piperonyl butoxide had little effect on the sulphone metabolite AUC and MRT values, and both inhibitors delayed the t_{max} values of the sulphone metabolite. The observed differences with the two inhibitors may also be explained by the difference in the hepatic enzyme contributions in the fenbendazole oxidative steps in the two species. Furthermore, MTZ is a substrate for the FMO system and competition with FBZ for binding sites on the enzyme system may be responsible for changes observed in the disposition kinetics of the sulphide and sulphoxide metabolites, as was seen with albendazole and albendazole sulphoxide in ruminants (Lanusse and Prichard, 1992a, b). In ruminants, MTZ is most likely to affect sulphoxidation, since the FMO system is the predominant system in these species (Delatour *et al.*, 1994). However, the involvement of MTZ with cytochrome P450 which is mostly responsible for sulphonation in ruminants, has also been demonstrated (Kedderis and Rickert, 1985). Disposition of the sulphone metabolite is, however, dependent on it's rate of formation and changes in the sulphoxide disposition induced by MTZ-impairment of the FMO system (Lanusse and Prichard, 1992a). The mean residence times and bioavailability of the inhibitors in plasma. Methimazole has a high bioavailability and a long half-life in plasma (Jansson *et al.*, 1985). The MRT of PB administered orally in sheep was 38.78 hours, with an AUC value of 649.8μg.h/ml (Benchaoui, 1994). There is currently no published literature on the pharmacokinetics of PB in the horse.

In conclusion, piperonyl butoxide was found to inhibit the S-oxidation of fenbendazole *in vitro* using equine liver microsomes and this finding may have important applications *in vivo* when aiming to enhance the efficacy of fenbendazole.

Chapter 4

General discussion

Feeding in the horse was demonstrated to affect the disposition kinetics of fenbendazole and its metabolites. Feeding principally slows the gut transit time which generally increases the absorption of drugs (Van Miert, 1982). It was difficult to make a simple assessment of the rate of absorption from the t_{max} and C_{max} values alone of fenbendazole in this study. A good estimate of the rate of absorption is given by the absorption half-life ($t_{1/2(a)}$ = 0.693ka) and the mean absorption time (MAT =$1/k_a$) based on the absorption rate constant (k_a). Unfortunately this could not be determined in this study because it required intravenous administration. Intravenous administration of fenbendazole, even in dimethylsulphoxide solutions is not possible in the horse because of poor tolerance, at least at therapeutic dose rates (Q.A. McKellar, unpublished data). Absorption half-life is given by 0.693 X MAT , which is based on the mean absorption time (Baggot, 1992). Absorption approximates a first-order process (i.e. the amount of drug administered is proportional to the amount absorbed in unit time) for most drugs. In comparison to unfed horses, the extent of absorption (bioavailability) of fenbendazole in fed horses was increased. However, the total bioavailability of both active compounds (fenbendazole and fenbendazole sulphoxide) was decreased by 50%. The sulphoxide in fed animals would not make much contribution to the anthelmintic effect as its concentrations were not only lowered but its MRT was also reduced (from 22.94 to 7.53 hours). The duration of residence of the inactive metabolite, fenbendazole sulphone, was extended following feeding. The time to C_{max}

for fenbendazole was increased from 3 to 6.5 hours probably due to a reduced rate of absorption.

Feeding, or the time of feeding, before or after oral administration of drugs in the horse has also been shown to affect the pharmacokinetics of several other drugs. Bogan *et al.*, (1984) showed that the mean peak plasma concentration and the systemic availability of trimethoprim was decreased following administration of trimethoprim-sulphadiazine oral paste after feeding. Varying the feeding schedules in ponies together with administration of phenylbutazone (aqueous suspension of a granular formulation) produced different t_{max} values (Maitho *et al.*, 1986); feeding delayed phenylbutazone absorption and yielded a non-statistically significant decrease in systemic availability. Post-prandial oral administration, as compared to pre-prandial administration of rifampin (5mg/kg) to horses decreased the systemic availability of the drug, but did not affect the rate of absorption (Baggot, 1992).

A number of studies have been carried out to improve the bioavailability and pharmacokinetic profiles of benzimidazole drugs and their active metabolites by combining them with possible potentiators. The bioavailability and anthelmintic efficacy of oxfendazole was improved when co-administered with another benzimidazole, parbendazole (Hennessy *et al.*, 1985). Parbendazole is believed to potentiate the action of oxfendazole by inhibiting its metabolism and excretion. In humans, the

co-administration of cimetidine with albendazole (Wen *et al.*, 1994) and mebendazole (Bekhti and Pirotte, 1987) has been shown to improve the clinical efficacy of the benzimidazoles against hepatic hydatid cysts by inhibiting metabolism, and thus the inactivation of the benzimidazoles. Methimazole is a flavin monooxygenase inhibitor and substrate, which has been shown to improve the bioavailability and disposition kinetics of albendazole sulphoxide after co-administration with the benzimidazole prodrug, netobimin, in sheep (Lanusse and Prichard, 1992a) and cattle (Lanusse and Prichard, 1992b). Piperonyl butoxide, an inhibitor of cytochrome P450, enhances the pharmacokinetic profile and anthelmintic efficacy of the benzimidazole, fenbendazole, in sheep and goats (Benchaoui and McKellar, 1996). Piperonyl butoxide is also used as a synergist for the ectoparasiticides, pyrethrum, pyrethroids and rotenone. Piperonyl butoxide was observed to improve the plasma concentration of fenbendazole in the horse (see Chapter 2).

