PHARMACOKINETICS OF PROPYLTHIO-BENZIMIDAZOLE ANTHELMINTICS: MODULATION OF LIVER BIOTRANSFORMATION IN SHEEP AND CATTLE

by

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TABLE OF CONTENTS

PREFACE

Adequate parasite control programs are a major contribution to efficient livestock production systems. Since the introduction of new anthelmintic molecules into the market is an extremely long and expensive approach, the more efficient use of available broad-spectrum compounds is desirable. The integration of available information on the host-parasite-environment relationship with a more complete understanding of the pharmacological properties of existing antiparasitic drugs, as well as of the factors that could affect their efficacy, should assist with more efficient parasite control and improved livestock production efficiency. This research project was established to characterize the pharmacokinetic behaviour and pattern of biotransformation of benzimidazole compounds in ruminants, and to modulate them in order to enhance anthelmintic efficacy.

The outcome of this research has been organized into 10 Chapters. Chapter 1 includes a general introduction and a literature review on the Pharmacology of benzimidazole anthelmintics. Chapters 2 to 9 report the experimental research undertaken. In Chapter 10, a general concluding discussion is presented. The experiments described in Chapter 9 were done in collaboration with Mr. Bakela Nare, B.Sc., a fellow graduate student at the Institute of Parasitology, who prepared the microsomal fractions and undertook the spectrophotometric determinations reported in that Chapter. The efficacy trial described in Appendix I was conducted in collaboration with Dr T.A. Yazwinski, University of Arkansas, and Schering-Plough Corporation, Kenilworth, NJ. This study was designed to assess, in terms of clinical efficacy, the findings of this thesis research on the pharmacological modulation of benzimidazole liver biotransformation. I have only participated in the experimental design, and in the analysis and interpretation of the results, which are included in Appendix I.

The references are cited in the text by first author where there are three or more authors, or by both authors where there are only two; they are arranged in alphabetical order in the References section. A glossary with the abbreviations used in the text is given in Appendix II. Appendix III gives a list of the publications arising from work reported in this thesis.

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CLAIMS OF ORIGINALITY

The following aspects and/or findings of the research described in this thesis are considered original contributions to scientific knowledge:

- The comparative pharmacokinetic behaviour and pattern of biotransformation of netobimin pro-drug and its albendazole metabolites in sheep and cattle following oral/intraruminal and parenteral administration, have been characterized for the first time.

- The complete pharmacokinetic analysis of parent drug and its various metabolites used throughout this thesis and based on both compartmental and non-compartmental methods of analysis, has been, to the best of my knowledge, utilized for the first time to describe plasma and different compartment disposition kinetics of an anthelmintic compound.

- Bioequivalence studies between a trisamine solution and a zwitterion suspension of netobimin, both after parenteral and oral administration to cattle, have been undertaken for the first time.

- The comparative profile of urinary excretion for netobimin parent drug and its metabolites after intraruminal and subcutaneous administration to sheep, was described for the first time.

- The characterization of the distribution of an anthelmintic parent compound and its various metabolites in different gastrointestinal compartments, and the relationship with their plasma disposition kinetics have been established for the first time.

- The identification of an amino-albendazole-sulphone metabolite in plasma and in abomasal and ileal fluids after

netobimin administration to cattle, and its pharmacokinetic characterization, are original contributions.

- The comparison between sheep and cattle is considered original on the following aspects: a) plasma disposition kinetics of albendazole metabolites after netobimin treatment, b) *in vitro* biotransformation of netobimin pro-drug, albendazole and albendazole sulphoxide by ruminal and ileal fluids, c) albendazole sulphoxidation by liver microsomal fractions, and d) inhibitory effect of methimazole on microsomal sulphoxidation of albendazole. The greater capacity of cattle gastrointestinal fluids to oxidize albendazole, the greater capacity of sheep gastrointestinal fluids to reduce albendazole sulphoxide, and the greater rate of albendazole sulphoxidation by sheep liver microsomes compared to those of cattle, have been reported for the first time.

- The *in vivo* pharmacological modulation of the liver benzimidazole biotransformation by oxidation-impairing compounds in sheep and cattle, is an original contribution in all its aspects, including the approach.

ABSTRACT

The aim of this research was to determine the influence of route of administration, drug formulation and modified-liver metabolism on the pharmacokinetic and metabolic patterns of benzimidazole anthelmintics in ruminants. Both route of administration and formulation dramatically affected the bioconversion of netobimin (NTB) pro-drug, N-methoxycarbonyl-N'-(2-nitro-5-propylphenylthio)-N"-(2-ethyl sulphonic acid) guanidine, and the bioavailability and disposition kinetics of its active albendazole (ABZ) metabolites in both sheep and cattle. The efficacy of NTB conversion by the gastrointestinal (GI) microflora, was markedly lower after subcutaneous (SC) administration of NTB pro-drug compared with enteral administrations in both species. Although trisamine and zwitterion formulations of NTB were bioequivalent after SC treatment, the zwitterion suspension was two-fold more bioavailable in terms of ABZ metabolites, after oral administration to cattle. ABZ sulphoxide (ABZSO) and ABZ sulphone ($ABZSO_2$), the main metabolites found in plasma, were reversibly exchanged between plasma and GI compartments and concentrated in the abomasum. ABZ, ABZSO and $ABZSO_2$ were detected in the GI tract for 72 h post-NTB administration to cattle. *In vitro*, ABZ was oxidized into ABZSO and $ABZSO_2$ by liver microsomes and ruminal and ileal fluids. However, only ABZSO was reduced (back to ABZ) by these GI fluids. The rate of ABZ sulphoxidation by liver microsomes was significantly lower in cattle compared to sheep. However, while the oxidizing activity was greater in GI fluids of cattle, the reducing activity was prevalent in those of sheep. This was consistent with the higher $ABZSO_2$/ABZSO ratio and the markedly faster disposition of both metabolites in cattle compared to sheep. The co-administration of NTB with different oxidation-impairing compounds, largely methimazole (MTZ), in both species, resulted in an increased bioavailability and/or markedly slower disposition kinetics of ABZ and/or ABZSO, which accounted for an improved efficacy against GI parasites. Overall, these findings are a major contribution to the pharmacology of antiparasitic drugs and parasite control.

RESUME

Cette recherche visait à évaluer l'effet de la formulation, de la voie d'administration et du métabolisme hépatique modifié sur la pharmacocinétique, le métabolisme et l'efficacité clinique des anthelminthiques benzimidazoles, chez les ruminants. La voie d'administration et la formulation ont beaucoup influencé la bioconversion de la pro-drogue nétobimin (NTB), de même que la biodisponibilité et le comportement cinétique de ses métabolites, chez l'ovin et chez le bovin. Comparée à l'administration par voie entérale, l'administration par voie sous-cutanée de la pro-drogue NTB diminua considérablement l'efficacité de la conversion de ce produit par la microflore intestinale. Chez les bovins, l'administration sous-cutanée de NTB démontra que la présentation sous forme triaminée et sous forme zwitterion étaient bioéquivalentes. Par voie orale, la suspension zwitterion doublait la biodisponibilité des métabolites de l'albendazole (ABZ). Les échanges réversibles entre le plasma et le tractus gastrointestinal (GI) des principaux métabolites trouvés dans le plasma, le sulfoxide (ABZSO) et le sulfone ($ABZSO_2$), et leur forte concentration dans l'abomasum justifient la présence d'ABZ, ABZSO et $ABZSO_2$ dans le système GI 72 heures après le traitement. In vitro, alors que l'oxidation de l'ABZ en ABZSO et $ABZSO_2$ par les microsomes hépatiques et par les liquides ruminal et iléal a été observée, seuls les liquides GI ont permis la réduction de ABZSO en ABZ. Le taux de sulfoxidation par les microsomes était significativement inférieur chez le bovin comparativement à l'ovin. Alors que l'activité oxidante était plus importante dans le liquide GI des bovins, l'activité réductrice était plus marquée dans le liquide GI de l'ovin. Ceci concorde bien avec le ratio $ABZSO_2$/ABZSO plus élevé et l'élimination plus rapide de ces métabolites chez le bovin. La co-administration du NTB et de substances interférant avec l'oxidation hépatique, tel le methimazole, augmenta la biodisponibilité et/ou ralentit sensiblement la cinétique de disposition de ABZ et/ou de ABZSO, responsables d'une efficacité anthelminthique plus grande. Ces résultats sont une contribution majeure à la pharmacologie des drogues antiparasitaires et à la lutte contre les parasites.

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LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1.: ECONOMIC IMPACT OF PARASITISM: ROLE OF CONTROL MEASURES

Parasitism is a primary cause of production losses in all cattle and sheep-producing countries of the world. Most losses are associated with gastrointestinal (GI) nematode infections, especially in young animals. Lungworm infections, however, also have a serious effect on ruminant productivity in many areas of temperate climate. The dramatic effects of heavy liver fluke infections leading to impaired growth and increased mortality rates are also well known. However, as with low level of nematode infections, moderate fluke burdens also seem to have a cumulative adverse economic impact on livestock production (Leaning and Guerrero, 1987).

The total economic impact of parasitism is extremely difficult to quantify accurately. The losses in animal production by clinical and subclinical helminth infections are only one part of the economic cost. A significant part of the economic impact of parasites is the **investment in control measures.** Adequate parasite control programs are a major contributor to efficient production systems. The economic importance of helminth infections in livestock nas long been recognized and it is probably for this reason that the most important advances in the chemotherapy of helminthiasis have come from the animal health area (Horton, 1990).

Although anthelmintics are used in all domestic animal species and man, the ruminant market is the largest one, where approximately $ 1.7 billion dollars are spent annually throughout the world, in an effort to reduce the deleterious effects of helminth parasites. **Unfortunately, the investment on control measures does not always result in the expected therapeutic success;** this represents an adverse economic effect of tremendous magnitude in many countries, where

climatic conditions and animal management strategies are extremely favourable for parasite transmission. This is even more relevant in developing countries whose economies are highly dependant on their livestock production based exclusively on grazing management. Among factors responsible for that therapeutic failure are: a) inadequate integration between management strategies and chemotherapy, b) incorrect use of anthelmintic drugs due to insufficient knowledge of their pharmacological features, c) insufficient understanding of the relationship between pharmacological properties, and several host-related factors that could lead to modifications on the pharmacokinetic behaviour and to a decreased antiparasite efficacy of the chosen drug. In addition, the availability of many compounds with a common mode of antiparasitic action and the indiscriminate use of these drugs have accounted for the widespread development of drug resistance, particularly in parasites of sheep and goats (Hennessy, 1989). Undoubtedly, drug resistance is a major factor responsible for therapeutic failure in parasite control and still requires investigation.

The introduction of new anthelmintics from chemical groups distinct from those currently used is likely to slow down because of the escalating cost and the prolonged development time (6-8 years) required to introduce a new molecule onto the market. A more economical approach is, however, to make more efficient use of available broad-spectrum drugs. Special research emphasis should be addressed to further understand the pharmacological properties of existing drugs and the influence of host-related factors on their clinical efficacy, as well as to improve drug delivery systems. Focusing research in these directions would permit a) improved efficacy against the most difficult parasites to control (i.e. arrested larval stages of GI nematodes, immature flukes, etc), b) reduced cost of the antiparasite treatment and c) avoidance of the problems of drug resistance. These potential improvements may notably decrease the overall economic losses caused by

parasitism in farm animals. Finally, integration of the available information on the host-parasite-environment relationship with a more complete understanding of the pharmacology of antiparasitic drugs and of the factors that could affect their efficacy, will account for both more efficient parasite control and improved livestock production.

1.2.: ANTHELMINTIC COMPOUNDS

Chemicals which can be used to remove endoparasites without undesirable side effects on host animals or man are difficult to discover and develop as useful drugs (Prichard, 1990). Different chemical compounds have been used since the beginning of this century, in an attempt to control helminth parasites. The limited antiparasite efficacy and the large number of side effects were among the main limitations of earlier compounds. In 1938, the discovery of the anthelmintic properties of phenothiazine was the first dramatic advancement to antinematodal therapy in ruminants and horses (Roberson, 1982). Since that time great progress toward the development of an "ideal" anthelmintic has been made, resulting in the discovery of many broad-spectrum compounds. This process has been particularly intensive since the introduction of thiabendazole in 1961 (Brown *et al.*, 1961). This was the first broad-spectrum anthelmintic and was the breakthrough that opened a new area in the treatment of the parasitic disease. Since then, many new drugs have been introduced, particularly of the benzimidazole family. Recent efforts have culminated in the introduction of the avermectins, which are not only broad-spectrum anthelmintics but also effective ectoparasiticides.

Desirable features for an ideal anthelmintic drug are: a) broad-spectrum activity at a reasonably inexpensive and safe dosage rate, b) easy and practical administration, c) low drug residues, which allow short withdrawal periods before treated animals could be used for human consumption. An effective anthelmintic must be toxic only to the parasite; this is an

important challenge in drug therapy research and may be achieved either because of the greater inherent susceptibility of the parasite cells compared with those of the host, or because of the achievement of toxic drug concentration only at the site of parasite location (Prichard, 1985a).

The current available broad-spectrum anthelmintics for use in livestock belong to the following families: 1) **imidazothiazoles** (levamisole, tetramisole), 2) **tetrahydropyrimidines** (morantel, pyrantel), 3) **avermectins** (ivermectin, abamectin, moxidectin, doramectin, etc) and 4) **benzimidazole and pro-benzimidazoles.** In addition, closantel is the only compound within the salicylanylide family that could be considered as a broad-spectrum anthelmintic. Since **benzimidazole** (BZD) and **pro-benzimidazole** (pro-BZD) compounds have been the subject of the experimental research reported in this thesis, the literature review that follows will focus exclusively on the pharmacology of these anthelmintic drugs.

1.3.: PHARMACOLOGY OF BENZIMIDAZOLE ANTHELMINTIC COMPOUNDS

The discovery and subsequent improvement of the BZD moiety has provided a major contribution to antiparasite chemotherapy. The search for new and more active BZD molecules in the last 30 years has been intensive and successful. However, the poor GI absorption and the lack of water solubility which reduces flexibility for drug formulation, for some compounds in the group, and the development of drug resistance still present exciting challenges for research in the area. Although BZD compounds are used in man and all domestic animals, this review will be mainly addressed to the ruminant species used as experimental animals in this thesis research.

1.3.1. Synthesis and chemistry

Since the discovery of TBZ in 1961, several thousand BZD molecules have been synthesized and screened for anthelmintic

activity (Townsend and Wise, 1990). However, no more than twenty of them have been commercially developed for use in domestic animals and man, either as BZD or pro-BZD. The BZD structure is a bicyclic ring system in which benzene has been fused to the 4- and 5- positions of the heterocycle (imidazole) (Figure 1.1). BZD compounds are white crystalline powders, with fairly high melting points and have limited to no solubility in water. Molecules that are unsubstituted on either of the imidazole nitrogen atoms possess both acidic and basic characteristics. The synthetic pathway to the various BZD molecules usually proceeds through two steps; first the construction of a benzene ring containing the desired substituents and a 1,2-diamine grouping, followed by the ring closure of the 1,2-diaminobenzene derivative to construct the imidazole ring (Townsend and Wise, 1990).

The BZD compounds currently marketed as anthelmintics can be grouped as follows; their chemical structures are shown in Figures 1.1 and 1.2:

a) **BZD thiazolyls:** thiabendazole (TBZ), cambendazole (CBZ).

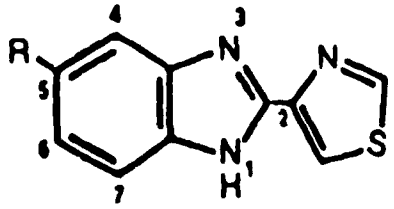
b) **BZD methylcarbamates:** parbendazole (PBZ), mebendazole (MBZ), flubendazole (FLBZ), ciclobendazole (CIBZ), oxibendazole (OBZ), luxabendazole (LBZ), albendazole (ABZ), ricobendazole (albendazole sulphoxide; ABZSO), fenbendazole (FBZ), oxfendazole (OFZ).

c) **Halogenated BZD thiols:** triclabendazole (TCBZ).

d) **pro-BZD:** thiophanate (TPT), febantel (FBT), netobimin (NTB)

Different modifications at positions 2- and 5- of the BZD ring system (Figures 1.1 and 1.2) have provided the most anthelmintically active drugs, especially with the discovery of sulphur-containing derivatives such as FBZ (Baeder *et al.*, 1974), OFZ (Averkin *et al.*, 1975) and ABZ (Theodorides *et al.*, 1976), which exhibit high efficacy against lungworms and inhibited larval stages of most GI nematodes. Studies involving atomistic molecular modeling have been recently carried out to understand the structure-activity relationship

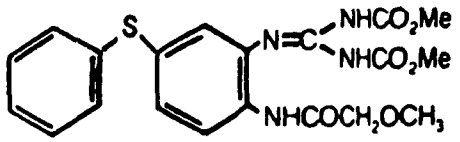
BENZIMIDAZOLE THIAZOLYLS



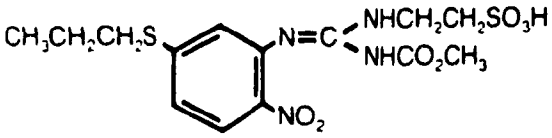
R = H- **THIABENDAZOLE**

R = $(CH_3)_2CHOCONH$- **CAMBENDAZOLE**

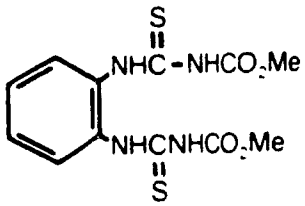
PRO-BENZIMIDAZOLES



FEBANTEL



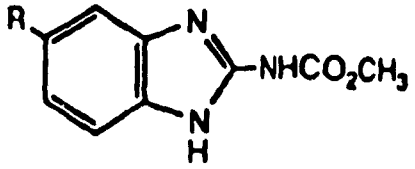
NETOBIMIN



THIOPHANATE(METHYL)

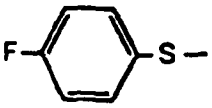
Figure 1.1.: Chemical structures of benzimidazole thiazolyls and pro-benzimidazoles.

BENZIMIDAZOLE METHYLCARBAMATES

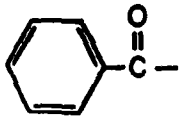


R = $CH_3CH_2CH_2CH_2$- PARBENDAZOLE

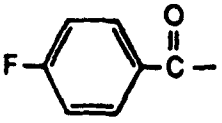
R = $CH_3CH_2CH_2O$- OXIBENDAZOLE

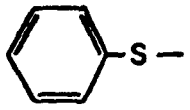
R =  LUXABENDAZOLE

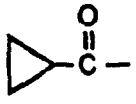
R = $CH_3CH_2CH_2S$- ALBENDAZOLE

R =  MEBENDAZOLE

R = RICOBENDAZOLE

R =  FLUBENDAZOLE

R =  FENBENDAZOLE

R =  CICLOBENDAZOLE

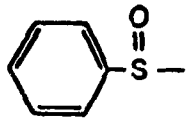
R =  OXFENDAZOLE

Figure 1.2: Chemical structures of benzimidazole methylcarbamates

of benzothiazole and BZD anthelmintics (McCracken and Lipkowitz, 1989, 1990). These authors claimed that the potency of the antiparasite activity for BZD compounds may depend upon the magnitude of the molecular dipole moment and the percentage of polar surface area. 2-thiazolyl and 2-methylcarbamates substituted BZD compounds showed structural and electronic congruence, suggesting that these groups behave similarly in transport to, and binding at, the active site. They proposed that BZD that have their substituents in position 5- twisted out-of-plane (ABZSO, OFZ) are more active than those with the 5- substituent group in a coplanar position (OBZ) with the central BZD ring. This is doubtful given the findings of Lubega and Prichard (1991b) that ABZSO and OFZ have lower binding affinity for nematode tubulin than their respective reduced thioethers, ABZ and FBZ. These different studies suggest, however, that not only the chemical substitution in the position 5- of the BZD ring, but also its conformational arrangement, are relevant in the access of the drug to the active site and in the resultant antiparasite efficacy.

The most recent advancement in the search for BZD moieties, has been the introduction of pro-BZD compounds; TPT, FBT and NTB are inactive pro-drugs that are enzymatically converted into active BZD methyl or ethyl carbamates in the host. The active molecule of TPT is an ethyl BZD carbamate derivative known as lobendazole (Gardiner *et al.*, 1974; Delatour *et al.*, 1988). FBT (Delatour and Euzeby, 1983) and NTB (Nafissi-Varchei, 1983; Delatour *et al.*, 1986) are guanidine derivatives, and they are metabolized in the host to FBZ and ABZ, respectively. A series of pro-BZD compounds have been synthesized in the last few years, but none of them has yet reached commercial development. A group of phenylthio-nitrophenyl guanidines (PNG) were tested as potential pro-drugs of FBZ and OFZ (Schering Plough Corp., NJ, USA, personal communication). Recently, a new phenyl thiourea derivative pro-BZD has been produced and tested against *Echinococcus*

multilocularis in a gerbil model (Walchshofer *et al.*, 1990); This pro-drug is thought to be converted into a different halogenated BZD molecule ([5-trifluoromethyl-1H-benzimidazole-2-yl]-carbamic acid methyl ester) (Walchshofer *et al.*, 1990). In addition, moieties with a piperazine group substitution on position 5- of the BZD ring system have recently been synthesized and tested as potential anthelmintics (Sanchez-Alonso *et al.*, 1989). TCBZ, an halogenated BZD thiol, have been developed for the treatment of all the stages of *Fasciola hepatica* (Boray *et al.*, 1983); however this BZD compound does not exhibit any activity against nematode parasites.

1.3.2. Mode of action

Energy generation in helminths involves anaerobic fermentation processes in which the ingested glucose is reduced to organic acids and alcohols (Rew 1978; Behm and Bryant, 1979). This energy is principally consumed in the parasite's motility and reproduction. Differences in the enzymes involved in respiratory and energy production systems between host and parasites are potential target for chemotherapy.

Initial studies of the mode of anthelmintic action for BZD compounds focused on their role in the carbohydrate metabolism of parasites. Prichard (1970) first demonstrated that TBZ inhibited fumarate reductase activity, an essential biochemical step in the anaerobic fermentation of many helminth parasites, and postulated this effect as a possible mode of action of TBZ. In comparative studies of BZD-resistant and susceptible strains of *Haemonchus contortus*, a differential inhibition of the fumarate reductase activity was observed (Prichard, 1973). Several other reports have confirmed the inhibition of this enzymatic system by different BZD compounds in nematodes and cestodes (Malkin and Camacho, 1972; Romanowski *et al.*, 1975; Barrowman *et al.*, 1984). In addition, other enzymes involved in the helminth's oxidative phosphorylation have been shown to be affected by BZD

molecules. For instance, the enzymatic activity of succinate dehydrogenase, phosphoenolpyruvate reductase and malate dehydrogenase from different helminth parasites was inhibited by various BZD compounds (Rahman and Bryant, 1977; Tejada *et al.*, 1987; Sharma *et al.*, 1989; Sanchez-Moreno *et al.*, 1989).

BZD have also been shown to inhibit glucose uptake both *in vitro* and *in vivo* in many helminth species, for example, *Ascaris suum, Trichinella spiralis* and *Moniezia expansa* (Prichard, 1987; Lacey, 1988), in some cases associated with a compensatory depletion of glycogen stores. Other effects of BZD anthelmintics on parasites, such as inhibition of acethycholinesterase secretion (Watts *et al.*, 1982; Sangster *et al.*, 1985) and inhibitory effects on serotonin metabolism (Lacey, 1988), membrane-bound monoamine oxidase activity (Moreno and Barret, 1979) and Na^+ uptake (Beames *et al.*, 1976), have been described. In a recent report (McCracken and Stillwell, 1991) it has been postulated that the anthelmintic activity of BZD compounds, in part, may be due to bioenergetic disruptions resulting from transmembrane proton discharge; BZD may behave as lipid soluble proton conductors that induce a reduction of the electrical resistance of bimolecular lipid membranes. It is clear that BZD compounds produce a large number of biochemical changes that induce important homeostatic modifications on the parasite; this leads to the expulsion of the parasite by the host's defense mechanisms, due to its inability to remain in its predilection site (Prichard, 1987; Lacey, 1988), rather than to a lethal direct effect of BZD drugs.

Coincidental to the study of the BZD effects on parasite's energy metabolism, Borgers and De Nollin (1975) postulated the involvement of MBZ in the disintegration of the normal microtubule structure in intestinal cells of *Ascaris suum*. Subsequent studies confirmed this effect of BZD anthelmintics on the tubulin-microtubules equilibrium of several helminth parasites (Verheyen *et al.*, 1976; Borgers *et al.*, 1975). In

all eukaryotes, the protein tubulin (a dimer of α and ß tubulin of 50 Kd each) undergoes a reversible polymerisation to form microtubules which are involved in cellular structure and function, particularly in cell division, transport of nutrients and excretion of waste products. Structurally, both α and ß-tubulins are heterogeneous proteins, products of multi-gene families as well as post-translational modifications (Lacey, 1990). Microtubules exist in dynamic equilibrium with tubulin, the microtubule subunit; the ratio of dimeric tubulin to polymeric microtubules is controlled by a range of endogenous regulatory proteins and co-factors (Lacey, 1988). Compounds which cause the alteration of this equilibrium are known as microtubule inhibitors. Some of the best characterized microtubule inhibitors are used in cancer chemotherapy (i.e. vincristine and vinblastine), and the search and characterization of these compounds with antimitotic properties is a growing area of research. Most of the inhibitor compounds bind to either mammalian, or fungal or parasite tubulin and shift the equilibrium between the tubulin subunit and microtubules, resulting in the depolymerisation of microtubules (Lacey, 1988).

In recent years, several reports have confirmed the hypothesis that BZD compounds inhibit the polymerisation of tubulin to microtubules (Lachette *et al.*, 1980; Friedman and Platzer, 1980; Lacey and Watson, 1985 a,b); microtubules are now recognized worldwide as the primary target for the antiparasite activity of BZD drugs. BZD methylcarbamates with strong inhibitory activity against mammalian tubulin were also shown to be potent inhibitors of egg hatch of *Haemonchus contortus*, while non-inhibitors failed to prevent hatching (Lacey *et al.*, 1987). BZD are thought to share the tubulin binding site with other well established microtubule inhibitors, such as colchicine and podophyllotoxin (Russel and Lacey, 1989; Lacey, 1990) and similarities between the binding of BZD and colchicine have been described (Lacey, 1988). The colchicine binding site has been identified in α-tubulin

(Schmitt and Atlas, 1976); however, the BZD binding site is still a matter of controversy. Although both tubulin dimers may be, to some extent, important for BZD binding, ß-tubulin may play a prevalent role. Studies with BZD susceptible and resistant strains have shown that changes on the ß-tubulin isoform pattern occur in BZD resistant isolates of *Haemonchus contortus* (Lubega and Prichard, 1991a). This has been confirmed by the different restriction patterns found for the ß-tubulin DNA of the resistant strain compared to those of the susceptible one (Roos, 1990). Furthermore, the receptors involved in the binding of different BZD molecules to parasite tubulin have been characterized as high and low affinity receptors (Lubega and Prichard, 1990).

The inhibitory concentration of different BZD compounds (IC_{50}) required to prevent [^{3}H] MBZ or [^{3}H] OBZ binding to tubulin and their recommend dose rate for *in vivo* use have been shown to correlate positively for most of the compounds investigated (Lacey, 1990; Lubega and Prichard, 1991b). Overall, BZD molecules that show higher *in vitro* IC_{50}, also require a higher therapeutic dose rate to achieve a satisfactory *in vivo* efficacy. However, host-related pharmacokinetic factors also play a relevant role in the differential pattern of clinical efficacy among BZD anthelmintics and their metabolites.