The concomitant administration of fenbendazole with piperonyl butoxide in the horse increased the AUC of FBZ by 10 times compared to that observed when fenbendazole was administered alone. The plasma concentration of the sulphoxide metabolite was only increased when the horses were also fed prior to treatment with the fenbendazole-piperonyl butoxide combination. This metabolic inhibitor also caused the time to mean peak plasma concentrations to be delayed. The fenbendazole/fenbendazole sulphoxide ratio (based on AUCs) was

increased from 0.17 to 4.28 following administration of piperonyl butoxide in the unfed horses whereas it remained more or less constant in the fed horses. Piperonyl butoxide effect on fenbendazole metabolism in equine hepatic microsomes was also investigated. The extent of metabolism of fenbendazole was reduced leaving smaller amounts of the metabolites. These results, if extrapolated to anticipated *in vivo* results, would indicate that the plasma concentration of fenbendazole would be increased with inhibition of metabolism.

The anthelmintic efficacy of the fenbendazole-piperonyl butoxide combination treatment in horses with helminthiosis has yet to be investigated. From the studies carried out *in vivo* and *in vitro* described in this thesis, it is most likely that the administration of fenbendazole in combination with the inhibitor will result in improved anthelmintic efficacy. The pharmacokinetics and metabolism of fenbendazole and its metabolites could be markedly altered during helminthiosis in the horse, and this could affect the expected drug efficacy. The bioavailability of fenbendazole has been shown to decrease during helminth infection in sheep (Marriner *et al.*, 1985) and goats (Bogan *et al.*, 1987). Helminthiosis can cause elevated gastric pH (Mostafa and McKellar *et al.*, 1989) and hyperplastic changes in the abomasal mucosal mass (Anderson *et al.*, 1988) which could affect the absorption and ionic-trap-mediated distribution of fenbendazole and its metabolites. Elevated pH causes reduced dissolution of fenbendazole in the GI tract and this would

contribute to reduced bioavailability. Helminth disease also affects gut transit times, which can influence the rate and extent of drug absorption. In addition, parasitism may cause altered drug metabolism and elimination if the liver is damaged. The interaction of helminthiosis, feed and piperonyl butoxide has not been investigated and even if there is some interaction it is not anticipated that parasitism will alter the overall conclusions reached in the present study with regard to food and metabolic inhibition.

The efficacy of both fenbendazole and fenbendazole sulphoxide co-administered with piperonyl butoxide was markedly improved against the benzimidazole-resistant strains of *Ostertagia circumcincta* (Benchaoui and McKellar, 1996). The efficacy of FBZ prior to PB treatment was only 8%, which was increased to 98% following co-administration with the inhibitor. The benzimidazole anthelmintic, parbendazole (PBZ) was shown to improve the efficacy of oxfendazole in sheep experimentally infected with thiabendazole-resistant *Haemonchus contortus* and *Trichostrongylus colubriformis* (Hennessy *et al.*, 1985). The improvement in efficacy of FBZ against *O. circumcincta* following FBZ-PB treatment was, however, greater than that achieved with OXF against *H. contortus* and *T.colubriformis* following potentiation of OXF with PBZ. Parbendazole has moderate anthelmintic potency but is a strong inhibitor of the polymerization of mammalian tubulin (Friedman and Platzer, 1978). By transiently binding to hepatic and kidney tubulin, PBZ reduces the rate of OXF biotransformation and also reduces elimination by decreasing the

biliary and urinary flow rates. This drug is also thought to increase anthelmintic action by increasing the extrabiliary secretion of OXF. The main limitation with the use of PBZ as a potentiator of OXF is the considerably short residence time and short time to reach mean maximum plasma concentrations of PBZ in relation to that of oxfendazole in sheep. Parbendazole improves the bioavailability, to varying degrees, of both FBZ and FBZSO, and thus anthelmintic efficacy of the anthelmintically active metabolites of FBZ and or FBZSO through altered metabolism and elimination. However, in contrast to PBZ, which causes metabolic inhibition by inhibiting microtubule assembly in mammalian hepatic or kidney cells, metabolic inhibition by PB is due to its interaction with the MFO system. This interaction results in the formation of metabolic intermediates which bind covalently to cytochrome P450 enzymes forming an inhibitory complex on enzymatic activity (Franklin, 1977). Piperonyl butoxide administered orally in sheep produces sustained plasma levels reaching a maximum concentration at 12-24 hours after administration. The mean residence time was about 39 hours and the drug was not detectable in plasma 13 days postadministration (Benchaoui, 1994). Piperonyl butoxide metabolic intermediates are probably more appropriate indicators of metabolic inhibition as they are directly involved in binding to and inhibiting cytochrome P450 enzymes. However, there is no published information on their plasma disposition in animals. Knowledge of the kinetic disposition of PB and its intermediates may be important in understanding some of the pharmacokinetic differences observed

between small ruminants and horses when benzimidazoles are administered in combination with the inhibitor. The C_{max} value of PB obtained in goats (Benchaoui, 1994) concurred with the C_{max} values of fenbendazole and oxfendazole, which range from 6 to 30 hours (see Chapter one, section 1.3.5). Thus a fairly adequate interaction of PB and benzimidazoles would be expected in goats. In horses, the pharmacokinetics of PB and its intermediates may be considerably different due to the rapid first pass effect in this species, which may result in fast elimination of PB from plasma with subsequently rapid formation of the PB intermediates which bind to cytochrome P450. If this assumption is correct, it may explain, at least in part, the greater metabolic inhibition observed in horses (see chapter 2) compared to sheep and goats (Benchaoui and McKellar, 1996).

Two other compounds (cimetidine and methimazole) used in the potentiation of benzimidazoles also showed significant metabolic inhibition with improved bioavailability of the benzimidazole active moieties, and in the case of cimetidine anthelmintic efficacy was also improved. The simultaneous treatment of human patients suffering from echinococcosis with mebendazole and the enzyme inhibitor, cimetidine, resulted in the complete resolution of previously unresponsive hydatid cysts (Bekhti and Pirotte, 1987). Cimetidine improved the serum concentrations of MBZ by reducing its biotransformation rate, and this resulted in improved therapeutic effect. The therapeutic effect in this case was determined by

reference to the change in the extent of $^{14}CO_2$ specific activity (SA) at 1 hour , which was reduced from 0.89 ± 0.32 to 0.57 ± 0.17% of the administered dose/kg. The antithyroid compound, methimazole, has been shown to inhibit microsomal oxidation of different xenobiotics (Tynes and Hodgson, 1983), and when co-administered with the benzimidazole prodrug, netobimin, improved the disposition kinetics of the active moieties (Lanusse and Prichard, 1992a, b; Lanusse *et al.*, 1995). However, the impact that these anthelmintically active metabolites with altered pharmacokinetics will have on parasites has yet to be determined. For the benzimidazole anthelmintics, effective 'toxic' concentrations in plasma must be presented to the parasite for a sufficient time to lead to irreversible damage (Lanusse and Prichard, 1993b). The basic cellular functions of the parasite are disrupted progressively with continued exposure to the active drug as a result of depolymerization of microtubules. The parasite may survive insufficiently sustained concentrations of the active drug. The period of time the parasite is exposed to the active drug concentrations is more important than the high peak concentrations that are followed by fast elimination (Lanusse and Prichard, 1992b). The *in vivo* studies on the interaction of feed, FBZ and PB in horses reported in chapter 2 showed that the MRT of FBZ was extended (from 11.68 to 17.51 hours) in unfed horses with mean maximum concentrations occurring at 17 hours. In the fed horses the MRT of FBZ was only extended by about one hour with t_{max} value at 13.6 hours. The co-administration of FBZ with the inhibitor slightly decreased the MRT

of the sulphoxide metabolite in unfed horses, but this was approximately doubled (from 7.53 to 13.56 hours) in the fed horses. The t_{max} values of the active metabolites/drugs occurred at about the same times in the fed horses, as was also the case in the unfed horses even though they occurred at an earlier time than in the unfed horses. In unfed horses, it was observed that the t_{max} value of FBZSO occurred at a considerably longer time (at 22.67 hours) than the t_{max} values of FBZSO (fed horses) and FBZ (fed or unfed horses), which ranged from 3 to 6.5 hours after co-administration of fenbendazole and PB. The reason why the MRT of FBZSO in unfed horses was not extended with concomitant administration of PB could be due to the disposition kinetics of PB and/ or intermediates in the plasma. It may be that PB is rapidly excreted and diminishing amounts with time in the plasma are insufficient to maintain metabolic inhibition of sulphonation. The MRT of FBZSO, even though not reduced with PB adiministration, is relatively high at 22.67 hours, and PB may have a much shorter MRT in the horse. Furthermore, PB is known to cause metabolic induction from 24-72 hours after administration (Skrinjaric-Spoljar *et al.*, 1971), and this may explain why the FBZSO MRT is not further extended.

The prolongation of the residence times of the metabolites in plasma by metabolic inhibition with PB may also increase the level of the anthelmintic residues in various tissues of the animal, which may jeopardise regulatory requirements. Obtaining approval from regulatory authorities for drug

combinations is difficult. Further research is required to determine the change in the amounts of anthelmintic residues in animal tissues caused by use of PB as a metabolic inhibitor. This is particularly important in animals that are used for food for human consumption. Piperonyl butoxide may also be widely distributed in animal tissues, and there is need to investigate its level of toxicity in ruminants and horses.

Considering the tremendous level of metabolic inhibition of FBZ when co-administered with PB after feeding, and the consequent considerable improvement in the disposition of the anthelmintically active drugs, this drug combination is most likely to be practical in anthelmintic chemotherapy. Furthermore, PB is a relatively inexpensive compound and its simultaneous use with benzimidazoles will be of economic benefit in terms of endoparasite control. A better assessment of the practicality of the combination would be better made after further research, e.g. anthelmintic efficacy studies and appropriate FBZ-PB dosage regimens that produce optimum efficacy at a lower cost.

In conclusion, fenbendazole should be administered without food in the horse, as this will cause increased bioavailability of active moieties and improve expected anthelmintic efficacy. The effect of piperonyl butoxide, whether administered with or without food, on fenbendazole disposition and kinetics was to increase its bioavailability.

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Appendix A

A-1 Recovery and coefficients of variation for fenbendazole metabolites following liquid phase extraction from plasma.

	Added Concentration (μg/ml)	Recovery (%)	Between-assay coefficient of variation (%)
FBZ	0.05	88.59 (n=6)	10.22
	0.10	82.02 (n=6)	13.24
	0.25	83.35 (n=6)	16.79
	0.50	81.14 (n=6)	11.73
	1.00	83.33 (n=6)	13.42
	Mean	83.68 (n=30)	13.08
FBZSO	0.05	93.60 (n=6)	14.96
	0.10	86.57 (n=6)	10.64
	0.25	92.85 (n=6)	10.50
	0.50	89.84 (n=6)	10.48
	1.00	90.55 (n=6)	10.72
	Mean	90.68 (n=30)	11.46
$FBZSO_2$	0.05	96.10 (n=6)	10.74
	0.10	92.26 (n=6)	10.94
	0.25	96.97 (n=6)	13.00
	0.50	90.50 (n=6)	8.72
	1.00	90.26 (n=6)	8.97
	Mean	93.22 (n=30)	10.47

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

A-2 Plasma concentrations of fenbendazole (FBZ)(μg/ml) following adminstration of fenbendazole (10mg/kg) alone with food in horses.