Demonstration of the selectivity of binding of BZD between helminth and mammalian tubulins provided a fundamental argument for tubulin as the site of BZD action (Lacey, 1990). Several reports have shown that BZD anthelmintics exhibit a higher affinity for parasite tubulin than for that of the mammalian host (Friedman and Platzer, 1978; Tang and Prichard, 1988). Therefore, differences in binding of the various BZD compounds to tubulin from parasites and mammalian tissues may account for the differential toxicity of these compounds between host and parasite cells (McKellar and Scott, 1990) and for their relatively wide margin of safety.

Tubulin may be a constituent protein of plasma and mitochondrial membranes in mammalian cells (Bernier-Valentin *et al.*, 1983). The role of tubulin in plasma membrane in nematodes is suggested by the observation that the classical tubulin binding drugs (colchicine, podophyllotoxin, MBZ, TBZ) inhibited 3-0 methylglucose uptake (membrane transport) in *Haemonchus contortus*. Furthermore, BZD-resistant strains of this nematode were less sensitive to 3-0 methyglucose inhibition than BZD-susceptible strains (Rew and Prichard, 1985). In addition, higher BZD concentrations are required to inhibit the activity of fumarate reductase in nematodes than those required to disrupt the tubulin-microtubules equilibrium (Dawson *et al.*, 1984). Considering the multiple role of microtubules in cell function, it is likely that the variety of metabolic and biochemical changes induced by BZD compounds to the parasites, are dependent upon an initial disruption of the polymerisation of tubulin to form microtubules. Therefore, there is now abundant evidence that the primary mode of action of BZD is a selective binding to parasite tubulin and that this tubulin may exist in solution, in microtubules or in membrane. The understanding of the relationship between mode of action and efficacy for these anthelmintics, which are used throughout the world, has progressed substantially; an integration of the various reported biochemical phenomena produced by BZD compounds in helminth parasites may be now feasible.

1.3.3. Pharmacokinetics

1.3.3.1. Absorption and Distribution

As a class, BZD anthelmintics have only limited water solubility and small differences in solubility may have a major influence on their absorption and on their resultant clinical efficacy. The lack of water solubility is an important limitation for the formulation of BZD compounds, which only allows their preparation as suspensions, pastes or granules for oral or intraruminal administration.

The mucous surface in the GI tract behaves as a lipid barrier for the absorption of active substances, so that absorption depends on lipid solubility and degree of ionisation at GI pH levels. However, the first kinetically distinct stage in absorption is, by necessity, solubilization of the BZD compound to facilitate its penetration through the GI mucosa. The dissolution rate of a enterally delivered compound influences the rate and extent of its absorption (systemic bioavailability), its maximal plasma concentration and its subsequent distribution and disposition kinetics. Drugs such as TBZ and CBZ, the most hydrosoluble moieties among BZD anthelmintics, are extensively dissolved in the aqueous ruminal fluid and rapidly absorbed (McKellar and Scott, 1990), reaching maximal plasma concentrations as early as 4 h post-administration to cattle (Tocco *et al.*, 1965; Prichard *et al.*, 1981).

Unlike TBZ, the newer BZD compounds show limited GI absorption due to their poor solubility in water. The dissolution rate, passage along the GI tract and absorption into the systemic circulation, of modified BZD, are markedly slower than those of TBZ. Such a phenomenon also results in extended residence times for the active metabolites of the substituted BZD compared with those of TBZ or CBZ. For instance, Prichard *et al.* (1981) demonstrated that maximal plasma concentration of TBZ in cattle occurs at 4 h post-treatment compared with 24 h for FBZ, and that FBZ persisted much longer than did TBZ in plasma and the GI tract. Consistently, TBZ and its metabolites were recovered in urine much more rapidly than were FBZ and its metabolites. This differential pharmacokinetic behaviour accounts, in part, for the greater anthelmintic potency of FBZ compared with TBZ.

In small domestic animals and man, the single dose administration of BZD compounds is much less effective than in herbivores. The small volume of gut contents and the low solubility of the most potent BZD drugs lead to limited

dissolution and therefore reduced availability in monogastrics (Marriner, 1986). Divided dose administration is, however, highly effective. The degree of absorption for BZD compounds in monogastrics has been shown to be dramatically affected by the dosing vehicle used, as well as by the diet. The absorption and bioavailability of ABZ metabolites (Lange et al., 1988) and MBZ in humans, were significantly improved by its administration with fatty meals (Welling, 1977; Munst *et al.*, 1980; Dawson and Watson, 1985; Edwards and Breckenridge, 1988). Furthermore, McKellar *et al.* (1990a) have postulated that a combination of poor solubility of FBZ and short gut transit time, could account for the small differences in the area under the curve for FBZ and its metabolites observed in dogs treated with 20 and 100 mg/kg, respectively. Consistently, a shorter time of residence of the digesta contents in the horse compared to ruminants could account for the lower absorption and the marked lower bioavailabilities of FBZ and OFZ in this species compared to ruminant species (McKellar and Scott, 1990).

The influence of the rumen as a compartment on the pharmacokinetic behaviour of many drugs is well documented. The rumen may substantially influence the absorption pattern and the resultant pharmacokinetic behaviour and antiparasite activity of enterally delivered BZD anthelmintics. The rumen accounts for about 20% of the animal's volume and never empties; the fermentation process is continuous with the contents being slowly mixed by waves of contraction of the musculature, which results in the stratification of the ruminal content (Hennessy, 1989). When a drug suspension is deposited in the rumen, solid particles mix and distribute through the digesta volume; this is likely to be influenced by the stratification of the rumen material. The large rumen volume and long digesta residence time assists absorption by delaying the rate of passage of drug down the GI tract (Hennessy, 1985). The rumen may act as a reservoir and prolong the duration of drug absorption and/or outflow down the GI

tract. The rumen has a very low area-to-volume ratio and absorption is by passive diffusion (Hennessy, 1989). The rumen/plasma pH gradient and the degree of ionization of a compound at the ruminal pH govern the absorption rate through the ruminal epithelium, which is only permeable to the lipophilic non-ionic form of a drug (Baggot, 1977). The influence of the rumen on BZD absorption was evidenced by the higher and more sustained concentration of FBZ and its metabolites in plasma obtained following oral treatment in sheep compared with the intra-abomasal administration of the compound (Marriner and Bogan, 1981b). Similarly, intraruminal administration of both FBZ and ABZ in sheep resulted in higher plasma area under the curves and maximal plasma concentrations of their major metabolites than those obtained after an intra-abomasal administration of both compounds (Hennessy and Steel, 1990).

Although the distribution of the drug in the stratified ruminal content may slow absorption and outflow down to the abomasum, the newer BZD compounds are less water soluble at ruminal pH than at abomasal pH. In addition, ABZ and, notably OFZ, are reported to be much more soluble than FBZ in abomasal fluid (McKellar and Scott, 1990). Considering the lower solubility of FBZ in abomasal fluid, it is likely that the rumen reservoir effect is more relevant for this compound than for ABZ or OFZ. The dissolution of FBZ parent drug in the ruminal fluid, or at least the delay of its outflow down to the abomasum, may be important in the resultant plasma bioavailability of FBZ and its metabolites. For instance, the intra-abomasal administration of FBZ in sheep resulted in reduced plasma residence time of the parent drug and its metabolites compared with that of the intraruminal administration (Prichard *et al.*, 1978a). In addition, as much as 70% of the intraruminally administered FBZ was absorbed from the rumen in cattle (Prichard *et al.*, 1981), and its rate of disappearance from the rumen was much slower than that of the more soluble TBZ compound. The ruminal sequestration of

FBZ in cattle has also been suggested by Short *et al.* (1987b); these authors found that only 44.6% of the total FBZ dose was eliminated by urine and faeces 6 days post-treatment, suggesting that the remainder may have been sequestered in the GI tract.

The relevance of water solubility and rates of dissolution in the GI tract lumen, on absorption and on resultant pharmacokinetic behaviour of BZD compounds, have been further demonstrated by altering the crystalline nature of the OFZ molecule. The production of an amorphous form of OFZ by treatment with acids, has resulted in a significant change in water solubility from approximately 4 μg/ml for the current available crystalline form to 11.3 μg/ml for the amorphous form at a pH of 7.6 (Chick *et al.*, 1987). The oral administration of amorphous OFZ resulted in a more rapid and complete absorption compared with the crystalline form. In the same experiments, it was shown that half the dose of the amorphous OFZ produced equivalent efficacy to that of the normal crystalline OFZ. Furthermore, it has been demonstrated that differences in the particle size of crystalline OFZ (Shastri *et al.*, 1980) and TCBZ (Richards *et al.*, 1987) affect its dissolution rate, GI absorption and plasma profiles in sheep.

Occasionally, reduced bioavailability and efficacy of BZD have been found after oral administration in comparison with intraruminal administration. It is possible that a portion of the orally administered anthelmintic can, on occasion, by-pass the rumen and rapidly enter the abomasum by closure of the oesophageal groove (Prichard and Hennessy, 1981); this results in a proportion of the dose directly entering the abomasum with resultant poor absorption from either this organ or the small intestine. This results in reduced plasma bioavailability of active metabolites (Prichard et al., 1985; Hennessy, 1989). Such an effect may indicate that the so called "reservoir" and "slow delivery" effects of the rumen

would be lost; although lag times between treatment and plasma detection of active metabolites could be shorter, the overall bioavailability of active BZD metabolites and their resultant efficacy may be significantly reduced (Prichard and Hennessy, 1981). After weaning, the oesophageal groove is considered to remain open; however, its spontaneous closure has been shown to occur following the administration of large volumes of liquid medications. It was found that 50% of cattle (McEvan and Oakley, 1978) and 42% of sheep (Prichard and Hennessy, 1981) orally treated with different anthelmintic suspensions had such a reflex. This phenomenon has been related to the variable efficacy of FBZ against resistant worms (Kelly *et al.*, 1977; Prichard *et al.*, 1978b). Ngomuo *et al.*, (1984) reported virtually no differences in the bioavailability of OFZ and its metabolites between oral and intraruminal treatments in cattle; however in another study, the sum of areas under the curves for OFZ metabolites obtained following intraruminal injection of OFZ was significantly higher than that obtained after oral administration of the compound (Prichard *et al.*, 1985). In spite of conflicting results and difficulties in assessing its practical implications, it is clear that upon spontaneous closure of the oesophageal groove a portion of an oral drench dose may by-pass the rumen, which consequently affects pharmacokinetic behaviour and clinical efficacy. It is also likely that this phenomenon is more relevant for compounds with a low solubility in abomasal fluid such as FBZ; thus, a significant proportion of the dose of orally administered FBZ would directly reach the abomasum, whereby FBZ insolubility and the short residence time of the digesta in this organ, may account for both an inefficient absorption and a significant proportion of the dose passing down the GI tract to be eliminated in faeces.

Because of modifications to the BZD structure, which reduce the rate of metabolism and elimination, the most recently developed compounds tend to be much less water soluble than members of the group developed earlier. These more lipophilic

substituted BZD compounds show slower plasma disposition kinetics (McKellar and Scott, 1990), and since it is assumed that an equilibrium exists between plasma and gastrointestinal tract, the period of time during which parasites are exposed to effective drug concentrations is extended (Prichard *et al.*, 1985).

Once an anthelmintic has been absorbed from the GI tract, or other administration site, it is rapidly distributed, by the circulatory system, throughout the entire body. During this time, the metabolic processes necessary to facilitate its elimination commence. Studies using whole-body autoradiography and liquid scintillation counting have shown the slow absorption of labeled MBZ and its distribution throughout the body of sheep treated orally (Bénard *et al.*, 1985). Radioactivity was observed in all the organs, including the central nervous system. However, in spite of its large distribution, MBZ and its metabolites seem to be concentrated in liver and melanin-containing tissues, where radioactivity residues can be found for 15 and 30 days post-treatment, respectively. Large volumes of distribution of ABZ and its metabolites in sheep have been recently reported (Galtier *et al.*, 1991). However, differential distribution patterns among BZD sulphides (FBZ, ABZ), sulphoxide (OFZ, ABZSO) and sulphone ($FBZSO_2$, $ABZSO_2$) metabolites, based on their differential lipophilicities, may be expected. The distribution rate, which is indicated by the apparent volume of distribution, depends on molecular weight, lipid solubility, and plasma protein binding of each drug metabolite. The majority of BZD compounds show a binding of less than 50% to plasma protein, relatively high volume of distribution, and a relatively fast elimination rate. However, TCBZ is strongly bound to plasma proteins, especially albumin, which reduces its distribution in the body and increases its elimination half-life (Mohammed Ali *et al.*, 1986; Hennessy *et al.*, 1987). Similarly, LBZ, a fluoro-derivative BZD, has been shown to be strongly bound to plasma proteins (95%) (Steel and Duwel, 1987), which could

account for its prolonged elimination half-life. The improved pharmacokinetic profile and the slow disposition kinetics of these two halogenated BZD (TCBZ and LBZ), compared to other substituted BZD (FBZ, OFZ), account for their high flukicidal activity both against mature and immature stages (Boray *et al.*, 1983; Mohammed Ali *et al.*, 1986; Duwel, 1987). The haematophagous behaviour of *Fasciola hepatica* probably determines the mechanism of uptake of TCBZ and LBZ active metabolites (Hennessy *et al.*, 1987). However, as pointed out by these authors the pharmacokinetic behaviour of TCBZ does not explain why the drug has specificity for flukes and not for other blood-sucking helminths. Undoubtedly, the flukicidal activity of TCBZ and LBZ is facilitated by their long residence times in the bloodstream; however, since LBZ is also active against nematodes and cestodes (Duwel, 1987; Abbott, 1987), a differential biochemical mechanism may mediate the antiparasite action of these two halogenated BZD compounds.

Maximal plasma concentrations of FBZ, FBZSO (OFZ) and $FBZSO_2$ occurred at 24, 30 and 36 hours, respectively, after oral dosing of FBZ to sheep (Marriner and Bogan, 1981b). In abomasal fluids the corresponding peak concentrations were obtained at 30, 48 and 72 hours, respectively. The pattern of metabolites in plasma of FBZ treated cattle was similar to that of sheep, except that the ratio of area under the curve for FBZ to OFZ was 0.75:1 (Prichard *et al.*, 1985) compared with 0.44:1 obtained in sheep. OFZ administration produces the same range of metabolites as does FBZ, but peak plasma levels of parent OFZ in sheep (0.76 µg/ml at 30 h post-treatment) after oral administration (Marriner and Bogan, 1981a), were higher than after the same 10 mg/kg dose rate of FBZ (Marriner and Bogan, 1981b). High concentrations of OFZ parent drug (peak concentration of 3.55 µg/ml at 20 h) were detected in abomasal fluid of sheep orally treated (10 mg/kg) and measurable levels lasted for 9 days post-treatment (Marriner and Bogan, 1981a). The poor performance of BZD compounds in goats compared to sheep has stimulated the study of the

comparative pharmacokinetics of some BZD between both species. The bioavailability of OFZ and its metabolites after oral administration was significantly lower in goats than in sheep (Bogan *et al.*, 1987b; Sangster *et al.*, 1990). However, the intravenous administration of OFZ in both species resulted in similar area under the curves values (Bogan *et al.*, 1987b). This may indicate that the differences between these two species are related to the GI absorption pattern rather than to tissue metabolism. This differential pattern between species has been also reported for ABZ, and it has been suggested that these BZD compounds should be used in goats, at double the recommended dose for sheep (Sangster *et al.*, 1990). In addition, FBZ was also more rapidly cleared from plasma in goats than in cattle and the detection of the sulphone metabolite ($FBZSO_2$) in plasma was delayed in cattle compared to goats (Short *et al.*, 1987a,b). A *p*-hydroxy FBZ metabolite (FBZOH), which did appear in plasma of goats but not in cattle plasma, was the major excretory product found in urine and faeces of both species following the administration of FBZ. Interestingly, despite the high plasma concentration of OFZ obtained in both species after intravenous administration of FBZ, little OFZ appeared in urine or faeces (Short *et al.*, 1987a). A potentiation of OFZ by its co-administration with PBZ in sheep, expressed as an increase in area under the curves, in maximal plasma concentration and in clinical efficacy, has been reported (Hennessy *et al.*, 1985). PBZ binds strongly to mammalian tubulin and this leads to a temporary decrease in hepatic metabolism and bile excretion. This PBZ-mediated effect results in reduced OFZ biotransformation and plasma clearance and accounts for an improved antiparasite efficacy for the co-administration compared with the administration of OFZ alone.

Following ABZ administration in sheep and cattle the parent compound was not detected in plasma, or only detected in trace amounts, and ABZSO and $ABZSO_2$ were the major metabolites found in plasma (Marriner and Bogan, 1980; Prichard *et al.*, 1985;

Delatour *et al.*, 1990a). The plasma concentration of ABZSO and $ABZSO_2$ metabolites were lower and more rapidly eliminated in cattle than in sheep, which is consistent with the poorer flukicidal activity of ABZ in cattle than in sheep (Fetterer *et al.*, 1982). Some preliminary reports (Steel *et al.*, 1985; Steel and Hennessy, 1987; Delatour *et al.*, 1986) suggest that NTB is a pro-drug and can be converted into metabolites like ABZ by metabolism in the host. The complete characterization of the pharmacokinetic and metabolic pattern for this modern anthelmintic pro-drug, is part of the experimental research presented in this thesis.

The anthelmintic efficacy of FBT pro-drug depends on its conversion into FBZ and OFZ (Delatour *et al.*, 1985; Prichard *et al.*, 1985). Following FBT administration in both sheep and cattle, the parent drug was not found in plasma, or was detected in low concentrations for only a short period of time (Delatour *et al.*, 1985; Prichard *et al.*, 1985); the sulphone metabolite ($FBZSO_2$) plasma concentrations were much higher after FBT administration than after administration of FBZ or OFZ. Despite these pharmacokinetic differences the efficacy of FBT against GI nematodes is similar to that of FBZ or OFZ. The antiparasite activity of TPT pro-drug depends on its biotransformation into an active ethyl BZD derivative metabolite (Delatour *et al.*, 1988); the rate of TPT metabolic conversion into its active metabolite has been found to be lower in sheep (34%) than in goats (52%) and cattle (57%). As reported for FBT (Beretta *et al.*, 1987), the bioactivation of TPT might take place both in the liver and in the GI tract (Delatour *et al.*, 1988).

1.3.3.2. Metabolism and elimination

Studies on metabolism and fate of BZD anthelmintics in different species have been useful to understand differences in pharmacokinetic behaviour, clinical efficacy and level of drug residues, among different BZD compounds. However, the biotransformation processes involved are complex and sites of

metabolic conversion, pathways and metabolic products, particularly for the most recent introduced drugs, need to be elucidated; although metabolic comparisons among species have been established for some compounds (Beretta *et al.*, 1987; Montesissa *et al.*, 1989; Short *et al.*, 1988a,b). Further research is required for different BZD in species in which pharmacokinetic profiles have been substantially different, and whereby extrapolation of dose rate data from one species to another, may be risky.

Overall, BZD anthelmintics are extensively metabolized in all mammalian species studied. As a common pattern among different BZD, the parent drug is short-lived and metabolic products predominate in plasma and all tissues and excreta of the host, as well as, in parasites recovered from BZD-treated animals (Fetterer and Rew, 1984). The primary metabolites usually are products of oxidation and hydrolysis processes and are all more polar and water soluble than the parent drug. In addition, phase II conjugation reactions are highly important in the detoxification of BZD-derived products; the oxidized and hydrolyzed metabolites are conjugated with glucuronide and/or sulphate to increase their polarities (Hennessy, 1985, 1989), which facilitates urinary or biliary excretion.

Metabolism of BZD and closely related compounds has been shown to be catalyzed by the enzymatic system of hepatic microsomal mixed function oxidases which are involved in such biotransformation reactions as sulphoxidation, demethylation and hydroxylation (Di Cuollo *et al.*, 1974; Gyurik *et al.*, 1981; Souhaili-El Amri *et al.*, 1987, 1988a,b; Short *et al.*, 1988a,b). The metabolism of BZD anthelmintics heavily depends on the substituent present on position 5- of the BZD ring system and involves a variety of reactions. Phase I reactions have been observed at this position; hydroxylation of TBZ (Tocco *et al.*, 1965), PBZ (Di Cuollo *et al.*, 1974), ABZ (Gyurik *et al.*, 1981) and FBZ (Short *et al.*, 1988a,b) have been demonstrated in different species of animals. The

reduction of MBZ (Allan and Watson, 1982), as well as the sulphoxidation of ABZ (Galtier *et al.*, 1986; Souhaili-El Amri *et al.*, 1987,1988a) and FBZ (Short *et al.*, 1988a,b) at the substituent group of the carbon 5- of the BZD nucleus have been also reported. The hydrolysis of the methlycarbamate group at position 2- of the BZD ring to form an amino-derivative product has been shown for MBZ (Allan and Watson, 1982; Behm *et al.*, 1983), FBZ (Short *et al.*, 1988a,b) and ABZ (Gyurik *et al.*, 1981). To a very limited extent, metabolic reactions involving the hydroxylation or N-methylation of the BZD ring itself (TBZ) have been shown to occur (Gottschall *et al.*, 1990).

The administration of TBZ results in a rapid conversion of the parent compound into a 5-hydroxy TBZ metabolite, formed by aromatic ring hydroxylation. Although this metabolite is sufficiently polar for a rapid urinary excretion, both its unconjugated and glucuronide and sulphate conjugated forms have been found in urine of TBZ treated animals (Tocco *et al.*, 1965; Gottschall *et al.*, 1990). The substitution of the BZD ring in position 5- has been particularly important in determining the metabolic fate of the newer BZD drugs. This position is metabolically labile and has permitted retardation of the biotransformation of 5-substituted BZD anthelmintics as well as improvement of their efficacy (Hennessy, 1989). The nature of this substitution in position 5- markedly influences the sequence of BZD liver metabolism. Aromatic BZD derivatives (Figure 1.2) such as FBZ, OFZ, MBZ and TCBZ require more extensive metabolism than aliphatic derivatives (ABZ, PBZ) to achieve sufficient polarity for excretion. Such a phenomenon accounts for the longer residence times and elimination half-lives of aromatic BZD and their metabolites compared to those of aliphatic derivatives. The aromatic substitution sterically hinders and slows the oxidation of the sulphur (FBZ, OFZ) or reduction of the keto (MBZ) linkage; further hydroxylation and the formation of glucuronide and sulphate conjugates results in soluble excretory metabolites,

which are eliminated more in bile (40-60% of the administerd dose) compared to urine (5-20% of the dose) (Hennessy, 1989).

Unlike aromatic substituted BZD, 5-aliphatic substituted BZD such as ABZ and PBZ, when oxidized are sufficiently polar to be largely excreted in urine rather than undergo further conjugation and secretion in bile (Hennessy, 1985, 1989). For instance, following ABZ treatment to sheep only 8% of the total dose was recovered in bile as unconjugated ABZSO and OH-ABZSO metabolites, and 6.3% as conjugated glucuronide and sulphate esters mainly of 2OH-ABZSO and 2OH-$ABZSO_2$ (Hennessy *et al.*, 1989); 59% of the dose was recovered in urine of ABZ-treated cattle (Gyurik *et al.*, 1981). On the other hand, the bile elimination of the aromatic substituted TCBZ in sheep, largely as conjugated metabolites, was approximately 45% of the administered dose while only 6.5% was eliminated by urine (Hennessy *et al.*, 1987). Similarly, the percentage of FBZ dose recovered in urine (2.5%) of orally treated cattle, was markedly lower than that recovered from faeces (42.1%), the latter being mainly the unchanged FBZ parent compound (Short *et al.*, 1987b).

The presence of a sulphur atom in the BZD substitution group at position 5- has a major effect on metabolism. For the commercially available thioether and sulphoxide BZD and pro-BZD compounds, liver microsomal sulphoxidation is a common pathway. However, biotransformation processes, characterized by the oxidation at this sulphur nucleophilic heteroatom, can be undertaken in other tissues. FBZ, ABZ and TCBZ administered as parent drug or as pro-drugs (FBT, NTB), are metabolized to their respective sulphoxide and sulphone entities by liver microsomal oxidation (Short *et al.*, 1988a,b; Souhaili-El Amri, *et al.*, 1987, 1988a; Hennessy *et al.*, 1987). Sulphoxidation appears to be a rapid and reversible process which forms an equilibrium with the respective thioether (Gyurick *et al.*, 1981), although the equilibrium favours metabolism towards sulphoxidation. A portion of the sulphoxide undergoes a

second, slower oxidative step which forms the sulphone metabolite; this latter metabolic step is irreversible (Averkin *et al.*, 1975). Two distinct microsomal enzymatic pathways have been proposed for the sequential ABZ sulphoxidation. The flavin-containing monooxygenase system (FMO) would be responsible for the ABZ first step oxidation in a NADPH-dependent reaction (Galtier *et al.*, 1986; Souhaili-El Amri *et al.*, 1987, 1988a), while the cytochrome P-450 system would be primarily involved in the oxidation of ABZSO to form the $ABZSO_2$ metabolite (Souhaili-El Amri *et al.*, 1988b). This biotransformation processes will be discussed further in this thesis with the experimental data.

Of particular interest are the reports that both ABZ and ABZSO can induce the enzymatic activity of different microsomal pathways, including their own metabolism in rats and humans (Souhaili-El Amri *et al.*, 1988b; Rolin *et al.*, 1989; Steiger *et al.*, 1990). In rats, enhanced sulphonation of the suphoxide metabolite and increased cytochrome P450c activity have been observed after pretreatment with ABZ. This suggests autoinduction (Souhaili-El Amri *et al.*, 1988b). In addition, in human hepatoma cells lines, ABZSO, and to a lesser extent $ABZSO_2$, induced the activity of cytochrome P-448 and UDP-glucuronyltransferase (Rolin *et al.*, 1989). An induced liver metabolism of ABZ and ABZSO may have a significant negative impact on the clinical efficacy of these compounds. However, more research is necessary to confirm these findings and to assess their practical implications under field conditions.

The sulphoxidation reaction is also an important step in the biotransformation of FBZ. As reported for ABZ, the sulphoxide (OFZ) and sulphone are the major metabolites found in plasma in the different species studied. However, the aromatic ring *p*-hydroxylation that leads to the formation of OH-FBZ in the liver seems also to be major pathway in the detoxification of FBZ in species other than sheep (Short *et al.*, 1987a,b, 1988a,b). This OH-FBZ metabolite, in its free and glucuronide

and sulphate-conjugate forms, has been shown to be the major excretory product found in the urine and faeces of goats and cattle treated with FBZ (Short *et al.*, 1987a,b). MBZ is thought to be the active anthelmintic rather than its metabolites (Gottschall *et al.*, 1990); it is biotransformed in the liver by the reduction of its ketone group to the secondary alcohol. The alcohol is the main metabolite; in bile it is found as the glucuronide and sulphate conjugates (Allan and Watson, 1982). This product and a MBZ-amino derivative formed by hydrolysis at position 2-, were the major products found in plasma of MBZ-treated sheep (Behm *et al.*, 1983).

1.3.4. Anthelmintic spectrum

BZD were introduced into the animal health market primarily for the control of GI nematodes, not only for use in livestock animals (cattle, sheep, goats, swine and poultry), but also for horses, dogs and cats. The use of BZD and pro-BZD compounds quickly became widespread because they offered major advantages over previously available drugs in terms of spectrum, efficacy against immature stages and safety for the host animal (Campbell, 1990). In addition, BZD anthelmintics have ovicidal activity which can be of importance under certain conditions when animals are moved at the time of anthelmintic treatment; thus eggs passing out on pasture following treatment are likely to be sterile (Prichard, 1986).