	Animal number					
Time(h)	1	2	3	4	5	6
0	0.000	0.000	0.000	0.000	0.000	0.000
1	0.016	0.029	0.024	0.000	0.030	0.019
2	0.017	0.037	0.037	0.004	0.033	0.036
3	0.013	0.016	0.023	0.004	0.078	0.021
4	0.000	0.056	0.038	0.008	0.032	0.019
6	0.000	0.016	0.028	0.013	0.041	0.023
8	ND	0.016	0.043	0.006	0.029	0.023
12	0.000	0.013	0.044	0.005	0.019	0.024
24	0.032	0.012	0.031	0.000	0.009	0.011
32	0.000	0.000	0.000	0.000	0.000	0.000
48	0.027	0.000	0.000	0.000	0.000	0.000
72	0.000	0.000	0.000	0.003	0.000	0.000
96	0.000	0.000	0.000	0.003	0.000	0.000

A-3 Pharmacokinetic parameters of fenbendazole (FBZ)(μg/ml) following adminstration of fenbendazole (10mg/kg) alone with food in horses.

	Animal number					
	1	2	3	4	5	6
AUC_{obs}·(μg.h/ml)	0.23	0.42	0.82	0.15	0.58	0.43
$AUMC_{obs}$ (μg.h/ml)	4.41	3.65	9.88	1.38	5.07	4.36
MRT (h)	19.17	8.69	12.05	9.52	8.82	10.14
C_{max} (μg/ml)	0.03	0.06	0.04	0.01	0.08	0.04
t_{max} (h)	24	4	2	4	3	2

A-4 Plasma concentrations of fenbendazole (FBZ)(μg/ml) following adminstration of fenbendazole (10mg/kg) alone without food in horses.

	Animal number					
Time(h)	1	2	3	4	5	6
0	0.000	0.000	0.010	0.000	0.000	0.000
1	0.023	0.000	0.036	0.009	0.000	0.000
2	0.027	0.000	0.039	0.010	0.017	0.000
3	0.031	0.000	0.040	0.000	0.014	0.000
4	0.018	0.000	0.041	0.008	0.000	0.000
6	0.024	0.000	0.029	0.006	0.011	0.000
8	0.023	0.000	0.029	0.009	0.000	0.000
12	0.000	0.000	0.019	0.013	0.000	0.022
24	0.018	0.000	0.010	0.012	0.000	0.017
32	0.000	0.000	0.008	0.008	0.000	0.000
48	0.000	0.000	0.000	0.000	0.000	0.000
72	0.000	0.000	0.000	0.000	0.000	0.000
96	0.000	0.000	0.009	0.000	0.000	0.029

A-5 Pharmacokinetic parameters of fenbendazole (FBZ)(μg/ml) following adminstration of fenbendazole (10mg/kg) alone without food in horses.

	Animal number					
	1	2	3	4	5	6
$AUC_{obs.}$ (μg.h/ml)	0.41	0.00	0.72	0.39	0.05	0.36
$AUMC_{obs.}$(μg.h/ml)	5.81	0.00	9.72	7.65	0.19	6.72
MRT (h)	14.17	0.00	13.59	19.87	3.80	18.67
C_{max} (μg/ml)	0.03	0.00	0.04	0.01	0.02	0.02
t_{max} (h)	2.00	0.00	1.00	1.00	2.00	12.00

A-6 Plasma concentrations of fenbendazole (FBZ)(μg/ml) following adminstration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) with food in horses.

	Animal number				
Time(h)	1	3	4	5	6
0	0.000	0.005	0.000	0.000	0.000
1	0.029	0.043	0.050	0.155	0.139
2	0.036	0.065	0.078	0.191	0.218
3	0.048	0.066	0.089	0.275	0.207
4	0.053	0.327	0.161	0.508	0.250
6	0.049	0.059	0.165	0.372	0.256
8	0.069	0.052	0.159	0.656	0.553
12	0.076	0.018	0.196	0.649	0.451
24	0.088	0.005	0.231	0.137	0.323
32	0.018	0.000	0.008	0.000	0.000
48	0.000	0.000	0.049	0.000	0.128
72	0.000	0.000	0.000	0.000	0.000
96	0.000	0.000	0.000	0.000	0.000

A-7 Pharmacokinetic parameters of fenbendazole (FBZ)(μg/ml) following adminstration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) with food in horses.

	Animal number				
	1	3	4	5	6
$AUC_{obs.}$ (μg.h/ml)	2.125	1.105	5.22	10.71	9.915
$AUMC_{obs.}$(μg.h/ml)	34.68	6.21	83	120.7	139
MRT (h)	16.32	5.62	15.9	11.27	14.02
C_{max} (μg/ml)	0.09	0.33	0.23	0.66	0.55
t_{max} (h)	24	4	24	8	8

A-8 Plasma concentrations of fenbendazole (FBZ)(μg/ml) following adminstration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) without food in horses.

	Animal number					
Time(h)	1	2	3	4	5	6
0	0.00	0.00	0.00	0.00	0.00	0.01
1	0.01	0.00	0.02	0.03	0.04	0.06
2	0.01	0.00	0.04	0.09	0.10	0.08
3	0.02	0.00	0.04	0.14	0.13	0.08
4	0.02	0.02	0.04	0.13	0.13	0.07
6	0.02	0.03	0.05	0.16	0.17	0.07
8	0.05	0.04	0.08	0.15	0.14	0.07
12	0.13	0.10	0.14	0.21	0.14	0.10
24	0.20	0.12	0.12	0.19	0.04	0.13
32	0.04	0.03	0.06	0.04	0.00	0.03
48	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
96	0.00	0.00	0.00	0.00	0.00	0.00

A-9 Pharmacokinetic parameters of fenbendazole (FBZ)(μg/ml) following adminstration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) without food in horses.

	Animal number					
	1	2	3	4	5	6
$AUC_{obs.}$ (μg.h/ml)	3.78	2.57	3.54	5.28	2.74	3.14
$AUMC_{obs.}$(μg.h/ml)	77.49	51.36	68.26	88.05	29.85	55.72
MRT (h)	20.50	19.98	19.28	16.66	10.87	17.74
C_{max} (μg/ml)	0.20	0.12	0.14	0.21	0.17	0.13
t_{max} (h)	24.00	24.00	12.00	12.00	6.00	24.00

A-10 Plasma concentrations of fenbendazole suphoxide (FBZSO)(μg/ml) following adminstration of fenbendazole (10mg/kg) alone with food in horses.

	Animal number					
Time(h)	1	2	3	4	5	6
0	0.000	0.000	0.000	0.000	0.008	0.000
1	0.003	0.004	0.000	0.000	0.009	0.000
2	0.004	0.006	0.011	0.004	0.010	0.005
3	0.003	0.006	0.006	0.004	0.020	0.004
4	0.016	0.015	0.010	0.008	0.011	0.002
6	0.004	0.005	0.002	0.013	0.024	0.000
8	ND	0.008	0.008	0.006	0.025	0.004
12	0.005	0.005	0.006	0.005	0.030	0.002
24	0.004	0.000	0.000	0.000	0.000	0.000
32	0.002	0.000	0.000	0.000	0.012	0.000
48	0.005	0.000	0.000	0.000	0.000	0.003
72	0.000	0.000	0.000	0.003	0.000	0.000
96	0.016	0.000	0.000	0.003	0.000	0.000

A-11 Pharmacokinetic parameters of fenbendazole suphoxide (FBZSO) (μg/ml) following adminstration of fenbendazole (10mg/kg) alone with food in horses.

	Animal number					
	1	2	3	4	5	6
$AUC_{obs.}$ (μg.h/ml)	0.11	0.09	0.15	0.15	0.43	0.01
$AUMC_{obs.}$(μg.h/ml)	1.08	0.47	1.31	1.38	3.99	0.02
MRT (h)	9.82	5.53	9.03	9.52	9.28	2.00
C_{max} (μg/ml)	0.02	0.01	0.01	0.01	0.03	0.01
t_{max} (h)	4	2	2	4	8	2

A-12 Plasma concentrations of fenbendazole sulphoxide (FBZSO)(μg/ml) following adminstration of fenbendazole (10mg/kg) alone without food in horses.

	Animal number					
Time(h)	1	2	3	4	5	6
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
3	0.00	0.00	0.04	0.00	0.02	0.00
4	0.00	0.00	0.04	0.00	0.02	0.00
6	0.00	0.00	0.04	0.00	0.02	0.00
8	0.00	0.01	0.04	0.00	0.03	0.00
12	0.06	0.04	0.04	0.00	0.03	0.00
24	0.05	0.05	0.05	0.00	0.05	0.00
32	0.07	0.05	0.04	0.00	0.06	0.00
48	0.06	0.06	0.04	0.00	0.05	0.00
72	0.00	0.00	0.00	0.00	0.02	0.00
96	0.00	0.00	0.00	0.00	0.00	0.00

A-13 Pharmacokinetic parameters of fenbendazole sulphoxide (FBZSO) (μg/ml) following adminstration of fenbendazole (10mg/kg) alone without food in horses.

	Animal number					
	1	2	3	4	5	6
$AUC_{obs.}$ (μg.h/ml)	3.02	2.65	2.40	0.00	3.12	0.00
$AUMC_{obs.}$(μg.h/ml)	102.24	92.88	71.40	0.00	121.62	0.00
MRT (h)	33.85	35.05	29.75	0.00	38.98	0.00
C_{max} (μg/ml)	0.07	0.06	0.05	0.00	0.06	0.00
t_{max} (h)	32	48	24	0	32	0

A-14 Plasma concentrations of fenbendazole sulphoxide (FBZSO)(μg/ml) following adminstration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) with food in horses.

	Animal number				
Time(h)	1	3	4	5	6
0	0.000	0.003	0.000	0.000	0.000
1	0.000	0.004	0.009	0.046	0.009
2	0.000	0.006	0.015	0.071	0.016
3	0.005	0.008	0.019	0.105	0.015
4	0.005	0.046	0.040	0.205	0.026
6	0.007	0.011	0.050	0.153	0.040
8	0.010	0.011	0.073	0.307	0.075
12	0.009	0.004	0.111	0.393	0.070
24	0.017	0.004	0.141	0.070	0.068
32	0.002	0.000	0.002	0.000	0.000
48	0.000	0.000	0.026	0.000	0.022
72	0.000	0.000	0.000	0.000	0.000
96	0.000	0.000	0.000	0.000	0.000

A-15 Pharmacokinetic parameters of fenbendazole sulphoxide (FBZSO) (μg/ml) following adminstration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) with food in horses.