The many BZD anthelmintic compounds do not have identical claims in terms of usages approved by governmental regulatory agencies (Campbell, 1990); however, with the exception of the halogenated BZD thiol, triclabendazole, which has only flukicidal activity against all stages of *Fasciola hepatica*, all BZD and pro-BZD compounds can be classified as broad-spectrum anthelmintic (Marriner, 1986; Bogan and Armour, 1987). However, earlier compounds (ie. TBZ) are not effective against lung worms nor against some larval stages of GI nematodes such as inhibited *Ostertagia ostertagi* in cattle.

The most recent introduced substituted BZD (ABZ, FBZ, OFZ) and pro-BZD (TPT, FBT, NTB) have an extended spectrum of activity being effective against adult, immature and arrested larval stages of important GI and lung nematodes, and also having activity against cestodes and trematodes. Of course, there are differences in spectrum and required dose rates among different compounds in the group. The anthelmintic spectrum and recommended dose rates in sheep and cattle, for the BZD and pro-BZD currently available in the market are summarized in Figure 1.3.

Although different reports show differences in the clinical efficacy of these newer compounds against inhibited *Ostertagia ostertagi* larvae in cattle (Elliot, 1977; Lancaster and Hong, 1977; Bogan and Armour, 1987; Reid, 1988), the activity against this larval stage is an important advance in cattle chemotherapy, because of its relevance from both clinical and epidemiological points of view. Reasons cited for this variability in efficacy include depth of nematode hypobiosis, degree of reduction in larval metabolism, and route of anthelmintic administration (Duncan *et al.*, 1977). In an efficacy trial in cattle, the intraruminal administration of OFZ was shown to be highly effective (93-98%) against both early and late 4^{th} stages of *Ostertagia ostertagi* larvae (Yazwinski *et al.*, 1986); these authors have attributed this consistent efficacy of OFZ against mature and immature GI nematodes in cattle, to the intraruminal injection which avoids the risk of ruminal by-pass.

MBZ, FBZ, OFZ, FBT and ABZ show good efficacy against tapeworms, particularly in sheep. ABZ and NTB at the higher recommended doses are active against adult *Fasciola hepatica*. NTB also shows activity on nematodes (Duncan *et al.*, 1985; Williams *et al.*, 1985), cestodes (Santiago *et al.*, 1985) and trematodes (Richards *et al.*, 1987) with differences in efficacy according to the chosen dose rate and route of administration (Prichard, 1987). Even though the broad

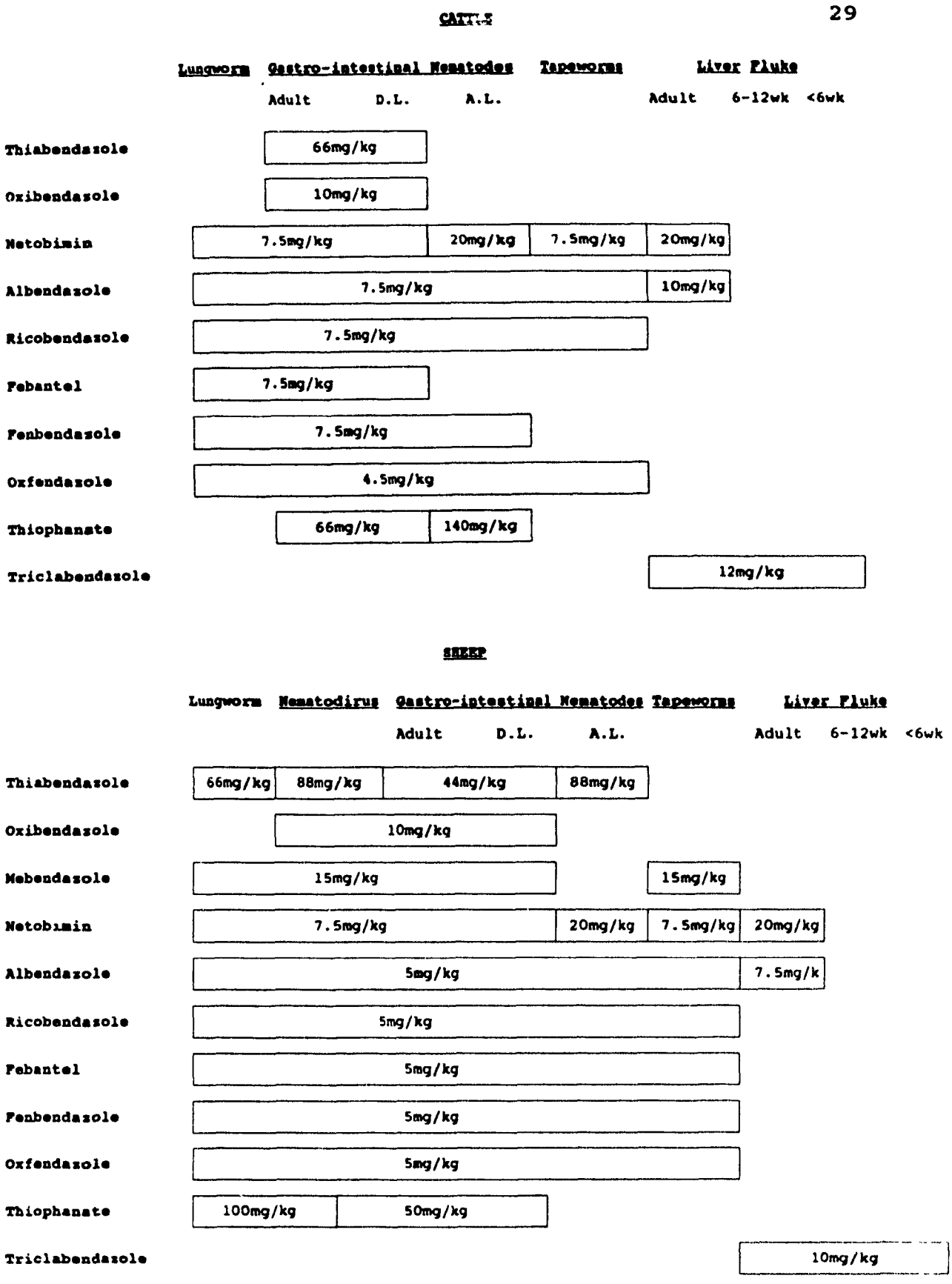


Figure 1.3: Anthelmintic spectrum and recommended dose rates for BZD and pro-BZD compounds in cattle and sheep (From McKellar and Scott, 1990, J. vet. Pharmacol. Therap., 13, 223-247). D.L.= developing larvae; A.L.= arrested larvae.

spectrum of NTB has been shown in both cattle and sheep, it seems that this pro-drug presents some efficacy advantages in sheep compared to cattle. A recent study has shown an extremely high efficacy of NTB, given orally at the higher recommended dose rate (20 mg/kg), against GI nematodes including inhibited trichostrongylds and *Moniezia expansa* in sheep naturally infected (Bauer and Hafner, 1990). It is also active against *Thysanosoma actinioides* (Santiago *et al.*, 1985) and *Dicrocoelium dendriticum* (Sanz *et al.*, 1985; Juste Jordán and García Perez, 1991) in sheep, which is killed by TPT (Onar, 1990), but not for other BZD compounds. It has been demonstrated that the greater potency and spectrum of activity of the newer BZD is largely a function of their pharmacokinetic behaviour rather than entirely due to their intrinsic differences in activity (Prichard *et al.*, 1981; Bogan and Armour, 1987). The moderate to good efficacy of the most modern BZD and pro-BZD against arrested and developing larval stages, depends on their slow GI absorption and the extended residence time of their active metabolites.

1.3.5. Factors affecting pharmacokinetics and efficacy

Some drug-related factors must be completely understood to optimize the anthelmintic efficacy of BZD compounds. The understanding of the complex relationships among route of administration, formulations, drug physicochemical properties and the resultant pharmacokinetic profiles is crucial in optimizing the efficacy of BZD anthelmintics. BZD compounds and/or their active metabolites must be delivered to the site of parasite location by the host, and they are, therefore, subjected to physiological and biochemical processes in the host (Prichard, 1985b). A number of host-related factors may affect the pharmacokinetic behaviour and the resultant clinical efficacy of BZD compounds. Nutritional status, feeding management and disease-related factors could influence the amount of active BZD drug which reaches the parasites and, even more important, the length of time that parasites are

exposed to active drug concentrations.

Absorption and biotransformation are two of the most important processes affected by the above mentioned factors. As stated earlier the spontaneous closure of the oesophageal groove may facilitate ruminal by-pass and substantially alter the absorption pattern of BZD anthelmintics in ruminants. Animal age, diet and other factors affecting ruminal development may have an important impact on the bioavailability and efficacy of BZD anthelmintics. Many dietary components have the potential for markedly affecting absorption of drugs and chemicals from the GI tract; physical and chemical interactions, dilutional effects and pH modifications in the rumen have been shown to affect the rate of absorption of different antibacterial drugs (Burrows and Egerton, 1989). The diet also influences the rate of passage of digesta and that of orally administered BZD drugs; this may in turn affect drug absorption (Prichard, 1985b). The binding of different BZD compounds to dietary fibre substantially modified the duration of the so called "rumen reservoir" effect, altering the overall bioavailability of BZD and their metabolites in the bloodstream (Hennessy, 1989). In addition, some BZD compounds have been shown to decrease the digestibilty of cellulose in the rumen of sheep (Jara *et al.*, 1984). Furthermore, the microbial biotransformation of pro-BZD and some BZD compounds in the GI tract, seems to be relevant for their anthelmintic efficacy. Changes in microflora populations based on dietary modifications may affect the GI metabolism of these drugs. This could lead to differences in pharmacokinetic behaviour and clinical efficacy between animals fed on grain-based diets and those reared exclusively on pasture.

Drug pharmacokinetic characterizations have traditionally been done in healthy animals. However, the presence of the parasite itself could induce important changes on the pharmacokinetic behaviour, side-effects and expected efficacy of the chosen anthelmintic for therapy. Parasite-mediated inflammatory

reaction with changes on mucosa permeability and in abomasal/intestinal pH could have an impact both on absorption and on ionic-trap-mediated distribution of different BDZ metabolites. The presence of parasites in the abomasum such as *Ostertagia ostertagi* (cattle) and *Ostertagia circumcincta* (sheep) causes elevation of plasma pepsinogen concentrations, increases in abomasal pH (Mostofa and McKellar, 1989; McKellar *et al.*, 1990b), and a constant stimulus for gastrin secretion that leads to hypergastrinaemia and pronounced hyperplastic changes in the abomasal mucosal mass (Anderson *et al.*, 1988). Some BZD absorption may occur in the rumen, but the most potent BZD compounds are poorly soluble at rumen pH. The acidic abomasal pH facilitates solubility and subsequent absorption in the lower GI tract. Marriner *et al.* (1984) observed that the presence of *Ostertagia circumcincta* infection in sheep reduced the solubilization of FBZ, subsequent plasma concentrations of FBZ and its metabolites, and reduced pH trapping in the abomasum and thus exposure of GI parasites to high BZD concentrations. Similarly, the OFZ bioavailability was reduced by 25% in goats infected with this parasite compared to uninfected animals (Bogan *et al.*, 1987b). In addition, the administration of FBZ has been shown to increase Na^+ concentrations in abomasum whilst the presence of abomasal parasite infection further increased this cation, abomasal transmural potential difference and pH, decreased K^+ concentrations, and stimulated HCO_3^- transfer (Dakkak *et al.*, 1985). Furthermore, GI parasitism alters gut transit times, which also may contribute to a modified pharmacokinetic pattern for BZD anthelmintics.

Liver disease and parasite-mediated liver damage with alteration of liver enzyme pattern could affect biotransformation and the resultant bioavailability of anthelmintically active BZD metabolites. A reduced enzymatic activity of different liver microsomal function oxidases has been reported in *Fasciola hepatica*-infected rats (Tekwani *et al.*, 1988) and sheep (Galtier *et al.*, 1987), which could lead

to altered patterns of drug/xenobiotic metabolism and clearance (Tufenkji *et al.*, 1988). Furthermore, enhanced clearance of antipyrine following infection of lambs with *Haemonchus contortus* has been recently reported (Kawalek and Fetterer, 1990). Amongst other speculations these investigators have correlated such an effect with a potential inductive response of hepatic P-450-mediated activities triggered by the infection. This might be caused by a generalized enhancement in hepatic protein synthesis, associated with the physiological response to replace plasma proteins and other blood components lost through GI haemorrhage due to the parasite's feeding. Although TCBZ pharmacokinetic behaviour was unaffected by *Fasciola hepatica* infection in goats (Kinabo and Bogan, 1988), it is likely that a potential modification in liver biotransformation will be more strongly reflected in the pharmacokinetics of those BZD with shorter residence times.

The nutritional status, pregnancy, and milk production may induce changes in adipose tissue deposition and in corporal fluids dynamics; these changes are likely to affect the pattern of distribution, plasma protein-binding and efficacy of BZD anthelmintics. Gestation has been shown to influence the pharmacokinetics of different sulphonamides in goats (Van Gogh *et al.*, 1990). Malnutrition due to a chronic deficiency of protein and iron may also alter plasma BZD concentrations and uptake by GI parasites (Prichard, 1985b).

There is sufficient evidence that changes in the pharmacokinetic behaviour and metabolism of BZD anthelmintics may lead to poorer clinical efficacy and facilitate selection for BZD resistant strains. The understanding of potential factors affecting the pharmacology of BZD, and other anthelmintics, in production animals is crucial to the optimization of their use and to reduced investment in parasite control. More research is required of those factors that affect drug efficacy in field conditions, whereby large

number of animals require frequent treatments to minimize losses caused by parasitism.

1.3.6. Resistance

Anthelmintic resistance is the presence of a heritable greater frequency of worms within a population able to tolerate recommended doses of a drug than in a normal population of the same species (Prichard *et al.*, 1980). The intensification of animal production systems has led to an increasing reliance on effective anthelmintic drugs to control helminth parasite diseases. The greatest problem with anthelmintic resistance in nematode parasites of livestock is associated with the BZD group. Resistance in sheep and goat nematodes, particularly of the trichostrongylid genera, to the effects of BZD is of economic importance in countries where climatic conditions and animal management strategies are highly favourable for parasite transmission and the spread of resistant populations. It is largely in the southern hemisphere, where animals graze all year round on the same pasture and receive regular suppressive treatments, that anthelmintic resistance reaches the highest prevalence rates. The incidence of resistance in cattle parasites to the effects of anthelmintics is low (McKellar and Scott, 1990), but an increasing number of reports are showing the emergence of cattle parasites resistant to BZD anthelmintics (Eagleson and Bowie, 1986; Jackson *et al.*, 1987). However, BZD resistance has not been a concern for parasite control in cattle so far.

Resistance to TBZ in the sheep parasite *Haemonchus contortus*, was reported shortly after the introduction of this compound into the market (Drudge *et al.*, 1964). Since then, resistance to different BZD compounds developed slowly but steadily in nematodes of sheep and goats (Prichard *et al.*, 1980), reaching now all the major genera of nematodes. Although originally described for TBZ, the resistance to the action of BZD anthelmintics has been extended to most of the compounds in

the group; in addition this resistance has been shown to cross among different BZD compounds. The appearance of parasites resistant to the effects of BZD anthelmintics has been extensively reported in the past few years and reviewed several times (Prichard, 1978, 1990; Coles, 1986; Waller, 1990; McKellar and Scott, 1990).

The biochemical basis of BZD resistance has been under intensive investigation in the past few years, and relevant progress in its understanding has been achieved. It now seems clear that the mechanism of resistance in nematodes is based on an alteration on the BZD-tubulin interaction. It has been observed that when given to nematode-infected animals at therapeutic dose rates, BZD anthelmintics cause microtubules to disappear from intestinal cells of BZD-susceptible, but not BZD-resistant, nematodes (Borgers *et al.*, 1975; Sangster *et al.*, 1985). Furthermore, *Haemonchus contortus* BZD-resistant strains were less sensitive to the inhibition of 3-O methylglucose uptake induced by classical microtubule inhibitor compounds such as colchicine and podophyllotoxin, as well as by TBZ and MBZ (Rew and Prichard, 1985). It was first reported that the mechanism of resistance in *Trichostrongylus colubriformis* involves a reduction in the association constant (Ka) of BZD compounds to the tubulin receptor of the resistant strain compared with that of the susceptible one (Sangster *et al.*, 1985). Tubulin from resistant mutants of *Haemonchus contortus* has been shown to bind less BZD drug than susceptible strains of the same parasite (Lacey and Prichard, 1986). Lubega and Prichard (1990) have recently shown that the binding of different BZD compounds to nematode tubulin could be resolved into a high affinity and a low affinity components; they did not observe changes in the Ka of BZD for tubulin of the resistant strain of *Haemonchus contortus* compared to the susceptible one, and they associated resistance with a loss of high affinity receptors to BZD drugs in tubulin from this mutant. The existence of at least three ß-tubulin isoforms has been described in a BZD-susceptible

strain of *Haemonchus contortus*, and changes in this isoform pattern in the BZD-resistant strain have been identified (Lubega and Prichard, 1991a). This pattern of isoforms for ß-tubulin is consistent with genetic analyses that indicated that more than one gene determined resistance to TBZ (Prichard, 1990). In addition, it has been found that ß-tubulin DNA from BZD-susceptible and resistant *Haemonchus contortus* shows different restriction patterns (Roos, 1990), which may be consistent with the differential patterns in ß-tubulin isoforms described above.

Despite attempts to introduce integrated control measures, parasite control still relies almost exclusively on anthelmintic treatments. Frequent treatment, underdosing and failure to alternate with other drug classes are commonly associated with occurrence of BZD resistance; however, there are other important management and epidemiological factors to be considered. For instance, frequent dosing during an episode of clinical parasitism, when larval availability on the pasture is high, may not be as strong a selector for resistant as fewer doses at a time when most parasites are within the host (Waller, 1990). Perhaps, the best strategy for delaying the emergency of resistance is to use as few anthelmintic treatments as possible each year. When resistance is not already a problem integrated systems of parasite control that rely on a minimal use of anthelmintic will help to prevent the build up of resistant genotypes (Taylor and Hunt, 1989). Rotation between BZD and other compounds with different mode of action, particularly those of a narrower spectrum, at yearly intervals has been suggested for decreasing the occurrence of BZD resistance (Prichard, 1987; Taylor and Hunt, 1989).

Without the availability of alternative drugs (except avermectins), an efficient broad-spectrum control of parasitic disease will require either a pharmacological-based improvement of existing compounds or the development of

improved formulations based on currently available anthelmintics. Certainly, a "responsible" use of slow release devices will be required to avoid parasites being exposed to low drug concentrations for extended periods of time. The development of resistance to anthelmintics in general, and to BZD in particular, is the single most important problem confronting the future control of helminth parasites in livestock. Further investigation needs to be carried out into many aspects of BZD resistance. The complete understanding, at the molecular level, of how parasites avoid the action of these drugs is still an important challenge for research. The existing in vitro techniques to detect BZD resistance in the field, namely, the egg-hatch assay, tubulin binding assay and a larval-development assay (Martin *et al.*, 1989; Johansen and Waller, 1989; Kerboeuf *et al.*, 1989) may require improvements to increase sensitivity. Meanwhile, practical management strategies and scientifically-based chemotherapy should be integrated in an attempt to overcome existing anthelmintic resistance and, particularly, to avoid its occurrence.

1.3.7. Modes of administration and formulations

As expressed earlier BZD anthelmintic compounds are virtually insoluble in water, which limits the formulation to suspension, paste, granule or powder for oral or intraruminal administration, or for administration in feed. For instance, FBZ was equally effective in the removal of common mature and immature cattle nematodes, administered as either oral drench or pellets or in feed premix (Blagburn et al., 1986). Drench formulations are most frequently used in ruminant species; pastes are often preferred for horses and tablets for dogs and cats. BZD have generally been administered to ruminants in the form of a single oral dose. This single dose treatment has traditionally been done by oral drench and more recently, by intraruminal injection; this latter approach is based on the design of a special syringe (intraruminal injector) that ejects the drug directly into the rumen cavity (Borgsteede and

Reid, 1982). This device is commercially available for the administration of a concentrated suspension of OFZ to cattle and was mainly designed to overcome the potential problems of the oesophageal groove closure after oral treatment.

To reduce the cost associated with treating large numbers of animals and with growing evidence that divided anthelmintic doses and prolonged administration increase anthelmintic efficacy (Prichard *et al.*, 1978a; Donald, 1985), several methods for drug delivery have been used in the past few years. The incorporation of drug into feed blocks, for ingestion in small amounts over a prolonged grazing period (Thomas, 1978), and the inclusion of drugs in drinking water have been used for therapeutic and prophylatic parasite control (Downey, 1987). Convenience and labour saving are obvious for these group medication systems, but at the same time no direct control is possible over the drug intake rate of individual animals (Donald, 1985).

The most versatile new technology in anthelmintic drug delivery has been the development of ruminal devices that when given to individual animals can deliver drugs for an extended period of time. These controlled-release devices or boluses more readly provide the conditions for increased anthelmintic efficacy, such as prolonged exposure of parasites to sustained concentrations of active parent drugs or their metabolites. After oral administration, the boluses remain in the rumen-reticulum and release the drug over a long period of time either in a sustained or pulsatile manner. Different controlled-release systems have been developed for the delivery of BZD anthelmintics. The OFZ pulse release bolus (Rowlands *et al.*, 1988) is based on the principle of releasing a series of individual therapeutic doses of OFZ at pre-determined intervals (20-21 days), which approximately coincide with the pre-patent period of the major parasite nematodes of cattle, for about four months. This is achieved by the continuous galvanic corrosion of a magnesium alloy rod

that periodically exposes an annular OFZ tablet to the ruminal fluid (Campbell, 1990). The reliability of this device in field conditions was confirmed by monitoring the plasma levels of OFZ and its metabolites (Bogan *et al.*, 1987a). These experiments indicated that the concentration of OFZ in plasma after each pulse is equivalent to that achieved with a normal therapeutic dose of 4.5 mg/kg. The periodic release of ABZ in cattle has been achieved by the development of a device that releases the drug in pulses controlled by an electronic system powered by small alkaline batteries (Campbell, 1990). This system is electronically timed to release three doses of ABZ at 31-day intervals. Pulse release devices allow the development of some parasites up to the late fourth and early fifth stages, which may be sufficient to stimulate an immune response. This may be an important advantage compared to sustained release bolus, and it is likely that cattle treated with a pulse release bolus system will acquire stronger immunity than those treated with a sustained release system, which could be of major relevance in the challenge of the next grazing season (McKellar, 1988).

An intraruminal slow release capsule which delivers, in the rumen of sheep and cattle, a low daily dose of ABZ for approximately three months has been recently designed (Delatour *et al.*, 1990a). The device is a hollow cylinder of non-biodegradable plastic which contains tablets of 3.85 g (sheep) and 18.46 g (cattle) of ABZ, and two external wings. After the administration, the wings spread out while the ruminal fluid starts to dissolve the first tablet, which releases the drug slowly in the rumen; a metal spring pushes six tablets toward the open end of the capsule. Recently, Delatour et al. (1990a) have characterized the plasma concentration profiles of ABZ metabolites following the administration of the aforementioned device in sheep and cattle; ABZ metabolites were found in plasma for 90 (cattle) and 105 (sheep) days post-capsule administration. Interesting parasite control results were obtained using this device in

sheep once a year, over a period of four years (Dorchies and Alzieu, 1990). In another efficacy trial, this ABZ slow release capsule was highly effective both in removing the existing burdens of mature and immature worms in the treated sheep, and in preventing egg excretion during the period in which the capsule was releasing ABZ (Barton *et al.*, 1990).

In an attempt to determine the delivery system that results in the most desirable availability and subsequent efficacy of ABZ, different infusion systems were studied in a sheep model (Kwan *et al.*, 1988). Confirming previous findings, these authors found that the percentage worm reduction increases from a single ABZ dose to divided daily doses (intermittent release) to continuous infusion. Undoubtedly, anthelmintic activity of BZD in general, and ABZ in particular, is influenced by the time of residence of anthelmintically active metabolites. However, some concern has been raised about the potential enzyme inductive effects of ABZ and its metabolites on microsomal oxidation systems (Souhaili-El Amri *et al.*, 1987, 1988a,b; Rolin *et al.*, 1989). The prolonged presence of ABZ metabolites in the bloodstream could induce liver microsomal enzymes, which may result in an even faster ABZ biotransformation sequence; this may lead to poorer efficacy and a higher risk of selection for resistant strains. In principle, controlled release of anthelmintics, and BZD in particular, substantially increases the risk of selecting for resistance compared with the ocasional single dosing (Donald, 1985). Although little empirical evidence is available, there is growing concern about the effect of controlled-release devices, particularly the sustained release formulations, on the selection pressure for drug-resistance populations (Zimmerman and Hoberg, 1988). Although the efficacy results obtained are encouraging, the implications of anthelmintic slow release delivery systems on drug resistance, individual immunity and potential enzyme induction effects require further investigation. Meanwhile, the search for alternative approaches to potentiate existing anthelmintic drugs, with

minimization of risks, must continue.

1.3.8. Safety and toxicity

BZD are probably the least toxic of all the anthelmintic drugs. The remarkable overall safety of BZD compounds has been a major factor in their successful worldwide use over three decades (Campbell, 1990). All BZD compounds are extremely well tolerated by domestic animals and man, and they are characteristically free of side effects at therapeutic doses even when administered to young, sick or debilitated animals (Roberson, 1982). It has been impossible to find out the LD50 for TBZ and FBZ (Bogan and Armour, 1987). Consecutive daily doses of FBZ at 45 mg/kg/day during 30 days are well tolerated in sheep; in ruminants and horses, OFZ does not cause detectable toxic effects at a single administration of 10 times the recommended dose or at 8 succesive administration of 3 times the therapeutic dose at 4-day intervals (Roberson, 1982). The high safety margins, particularly for the most potent substituted BZD, is thought to be correlated with their low solubility in the GI fluids; this accounts for a low absorption rate and for insufficient drug reaching the bloodstream to have a toxic effect (McKellar and Scott, 1990).

However, some members of the group such PBZ, CBZ, OFZ and ABZ have been reported to be teratogenic at approximately 4 times their recommended doses; this limits their use in the early stages of pregnancy and sheep seem to be especially sensitive compared to other species (Marriner, 1986; McKellar and Scott, 1990). These species differences in the sensitivity to the teratogenic activity may be related to differences in the pharmacokinetics and metabolism of BZD among species. PBZ and CBZ exert a teratogenic effect when given to pregnant ewes during the 2^{nd}-4^{th} weeks of gestation. The period of greatest embryonic susceptibility to these drugs coincides with the time that normal embryonic limb development begins (approximately on day 20^{th} of pregnancy); the principal

malformations observed following treatment in this stage of pregnancy have been deformities of the limbs and overflexion of the carpal joints (Roberson, 1982). Reports of congenital malformations resulting from administration of other BZD compounds (OFZ, ABZ, FBT) to ewes in early gestation have been also made. The mechanism of BZD teratogenicity seems to be related to their effects on the disruption of the tubulin-microtubules equilibrium dynamics in mammalian cells. Other tubulin-binding drugs such as colchicine and vinblastine, which are not anthelmintics, do cause teratogenic effects in rats (Delatour *et al.*, 1984). In addition, many anti-microtubular drugs are antimitotic and used in cancer chemotherapy (Cabral and Barlow, 1989). Microtubules are responsible for the formation of the mitotic spindle and they are relevant for cell division (Lacey, 1988). BZD compounds may exert their teratogenic and embryotoxic effects by disruption of microtubule dynamics and subsequent alteration in cell division. Recently, studies using human-hepatoma cell lines have shown that ABZ and its metabolites cause a drop in cell proliferation and a rise in the mitotic index resulting from cell division blockage (Rolin *et al.*, 1989). These investigators found that ABZ blocked the cells at the early metaphase stage with a rather toxic and necrotic effect after prolonged in vitro exposure (24 h), whereas the ABZSO and $ABZSO_2$ metabolites induced metaphase blockage accompanied by few anaphase abnormalities and anarchic chromosomal dispersions. From these studies it was also suggested that ABZ parent drug is more cytotoxic than its metabolites, which is inconsistent with previous reports that attributed teratogenic effects in rats to the sulphoxides metabolites (OFZ and ABZSO) found in high concentrations in plasma (Delatour *et al.*, 1984). Assuming that the more lipophilic parent thioethers (ABZ, FBZ) are more toxic than their sulphoxides and sulphones metabolites, the rapid bioconversion and removal of the parent drug from the bloodstream may also account for large safety margin of these BZD drugs in non-pregnant animals. Because of tissue and milk residues, slaughter clearance times are

required after treatment with substituted BZD, and it is recommended that milk of treated animals not be used for human consumption. Withdrawal periods for the BZD vary from zero in the case of TBZ for both meat and milk, up to 14 days for meat in the case of the more modern drugs (Marriner,1986).