	Animal number				
	1	3	4	5	6
$AUC_{obs.}$ (μg.h/ml)	0.34	0.15	2.69	5.60	1.65
$AUMC_{obs.}$(μg.h/ml)	6.15	0.71	46.77	65.26	25.97
MRT (h)	18.09	4.90	17.39	11.66	15.79
C_{max} (μg/ml)	0.02	0.05	0.14	0.39	0.07
t_{max} (h)	24	4	24	12	8

A-16 Plasma concentrations of fenbendazole sulphoxide (FBZSO)(μg/ml) following adminstration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) without food in horses.

	Animal number					
Time(h)	1	2	3	4	5	6
0	0.000	0.000	0.000	0.000	0.000	0.000
1	0.000	0.000	0.000	0.000	0.000	0.000
2	0.000	0.000	0.000	0.000	0.000	0.000
3	0.000	0.000	0.000	0.012	0.011	0.000
4	0.000	0.000	0.000	0.011	0.016	0.010
6	0.000	0.000	0.000	0.020	0.031	0.010
8	0.000	0.000	0.000	0.022	0.033	0.037
12	0.009	0.013	0.018	0.044	0.027	0.050
24	0.046	0.037	0.029	0.047	0.000	0.020
32	0.000	0.000	0.000	0.013	0.000	0.047
48	0.000	0.000	0.000	0.019	0.000	0.008
72	0.000	0.000	0.000	0.000	0.000	0.000
96	0.000	0.000	0.000	0.000	0.000	0.000

A-17 Pharmacokinetic parameters of fenbendazole sulphoxide (FBZSO) (μg/ml) following adminstration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) without food in horses.

	Animal number					
	1	2	3	4	5	6
AUC_{obs}. (μg.h/ml)	0.53	0.48	0.46	1.46	0.43	1.55
$AUMC_{obs}$. (μg.h/ml)	11.90	10.56	9.12	39.69	4.11	39.54
MRT (h)	22.38	22.00	19.83	27.09	9.56	25.43
C_{max} (μg/ml)	0.05	0.04	0.03	0.05	0.03	0.05
t_{max} (h)	24	24	24	24	6	12

A-18 Plasma concentrations of fenbendazole suphone ($FBZSO_2$)(μg/ml) following adminstration of fenbendazole (10mg/kg) alone with food in horses.

	Animal number					
Time(h)	1	2	3	4	5	6
0	0.000	0.000	0.000	0.005	0.015	0.006
1	0.013	0.029	0.009	0.012	0.039	0.037
2	0.037	0.065	0.036	0.021	0.054	0.014
3	0.037	0.063	0.028	0.027	0.077	0.019
4	0.067	0.138	0.054	0.038	0.066	0.009
6	0.018	0.056	0.045	0.059	0.119	0.015
8	ND	0.038	0.079	0.057	0.095	0.019
12	0.038	0.048	0.054	0.033	0.087	0.024
24	0.033	0.019	0.030	0.017	0.036	0.029
32	0.009	0.007	0.008	0.021	0.021	0.029
48	0.061	0.012	0.015	0.013	0.017	0.004
72	0.006	0.000	0.000	0.008	0.004	0.006
96	0.009	0.010	0.000	0.003	0.025	0.006

A-19 Pharmacokinetic parameters of fenbendazole suphone ($FBZSO_2$) (μg/ml) following adminstration of fenbendazole (10mg/kg) alone with food in horses.

	Animal number					
	1	2	3	4	5	6
$AUC_{obs.}$ (μg.h/ml)	1.55	1.41	1.40	1.18	2.32	0.99
$AUMC_{obs.}$(μg.h/ml)	39.51	20.15	22.68	21.74	38.00	21.42
MRT (h)	25.57	14.29	16.26	18.42	16.41	21.64
C_{max} (μg/ml)	0.07	0.14	0.08	0.06	0.12	0.04
t_{max} (h)	4	4	8	6	6	1

A-20 Plasma concentrations of fenbendazole ($FBZSO_2$)(μg/ml) following adminstration of fenbendazole (10mg/kg) alone without food in horses.

	Animal number					
Time(h)	1	2	3	4	5	6
0	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	0.03	0.02	0.07	0.00	0.01	0.00
3	0.04	0.02	0.10	0.00	0.02	0.00
4	0.03	0.02	0.11	0.00	0.02	0.00
6	0.03	0.01	0.11	0.01	0.03	0.00
8	0.04	0.01	0.10	0.02	0.02	0.00
12	0.02	0.02	0.05	0.00	0.04	0.00
24	0.02	0.01	0.02	0.03	0.04	0.00
32	0.01	0.00	0.02	0.03	0.03	0.00
48	0.00	0.00	0.03	0.01	0.02	0.01
72	0.00	0.00	0.03	0.01	0.00	0.00
96	0.00	0.00	0.02	0.00	0.00	0.00

A-21 Pharmacokinetic parameters of fenbendazole ($FBZSO_2$)(μg/ml) following adminstration of fenbendazole (10mg/kg) alone without food in horses.

	Animal number					
	1	2	3	4	5	6
$AUC_{obs.}$ (μg.h/ml)	0.79	0.39	3.50	1.18	1.66	0.20
$AUMC_{obs.}$(μg.h/ml)	12.25	4.91	148.82	46.20	45.20	9.60
MRT (h)	15.61	12.59	42.58	39.15	27.23	48.00
C_{max} (μg/ml)	0.00	0.02	0.11	0.03	0.04	0.01
t_{max} (h)	3	2	4	24	12	48

A-22 Plasma concentrations of fenbendazole ($FBZSO_2$)(μg/ml) following adminstration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) with food in horses.

	Animal number				
Time(h)	1	3	4	5	6
0	0.000	0.012	0.000	0.000	0.000
1	0.000	0.010	0.000	0.000	0.000
2	0.000	0.007	0.000	0.009	0.000
3	0.000	0.006	0.000	0.009	0.000
4	0.000	0.017	0.000	0.015	0.000
6	0.012	0.015	0.000	0.008	0.009
8	0.013	0.015	0.007	0.025	0.018
12	0.023	0.057	0.017	0.041	0.027
24	0.139	0.014	0.079	0.213	0.066
32	0.037	0.009	0.016	0.000	0.013
48	0.000	0.008	0.137	0.045	0.166
72	0.006	0.004	0.000	0.000	0.000
96	0.005	0.007	0.000	0.000	0.000

A-23 Pharmacokinetic parameters of fenbendazole ($FBZSO_2$)(μg/ml) following adminstration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) with food in horses.

	Animal number				
	1	3	4	5	6
AUC_{obs}.(μg.h/ml)	1.77	0.74	1.07	2.57	1.06
$AUMC_{obs}$.(μg.h/ml)	41.00	9.98	23.92	55.19	21.56
MRT (h)	23.16	13.58	22.36	21.52	20.34
C_{max} (μg/ml)	0.14	0.06	0.08	0.21	0.07
t_{max} (h)	24	12	24	24	24

A-24 Plasma concentrations of fenbendazole ($FBZSO_2$)(μg/ml) following adminstration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) without food in horses.

	Animal number					
Time(h)	1	2	3	4	5	6
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	0.01	0.00
3	0.00	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00	0.02	0.00
6	0.00	0.00	0.00	0.00	0.00	0.00
8	0.00	0.00	0.03	0.00	0.02	0.00
12	0.00	0.05	0.00	0.01	0.02	0.00
24	0.00	0.08	0.02	0.05	0.06	0.06
32	0.00	0.00	0.03	0.07	0.02	0.08
48	0.00	0.00	0.02	0.00	0.01	0.00
72	0.00	0.00	0.03	0.00	0.01	0.00
96	0.00	0.00	0.00	0.00	0.01	0.00

A-25 Pharmacokinetic parameters of fenbendazole ($FBZSO_2$)(μg/ml) following adminstration of fenbendazole (10mg/kg) in combination with piperonyl butoxide (PB) (63mg/kg) without food in horses.

	Animal number					
	1	2	3	4	5	6
$AUC_{obs.}$ (μg.h/ml)	0.00	1.20	1.77	1.42	1.78	1.56
$AUMC_{obs.}$(μg.h/ml)	0.00	24.00	88.08	39.84	74.54	45.12
MRT (h)	0.00	20.00	49.76	28.06	41.88	28.92
C_{max} (μg/ml)	0.00	0.08	0.03	0.07	0.06	0.08
t_{max} (h)	0	24	8	32	24	32

Appendix B

Appendix B-1. Quantities of components of the NADPH-generating cofactor solution sufficient for ten assays.

Component	Amount required per ten assays
Tris buffer, pH 7.4 , 0.1M	8.5 ml
$MgCl_2$, 0.15M	1.0 ml
Nicotinamide, 0.5M	1.0 ml
Trisodium isocitrate	40 mg
Isocitrate dehydrogenase	2 units
$NADP^+$	8 mg

N.B. (a) 1.0 ml of the above solution is usually required per assay.
(b) The components should be thoroughly mixed and dissolved prior to use.
(c) The mixed solution should not be allowed to stand for more than a few minutes, otherwise the generated NADPH will break down.

Appendix B-2. Calculated amounts of microsomal protein/suspension used.

	A	B	C	D	E	F
Microsomal protein (mg/g of liver)	5.56	2.30	1.52	6.05	3.51	5.98
Microsomal protein (mg/ml of microsome suspension)	8.82	2.55	3.38	11.20	5.48	9.06
Weight of liver used (g)	100	71.43	100	100	100	100
Final volume of microsome suspension (ml)	63	64.5	45	54	64	66
Volume of microsome suspension (ml) per 4 mg protein	0.454	1.567	1.185	0.357	0.730	0.442

Appendix B-3. Microsomal protein used in incubation of microsomes with oxfendazole in equine liver (n=4).

	A	B	C	D	Mean ± SEM
Microsomal protein (mg/g of liver)	5.56	2.30	1.52	6.05	3.86 ± 1.14

Appendix B-4. Microsomal protein used in incubation of microsomes with fenbendazole in equine liver (n=4).

	A	D	E	F	Mean ± SEM
Microsomal protein (mg/g of liver)	5.56	6.05	3.51	5.98	5.28 ± 0.60

Appendix B-5. Amount of drug and metabolites recovered (nmoles/ml) after incubation without microsomes.

	Sulphide	Sulphoxide	Sulphone
OXF (n=3)	0.00	18.36	0.26
FBZ (n=3)	55.06	0.00	0.00

Appendix B-6. Amount of oxfendazole (OXF) (nmoles/ml) remaining in the microsomal reaction mixture after incubation with OXF.