1.4.: PRINCIPLES OF PHARMACOKINETICS

Pharmacokinetics is the study of the time course of drug and metabolite concentrations in various body fluids, tissues, and excreta and the construction of suitable models to interpret these data (Wagner, 1968), or more specifically, the study of the kinetics of absorption, distribution, metabolism and elimination of drugs and their pharmacologic, therapeutic or toxic response in man and animals (Notari, 1987). Bioavailability indicates the rate and relative amount of the administered drug which reaches the general circulation (Notari, 1987). Maximum bioavailability is achieved when a drug is administered into the vascular system or when a non-vascular administered drug, is completely absorbed from the site of administration into the bloodstream. In the case of any other oral or parenteral route of administration, the bioavailable dose will generally be less than the administered dose. Bioavailability is estimated by comparing the areas under the concentration-time curves (AUC) obtained after the administration of a drug by a non-vascular and a vascular routes (absolute bioavailability), or after the administration of two different formulations of the same drug by the same or different routes and at equal dose rates (relative bioavailability) (Sietsema, 1989). Biopharmaceutics studies the factors influencing the bioavailability of a drug or its metabolites and the use of this information to optimize therapeutic activity of drug products in clinical application. The knowledge of the amount of active drug that reaches the site of action as a function of time is crucial to maximize drug efficacy. The concepts of pharmacokinetics and bioavailability are important in biomedical drug research,

pharmaceutical product development and in the optimal use of drug formulations.

Release from dosage form and absorption precede entry of a drug into the bloodstream, which serves as the tissue in which drug and metabolite molecules are conducted to various parts of the body. Within the bloodstream, a fraction of most drugs binds reversibly to plasma proteins, and the remainder undergoes simultaneous distribution, metabolism and excretion (Baggot, 1978). The access of drug molecules to intracellular sites depends upon their ability to penetrate the capillary endothelium and to cross the cell membrane. A schematic representation of the various processes that control the movement of the drug in the body and the duration of the pharmacological response is presented in Figure 1.4. Most drugs are weak organic acids or bases and exist in solution, at physiological pH, as both non-ionized and ionized forms (Baggot, 1982); while the poor lipophilicity of the ionized molecules excludes them from passive diffusion, the lipid-soluble non-ionized moieties passively diffuse across biological membranes until an equilibrium is established. The ratio of non-ionized to ionized forms depends upon the pKa of the drug and the pH of the fluid in which the drug is dissolved, and the pH gradient between plasma and different tissues dictates the concentrations of drug/metabolite at either side of the separating cell membranes; at equilibrium, there will be a higher total concentration of the drug on the side of the membrane where the degree of ionization is greater (Baggot, 1978, 1982).

The most commonly used approach to the pharmacokinetic characterization of a drug is to represent the body as a system of compartments, even though these compartments usually have no physiologic or anatomic reality, and to assume that the rate of transfer between compartments and the rate of elimination follows first-order kinetics (Gibaldi and Perrier, 1982). In general, such compartmental models can be classified

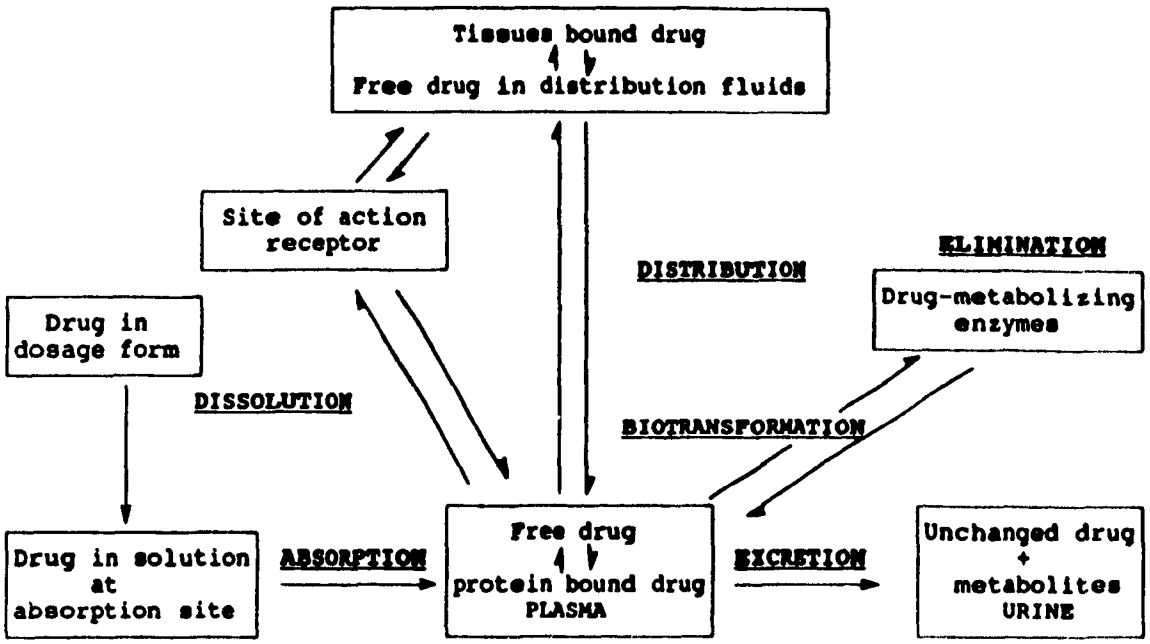


Figure 1.4.: Schematic representation of the various processes that influence uptake, access to the site of action, and removal of a drug and its metabolites from the body (from Baggot, J., 1978, J. vet. Pharmacol. Therap., 1, 5-18).

as single or multi-compartmental; the one-compartment model, the simplest one, assumes that the body is homogeneous with respect to the drug distribution and that the drug distributes rapidly throughout the body. Th most commonly used kinetic model is the two compartment model which assumes that the drug is homogeneously and instantaneously distributed through the central compartment (blood and extra-cellular fluids of well perfused organs such as liver and kidneys) and that this compartment equilibrates more slowly with a peripheral compartment (less well perfused organs) (Baggot, 1978). Therefore, when the logarithm concentration-time curve, after intravenous injection of a drug which follows a two-compartment model is examined, two different phases are seen: 1) distributive phase (rapid decline in plasma levels), and 2) elimination phase (slow linear decline in plasma levels). Often tri-compartmental models are required to describe the disposition kinetics of some compounds, notably those with extensive tissue distribution.

In addition to compartmental-based models, other different approaches have recently been made to characterize drug disposition kinetics; among them are: non-compartmental, physiological, population pharmacokinetic and pharmacokinetic-pharmacodynamic models (Kinabo and McKellar, 1989). The choice of the "ideal" model is complex and many factors must be rationally evaluated, particularly those affecting the time course of drug in the body. Physiologic-based kinetic models utilize anatomical (real) tissue volumes, blood flow rates and other physiological and biochemical data, to characterize drug disposition (Nakashima and Benet, 1988). Although very useful to describe the dynamics of drug distribution, these models require a large amount of data, which make them difficult to develop (Kinabo and McKellar, 1989). Investigators in veterinary pharmacology, largely an applied science, have generally used kinetic models rather than developed them (Kinabo and McKellar, 1989); according to these authors 90% of the pharmacokinetic analyses reported in veterinary journals

were based on classical compartmental models. However, I have personally noticed a gradual change from compartmental towards non-compartmental methods of analysis in a growing number of articles regarding drug pharmacokinetics in domestic animals. Nonetheless, this is not the case for anthelmintic drugs where pharmacokinetic reports have been mostly restricted to plasma concentration-time data. The experimental data generated throughout this thesis research, for parent drugs and/or their metabolites, have been analyzed using either compartmental or non-compartmental approaches, or both, according to different situations and in order to optimize the characterization of drug disposition kinetics. While the linear regression theory (compartmental models) relies exclusively upon the estimation procedure known as least squares, the non-compartmental approach uses an estimation procedure known as the method of moments (Powers, 1990); both of these approaches lead to estimates of the pharmacokinetic parameters, which are the quantitative tools to define the drug kinetics behaviour. Non-compartmental methods are based on the statistical moment theory (SMT) (Yamoaka *et al.*, 1978) and do not require the assumption of a specific compartmental model; these models can be applied to virtually any compartmental model provided that linear pharmacokinetics can be assumed (Gibaldi and Perrier, 1982), and have been also called "independent models" (Notari, 1987). However, this is a debatable topic and some authors have indicated that non-compartmental models require even a more restricted structure than multicompartmental methods (Powers, 1990). The mean residence time (MRT), obtained from non-compartmental analysis, is an estimate of the average amount of time required for an intact drug/metabolite molecule to move trough the body (Mahon *et al.*, 1987). The MRT is a composite of all kinetics processes including absorption, distribution, metabolism and elimination, and is a good indicator for comparative characterization of the overall pharmacokinetic behaviour of drug/metabolites under different experimental or clinical conditions. Although the whole outcome of the kinetic analysis, regardless of the chosen

model, is important in the characterization of drug/metabolites behaviour, the big "challenge" is the adequate interpretation of those parameters with clinical and practical relevance.

1.5.: CONCEPTS OF DRUG BIOTRANSFORMATION

The biotransformation processes in the body tend to transform drugs/xenobiotics into less lipid soluble and more polar metabolites for easier and faster elimination. The fate of a drug is largely determined by certain of its physicochemical properties, specifically lipid solubility and degree of ionization. A certain degree of lipid solubility appears to be a prerequisite for drug biotransformation by the hepatic microsomal enzyme system (Baggot, 1978, 1982). Hydrophilic drugs and the majority of drug-metabolism products (metabolites) are sufficiently polar to be eliminated, largely by the kidneys. Polarity is a requisite to render metabolites suitable for carrier-mediated excretion processes.

The hepatic microsomal fraction is, quantitatively, the main site for drug biotransformation. The general pattern of drug metabolism is biphasic. Phase I reactions (oxidation, reduction, hydrolysis) usually convert the parent drug to a more polar metabolite by introducing or unmasking a functional group (-OH, -NH2, -SH). Often these metabolites are inactive, although in some instances activity is increased or modified (ie. BZD biotransformation). These functional groups enable the compounds to undergo conjugation (phase II reactions) with endogenous substances such as glucuronic acid, acetate, sulphate, aminoacids, bile salts or glutathione. These phase II reactions (synthetic reactions) produce inactive and water soluble products which facilitates their elimination in bile or urine (Baggot, 1977). Phase I drug metabolism tends to be catalysed by two major microsomal enzyme complexes, including the heme-associated cytochrome P-450 and non-heme-containing flavin monooxygenase (FMO) systems. Some years ago, all NADPH-

dependent phase I oxidations were believed to be catalysed by cytochrome P-450; however subsequent studies with secondary and tertiary amines resulted in the purification of the microsomal FMO system (Gottschall *et al.*, 1990), which catalysed the oxidation of nucleophilic nitrogen and sulphur-containing compounds. The FMO system was found to contain approximately 15 nmole flavin-adenin-dinucleotide (FAD)/mg of protein and to have a molecular weight of 65 000; the enzyme mechanism involves the two-electron reduction of the flavin by NADPH, followed by binding of molecular oxygen to form a flavin hydroperoxide. The substrate binds, oxygen transfer occurs, and both oxidized substrate and $NADP^+$ are released in preparation for another cycle (Baggot, 1977; Gottschall *et al.*, 1990). Cytochrome P-450 is the enzyme of primary importance, in phase I biotransformation in particular, and in the metabolism of all foreign compounds in general. It is a family of isoenzymes with distinctive and overlapping substrate specificities. P-450 isoenzymes are membrane bound and located in the microsomal fraction. Their minimum molecular weight is 49 000, and each polypeptide chain contains one heme iron. The enzyme mechanism involves the binding of a drug substrate to cytochrome P-450; the oxidized cytochrome P-450-substrate complex is subsequently reduced by transfer of two electrons from NADPH, via cytochrome P-450 reductase. Then, molecular oxygen binds to form a cytochrome P-450-O_2-substrate complex. The oxidized product then dissociates, and the regenerated oxidized cytochrome P-450 is ready for another cycle (Baggot, 1977; Hennessy, 1985). Unlike the FMO system, P-450 isoenzymes can be induced by different agents which are themselves metabolized by the microsomal enzymatic fraction. Increased concentrations of different P-450 isoenzymes and the proliferation of the smooth endoplasmic reticulum are common features of liver enzymatic induction, which has been extensively studied in the past few years. This phenomenon leads to an enhanced metabolizing capacity for endogenous or exogenous compounds. Overall, enzyme induction indicates increased drug metabolic rates, faster elimination

and decreased pharmacologic effects; however, the influence of induction on pharmacologic action depends on the relative activity of the parent drug and its phase I oxidized metabolites. Conversely, the rate of drug metabolism can be decreased by drug-mediated inhibition processes. Lower metabolic rates, delayed drug/metabolites elimination and improved pharmacologic response are direct consequences of enzyme inhibition. This concept is one of the basic principles of the research carried out in this project and will be extensively discussed in the chapters ahead.

Although the liver is the primary organ for drug/xenobiotic biotransformation and detoxification, drug metabolism also takes place in other tissues such as blood, plasma, lungs, kidneys and GI tract. The particular relevance in ruminant therapeutics, is the drug metabolism carried out by the microflora of the rumen and intestine. By comparison to the liver, in which oxidative metabolism predominates, the GI microflora by virtue of the low redox potential of the organ's lumen, are very active in reductive reactions (Rowland, 1986). Reduction by the GI microflora plays an important role in the metabolism and toxicity of a number of drugs and other foreign compounds, particularly those containing nitro (Rowland, 1986) and sulphoxide (Renwick *et al.*, 1986) groups. The GI metabolism of such drugs may be modified by a variety of factors which influence the bacterial reductive capacity, including diet. Clearly, orally administered compounds which are poorly absorbed from the GI tract will stand the greatest chance of undergoing metabolism by the microflora, although a large number of drugs/metabolites gain entry to the gut via biliary secretion or plasma-GI exchange and will also be exposed to microbial activity. Hydrolysis is another very common reaction that occurs in the GI tract. The biodegradation of active drugs by microflora of the ruminant forestomachs leading to poor bioavailability, has been described for several drugs of high therapeutic relevance in veterinary medicine (Ladage *et al.*, 1989), such as

norgestagens, digitalis glycosides, trimethoprim, chloramphenicol and ivermectin.

1.6.: BASIC LINKAGES BETWEEN PHARMACOKINETIC BEHAVIOUR AND ANTHELMINTIC EFFICACY

Internal parasites live on the mucosal surface of the GI tract, the lungs, bile ducts or within other tissues. Thus, it is necessary for an anthelmintic to be absorbed and the parent drug and/or active metabolites transported to the site of infection via the circulation. BZD and pro-BZD anthelmintics while usually highly efficacious against adult GI nematodes, are less so against immature and tissue dwelling parasites in man and animals. BZD anthelmintic activity not only depends on their affinity for parasite tubulin but also on their ability to reach high and sustained concentrations at the site of parasite location; this in turn depends on the pharmacokinetic behaviour and metabolic pattern of these compounds in the host. The relationship between pharmacokinetics and clinical efficacy for anthelmintic compounds is now well substantiated.

The development of specific analytical methodology, notably high performance liquid chromatography (HPLC), combined with efficient drug extraction techniques, has meant that it is now possible to measure drugs and their metabolites with high accuracy in different biological fluids. Thus, pharmacokinetic profiles and disposition kinetics patterns for parent drugs and metabolites can be characterized, and related to dose rates and efficacy data. In addition, the availability of modern methodology for *in vitro* characterization of drug/xenobiotic metabolism, has positively contributed to the understanding of the pharmacology of antiparasitic drugs. Furthermore, the identification of different BZD metabolites by molecular chirality, using a HPLC on chiral stationary phase technique has been recently described (Delatour *et al.*, 1990b,c,1991a,b). Chirality is a structural characteristic

which results in a molecule being asymmetric and consequently non-superimposible with its mirror image (Lee and Williams, 1990). Since many steps in drug metabolism have been shown to be chiral-dependant, the identification of different BZD enantiomers in plasma is a significant contribution to the pharmacology of these compounds.

A toxic anthelmintic concentration must be presented to the parasite for sufficient time to lead to irreversible damage. The intrinsic action of the BZD molecule on the parasite is based on the disruption of basic cell functions that requires a "lag time" until the parasite's survival is threatened. The parasite may be able to survive in the short term, but if the impairment of essential functions is maintained for a sufficiently long time, the ability of the parasite to survive at its predilection site will be affected. Thus, anthelmintic concentrations above the required minimum inhibitory concentration (MIC) and a slow decline of the active drug/metabolite elimination phases, leading to an extended time of parasite exposure, are clinically important.

The rate of absorption, metabolism and excretion of BZD anthelmintics varies from drug to drug, with slower absorption and prolonged recycling between enteral and parenteral tissues being relevant to enhance efficacy. Worms attached to the lining of the gut may be more exposed to this recycling drug than to that actually passing down the GI tract in food which is being digested (Prichard, 1985a). Moreover, the absorbed BZD drug may be more important than unabsorbed drug passing down the gastrointestinal tract, even against gastrointestinal nematodes (Hennessy and Prichard, 1981). These researchers compared single oral OFZ administration with a variety of intravenous OFZ administration regimens, all at the same dose rate (5 mg/kg), and found intravenous administration was equally or more effective than oral administration against BZD-resistant *Haemonchus contortus* in the abomasum and *Trichostrongylus colubriformis* in the small intestine. It is

clear then, that the plasma concentration of anthelmintically active moieties reflects the pattern of exposure of worms in, or on, the GI mucosa, as well as, of those located in other tissues; thus the characterization of the plasma pharmacokinetic profiles and disposition kinetics of a parent drug and its metabolites, is critical to predict and optimize anthelmintic efficacy.

1.7. GOALS OF RESEARCH

The complete comprehension of the pharmacokinetic and metabolic behaviour of BZD and pro-BZD compounds in the host as well as of those factors affecting them, is highly important to maximizing their anthelmintic efficacy. Netobimin (NTB), a modern pro-BZD, was the compound largely used as a BZD model throughout this research project. The reasons for that choice were: a) the area of pro-drug research is relatively new in the anthelmintic field, and has great promise, b) NTB as a salt is freely soluble in water, which allows a great flexibility of formulation for both oral and parenteral administration; this a major practical advantage compared to other insoluble BZD drugs, c) NTB is a broad-spectrum anthelmintic and exerts its action by conversion into ABZ-like moieties; thus, NTB appropriately represents the BZD group and, d) all these features make NTB a pharmacologically interesting compound, being the ideal anthelmintic molecule to study the influence of drug formulation and route of administration on pharmacokinetic behaviour and clinical efficacy in different species.

The sequential metabolic oxidations of BZD and pro-BZD thioethers in the host leads to more polar and less active metabolites. In terms of parasite uptake and binding to parasite tubulin, the parent thioethers are more potent than the sulphoxide metabolites, while the sulphone metabolites are inactive (Lacey *et al.*, 1987; Lubega and Prichard, 1991b). The

oxidized sulphur atom present in both sulphoxides (S=O) and sulphones (O-S-O), tends to make then more polar than the parent thioether. This may result in decreased parasite uptake and more difficult tissue distribution, facilitating a faster elimination. Therefore, the metabolism (sulphoxidation) of the parent thioether into its sulphoxide, and even more so into its sulphone, results in a considerable reduction in anthelmintic efficacy. This liver microsomal sulphoxidation is then, relevant for the pharmacokinetic behaviour and clinical efficacy of BZD and pro-BZD thioethers and it is a research target in this project. Therefore, by manipulation of the pharmacokinetics and biotransformation patterns for BZD and pro-BZD drugs, it may be possible to improve their clinical efficacy against GI and tissue-dwelling parasites; this could be particularly important in improving efficacy against the most difficult parasites to control in livestock, such as arrested *Ostertagia ostertagi* larval stage, immature stages of *Fasciola hepatica* or BZD-resistant strains of *Haemonchus contortus*, which are only susceptible to active drug concentrations presented for long periods of time.

To further understand the pharmacokinetic behaviour and patterns of metabolism for BZD and pro-BZD compounds in ruminants, and to maximize their anthelminic efficacy, the overall goals of research in this project were:

1) to characterize the basic pharmacokinetic behaviour and pattern of biotransformation for NTB and its metabolites in sheep and cattle.

2) to determine the influence of the route of administration, formulation and animal species on the bioconversion of NTB parent drug, and on the disposition kinetics of the resultant metabolites.

3) to introduce potential changes to the biotransformation and

pharmacokinetic behaviour of BZD and pro-BZD thioethers compounds and their metabolites, by their co-administration with liver oxidation-impairing compounds in sheep and cattle.

CHAPTER 2

PHARMACOKINETIC BEHAVIOUR OF NETOBIMIN AND ITS METABOLITES IN SHEEP

2.1.: INTRODUCTION

Poor drug absorption and the lack of water solubility are important limitations for the formulation, pharmacokinetics and efficacy of BZD methylcarbamate anthelmintics. Anthelmintic pro-drugs have now been developed in an attempt to overcome these problems. Pro-BZD are appropriately substituted benzene molecules that are enzymatically converted to an active BZD methylcarbamate in the host. Netobimin (NTB), N-methoxycarbonyl-N'-(2-nitro-5- propylphenylthio)-N"-(2-ethyl sulphonic acid) guanidine (Figure 2.1) is the most recent developed pro-BZD anthelmintic. The synthesis of NTB is accomplished by treatment of a 5-methylisothiourea derivative with taurine (Townsend and Wise, 1990). The presence of sulphonic acid in the taurine residue of the NTB structure enhances its water solubility. As a salt, NTB is freely soluble in water which permits its formulation as either an aqueous solution or aqueous suspension, for oral and parenteral administration. This allows great flexibility in the administration of NTB, which is an important practical advantage compared with insoluble BZD methylcarbamate anthelmintics.

Preliminary reports (Delatour *et al.*, 1986; Steel and Hennessy, 1987) have shown that NTB is converted into the corresponding propylthio benzimidazole, albendazole (ABZ), and its metabolites in the host. The putative sequence of NTB biotransformation is shown in Figure 2.1. NTB is a broad-spectrum pro-BZD, which is effective against nematodes (Duncan *et al.*, 1985; Williams *et al.*, 1985), cestodes (Bauer and Hafner, 1990) and trematodes (Richards *et al.*, 1987); its activity against *Fasciola hepatica* (Richards *et al.*, 1987),

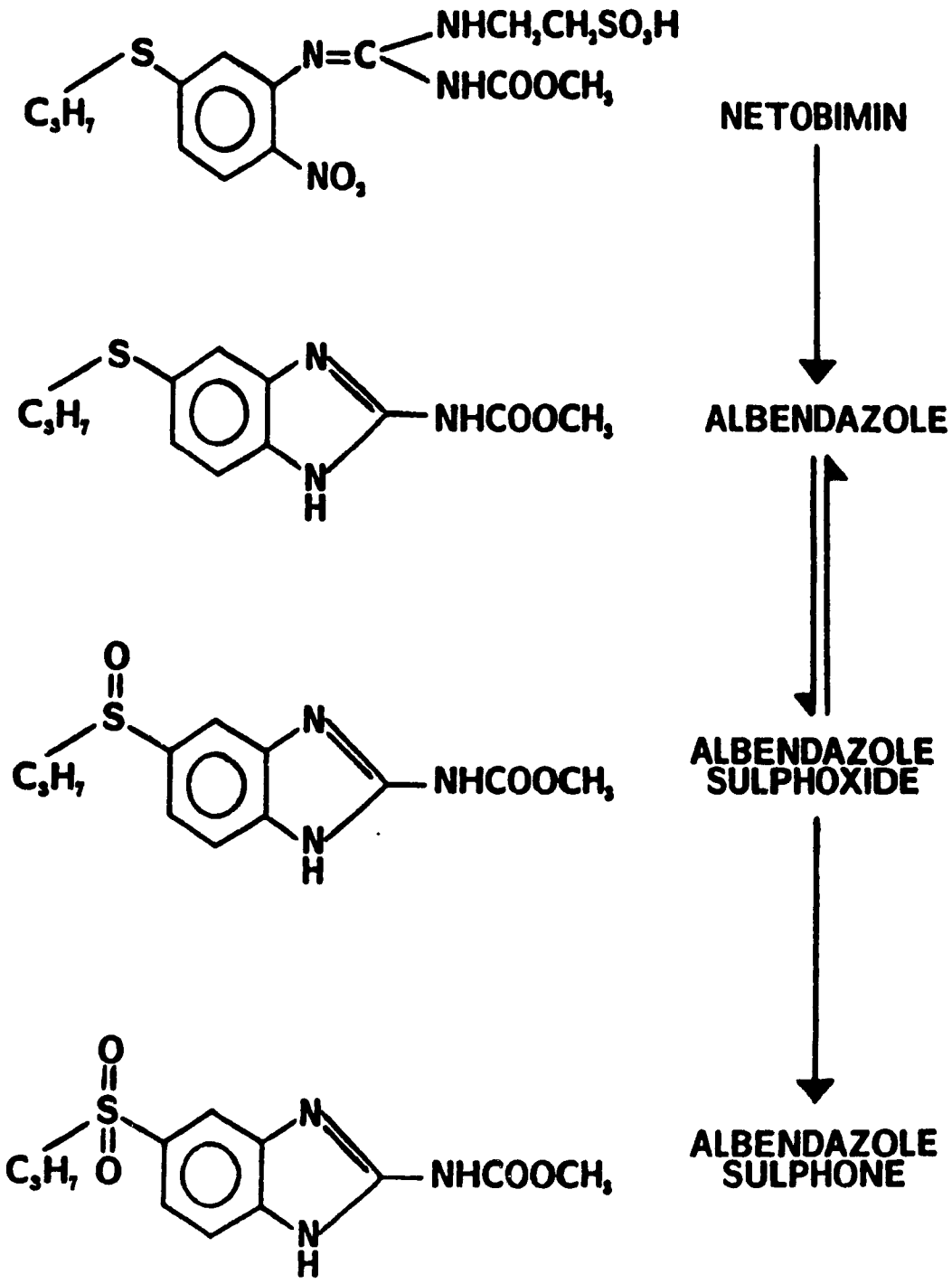


Figure 2.1.: Putative biotransformation pathway for netobimin.