		OXF (nmoles/ml)				
	Conc. (μM)	A	B	C	D	Mean ± SEM
With PB	5 (n=3)	5.81	6.78	-	16.74	9.78 ± 3.47
	10 (n=3)	15.10	15.54	-	38.06	22.90 ± 6.56
	25 (n=4)	42.83	42.53	27.30	125.89	59.64 ± 23.53
	50 (n=4)	64.85	64.38	59.87	189.34	94.61 ± 34.59
Without PB	5 (n=4)	6.52	6.75	9.46	17.84	10.14 ± 3.23
	10 (n=3)	13.73	13.2	-	26.80	17.91 ± 4.45
	25 (n=3)	31.97	32.21	-	77.35	47.18 ± 27.24
	50	-	-	-	-	-

Appendix B-7. Amount of fenbendazole (FBZ) (nmoles/ml) remaining in the microsomal reaction mixture after incubation with FBZ.

		FBZ (nmoles/ml) (n=4)				
	Conc. (μM)	A	D	E	F	Mean ± SEM
With PB	5	5.38	5.51	9.22	5.18	6.32 ± 1.64
	10	8.39	8.62	20.11	7.89	11.25 ± 3.59
	25	25.33	26.16	50.02	23.66	31.29 ± 8.85
	50	38.39	39.79	58.64	42.00	44.70 ± 10.79
Without PB	5	7.22	0.67	6.01	3.91	4.45 ± 1.59
	10	9.02	1.34	8.69	8.69	6.93 ± 2.24
	25 (n=3)	44.24	2.64	-	25.46	24.11 ± 12.03
	50	35.52	3.51	72.10	37.45	37.15 ± 14.71

Appendix B-8. Amount of fenbendazole sulphone ($OXFSO_2$) (nmoles/mg protein/hour) formed in microsomal reaction mixture after incubation of oxfendazole with or without piperonyl butoxide (PB) in equine liver.

		$OXFSO_2$ formed (nmoles/mg protein/hour) (n=4)				
	Conc. (μM)	A	B	C	D	Mean ± SEM
With PB	5 (n=3)	0.02	0.03	-	0.03	0.03 ± 0.01
	10 (n=3)	0.10	0.05	-	0.06	0.07 ± 0.02
	25	0.13	0.18	0.22	0.21	0.18 ± 0.04
	50	0.14	0.29	0.29	0.30	0.26 ± 0.07
Without PB	5	0.18	0.04	0.18	0.05	0.11 ± 0.04
	10	0.19	0.07	6.65	0.07	1.75 ± 1.47
	25	0.15	0.20	10.47	0.20	2.76 ± 2.31
	50	0.08	0.01	16.43	0.35	4.22 ± 3.65

Appendix B-9. Amount of fenbendazole (FBZ) (nmoles/mg protein/hour) formed in microsomal reaction mixture after incubation of oxfendazole with or without piperonyl butoxide (PB) in equine liver.

		FBZ formed (nmoles/mg protein/hour) (n=4)				
	Conc. (μM)	A	B	C	D	Mean ± SEM
With PB	5 (n=3)	0.02	0.05	-	0.11	0.06 ± 0.03
	10 (n=3)	0.02	0.07	-	0.16	0.08 ± 0.04
	25	0.06	0.16	0.65	0.08	0.24 ± 0.13
	50	0.09	0.15	0.59	0.32	0.29 ± 0.12
Without PB	5	0.71	0.12	1.04	0.12	0.50 ± 0.23
	10	1.49	0.22	20.41	0.24	5.59 ± 4.46
	25	3.47	0.38	42.98	0.48	11.83 ± 9.39
	50	0.01	0.00	74.60	0.64	18.81 ± 16.65

Appendix B-10. Amount of fenbendazole sulphoxide (FBZSO) (nmoles/mg protein/hour) formed in microsomal reaction mixture after incubation of fenbendazole with or without piperonyl butoxide (PB) in equine liver.

		FBZSO formed (nmoles/mg protein/hour) (n=4)				
	Conc. (μM)	A	D	E	F	Mean ± SEM
With PB	5	0.03	0.03	0.07	0.03	0.04 ± 0.01
	10	0.04	0.04	0.16	0.06	0.07 ± 0.03
	25	0.08	0.07	0.15	0.11	0.10 ± 0.03
	50	0.09	0.09	0.15	0.09	0.10 ± 0.03
Without PB	5	0.08	2.22	0.14	0.03	0.62 ± 0.48
	10	0.11	3.34	0.16	0.05	0.91 ± 0.73
	25	0.17	9.63	0.28	0.13	2.55 ± 2.12
	50	0.50	20.19	0.35	0.16	5.21 ± 4.48

Appendix B-11. Amount of fenbendazole sulphone ($FBZSO_2$) (nmoles/mg protein/hour) formed in microsomal reaction mixture after incubation of fenbendazole with or without piperonyl butoxide (PB) in equine liver.

		$FBZSO_2$ formed (nmoles/mg protein/hour) (n=4)				
	Conc. (μM)	A	D	E	F	Mean ± SEM
With PB	5	0.04	0.03	0.03	0.06	0.04 ± 0.01
	10	0.04	0.08	0.10	0.06	0.07 ± 0.01
	25	0.04	0.05	0.06	0.08	0.06 ± 0.01
	50	0.05	0.06	0.10	0.08	0.07 ± 0.02
Without PB	5	0.04	0.06	0.02	0.00	0.03 ± 0.01
	10	0.11	0.09	0.00	0.04	0.06 ± 0.03
	25	0.11	0.25	0.07	0.05	0.12 ± 0.05
	50	0.13	0.43	0.07	0.06	0.17 ± 0.08