Dicrocoelium dendriticum (Sanz *et al.*, 1985; Juste Jordán and García Perez, 1991) and *Thysanosoma actinoides* (Santiago *et al.*, 1985) in sheep is of considerable interest. However, from the various efficacy studies it is apparent that the route of administration may affect the efficacy profile and this may result from different pharmacokinetic profiles of the active metabolites. The main goal of the experiments reported in this chapter was to characterize the pharmacokinetic behaviour and profile of urinary excretion for NTB and its metabolites, after intraruminal (IR) or subcutaneous (SC) administration at equivalent dose rates of NTB to adult sheep.

2.2.: MATERIALS AND METHODS

2.2.1. Experimental animals

Four adult male Finn Dorset crossbred sheep weighing 58-70 kg were used in this study. The sheep were housed in individual metabolism cages and given a pelleted sheep ration of 2000 g per day. Water was offered *ad libitum*. The health of all animals was monitored prior to and throughout the experimental periods.

2.2.2. Experimental design

In the first phase of this study four sheep received 20 mg/kg of netobimin as an oral suspension (zwitterion, 150 mg/ml) (SCH-32481, Schering Plough, NJ, USA) by IR administration through an 18 G needle. After a four week wash-out period, a dose of 20 mg/kg of netobimin as an aqueous solution of trisamine salt (250 mg/ml) (SCH-32481 - Schering Plough, NJ, USA) was given by SC administration in the shoulder area to the same four animals. Adverse reactions in the injection site were not observed.

For both treatments, blood samples (10 ml) were taken from the jugular vein in vacutainers with sodium heparin, prior to the treatment and at 0.5, 1, 2, 4, 6, 8, 10, 12, 18, 24, 30, 36, 48, 72, 96 and 120 hours post-treatment. Plasma was separated

by centrifugation at 3000 *g* for 15 minutes. Total urine was collected in a container (placed under the bottom of the metabolism cages) at the above indicated times, the first sample covering the period 0-6 hours post-administration. The urine was filtered using a sieve (150 μm pore size); the total volume was measured and an aliquot was taken for analysis. Plasma and urine samples were frozen at -20 C until analyzed within two months.

2.2.3. Analytical procedures

After thawing, plasma and urine samples (1 ml) were spiked with an internal standard (oxibendazole, 1 μg/10 μl methanol) and prepared (drug/metabolite extraction) for high performance liquid chromatographic (HPLC) analysis.

The sample extraction was as described by Allan *et al.* (1980) and modified by Hennessy *et al.* (1985), using C_{18} Sep Pak cartridges (Part No. 51910, Waters Associates, Milford, Mass., USA). Each cartridge was conditioned by washing with 5.0 ml HPLC grade methanol (Fisher Scientific, Canada) followed by 5.0 ml 0.017 M ammonium dihydrogen phosphate buffer pH 5.5. After application of spiked plasma or urine the cartridge was successively washed with 20 ml distilled water, 0.5 ml methanol (40%), 0.4 ml methanol (100%) and 2.5 ml methanol (100%). NTB and ABZ metabolites eluted in the last 2.5 ml of methanol were concentrated to approximately 0.45-0.5 ml under a stream of nitrogen and refrigerated until analyzed by HPLC. Standard solutions and extracted metabolites from unknown samples were quantified on a LKB Bromma HPLC System (LKB, Bromma, Sweden) using an autosampler (LKB, Model 2153), Bondex 10 C_{18} reverse phase column (Phenomenex, CA, USA), LKB 2150 solvent delivery pumps, and a LKB spectral variable wavelength absorbance detector (Model 2140) reading at 292 nm (ABZ metabolites) and 320 nm (NTB). For both plasma and urine metabolites, the mobile phase was an acetonitrite/0.025 M ammonium acetate gradient in the following proportions: 28:72 (9 min), 42:58 (15 min) and 28:72 (9 min). The flow rate was

of 1 ml/min. A typical chromatographic separation for NTB and its metabolites in a spiked plasma sample is shown in Figure 2.2.

Identification of each metabolite was undertaken by comparison with the retention time of the pure reference standards (97-98.5 % pure) (donated by Schering Plough, NJ, USA), which were also used to prepare standard solutions in order to make calibration curves for each metabolite in either fluid. The linear regression lines for the standard curves for each analyte over a range of 0.02 to 3 μg/ml showed correlation coefficients between 0.980 and 0.994. Unknown concentrations were calculated by comparison of each analyte and internal standard peak area using a Nelson Analytical Software, model 2600 (Nelson Analytical, Inc., CA, USA) on an IBM-AT computer. The limits of detection (μg/ml) were as follows: 0.040 (NTB), 0.020 (ABZ and ABZSO) and 0.025 ($ABZSO_2$).

2.2.4. Pharmacokinetic analysis

An interactive computer program (PKCALC) (Schumaker, 1986), coupled to an augmented copy of the stripping program ESTRIP (Brown and Manno, 1978) was used to determine the least squares best fit curve for the concentration versus time data. The data were fit, for NTB and all detected metabolites, for each animal individually after both treatments. Both after IR and SC treatments, the plasma concentration profile of NTB and its metabolites was well described by a one-compartment model with a first-order absorption (NTB parent drug) or a first-order rate of metabolite formation (ABZ metabolites) (Gibaldi and Perrier, 1982), based on the following equation:

$$Cp = Be^{-\beta t} - Be^{-Kt}$$

Cp = plasma concentration at time t after administration (μg/ml); B = concentration at time zero extrapolated from the elimination phase (μg/ml); e = the base of the natural logarithm; ß = the elimination rate constant (h^{-1}) and K

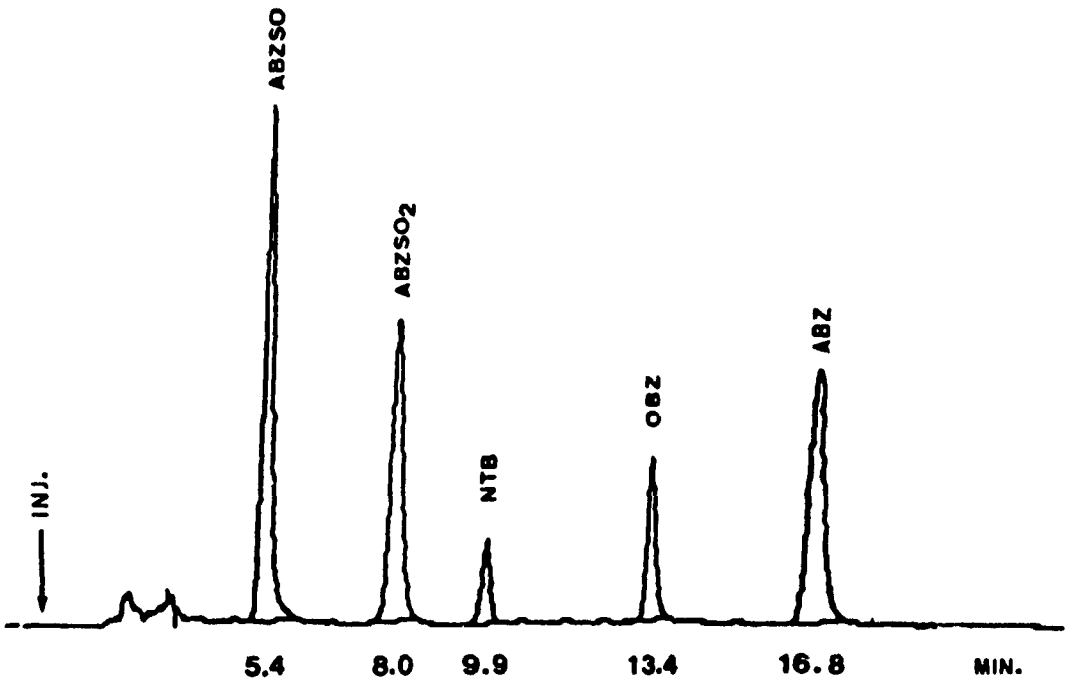


Figure 2.2.: Typical chromatographic separation for NTB and its metabolites in a spiked plasma sample extracted and processed as described in 2.2.3, using oxibendazole (OBZ) as internal standard.

represents either the absorption rate constant (Kab) (NTB parent drug) or first-order rate constant of metabolite formation (Kf) (ABZ metabolites). The choice of this model was based on the values of the coefficient of determination (r^2) for data from individual animals together with the distribution of data points between both phases in the plasma concentration-time curve.

Kab (NTB) or Kf (ABZ metabolites) were obtained by computer analysis of the plasma concentration data, using the method of residuals (Gibaldi and Perrier, 1982). The slope of the regression of the terminal phase of concentration against time (ß) was calculated by least squares analysis. Kab and ß were used either to calculate the absorption half-life (T ½ ab) (NTB parent drug) or elimination half-life (T ½ ß) (NTB and all metabolites) as ln 2/Kab or ln 2/ß, respectively. The peak plasma concentration (Cmax) and the time of the peak (Tmax) were read from the plasma concentration profile of each metabolite in each animal.

The area under the plasma concentration-time curve (AUC) was calculated by trapezoidal rule (Baggot, 1977) being extrapolated to infinity by dividing the last measured plasma concentration by the elimination rate constant (ß). Since the intravenous route was not used, the total body clearance (Cl_b) and volume of distribution (method of the area) (Vd_{area}) for NTB parent drug represent their true values divided by the bioavailability (F) (Gibaldi and Perrier, 1982). Therefore, these parameters are reported and calculated as follows:

$$Cl_b/F = \frac{Dose}{AUC} \qquad Vd_{area}/F = \frac{Dose}{AUC.ß}$$

In addition, the individual concentration-time data were analyzed by non-compartmental analysis based on statistical moment theory using a different option in the PKCALC computer

program. The mean residence time (MRT) was obtained for each metabolite as follows (Perrier and Mayersohn, 1982):

$$MRT = \frac{AUMC}{AUC} - \frac{1}{K}$$

where AUC and K are as defined previously and AUMC is the area under the curve of the product of time and the plasma drug concentration versus time from zero to infinity (Gibaldi and Perrier, 1982).

2.2.5. Statistical analysis

Paired t-test was used in order to establish the level of significance between mean pharmacokinetic parameters, a value of $P < 0.05$ being considered significant. The pharmacokinetic parameters are reported as mean ± S.E.M.

2.3.: RESULTS

The mean plasma concentration-time curves for NTB and its metabolites obtained after IR and SC administration are shown in Figures 2.3 and 2.4, respectively.

The pharmacokinetic parameters for NTB parent drug obtained after IR and SC administration are shown in Table 2.1. After both treatments, NTB showed a very fast absorption with a $T_{\frac{1}{2}ab}$ of 0.45 ± 0.21 h (IR) and 0.36 ± 0.11 h (SC), measured between 0.5 and 12 h post-treatment. The C_{max} values were close (1.36 ± 0.59 and 1.11 ± 0.13 μg/ml for IR and SC treatments, respectively), whereas T_{max} was significantly earlier ($P < 0.05$) for the SC administration (0.88 ± 0.13 compared with 2.75 ± 0.75 h for the IR administration).
Most of the parameters which define the pharmacokinetic profile of NTB were not statistically different between treatments (Table 2.1).

While ABZ was not detected in plasma at any time after the SC

administration of NTB, low levels were detected between 0.5 and 8 h after IR administration (Figure 2.3). The ABZ pharmacokinetic analysis is presented in Table 2.2. ABZ showed a low AUC (0.87 ± 0.12 μg.h/ml) and a rapid disappearance from plasma ($T_{½}$ β= 1.56 ± 0.27 h). The C_{max} was 0.20 ± 0.04 μg/ml achieved at T_{max}= 3.00 ± 0.58 h.

In Table 2.2 are shown the pharmacokinetic parameters for ABZSO obtained after IR and SC administration of NTB. While low ABZSO plasma levels were detected only between 6 and 30 h after SC treatment, high ABZSO plasma concentrations were detected after IR treatment from 0.5 to 96 h. The peak plasma concentration was significantly higher (P< 0.05) after IR administration (2.44 ± 0.67 μg/ml) than after SC administration (0.15 ± 0.01 μg/ml) and was achieved at T_{max} of 17.50 ± 4.50 h and 10.0 ± 0.82 h, respectively. The AUC of ABZSO obtained after IR administration of NTB (94.92 ± 30.21 μg.h/ml) was significantly higher (P< 0.05) than that obtained after SC injection of NTB (3.24 ± 0.22 μg.h/ml).

The pharmacokinetic values obtained for $ABZSO_2$ after IR and SC treatments are shown in Table 2.2. Low plasma levels of this metabolite were detected between 8 and 30 h after SC administration. The AUC (4.30 ± 0.47 μg.h/ml) was significantly lower (P< 0.05) compared with IR administration (32.83 ± 7.38 μg.h/ml). After IR administration, $ABZSO_2$ was detected from 0.5 up to 96 h post-administration. The C_{max} value for $ABZSO_2$ was significantly higher (P< 0.01) in the IR treatment (0.70 ± 0.08 μg/ml) than in the SC treatment (0.18 ± 0.03 μg/ml), and was achieved (T_{max}= 11.50 ± 0.50 h) significantly earlier (P< 0.05) than after IR treatment (25.50 ± 5.12 h). The ratios of AUC for $ABZSO_2$/ABZSO metabolites were 0.35 and 1.33 after IR and SC administration of NTB, respectively.

The mean cumulative percentages of total NTB dose excreted in urine as NTB parent drug or ABZ, ABZSO and $ABZSO_2$ after the IR

and SC treatments are shown in Figures 2.5.a and 2.5.b, respectively. The mean cumulative percentage of total dose excreted in urine as these analytes, expressed as the sum of all measured analytes was 17.05 ± 7.21% after IR and 8.16 ± 2.12% after SC administration of NTB. The percentages of total urinary excretion eliminated as NTB parent drug or the sum of ABZ metabolites (ABZ, ABZSO and $ABZSO_2$) in the 120 h-collection period after the IR and SC treatments with NTB are shown in Table 2.3.

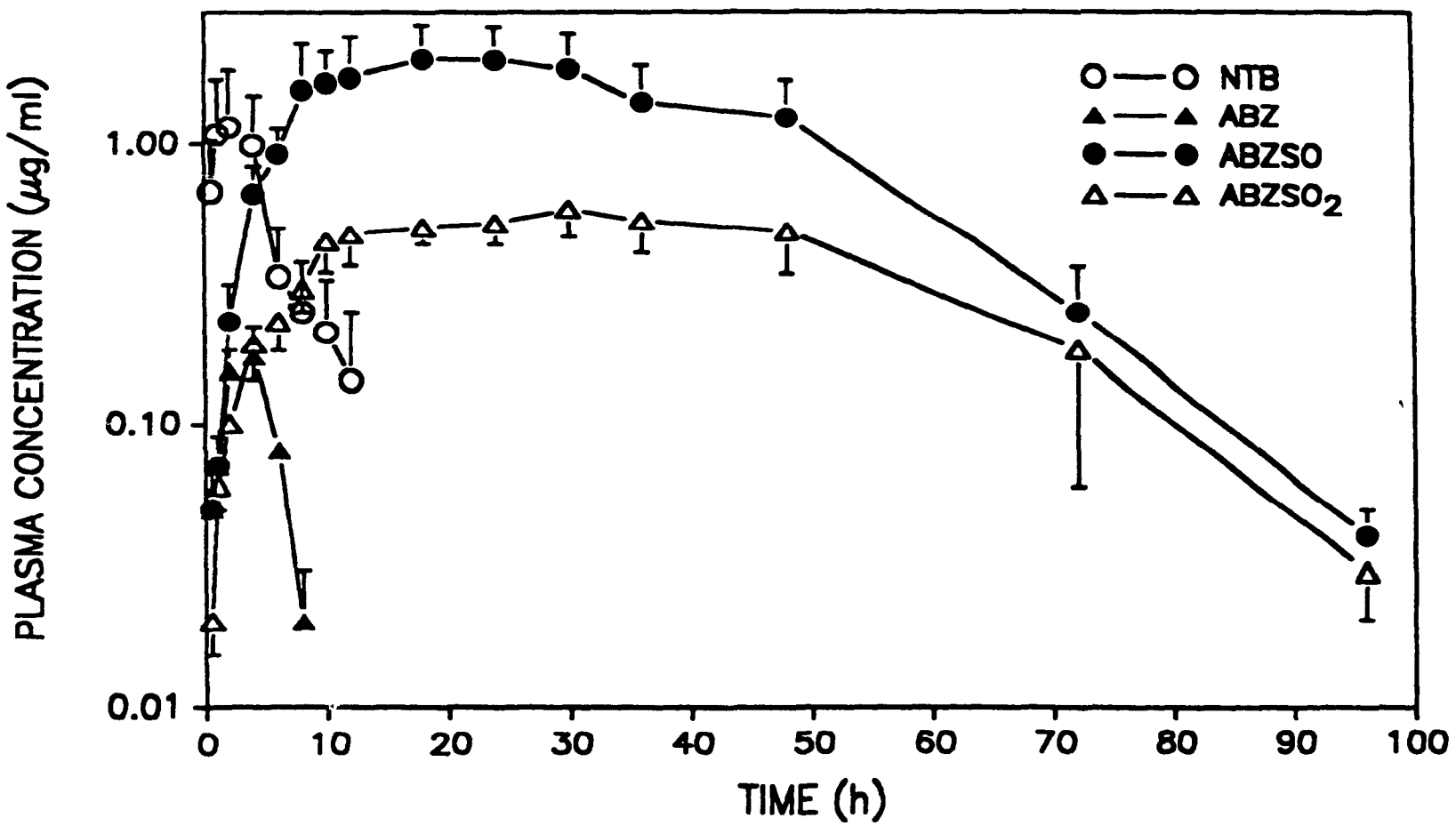


Figure 2.3.: Mean plasma concentration of NTB parent drug, ABZ, ABZSO and $ABZSO_2$ in sheep (n=4) following IR administration of NTB at 20 mg/kg.

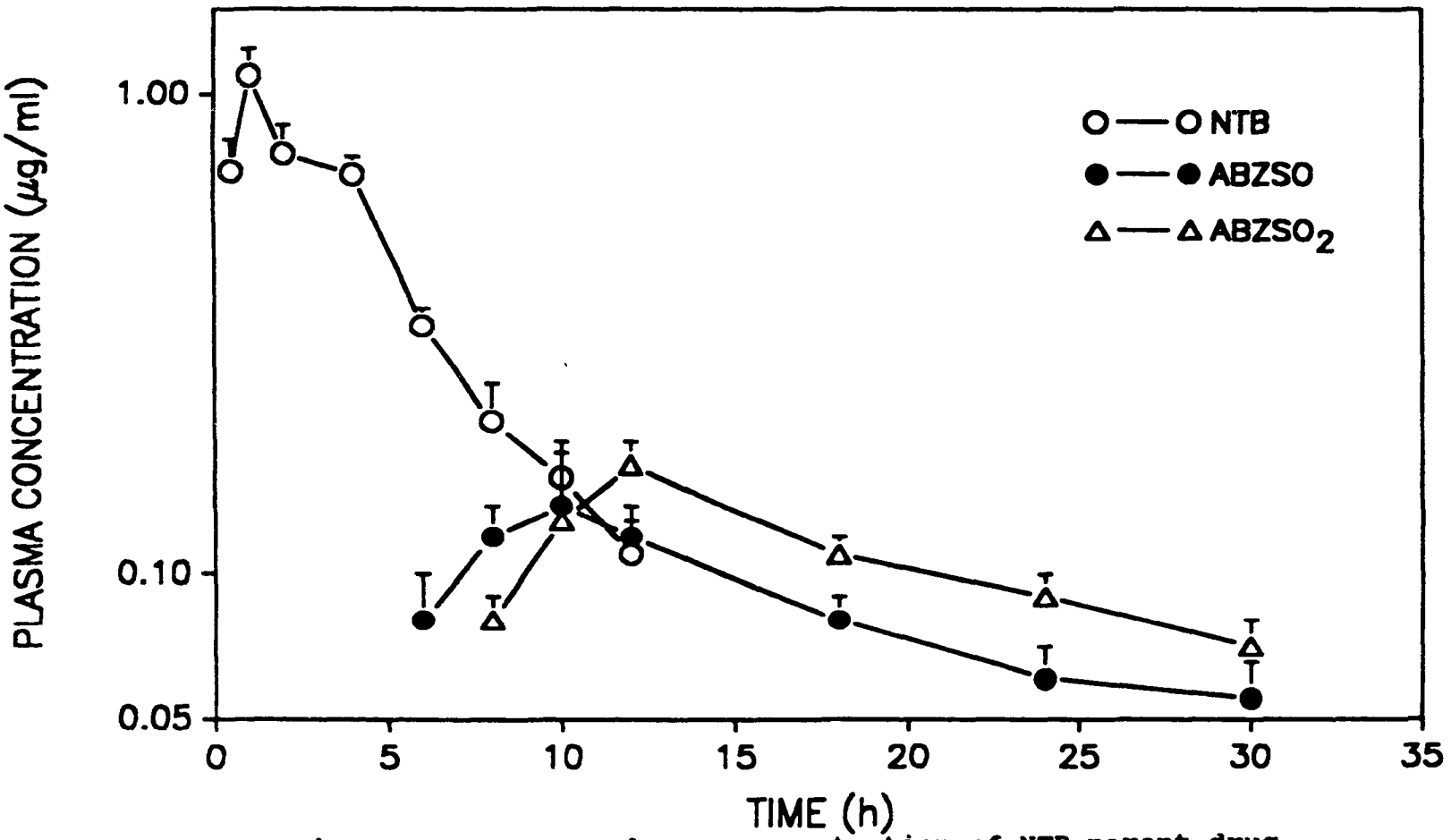


Figure 2.4.: Mean plasma concentration of NTB parent drug, ABZSO and $ABZSO_2$ in sheep (n=4) following SC administration of NTB at 20 mg/kg.

Table 2.1.: Pharmacokinetic parameters (mean ± SEM) for NTB parent drug obtained after its administration IR and SC (20 mg/kg) to sheep (n=4).

		ROUTE	
Parameter	Unit	IR	SC
K_{ab}	h^{-1}	2.59 ± 0.79	2.73 ± 0.92
T ½ ab	h	0.45 ± 0.21	0.36 ± 0.11
ß	h^{-1}	0.20 ± 0.04	0.24 ± 0.04
T ½ ß	h	4.11 ± 1.17	3.13 ± 0.55
C_{max}	µg/ml	1.36 ± 0.59	1.11 ± 0.13
T_{max}	h	2.75 ± 0.75	0.88 ± 0.13*
AUC	µg.h/ml	8.18 ± 4.78	5.60 ± 0.22
MRT¶	h	5.45 ± 1.15	4.10 ± 0.95
$Vd_{(area)}/F$	l/kg	29.8 ± 11.2	15.7 ± 2.06
Cl_b/F	ml/h/kg	6788 ± 3146	3586 ± 134

*: Significantly different from IR administration at $P< 0.05$.

¶: Value obtained by non-compartmental analysis of the data based on the statistical-moment theory.

Table 2.2.: Pharmacokinetic parameters (mean ± SEM) for ABZ, ABZSO and $ABZSO_2$ obtained after IR and SC administration of NTB (20 mg/kg) to sheep (n=4).

Parameter	Unit	ABZ#	ABZSO		$ABZSO_2$	
		IR	IR	SC	IR	SC
β	h^{-1}	0.50±0.10	0.052±0.008	0.057±0.009	0.049±0.006	0.057±0.016
$T_{1/2}\ \beta$	h	1.56±0.27	14.77±2.79	12.95±1.89	14.44±0.83	15.55±4.25
C_{max}	µg/ml	0.20±0.04	2.44±0.67	0.15±0.01*	0.70±0.08	0.18±0.03**
T_{max}	h	3.00±0.58	17.5±4.50	10.0±0.82	25.5±5.12	11.5±0.50‡
AUC	µg.h/ml	0.87±0.12	94.92±30.21	3.24±0.22‡	32.83±7.38	4.30±0.47*
MRT¶	h	2.92±0.17	24.9±3.24	18.82±2.67	25.11±2.10	21.51±5.89

#:ABZ was not detected after the SC treatment.

*:Significantly different from IR administration at P< 0.05.

‡:Significantly different from IR administration at P< 0.05 with n=3. One animal was not included for statistical comparison because of the spurious results obtained after the IR treatment.

**:Significantly different from IR administration at P< 0.01.

¶:Value obtained by non-compartmental analysis of the data based on statistical-moment theory.

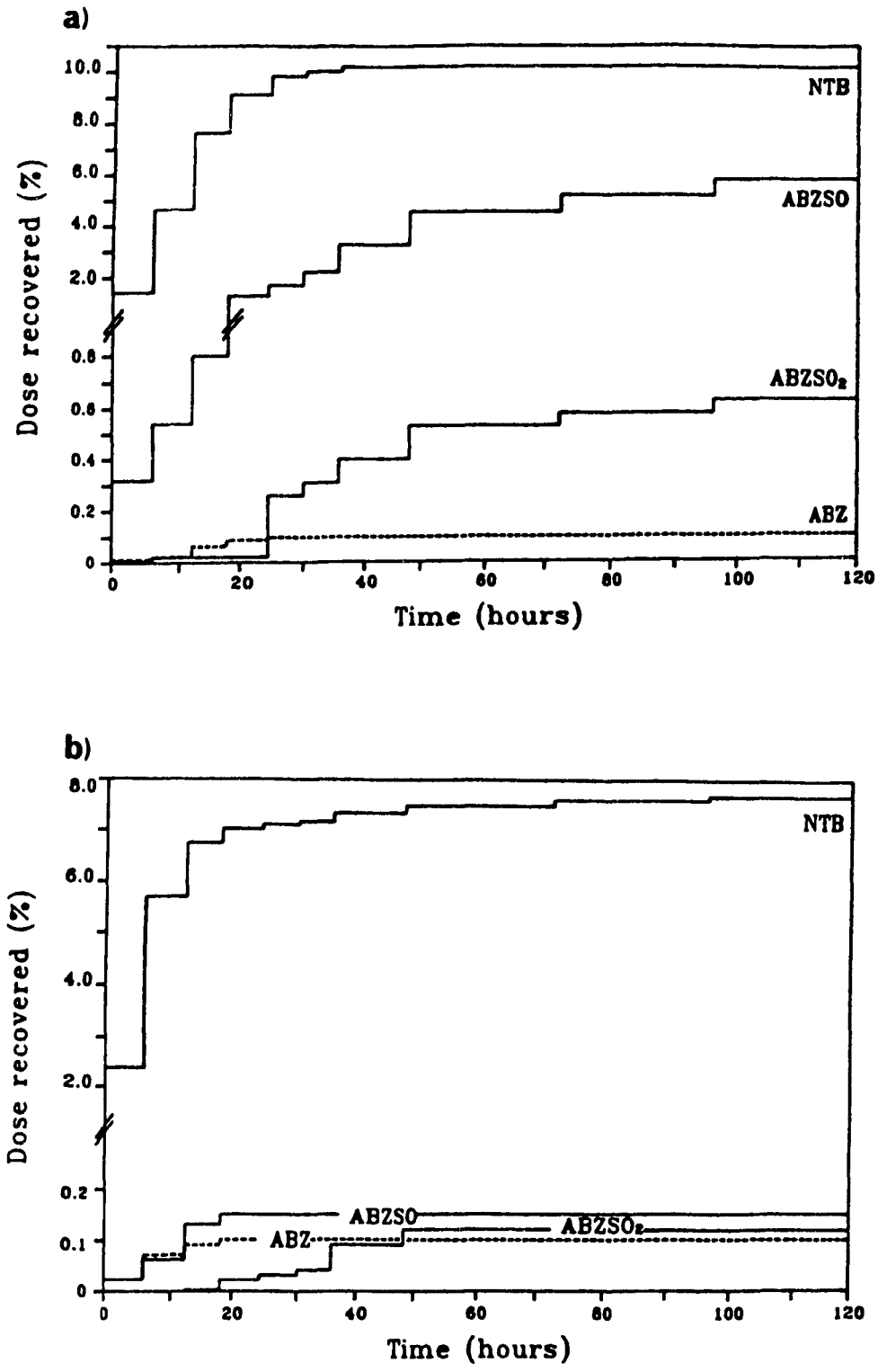


Figure 2.5.: Mean cumulative percentage of total dose excreted in urine as parent drug and metabolites after (a) IR and (b) SC administration of NTB (20 mg/kg) in four sheep.

Table 2.3.: Percentage of total urine excretion eliminated as NTB or the sum of ABZ metabolites after IR and SC administration of NTB (20 mg/kg) in four sheep.

	SHEEP							
	A		B		C		D	
	IR	SC	IR	SC	IR	SC	IR	SC
NTB	40.3	95.4	5.5	97.1	10.8	94.3	92.6	94.5
ABZ metab.	59.7	4.6	94.5	2.9	89.2	5.7	7.4	5.5

Data expressed as percentage of total amount eliminated by urine in a 120-h period post-administration.

2.4.: DISCUSSION

NTB was rapidly absorbed in sheep after both IR and SC treatment. High NTB plasma levels were detected in the samples at 0.5 h post-administration. The absorption half-life showed similar values after both treatments, but the T_{max} was significantly earlier ($P < 0.05$) after the SC administration. It is possible that this earlier peak plasma concentration is due to the higher water solubility of the trisamine aqueous solution which facilitated a faster absorption from the site of SC injection compared with the absorption of the intraruminally administered zwitterion suspension from the GI tract.

An early C_{max}, short elimination half-life, relatively high body clearance, low AUC and the detection of plasma levels up to 12 h post-administration indicated a similar pharmacokinetic profile for NTB parent drug after both treatments. However, the greater extent of NTB conversion into ABZ metabolites after IR treatment indicates a different pattern of distribution of the parent compound between the two routes of administration.

After SC administration of NTB, ABZ was not detected in plasma, but low levels were found in the urine samples between 6 and 24 h post-administration. Following IR administration of the NTB zwitterion suspension at 20 mg/kg, ABZ could be detected in plasma between 0.5 and 8 h post-treatment, resulting in a low AUC and short elimination half-life (1.56 ± 0.27 h). ABZ has not previously been detected in plasma after the administration of NTB in sheep (7.5 mg/kg, IR, Steel *et al.*, 1985; 8.4 mg/kg, oral, Delatour *et al.*, 1986), after SC administration of NTB in cattle (Steel and Hennessy, 1987), or after the oral administration of ABZ itself to cattle (Prichard *et al.*, 1985). The transient appeareance of ABZ in plasma has been reported after ABZ administration to sheep (10 mg/kg) (Marriner and Bogan, 1980). The pharmacokinetic

analysis of ABZSO and $ABZSO_2$ showed important differences between IR and SC administrations of NTB. C_{max} and AUC for both metabolites were significantly higher after IR treatment. These results demonstrate a quite different pattern of biotransformation of NTB according to the route of administration. In both treatments the ABZSO C_{max} was reached earlier than the $ABZSO_2$ C_{max} confirming the sequential oxidative steps after the NTB cyclisation into ABZ, proposed by Delatour *et al.* (1986).

It appears that after IR administration a more efficient conversion of NTB into ABZ metabolites takes place in the GI tract than after SC administration, resulting in a greater amount of ABZSO reaching the systemic circulation which would explain the lower ratio of AUC $ABZSO_2$/ABZSO obtained after IR treatment (0.35) compared with the SC treatment (1.33).

There is evidence that ABZ and ABZSO are active in binding to nematode tubulin, the putative mechanism of action of benzimidazole anthelmintics, whereas $ABZSO_2$ may be inactive (Lacey 1990; Lubega and Prichard, 1991b). Therefore, the detection of ABZ in plasma and the detection of high plasma levels of ABZSO from 0.5 up to 96 h post-treatment and the low ratio of sulphone/sulphoxide obtained after IR administration of NTB are of interest for anthelmintic efficacy.

The cumulative percentage of total dose excreted in urine as NTB, ABZ, ABZSO and $ABZSO_2$ was higher following IR (17.05%) than following SC (8.16%) administration of NTB. After both treatments, NTB parent drug was the analyte recovered in the largest proportion over the 120 h collection period. The cumulative percentage of ABZSO was lower after SC injection (0.16%) compared with IR administration (6.01%). In addition, when the animals received NTB by SC injection a range of 94.3 to 97.1% of the total analytes measured in urine was unchanged parent drug and only from 2.9 to 5.7% was measured as ABZ metabolites. Following the IR treatment, these results were

reversed and the percentage of measured ABZ metabolites was greater in 3 of the 4 sheep (Table 2.3). The exception (sheep #D) showed an elimination pattern similar to the subcutaneously injected animals after the IR treatment. Administration into the rumen was confirmed in all animals by the elimination of rumen gases through the injecting needle.

The low levels of ABZ metabolites and the high concentration of unchanged parent drug recovered in urine after SC administration of NTB are correlated with the significantly lower C_{max} and AUC values obtained for ABZSO and $ABZSO_2$ following this treatment. These results indicate a low efficacy in the NTB conversion after its parenteral administration. In addition, the early detection of ABZ, ABZSO and $ABZSO_2$ in plasma after the IR treatment (0.5 h) suggests that the digestive flora play a predominant role in the rapid reduction of NTB. The delayed appearance of detectable plasma levels of ABZSO and $ABZSO_2$ (6-8 h) after SC administration also suggest that for parenterally administered NTB, the GI tract may be the principal site of conversion; thus, when NTB is administered parenterally, the efficiency of its transformation to active metabolites may depend on the amount of NTB which reaches the GI tract.

In conclusion, after both IR and SC administration, NTB parent drug is rapidly absorbed, distributed and cleared from the body or converted into ABZ metabolites. The plasma profile of ABZ metabolites depends on the efficacy by which NTB is reduced and cyclised, probably, by the GI flora. This efficacy of conversion is markedly lower after parenteral administration of NTB. The IR treatment at 20 mg/kg resulted in the detection of ABZ in plasma and in detectable levels of ABZSO for up to 96 h post-administration. There was a high AUC for ABZSO, and low ratio of AUC sulphone/sulphoxide after IR administration. The good pharmacokinetic profile of ABZSO after IR administration may account for the high clinical efficacy of NTB.

CHAPTER 3

PHARMACOKINETIC BEHAVIOUR OF NETOBIMIN AND ITS METABOLITES IN CATTLE: BIOEQUIVALENCE OF TWO DIFFERENT FORMULATIONS

3.1.: INTRODUCTION

Since NTB parent drug is anthelmintically inactive (Delatour, *et al.*, 1986), the overall efficacy of this pro-BZD compound depends on its rate of conversion into active metabolites in the host. As showed in Chapter 2, the broad-spectrum activity of NTB in ruminants relies on the efficiency of its biotransformation into ABZ-related moieties. BZD compounds exert their anthelmintic effects by binding to parasite tubulin; since only ABZ and ABZSO have been shown as active molecules (Lacey, 1990; Lubega and Prichard, 1991b), it is likely that the efficacy of NTB not only depends on its bioactivation, but also on the bioavailability and disposition kinetics of these active metabolites in the host.

NTB contains a solubilizing taurine moiety that allows its formulation either as a water soluble salt (aqueous trisamine solution) or as an insoluble zwitterion (aqueous zwitterion suspension). This property results in an interesting practical advantage and permits both parenteral and oral/intraruminal administration. However, from the results obtained in sheep (Chapter 2), it is evident that the route of administration plays a significant role in the conversion of NTB and the resultant bioavailability of its anthelmintically active metabolites.

To characterize the basic pharmacokinetic and metabolic pattern of NTB in cattle, and in order to identify the formulation of the pro-drug that results in the most favourably bioavailability and disposition kinetics for active metabolites, the purposes of the studies reported in this chapter were:

a) to investigate the basic pharmacokinetic behaviour of netobimin and/or its metabolites in cattle, following parenteral and oral administration at recommended dose rates.
b) to determine the bioequivalence between a trisamine solution and a zwitterion suspension formulation of NTB administered both subcutaneously and orally to cattle.

3.2.: PHARMACOKINETICS OF NETOBIMIN AFTER SUBCUTANEOUS ADMINISTRATION: BIOEQUIVALENCE STUDY BETWEEN TRISAMINE AND ZWITTERION FORMULATIONS

3.2.1. MATERIALS AND METHODS

3.2.1.1. Experimental animals

Eight Holstein Fresian calves weighing between 100 and 120 kg were used. High-quality hay and water were available ad libitum, and calves were allotted to two groups of four each. The health of all the calves was closely monitored prior to and throughout the course of the study. A two week wash-out period transpired before the same calves participated in the second phase of the study.

3.2.1.2. Experimental procedures

The experimental design consisted of a 4 x 4 crossover sequence, using the two NTB formulations. Treatments were given as follows:

For phase 1, calves were treated with a "wet/dry" formulation of NTB (aqueous solution of trisamine salt, 250 mg/ml) (donated by Schering Plough, NJ, USA) by SC injection (left shoulder) at dosage of 12.5 mg/kg of body weight (group A) or were given a "wet/wet" formulation of NTB (aqueous zwitterion suspension, 417 mg/ml) by SC administration (left shoulder) at the same dosage (group B).

For phase 2, the formulations were reversed for each group and were administered via the same route (right shoulder) at the

aforementioned dose rates. In each experimental phase, samples of blood were drawn from the jugular vein into heparinized tubes prior to treatment and at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12, 16, 18, 20, 24, 32, 48 and 72 hours after treatment. Blood samples were placed on ice before centrifugation. Plasma was separated by centrifugation at 2500 *g* for 15 minutes, placed in plastic vials, and immediately stored at -20 °C until analyzed.

3.2.1.3. Analytical procedures

Unknown and spiked plasma samples were extracted and analyzed by HPLC, for NTB and its ABZ metabolites, using the analytical procedures, conditions and equipment described in Chapter 2, section 2.2.3. Calibration curves for each analyte were obtained by triplicate analysis of spiked plasma samples over a range of 0.02 to 3 μg/ml. Correlation coefficient values were between 0.970 and 0.982. Identification of each metabolite was made by comparison with the retention time of pure reference standard (97-98.5% pure). Concentrations were calculated by comparison of each metabolite and internal standard peak area using Nelson Analytical Software, model 2600 (Nelson Analytical, Inc., CA, USA).

3.2.1.4. Pharmacokinetic and statistical analyses

The least squares best fit curves and all pharmacokinetic parameters for NTB and all detected metabolites were obtained as explained in 2.2.4. The plasma concentration-time profile for NTB parent drug, ABZSO and $ABZSO_2$ could be represented by a one-compartment open model. The pharmacokinetic variables for the various metabolites were calculated for each calf and mean ± SEM were determined for each formulation in each phase (n=4). Overall mean ± SEM were determined for each formulation (n=8) regardless of phase.

All pharmacokinetic variables were subjected to the W statistical test for normality (Shapiro and Wilk, 1965). Analysis of variance for crossover bioequivalence studies

(Bolton, 1984) was used to establish the level of significance between pharmacokinetic variables from different calves, experimental phases, and drug formulations, which were normally distributed. The Wilcoxon rank sum and Kruskal-Wallis tests (Sokal and Rohlf, 1981) were used to compare variables that were not normally distributed. A value of $P< 0.05$ was considered significant.

3.2.2.: RESULTS

The mean plasma concentration ± SEM obtained during each experimental phase and the overall average for both NTB formulations after SC administration in calves are seen in Tables 3.1 (NTB parent drug), 3.2 (ABZSO) and 3.3 ($ABZSO_2$). The pharmacokinetic parameters obtained during each experimental phase and the overall average after the administration of both formulations are given in Tables 3.4 (NTB parent drug) and 3.5 (ABZSO and $ABZSO_2$). The overall mean plasma concentration obtained for NTB and its metabolites after SC administration of either formulation are shown in Figure 3.1. The only pharmacokinetic parameter found to be not normally distributed was T_{max} for ABZSO; thus analysis was performed by the Wilcoxon rank sum (phase and formulation effects) and Kruskal-Wallis (calves) tests. All other variables were analyzed by use of analysis of variance (Table 3.6).

NTB was quickly absorbed after SC administration, being detected between 0.25 and 12 hours after treatment (Figure 3.1). Overall, the C_{max} was 2.20 ± 0.36 μg/ml achieved at 0.75 ± 0.07 h (trisamine formulation) and 1.37 ± 0.21 μg/ml at 0.81 ± 0.06 h (zwitterion formulation) (Table 3.4). This C_{max} value was significantly ($P< 0.01$) higher for the trisamine formulation. A statistically significant ($P< 0.01$) difference was evident for C_{max} between experimental phases 1 and 2. Statistical differences in AUC and $T_{\frac{1}{2}\beta}$ values were not observed for NTB parent drug, using either formulation

(Table 3.6).

Although ABZ was not detected at any time after SC administration of NTB, ABZSO was detected between 4 and 20 hours (trisamine) and between 6 and 24 hours (zwitterion) after drug administration (Figure 3.1). Peak plasma concentrations, 0.48 ± 0.06 and 0.46 ± 0.09 μg/ml for trisamine and zwitterion, respectively, were reached at 9.50 ± 0.50 h (trisamine) and 11.30 ± 0.49 h (zwitterion) after NTB administration (Table 3.5). The ABZSO AUC values were 3.86 ± 0.37 μg.h/ml (trisamine) and 4.40 ± 1.14 μg.h/ml (zwitterion); T ½ ß was 3.05 ± 0.35 hours (trisamine) and 3.90 ± 0.51 h (zwitterion). Statistical differences between formulations for ABZSO pharmacokinetic variables were not evident, except for T_{max}. However, AUC and C_{max} were significantly different ($P< 0.05$) between experimental phases (Table 3.6).

$ABZSO_2$ was detected from 4 (trisamine) and 6 hours (zwitterion) up to 24 hours after treatment (Figure 3.1). The AUC was 6.98 ± 0.57 μg.h/ml (trisamine) and 10.51 ± 2.62 μg.h/ml (zwitterion), but these values were not significantly different. The C_{max} for $ABZSO_2$ was 0.76 ± 0.07 μg/ml at T_{max} of 12.0 ± 0.65 h (trisamine) and 0.70 ± 0.12 μg/ml at T_{max} of 12.50 ± 0.82 h (zwitterion); T ½ ß was significantly longer ($P< 0.05$) for the zwitterion formulation (7.77 ± 1.67 h) than for trisamine (2.87 ± 0.22 h). The large SEM found for the zwitterion formulation in ABZSO and $ABZSO_2$ plasma concentration values were attributable to high values detected in one calf during experimental phase 1. A comparison of the overall AUC obtained for NTB, ABZSO and ABZSO after SC treatment with both formulations is shown in Figure 3.2. The AUC ratio for albendazole sulfone/sulfoxide was 1.8 and 2.4 for trisamine and zwitterion treatments, respectively.

Table 3.1.: Mean plasma concentration (μg/ml) of NTB parent drug after its SC administration (12.5 mg/kg) as trisamine and zwitterion injectable formulations in calves.

Time (h)	Trisamine			Zwitterion		
	Phase 1 (n=4)	Phase 2 (n=4)	Overall (n=8)	Phase 1 (n=4)	Phase 2 (n=4)	Overall (n=8)
0.25	1.28±0.27	0.74±0.12	1.01±0.17	0.70±0.17	0.55±0.15	0.62±0.11
0.5	2.54±0.26	1.10±0.15	1.82±0.31	1.01±0.14	0.67±0.23	0.84±0.14
0.75	2.53±0.53	1.21±0.07	1.87±0.35	1.57±0.37	0.94±0.14	1.26±0.22
1	2.35±0.54	1.19±0.11	1.77±0.34	1.59±0.15	0.79±0.15	1.19±0.21
2	1.38±0.44	0.94±0.07	1.16±0.24	0.99±0.07	0.72±0.14	0.85±0.09
4	0.77±0.36	0.74±0.04	0.76±0.17	0.87±0.03	0.67±0.12	0.77±0.06
6	0.52±0.14	0.42±0.09	0.47±0.08	0.68±0.07	0.40±0.04	0.54±0.06
8	0.27±0.07	0.24±0.03	0.25±0.04	0.37±0.11	0.23±0.04	0.30±0.04
10	0.13±0.01	0.13±0.03	0.13±0.01	0.21±0.07	0.20±0.04	0.20±0.04
12	0.07±0.01	ND	NA	0.12±0.01	0.13±0.03	0.12±0.02
16	ND	ND	NA	ND	ND	NA

ND= not detected

NA= not applicable

Data are expressed as mean ± SEM.

Table 3.2.: Mean plasma concentration (μg/ml) of ABZSO after SC administration of NTB (12.5 mg/kg) as trisamine and zwitterion injectable formulations in calves.

Time (h)	Trisamine			Zwitterion		
	Phase 1 (n=4)	Phase 2 (n=4)	Overall (n=8)	Phase 1 (n=4)	Phase 2 (n=4)	Overall (n=8)
1	ND	ND	NA	ND	ND	NA
2	0.08±0.04	ND	NA	ND	ND	NA
4	0.12±0.03	0.07±0.01	0.11±0.02	0.05±0.01	ND	NA
6	0.26±0.06	0.12±0.01	0.19±0.04	0.18±0.05	0.08±0.02	0.14±0.03
8	0.46±0.12	0.26±0.05	0.36±0.07	0.23±0.01	0.16±0.07	0.19±0.06
10	0.55±0.07	0.34±0.07	0.44±0.06	0.50±0.10	0.22±0.08	0.36±0.08
12	0.30±0.06	0.24±0.05	0.27±0.12	0.52±0.13	0.28±0.11	0.40±0.09
16	0.15±0.03	0.12±0.03	0.13±0.02	0.29±0.11	0.09±0.02	0.19±0.06
18	0.09±0.02	0.06±0.02	0.08±0.01	0.19±0.09	0.07±0.01	0.13±0.05
20	0.04±0.01	0.04±0.01	0.04±0.01	0.12±0.06	0.05±0.01	0.09±0.03
24	ND	ND	NA	0.12±0.06	0.03±0.00	0.08±0.03
32	ND	ND	NA	ND	ND	NA

ND= not detected

NA= not applicable

Data are expressed as mean ± SEM

Table 3.3.: Mean plasma concentration (μg/ml) of $ABZSO_2$ after SC administration of NTB (12.5 mg/kg) as trisamine and zwitterion injectable formulations in calves.

Time (h)	Trisamine			Zwitterion		
	Phase 1 (n=4)	Phase 2 (n=4)	Overall (n=8)	Phase 1 (n=4)	Phase 2 (n=4)	Overall (n=8)
1	ND	ND	NA	ND	ND	NA
2	0.12±0.03	ND	NA	ND	ND	NA
4	0.22±0.07	0.10±0.04	0.17±0.05	ND	ND	NA
6	0.26±0.06	0.18±0.03	0.22±0.04	0.19±0.04	0.13±0.03	0.15±0.02
8	0.31±0.05	0.46±0.05	0.38±0.04	0.23±0.04	0.26±0.10	0.24±0.05
10	0.55±0.07	0.64±0.06	0.59±0.05	0.39±0.05	0.37±0.07	0.38±0.04
12	0.65±0.10	0.77±0.16	0.71±0.09	0.67±0.07	0.58±0.18	0.62±0.09
16	0.29±0.06	0.44±0.11	0.36±0.06	0.51±0.08	0.31±0.09	0.41±0.07
18	0.12±0.03	0.24±0.09	0.18±0.05	0.50±0.18	0.22±0.08	0.39±0.10
20	0.09±0.02	0.12±0.04	0.10±0.02	0.48±0.15	0.18±0.06	0.31±0.09
24	0.05±0.01	0.05±0.01	0.05±0.01	0.34±0.09	0.07±0.01	0.20±0.06
32	ND	ND	NA	ND	ND	NA

ND= not detected

NA= not applicable

Data are expressed as mean ± SEM

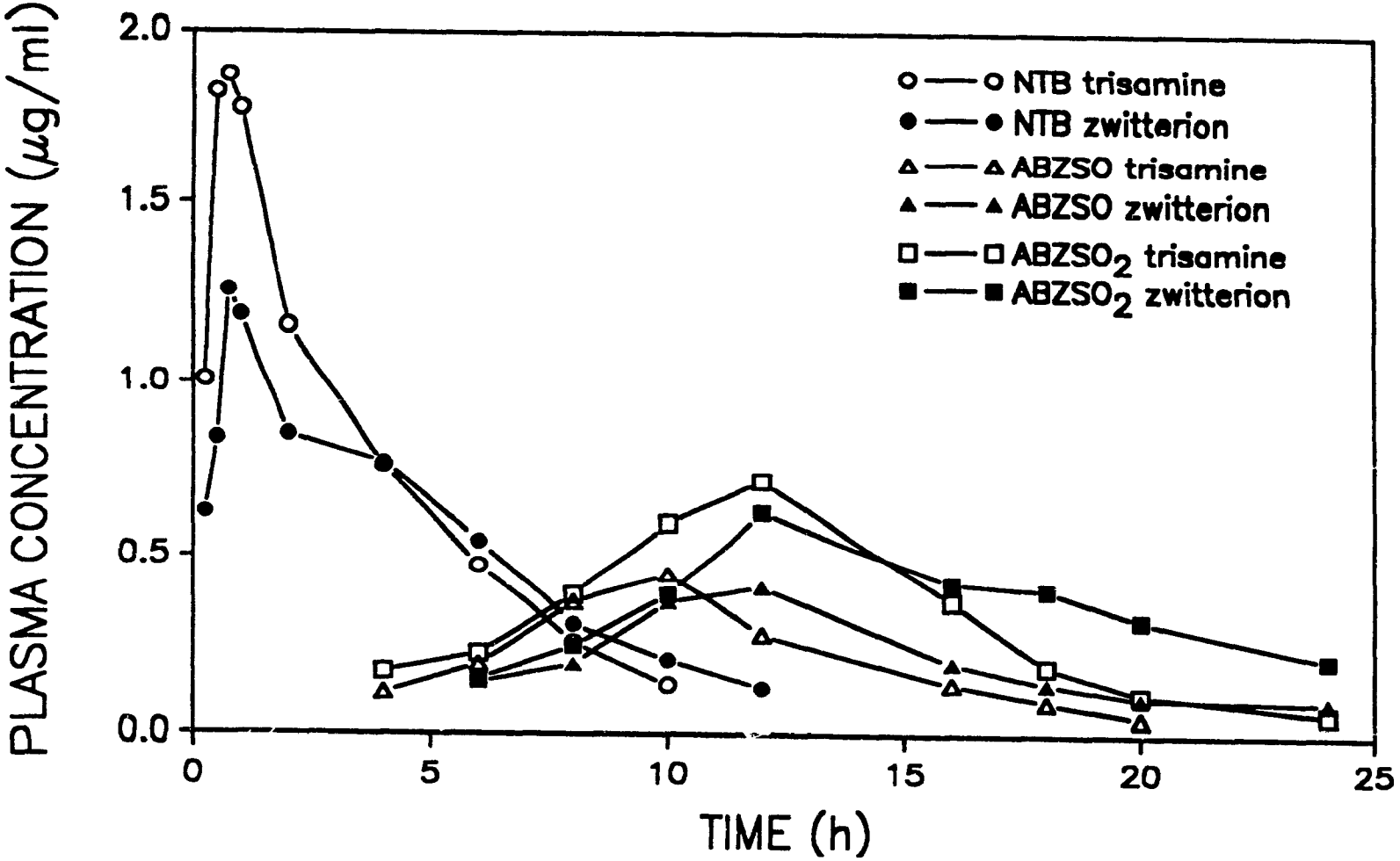


Figure 3.1.: Mean plasma concentration (n=8) for NTB parent drug, ABZSO and $ABZSO_2$ obtained after SC administration of NTB (12.5 mg/kg) as trisamine solution (open symbols) and zwitterion suspension (closed symbols) injectable formulations in calves.

Table 3.4.: Pharmacokinetic parameters for NTB obtained after its SC administration (12.5 mg/kg) as trisamine and zwitterion formulations in calves.

Parameter	Unit	Phase	Formulation	
			Trisamine	Zwitterion
T ½ ß	h	1	2.18±0.18	3.01±0.54
		2	3.01±0.28	4.13±0.92
		x	2.59±0.22	3.57±0.51
AUC	µg.h/ml	1	8.73±2.11	8.06±0.63
		2	6.45±0.56	5.77±0.26
		x	7.59±1.10	6.92±0.53
C_{max}	µg/ml	1	3.10±0.23	1.77±0.27
		2	1.31±0.11	0.98±0.15
		x	2.20±0.36	1.37±0.21
T_{max}	h	1	0.75±0.10	0.88±0.07
		2	0.75±0.10	0.75±0.10
		x	0.75±0.07	0.81±0.06

Data are expressed as mean ± SEM with n=4 for experimental phases (1 and 2) and n=8 for overall mean (x).

Table 3.5.: Pharmacokinetic parameters for ABZSO and $ABZSO_2$ obtained after SC administration of NTB (12.5 mg/kg) as trisamine and zwitterion injectable formulations in calves.

Parameter	Phase	Trisamine		Zwitterion	
		ABZSO	$ABZSO_2$	ABZSO	$ABZSO_2$
T ½ ß (h)	1	2.63±0.11	3.08±0.36	4.51±0.88	10.71±2.46
	2	3.46±0.44	2.67±0.23	3.30±0.44	4.82±1.04
	x	3.05±0.35	2.87±0.22	3.90±0.51	7.77±1.67
AUC (µg.h/ml)	1	4.63±0.33	6.43±0.84	6.25±1.84	15.00±4.14
	2	3.09±0.36	7.52±0.75	2.56±0.69	6.03±1.16
	x	3.86±0.37	6.98±0.57	4.40±1.14	10.51±2.62
C_{max} (µg/ml)	1	0.60±0.05	0.69±0.09	0.60±0.12	0.79±0.07
	2	0.37±0.06	0.82±0.13	0.31±0.11	0.62±0.15
	x	0.48±0.06	0.76±0.07	0.46±0.09	0.70±0.12
T_{max} (h)	1	10.00±0.81	11.50±0.50	11.00±0.57	13.50±1.50
	2	9.00±0.40	12.50±1.25	11.50±0.50	11.50±0.50
	x	9.50±0.50	12.00±0.65	11.30±0.49	12.50±0.82

Data are expressed as mean ± SEM with n=4 for experimental phases (1 and 2) and n=8 for overall mean (x).

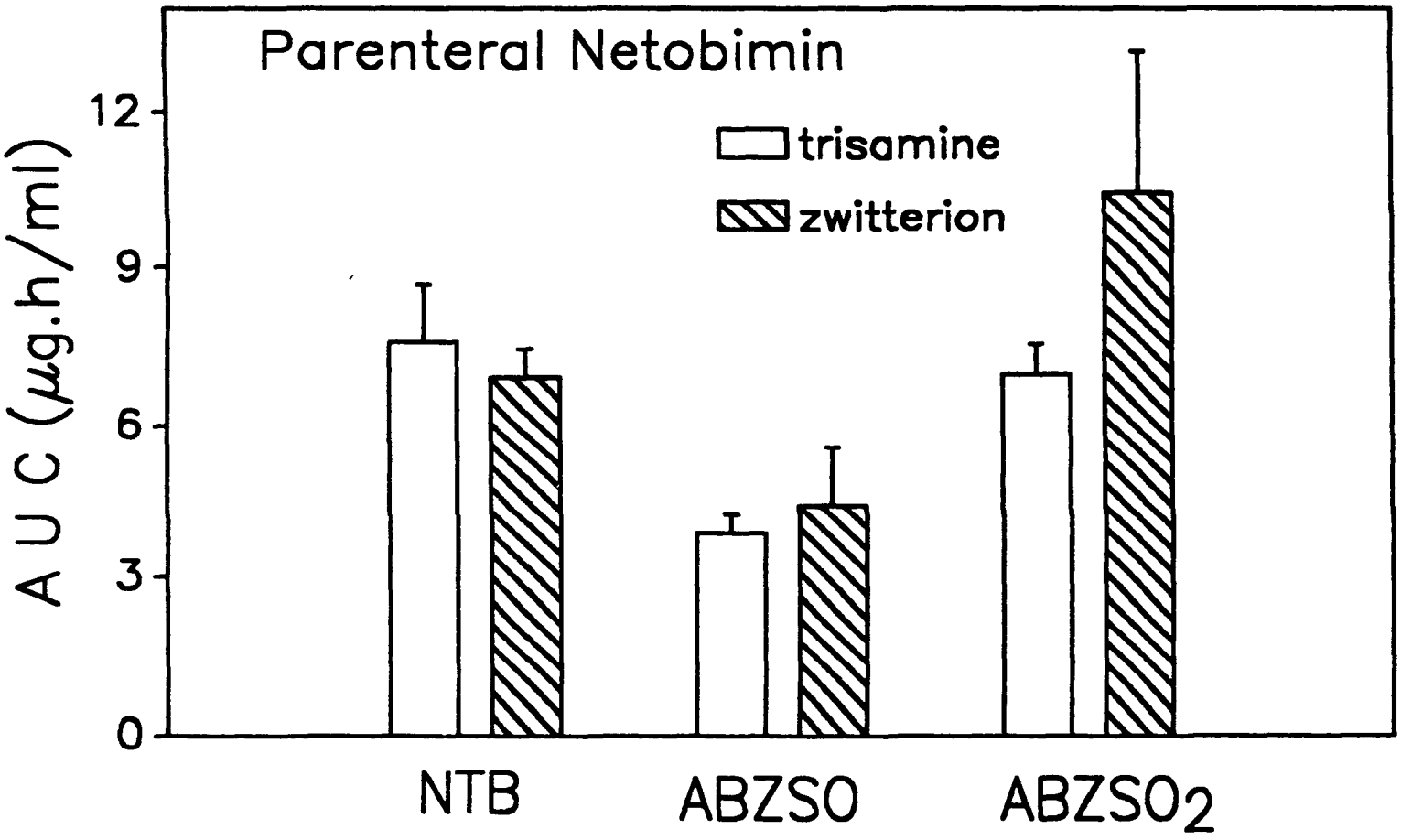


Figure 3.2.: Comparative area under the plasma concentration-time curves for NTB, ABZSO and $ABZSO_2$ obtained after SC administration of NTB as trisamine and zwitterion formulations in cattle.

Table 3.6.: Results of statistical analysis for the pharmacokinetic parameters obtained for NTB and its metabolites after SC administration as trisamine and zwitterion formulations in calves#.

Parameter	Source	NTB	ABZSO	$ABZSO_2$
T ½ ß	Calves	0.4639	0.7629	0.4473
	Phases	0.1006	0.7898	0.0740
	Formulations	0.1006	0.2530	0.0153*
AUC	Calves	0.4647	0.4551	0.2034
	Phases	0.0768	0.0380*	0.1034
	Formulations	0.5526	0.6009	0.1355
C_{max}	Calves	0.1348	0.3535	0.2810
	Phases	0.0004**	0.0146*	0.8304
	Formulations	0.0038**	0.7345	0.6204
T_{max}	Calves	0.5947	0.8088@	0.0476*
	Phases	0.5370	0.8208‡	0.1466
	Formulations	0.5370	0.0235‡*	0.1466

#: Data were evaluated by analysis of variance, except: ‡ analyzed by the Wilcoxon rank sum test and @ analyzed by the Kruskal-Wallis test.

*: Statistically different at P< 0.05; **: Statistically different at P< 0.01.

Degrees of freedom = calves, 7; phases, 1; formulations, 1; residual, 6; total, 15.

Data are expressed as probability (P) > F values.

3.3.: PHARMACOKINETICS OF ALBENDAZOLE METABOLITES AFTER ORAL ADMINISTRATION OF TRISAMINE AND ZWITTERION FORMULATIONS OF NETOBIMIN TO CATTLE.

3.3.1.: MATERIALS AND METHODS

3.3.1.1. Experimental animals

Eight Holstein Fresian bull calves, in parasite-free condition, weighing between 130-150 kg, were used in this study. High quality hay and water were available to them *ad libitum*. These animals had never received anthelmintic treatment before this experiment. The calves were divided into two groups of four animals each. The health of all the calves was closely monitored prior to and throughout the course of the experiment.

3.3.1.2. Experimental procedures

The experimental design consisted of a 4 x 4 crossover sequence of the two oral formulations of NTB. The treatments were as follows.

Phase 1:

Group A. Animals were treated with trisamine salt solution of netobimin (50 mg/ml) (Hapadex oral powder, batch # 22277-082, Schering Plough, NJ, USA) by oral drench at a dosing rate of 20 mg/kg.

Group B. Animals were treated with a micronized suspension of the netobimin zwitterion (50 mg/ml) (Hapadex oral suspension, batch # 22277-081, Schering Plough, NJ, USA) by oral drench at a dosing rate of 20 mg/kg.

Phase 2:

After a two week wash-out period, the formulations were reversed for each group and administered by the same route and dose as mentioned above.

In each experimental phase, samples of blood (10 ml) were taken from the jugular vein into heparinized Vacutainer tubes

(Becton Dickinson, Canada) prior to treatment and at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12, 16, 20, 24, 32, 48 and 72 hours post-administration. Blood samples were placed on ice before centrifugation. Plasma was separated by centrifugation at 2500 *g* for 15 minutes, placed in plastic vials and immediately stored frozen at -20 °C until analyzed.

3.3.1.3. Analytical procedures

Sample extraction procedures, HPLC equipment and analysis conditions were as previously described in sections 2.2.3 and 3.2.1.3.

3.3.1.4. Pharmacokinetic and statistical analyses

The interactive computer program (PKCALC) (Shumaker, 1986), coupled to an augmented copy of the stripping program ESTRIP (Brown and Manno, 1978) was used to determine the least squares best fit curve for the concentration versus time data. The data for the detected metabolites (ABZSO and $ABZSO_2$) were fitted for each animal individually after the administration of both formulations. After both oral treatments, the plasma concentration profile of ABZSO and $ABZSO_2$ was well described by a one-compartment kinetic model based on the biexponential equation described in section 2.2.4. Pharmacokinetic parameters were obtained by the equations previously presented (2.2.4). In addition, the individual concentration-time data were analyzed by non-compartmental analysis based on statistical moment theory.

A typical analysis of variance for crossover bioequivalence studies (Bolton, 1984) was used in order to establish the level of significance between mean pharmacokinetic parameters in different animals, experimental phases and formulations. A value of $P< 0.05$ was considered significant. A range of bioequivalence between both oral formulations was calculated by establishing a 95% confidence interval for the differences of the AUC (Bolton, 1984).

The pharmacokinetic parameters for different metabolites were calculated for each animal and means ± SEM determined for each formulation in each phase (n= 4) and, finally, overall means ± SEM determined for each formulation (n= 8) regardless of phase.

3.3.2.: RESULTS

Low concentrations of NTB parent drug were detected in a few samples in some of the experimental animals between 0.5 and 1 h post-treatment. These results are not presented because their occasional appearance in this short interval precludes any pharmacokinetic analysis. ABZ was not detected in plasma at any time after administration of either NTB oral formulation. The mean plasma concentrations ± SEM of ABZSO and $ABZSO_2$ in each experimental phase and the overall means obtained for both oral formulations are presented in Tables 3.7 and 3.8, respectively. Figure 3.3 shows the plot of the overall mean plasma concentrations of these metabolites obtained after the oral administration of NTB as trisamine solution and zwitterion suspension. The results of the pharmacokinetic analysis for ABZSO and $ABZSO_2$ in each phase, and the overall mean, after both treatments, are summarized in Tables 3.9 and 3.10, respectively. Table 3.11 shows the results of the statistical analysis of the differences between pharmacokinetic parameters obtained for each formulation.

ABZSO was detected in plasma from 0.5 or 0.75 h up to 32 h post-treatment resulting in both a C_{max} (1.21 ± 0.13 μg/ml) and AUC (18.55 ± 1.45 μg.h/ml) for the zwitterion suspension which were significantly higher ($P< 0.01$) than for the trisamine solution (C_{max}= 0.67 ± 0.12 μg/ml; AUC= 8.57 ± 0.91 μg.h/ml) (Table 3.9). The peak concentration was achieved at T_{max}= 10.75 ± 0.53 (zwitterion suspension) and 10.0 ± 0.65 h (trisamine solution) post-administration. ABZSO was cleared from the bloodstream with a $T_{½ \beta}$ of 5.45 ± 0.25 (zwitterion suspension) and 5.81 ± 0.46 h (trisamine solution), and MRT of

9.74 ± 0.40 (suspension) and 9.56 ± 0.56 h (solution). Neither parameter showed statistical differences between formulations (Table 3.11).

$ABZSO_2$ was detected between 0.75 and 48 h post-administration of both formulations. The resultant AUC (Table 3.10; Figure 3.4) was significantly higher ($P< 0.01$) for the zwitterion suspension (51.67 ± 1.95 μg.h/ml) than for the trisamine solution (22.77 ± 1.09 μg.h/ml). Also, in the treatment with the zwitterion suspension, the AUC for $ABZSO_2$ was significantly lower ($P< 0.05$) in phase 2 than in phase 1 (Table 3.11). The C_{max} for $ABZSO_2$ was significantly higher ($P<0.01$) for the zwitterion suspension (2.91 ± 0.10 μg/ml) than for the trisamine solution (1.67 ± 0.11 μg/ml) and reached T_{max} at 16.50 ± 0.50 (suspension) and 13.75 ± 0.88 h (solution) post-treatment, respectively. The $T_{½ ß}$ for $ABZSO_2$ was 5.93 ± 0.29 (suspension) and 5.16 ± 0.56 h (solution); these values were not statistically different (Table 3.11). However, the $ABZSO_2$ MRT was significantly longer ($P<0.01$) for the zwitterion suspension.

The mean k values for ABZSO, representing the first-order rate constant of either absorption or metabolite formation, were 0.250 ± 0.015 (zwitterion suspension) and 0.267 ± 0.020 h^{-1} (trisamine solution) with lag times of 0.95 ± 0.18 and 0.73 ± 0.20 h, respectively. For the metabolite $ABZSO_2$, k was 0.182 ± 0.008 (zwitterion suspension) and 0.234 ± 0.013 h^{-1} (trisamine solution), with lag times of 2.07 ± 0.17 and 1.36 ± 0.13 h, respectively.

The ratios of AUC for $ABZSO_2$/ABZSO were 2.92 ± 0.26 and 2.80 ± 0.20 for the zwitterion and trisamine, respectively. Based on the AUC data for ABZSO, the NTB zwitterion suspension resulted in a 90 to 144% greater bioavailability (at 95% confidence intervals) than the trisamine solution. The overall AUC values for ABZSO and $ABZSO_2$ obtained for either formulation are compared in Figure 3.4.

Table 3.7.: Plasma concentrations of ABZSO (μg/ml) obtained after oral administration of NTB (20 mg/kg) as trisamine solution and zwitterion suspension formulations in calves.

	Trisamine Solution			Zwitterion Suspension		
Time (h)	Phase I (n=4)	Phase II (n=4)	x (n=8)	Phase I (n=4)	Phase II (n=4)	x (n=8)
0.25	ND	ND	ND	ND	ND	ND
0.5	ND	ND	ND	ND	0.05±0.01	0.05±0.01
0.75	0.06±0.02	0.07±0.02	0.07±0.01	0.09±0.01	0.11±0.01	0.19±0.01
1	0.08±0.01	0.11±0.03	0.09±0.02	0.12±0.03	0.18±0.02	0.15±0.02
2	0.17±0.03	0.15±0.05	0.16±0.03	0.29±0.06	0.43±0.08	0.36±0.06
4	0.44±0.12	0.34±0.11	0.39±0.08	0.54±0.07	0.90±0.14	0.72±0.10
6	0.55±0.10	0.38±0.10	0.47±0.07	0.66±0.11	1.08±0.09	0.87±0.10
8	0.80±0.23	0.39±0.06	0.59±0.14	0.82±0.12	1.18±0.06	1.00±0.09
10	0.74±0.12	0.46±0.09	0.60±0.09	0.83±0.11	1.46±0.15	1.14±0.15
12	0.55±0.07	0.45±0.10	0.50±0.06	0.88±0.11	1.16±0.16	1.02±0.11
16	0.23±0.01	0.26±0.06	0.25±0.03	0.68±0.05	0.80±0.06	0.74±0.04
20	0.15±0.02	0.13±0.01	0.14±0.01	0.54±0.07	0.49±0.08	0.51±0.04
24	0.10±0.01	0.08±0.01	0.09±0.01	0.19±0.03	0.22±0.05	0.21±0.03
32	0.06±0.00	0.05±0.01	0.06±0.00	0.10±0.01	0.08±0.01	0.09±0.01
48	ND	ND	ND	ND	ND	ND

Data are expressed as mean ± SEM.

ND = not detected

Table 3.8.: Plasma concentrations of $ABZSO_2$ (µg/ml) after oral administration of NTB (20 mg/kg) as trisamine solution and zwitterion suspension formulations in calves.

Time (h)	Trisamine Solution			Zwitterion Suspension		
	Phase I (n=4)	Phase II (n=4)	x (n=8)	Phase I (n=4)	Phase II (n=4)	x (n=8)
0.5	ND	ND	ND	ND	ND	ND
0.75	0.10±0.03	0.07±0.02	0.08±0.02	0.11±0.02	0.10±0.02	0.11±0.01
1	0.14±0.01	0.13±0.02	0.14±0.01	0.14±0.01	0.16±0.02	0.15±0.01
2	0.28±0.04	0.21±0.01	0.24±0.02	0.38±0.05	0.26±0.03	0.32±0.03
4	0.63±0.04	0.45±0.05	0.54±0.05	0.57±0.08	0.65±0.05	0.61±0.05
6	0.77±0.12	0.64±0.06	0.70±0.07	1.05±0.13	0.78±0.07	0.92±0.09
8	1.23±0.75	0.76±0.04	1.00±0.15	1.40±0.16	1.12±0.11	1.26±0.11
10	1.56±0.19	1.16±0.03	1.36±0.12	1.51±0.19	1.63±0.16	1.58±0.12
12	1.79±0.08	1.30±0.09	1.55±0.11	2.47±0.08	1.92±0.12	2.20±0.12
16	1.42±0.15	1.43±0.12	1.43±0.09	3.04±0.18	2.55±0.20	2.80±0.12
20	0.53±0.10	0.66±0.14	0.60±0.08	2.92±0.15	2.53±0.18	2.73±0.13
24	0.18±0.04	0.18±0.02	0.18±0.02	1.49±0.31	1.34±0.37	1.41±0.22
32	0.09±0.02	0.09±0.01	0.09±0.01	0.31±0.10	0.31±0.04	0.31±0.05
48	0.07±0.00	0.05±0.01	0.06±0.01	0.09±0.02	0.11±0.01	0.10±0.01
72	ND	ND	ND	ND	ND	ND

Data are expressed as mean ± SEM.

ND = not detected

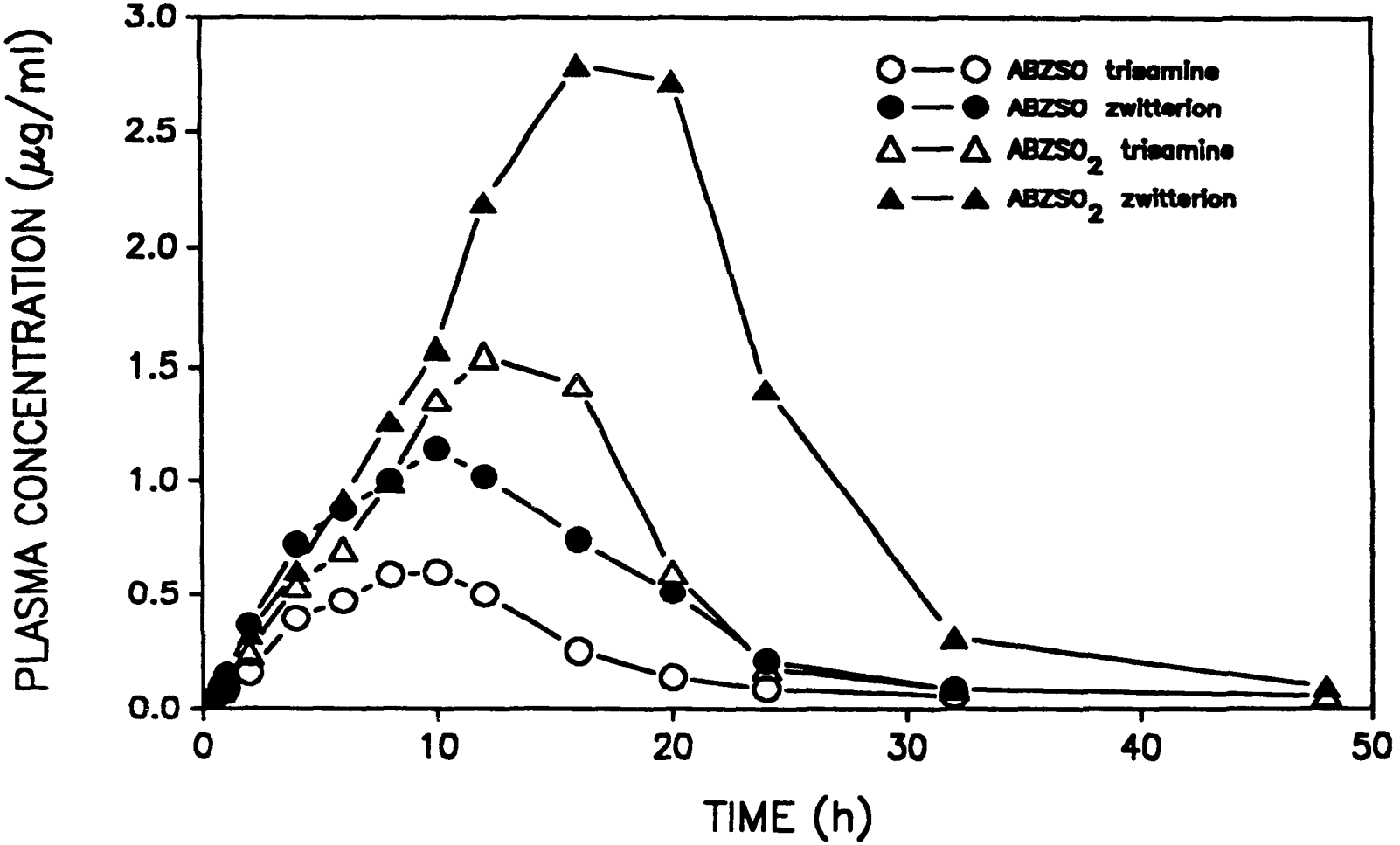


Figure 3.3.: Mean plasma concentration (n=8) for ABZSO and $ABZSO_2$ obtained after oral administration of NTB (20 mg/kg) as trisamine solution (open symbols) and zwitterion suspension (closed symbols) formulations in calves.

Table 3.9.: Pharmacokinetic parameters for ABZSO obtained after oral administration of NTB (20 mg/kg) as trisamine solution and zwitterion suspension formulations in calves.

Parameter	Trisamine solution			Zwitterion suspension		
	Phase I (n=4)	Phase II (n=4)	x (n=8)	Phase I (n=4)	Phase II (n=4)	x (n=8)
β (h^{-1})	0.126±0.016	0.121±0.011	0.123±0.010	0.120±0.003	0.142±0.007	0.131±0.01
$T_{\frac{1}{2}}\beta$ (h)	5.71±0.69	5.91±0.71	5.81±0.46	5.79±0.17	5.11±0.43	5.45±0.25
AUC (μg.h/ml)	9.83±1.16	7.31±1.23	8.57±0.91	16.16±1.90	20.94±1.57	18.55±1.45
C_{max} (μg/ml)	0.87±0.19	0.48±0.09	0.67±0.12	0.96±0.11	1.46±0.15	1.21±0.13
T_{max} (h)	8.50±0.50	11.50±0.50	10.0±0.65	11.0±1.00	10.5±0.50	10.75±0.53
MRT‡ (h)	8.86±0.85	9.76±0.97	9.56±0.56	10.28±0.47	9.21±0.58	9.74±0.40

Data are expressed as mean ± SEM. x: overall mean (n=8).

‡: Value obtained by non-compartmental analysis of the data based on the statistical moment theory.

Table 3.10.: Pharmacokinetic parameters for $ABZSO_2$ obtained after oral administration of NTB (20 mg/kg) as trisamine solution and zwitterion suspension formulations in calves.

Parameter	Trisamine solution			Zwitterion suspension		
	Phase I (n=4)	Phase II (n=4)	x (n=8)	Phase I (n=4)	Phase II (n=4)	x (n=8)
β (h^{-1})	0.152±0.021	0.136±0.018	0.144±0.013	0.127±0.009	0.111±0.009	0.119±0.01
T ½ β (h)	4.94±0.95	5.38±0.72	5.16±0.56	5.54±0.41	6.31±0.35	5.93±0.29
AUC (µg.h/ml)	24.41±1.26	21.4±1.48	22.77±1.09	54.86±2.93	48.48±1.53	51.67±1.95
C_{max} (µg/ml)	1.90±0.08	1.44±0.11	1.67±0.11	3.05±0.18	2.78±0.07	2.91±0.10
T_{max} (h)	12.50±1.26	15.0±1.00	13.75±0.88	16.0±0.00	17.0±1.00	16.50±0.50
$MRT^{\ddagger}$ (h)	9.74±0.68	10.05±0.70	9.86±0.46	13.27±0.57	12.88±0.55	13.07±0.37

Data are expressed as mean ± SEM. x: overall mean (n=8).

‡: Value obtained by non-compartmental analysis of the data based on the statistical moment theory.

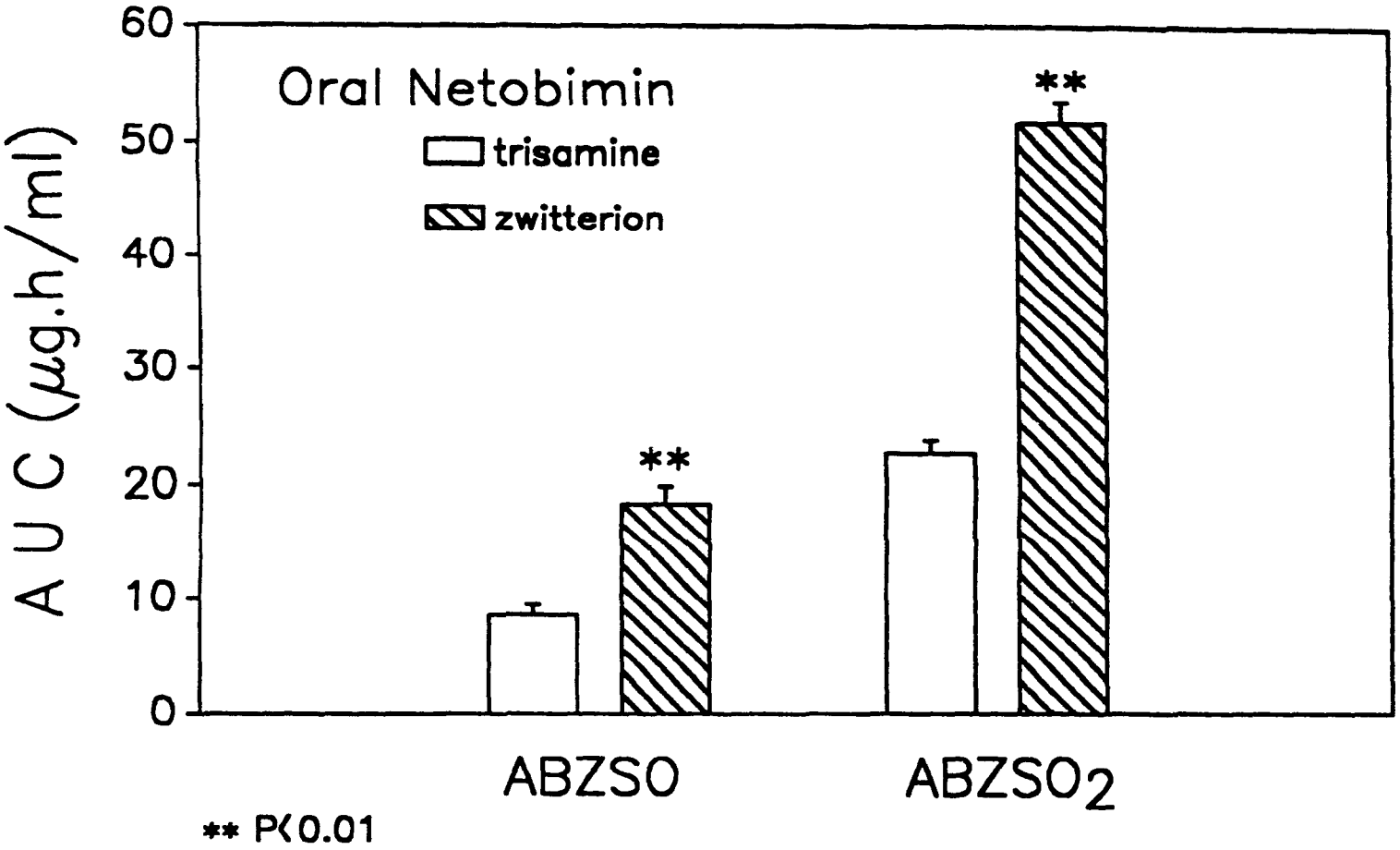


Figure 3.4.: Comparative area under the plasma concentration-time curves for ABZSO and $ABZSO_2$ obtained after oral administration of NTB as trisamine and zwitterion formulations in cattle.

Table 3.11.: Analysis of variance of the pharmacokinetic parameters obtained for ABZSO and $ABZSO_2$ after oral administration of NTB as trisamine and zwitterion formulations in calves.

Parameter	Source	ABZSO P > F	$ABZSO_2$ P >F
ß	Phases	0.4371	0.1939
	Formulations	0.5174	0.0634
T ½ ß	Phases	0.6440	0.2808
	Formulations	0.4932	0.1821
AUC	Phases	0.2720	0.0341*
	Formulations	0.0001**	0.0001**
Cmax	Phases	0.6293	0.0588
	Formulations	0.0035**	0.0002**
Tmax	Phases	0.0401*	0.1775
	Formulations	0.1682	0.0533
MRT	Phases	0.6567	0.9520
	Formulations	0.8063	0.0021**

Data expressed as probability (P) > F values

* Statistically different at P < 0.05

** Statistically different at P < 0.01

Degrees of freedom: animal = 7; phases = 1; formulations = 1, residual = 6; total = 15

3.4.: DISCUSSION

The early detection of NTB in plasma and the early NTB C_{max} obtained for both formulations after the SC treatment indicate that the NTB parent drug is rapidly absorbed from the SC injection site. The earlier appearance (Figure 3.1) and higher NTB peak concentration ($P< 0.01$) obtained for the trisamine solution, compared with the zwitterion suspension, can be explained in terms of the greater water solubility of the trisamine formulation, which could permit easier absorption from the SC administration site, compared with the zwitterion injectable suspension. In addition, the short elimination half-lives obtained for NTB using both formulations indicate that, after absorption, the parent compound is rapidly lost or cyclized into ABZ. The detection of low ABZSO plasma concentration, resulting in low AUC for both injectable formulations, and the short elimination half-life for this metabolite (Table 3.5) would indicate either rapid oxidation to $ABZSO_2$ and/or low rate of conversion of NTB into its anthelmintically active metabolites. The latter would be consistent with the rapid disposition of the parent drug. The two injectable formulations of NTB seem to be bioequivalent, with similar pharmacokinetic profiles and without any statistical differences in the AUC for the various metabolites. The pharmacokinetic variables that had statistical differences between formulations (C_{max} for NTB, T_{max} for ABZSO and T ½ ß for $ABZSO_2$) are probably not relevant in terms of anthelmintic efficacy.

It was found that C_{max} for NTB and ABZSO, as well as AUC for ABZSO, were significantly higher during experimental phase 1 (Tables 3.6). These results can be appreciated by looking at the lower concentrations for all metabolites with both formulations during phase 2 (Tables 3.1, 3.2 and 3.3) and seem to indicate increased metabolic rate in the calves during phase 2. Coincidentally, recently published reports indicate that ABZ can have an inductive effect on some liver microsomal

enzymes in rats (Souhaili-El Amri *et al.*, 1988a,b) which might explain the differences between the phases for results obtained in this study. Although this hypothesis must be confirmed, it is possible that calves given a primary NTB dose might have experienced induction of liver enzymes that would have affected the biotransformation of NTB and ABZ metabolites during experimental phase 2, conducted two weeks after the first dose of NTB.

The T_{max} for $ABZSO_2$ was delayed compared with that of ABZSO in cattle treated with NTB both orally and subcutaneously; the same results were observed in sheep following IR and SC administrations. This confirms a relatively slower oxidation rate of ABZSO into $ABZSO_2$ in comparison with ABZ oxidation. This is consistent with the two distinct enzymatic pathways proposed for the liver microsomal biotransformation of ABZ (Souhaili-El Amri *et al.*, 1988b). According to these authors, a flavin containing monooxygenase (FMO) would be reponsible for the ABZ first step oxidation, while the cytochrome P-450 system would be involved in the $ABZSO_2$ formation. In addition, this latter sulphonation reaction could be the consequence of two enzyme systems, one characterized by low affinity and high capacity, the other by high affinity and low capacity. The longer lag time necessary to resolve the $ABZSO_2$ concentration-time curve into its exponential components by the stripping technique after oral treatment, compared with that for ABZSO, would also confirm a slower rate of $ABZSO_2$ formation.

The ratios of AUC for $ABZSO_2$/ABZSO obtained after both oral (2.80-2.92) and parenteral (1.80-2.40) administrations of NTB in calves, were higher than those found in sheep after intraruminal (0.35) and SC (1.33) treatments. These results are consistent with those obtained after oral administration of ABZ itself in cattle (Prichard *et al.*, 1985) and sheep (Marriner and Bogan, 1980) and would indicate a differential pattern of oxidation between cattle and sheep.

Comparison of the pharmacokinetic profiles of NTB metabolites between the zwitterion oral suspension and the trisamine oral solution, in calves, was interesting. There were no statistical differences for either formulation in terms of T ½ ß for ABZSO and $ABZSO_2$. The pharmacokinetic analysis for the ABZSO metabolite showed a C_{max} and AUC significantly higher ($P< 0.01$) for the zwitterion suspension (Tables 3.7 and 3.11). Similarly, $ABZSO_2$ C_{max} and AUC were significantly higher ($P< 0.01$) for the zwitterion suspension than for the trisamine solution (Table 3.8 and 3.11). Determination of the MRT by non-compartmental analysis of the concentration-time data provided an additional independent parameter for the comparative characterization of each metabolite's disposition kinetics after the administration of both formulations. MRT for $ABZSO_2$ was significantly longer ($P< 0.01$) for the zwitterion suspension formulation. Taken together, these pharmacokinetic data demonstrate that the zwitterion suspension resulted in a greater bioavailability of NTB metabolites than the trisamine solution. In terms of ABZSO, which is perhaps the most relevant metabolite in regard to the anthelmintic efficacy of NTB, the zwitterion suspension was from 90 to 144% more bioavailable than the trisamine solution. These results suggest a more efficient reduction of NTB with ring closure and oxidation to form ABZSO and $ABZSO_2$ after administration of the oral suspension. At present, one can only speculate on possible explanations for the differences between the two formulations. Possibly, the lower pH of the zwitterion suspension (4.5-5.0), compared with the trisamine solution (pH 8.0-8.8), may have facilitated more rapid bacterial reduction of NTB. It is also possible that the differences in dissolution rate between the micronized suspension and solution may have delayed passage of the untransformed parent drug, allowing more time for bacterial reduction of the suspension in the rumen. These, and other possible explanations for the difference in the kinetic profiles of NTB metabolites between the two formulations, require further investigation, which in part, is also

presented in this thesis.

While NTB parent drug was consistently detected only after subcutaneous treatment, ABZ was not found in plasma at any time after either subcutaneous or oral administrations. The appearance of detectable plasma levels of ABZSO and $ABZSO_2$ was delayed after the subcutaneous treatment (2-4 h) in comparison with the oral administration (0.5-0.75 h), regardless of the formulation used. This was evidenced by the significantly longer lag times obtained for both metabolites after oral treatment compared with those after SC treatment (Figures 3.5 and 3.6). As suggested for sheep in Chapter 2, the GI tract seems to be the principal site of NTB reduction and cyclisation into ABZ, both after oral and parenteral administration. Either an oxidation in the GI mucosa before absorption or a first-pass oxidation in the liver microsomal fraction may account for ABZ not being detected in plasma after both oral and subcutaneous treatments with NTB in calves. In these studies, using recommended doses of NTB, the oral administration resulted in a significantly different pharmacokinetic profile and disposition kinetics for both ABZSO and $ABZSO_2$ metabolites than did the SC treatment. Although the recommended dose rates used in these experiments were different, the comparison of the overall disposition kinetics for NTB metabolites and the pattern of pro-drug conversion, according to the route of administration are valid. $T_{\frac{1}{2}\beta}$ and MRT for both ABZSO and $ABZSO_2$ were significantly longer ($P< 0.05$) after the oral treatment (Figures 3.5 and 3.6). These pharmacokinetic differences are due to a less efficient NTB cyclisation into ABZ metabolites following SC administration, as demonstrated in sheep. The parenterally administered NTB must be absorbed, distributed and, by secretion or biliary elimination, reach the GI tract. The rapid elimination of the hydrophilic parent compound after SC administration may also account for a less efficient conversion into its cyclized metabolites compared with the oral treatment. After oral administration, the absence of NTB

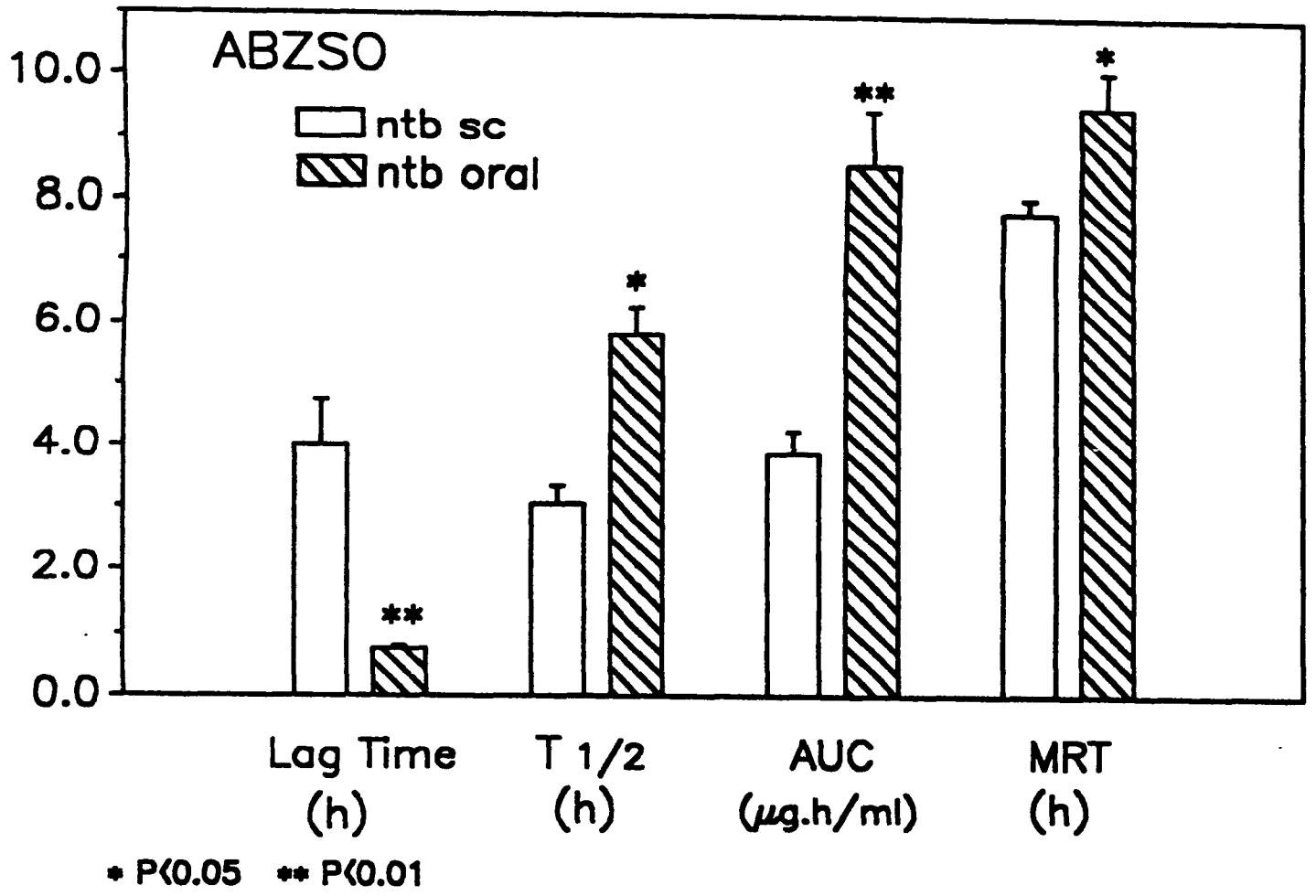
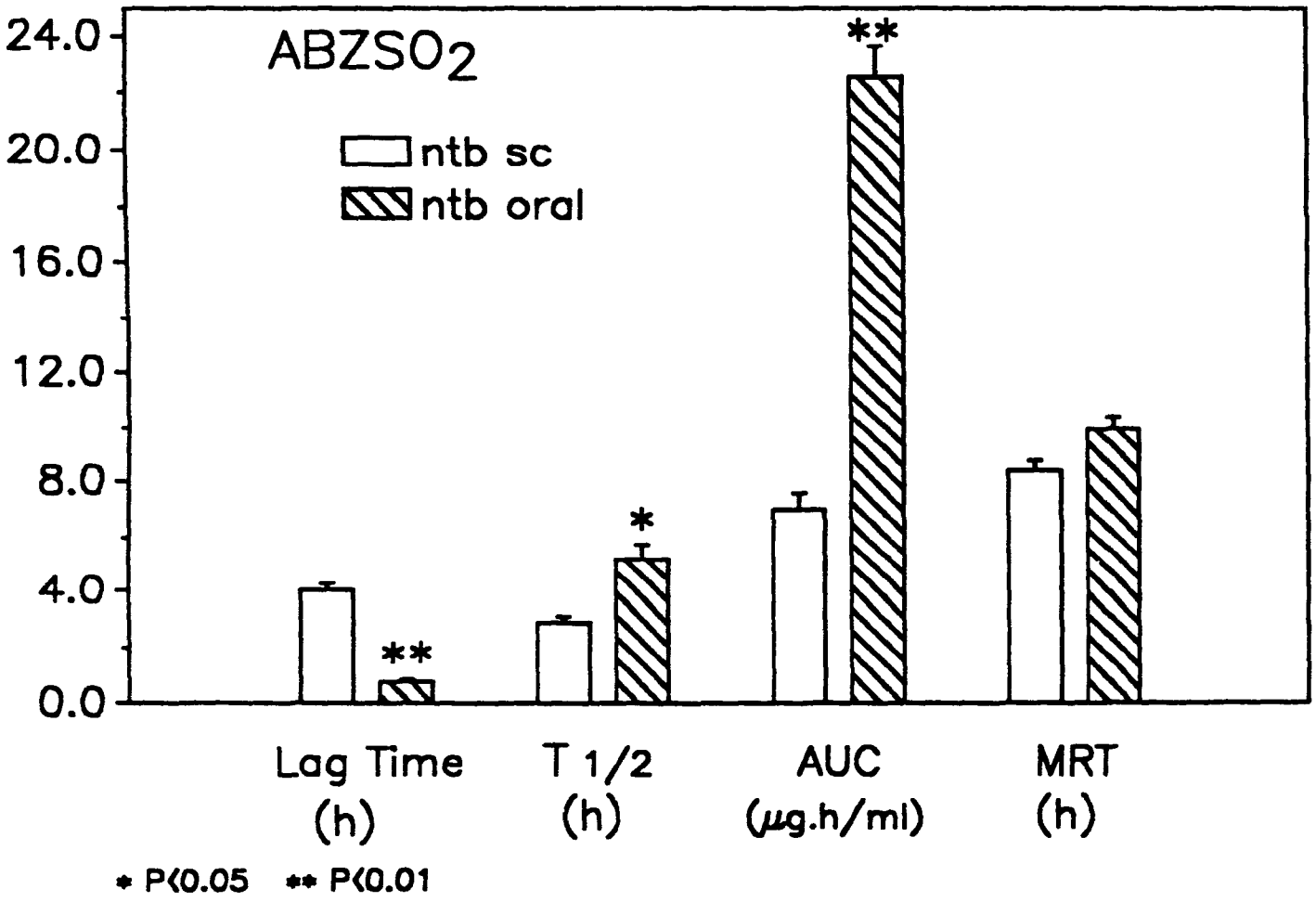


Figure 3.5.: Comparative disposition kinetics for ABZSO obtained after the SC and oral administration of NTB (trisamine solution) at recommended doses to cattle.



* P<0.05 ** P<0.01

Figure 3.6.: Comparative disposition kinetics for $ABZSO_2$ obtained after the SC and oral administration of NTB (trisamine solution) at recommended doses to cattle.

or its occasional detection in trace amounts, and the high profile of its metabolites in plasma indicate a greater degree of conversion to ABZ metabolites.

In conclusion, these experiments have demonstrated that after SC administration in cattle, NTB is rapidly absorbed and cleared from the body or metabolized to ABZSO and $ABZSO_2$. Low ABZSO AUC, fast elimination or oxidation of this active metabolite and high $ABZSO_2$ to ABZSO ratios indicate that the anthelmintic activity of NTB after SC administration depends on the rate of formation and time of residence of ABZSO, which in turn relies on the amount of pro-drug that escapes the renal elimination and reaches the GI tract to be bioactivated. Conversely, after oral administration, NTB is rapidly and more efficiently converted into ABZ metabolites. As demonstrated in sheep, the oral administration results in an improved pharmacokinetic profile of ABZ metabolites compared with the SC injection of NTB, which could account for advantages in anthelmintic efficacy. Finally, although both formulations were bioequivalent after parenteral treatment, the zwitterion suspension was significantly more bioavailable (approximately two-fold) than the trisamine solution in terms of ABZSO and $ABZSO_2$ metabolites following oral administration to cattle.

CHAPTER 4

GASTROINTESTINAL DISTRIBUTION OF ALBENDAZOLE METABOLITES FOLLOWING NETOBIMIN ADMINISTRATION TO CATTLE: RELATIONSHIP WITH THEIR PLASMA DISPOSITION KINETICS

4.1.: INTRODUCTION

The pharmacokinetic behaviour and metabolism in the host can affect the spectrum of activity of BZD and pro-BZD anthelmintic compounds. As discussed earlier the nitro-reduction and cyclisation of NTB pro-drug in the host are crucial for both the pharmacokinetic profile of its metabolites and its anthelmintic activity.

The characterization of the plasma disposition kinetics of NTB parent drug and its ABZ metabolites in sheep and cattle, described in Chapters 2 and 3, has revealed that both formulation and route of administration may dramatically affect the rate of NTB conversion and the bioavailability and disposition of its main plasma metabolites. The antiparasite efficacy of BZD anthelmintics not only depends on their affinity for parasite tubulin, but also on their ability to reach high and sustained concentrations at the site of parasite location; this, in turn, depends on host-related pharmacokinetic, metabolic and tissue distribution factors. The efficacy of BZD and pro-BZD compounds fully depends on the availability of anthelmintically active metabolites at the sites where the parasites are located. The present study was carried out to determine the pharmacokinetic profiles of NTB and its ABZ metabolites in different gastrointestinal (GI) compartments in cattle, correlate them with their plasma disposition kinetics and characterize the plasma-GI tract exchange.

4.2.: METHODS

4.2.1. Experimental animals

Eight parasite-free Holstein steers (150-180 kg) were surgically fitted with permanent cannulae in the rumen, abomasum and ileum. The animals were fasted for 48 h before surgery. Surgery was performed under general anaesthesia with Halothane® administered in a closed circuit inhalation unit with previous administration of xylazine hydrochloride (0.1 mg/kg) as a preanaesthetic. The cannulae (Bar Diamond Inc., Parma, ID, USA) were inserted in the dorsal sac of the rumen, in the pyloric region of the abomasum and at the distal region of the ileum as described by Komarek (1981a,b) and McSweeney (1989). The animals were allowed a six week post-surgery recovery period before starting the pharmacokinetic trial. The animals were housed in individual pens in an indoor cattle facility. They were handled and observed daily to ensure their good health prior to and throughout the experimental period. High quality hay and water were available *ad libitum*.

4.2.2. Treatment and sampling procedures

The eight cannulated animals were treated with a micronized suspension of zwitterion netobimin (400 mg/ml) (batch # 23363-078, Schering Plough, NJ, USA) by oral drench at a dose rate of 20 mg/kg. Samples of ruminal, abomasal and ileal fluids (via cannulae) and jugular blood were drawn prior to treatment and at 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 10, 12, 18, 24, 30, 36, 48, 72 and 96 h post-treatment. At some of the scheduled times, no ileal fluid could be obtained from the ileal cannula, particularly at the earliest stages of the sampling schedule. Digesta samples (approximately 10 ml) were collected into labelled vials. Blood samples were taken in sterile Vacutainer® tubes (Becton Dickinson, Canada) containing heparin and placed on ice before centrifugation at 2500 *g* for 15 min. The extracted plasma, as well as all digesta samples, were frozen at -20°C until analyzed. The

stability of NTB, and its metabolites, in stored samples was corroborated by HPLC determination of samples of the different fluids fortified with each analyte, which had been kept for an equivalent period as the experimental samples under the same conditions.

4.2.3 Analytical procedures

4.2.3.1. Sample extraction

An aliquot (1 ml) of each plasma, and ruminal, abomasal and ileal fluid sample was spiked with 1 μg of oxibendazole (1000 μg/ml; 99.2% pure) as an internal standard. NTB and its metabolites were extracted from plasma and digesta samples using C_{18} Sep Pak cartridges (Waters Associates, Milford, MA, USA), which had previously been conditioned with 5.0 ml of methanol (HPLC grade, Fisher Scientific, Canada) followed by 5.0 ml of 0.017 M ammonium dihydrogen phosphate (pH 5.5). All samples were applied into the cartridge and sequentially eluted, concentrated and prepared for HPLC analysis as previously described (Chapter 2, section 2.2.3). For ruminal, abomasal and ileal fluid samples, there was a solvent-mediated extraction before Sep Pak clean-up. The procedure was a modification of that described by Hennessy (1985), in which the internal standard fortified digesta samples were made alkaline with sodium bicarbonate (1 ml, saturated solution), mixed with 15 ml of ethyl acetate (HPLC grade, Fisher Scientific, Canada) and shaken on a mechanical shaker for 15 min. This extraction step was repeated 3 times and the combined ethyl acetate extracts were evaporated under a stream of nitrogen. The residue was redissolved with 1 N hydrochloric acid (4 ml), mixed with 5 ml of 0.017 M ammonium dihydrogen phosphate (pH 5.5), the final pH adjusted to 5.0 with sodium hydroxide and finally injected into a conditioned Sep Pak cartridge as indicated above.

4.2.3.2. Drug analysis

Experimental and fortified samples were analyzed by HPLC for NTB, ABZ, ABZSO, $ABZSO_2$, amino-albendazole sulphone (NH_2ABZSO_2)

and for a NTB-hydrolysis product. The HPLC equipment and conditions were as described in section 2.2.3. NTB and the NTB-hydrolysis product were read at 320 mm, and all other ABZ metabolites at 292 mm, in a variable wavelength absorbance detector. The retention times were approximately as follows: NH_2ABZSO_2 (4.0 min), ABZSO (5.40 min), NTB-hydrolysis product (6.50 min), $ABZSO_2$ (8.10 min), NTB (10.0 min), oxibendazole (internal standard) (13.50 min) and ABZ (16.90 min).

Identification of each analyte was undertaken by comparison with retention times of pure reference standards (96-98.5% pure) (supplied by Schering Plough, NJ, USA). These standards were also used to establish calibration curves for each analyte in each compartment. Aliquots of cattle plasma and ruminal, abomasal and ileal fluids were spiked with known amounts of each analyte in a range of 0.01 to 5 µg/ml, extracted and then analyzed by HPLC (triplicate determinations) in order to establish the percentage of recovery for each fluid. Concentrations were calculated by comparison of unknown metabolites and internal standard peak area, using Nelson Analytical Software, model 2600 (Nelson Analytical, Inc., CA, USA) on an IBM-compatible AT computer. The limits of HPLC detection, percentage of recoveries and correlation coefficients of calibration curves for NTB and its metabolites in fortified cattle plasma and ruminal, abomasal and ileal fluid samples are summarized in Table 4.1.

4.2.4. Pharmacokinetic analysis of data

The concentration vs. time curves for NTB and/or its metabolites for each individual animal and for each sample compartment were fitted with the PKCALC computer program (Shumaker, 1986). The equation shown in section 2.2.4 was used to describe the biexponential concentration-time curves for the different metabolites in both plasma and gastrointestinal compartments. However, the profiles for ABZSO and $ABZSO_2$ in both abomasal and ileal fluids were best fitted using a triexponential equation.

Table 4.1: Validation of the HPLC methodology for analysis of NTB and its metabolites in fortified samples of cattle plasma and ruminal, abomasal and ileal fluids.

		Plasma	Rumen	Abomasum	Ileum
	d.l.	0.040	0.040	0.040	0.050
NTB	% rec.	89.6	85.3	85.5	81.10
	r	0.9713	0.9834	0.9848	0.9968
	d.l.	0.010	0.020	0.010	0.040
ABZ	% rec.	93.8	89.8	86.4	89.6
	r	0.9895	0.9921	0.9938	0.9927
	d.l.	0.010	0.020	0.010	0.030
ABZSO	% rec.	92.3	90.0	85.2	86.8
	r	0.9886	0.9923	0.9895	0.9966
	d.l.	0.010	0.020	0.010	0.030
$ABZSO_2$	% rec.	93.0	82.5	81.8	80.4
	r	0.9885	0.9945	0.9837	0.9985
	d.l.	0.025	0.025	0.025	0.030
NH_2ABZSO_2	% rec.	82.5	84.5	86.0	85.8
	r	0.9823	0.9799	0.9654	0.9918

d.l.= detection limit (µg/ml); % rec.= percentage of recovery; r = correlation coefficient.

Percentage of recovery (expressed as mean values) and calibration curves were obtained after triplicate determination of spiked samples of each analyte in a range of 0.01 to 5 µg/ml for either fluid. Calibration curves were established by plotting the unknown/internal standard peak area ratio vs. known concentrations of each compound.

The elimination half-life (T ½ ß) and absorption (T ½ ab) or metabolite formation half-lives (T ½ for) were calculated as ln 2/ß and ln 2/k, respectively. The peak concentration (C_{max}) and time to peak concentration (T_{max}) were read from the plotted concentration-time curve of each metabolite in the different compartments. The areas under the concentration-time curves (AUC) were calculated by trapezoidal rule (Gibaldi and Perrier, 1982). The plasma AUC was further extrapolated to infinity by dividing the last experimental plasma concentration by the terminal slope (ß). The AUC values for NTB parent compound and its metabolites in the different GI compartments represent the period from drug administration up to 72 h post-treatment. Statistical moment theory was applied to calculate the mean residence time (MRT) for NTB metabolites in plasma as previously indicated.

4.2.5. Statistical analysis

Pharmacokinetic parameters are expressed as mean ± SEM (n= 8); data for ileum were obtained with n= 6. Statistical comparison of mean pharmacokinetic parameters obtained for each metabolite in different compartments was performed by analysis of variance. Where a significant F value was obtained, Duncan's multiple range test (Steel and Torrie, 1960) was applied to indicate order of significance.

4.3.: RESULTS

Neither NTB parent drug nor ABZ were detected in plasma at any time after the oral administration of NTB in cattle in this experiment. Figure 4.1. shows the plot of the mean plasma concentration of ABZSO, $ABZSO_2$ and NH_2ABZSO_2. ABZSO and $ABZSO_2$ were the main metabolites found in plasma and they were detected between 0.25 and 30-36 h post-treatment. Low concentrations of the NH_2ABZSO_2 metabolite were found in plasma from 12-18 h up to 48 h post-treatment. The plasma disposition kinetics data for these metabolites are shown in Table 4.2. The outcome of the pharmacokinetic analysis for ABZSO and

$ABZSO_2$ in plasma was very similar. Short formation half-lives (between 1.84 and 1.87 h) and close C_{max}, AUC and elimination half-life values show a similar pharmacokinetic pattern for these two metabolites. However, the T_{max} for $ABZSO_2$ (17.25 h) was delayed compared with that of ABZSO (9.25 h). A long formation half-life (5.17 h) with a low AUC and a low C_{max} value reached at 25.50 h post-treatment characterized the plasma kinetics of the NH_2ABZSO_2 metabolite.

The profiles of NTB and its metabolites in ruminal, abomasal and ileal fluids are plotted in Figures 4.2, 4.3 and 4.4, respectively. NTB parent drug showed an early peak and was rapidly removed (12-18 h) from all sampled GI compartments. ABZ, ABZSO and $ABZSO_2$ were found in the rumen, abomasum and ileum from 0.25-0.5 h to 72 h post-NTB administration. The results of the pharmacokinetic analyses for the parent drug and each metabolite in the different GI compartments are summarized in Table 4.3. The comparative area under the concentration-time curves obtained for NTB and its metabolites in different compartments are shown in Figure 4.5. The comparative profiles of ABZSO in plasma and different GI compartments are shown in Figure 4.6.

ABZ, ABZSO and $ABZSO_2$ AUC and C_{max} values were significantly higher ($P< 0.01$) in abomasal than in either ruminal or ileal fluid. However, the AUC and C_{max} for the NH_2ABZSO_2 metabolite were significantly lower ($P< 0.01$) in both abomasal fluid and plasma than in ileal fluid. Table 4.4 summarizes the results of the statistical comparison of AUC values for each metabolite in the different sampled compartments. While there were no statistical differences for ABZ T ½ ß and T_{max} among the different compartments, the ABZSO T ½ ß was significantly longer ($P< 0.01$) in both abomasum and ileum than in plasma and rumen, respectively. Similarly, the $ABZSO_2$ and NH_2ABZSO_2 T ½ ß were significantly longer ($P< 0.01$) in abomasal fluid than in plasma.

Low concentrations of a NTB hydrolysis product were occasionally detected in some of the plasma and ruminal fluid samples of some animals. The lack of consistency precluded any pharmacokinetic analysis and, therefore, these results are not included.

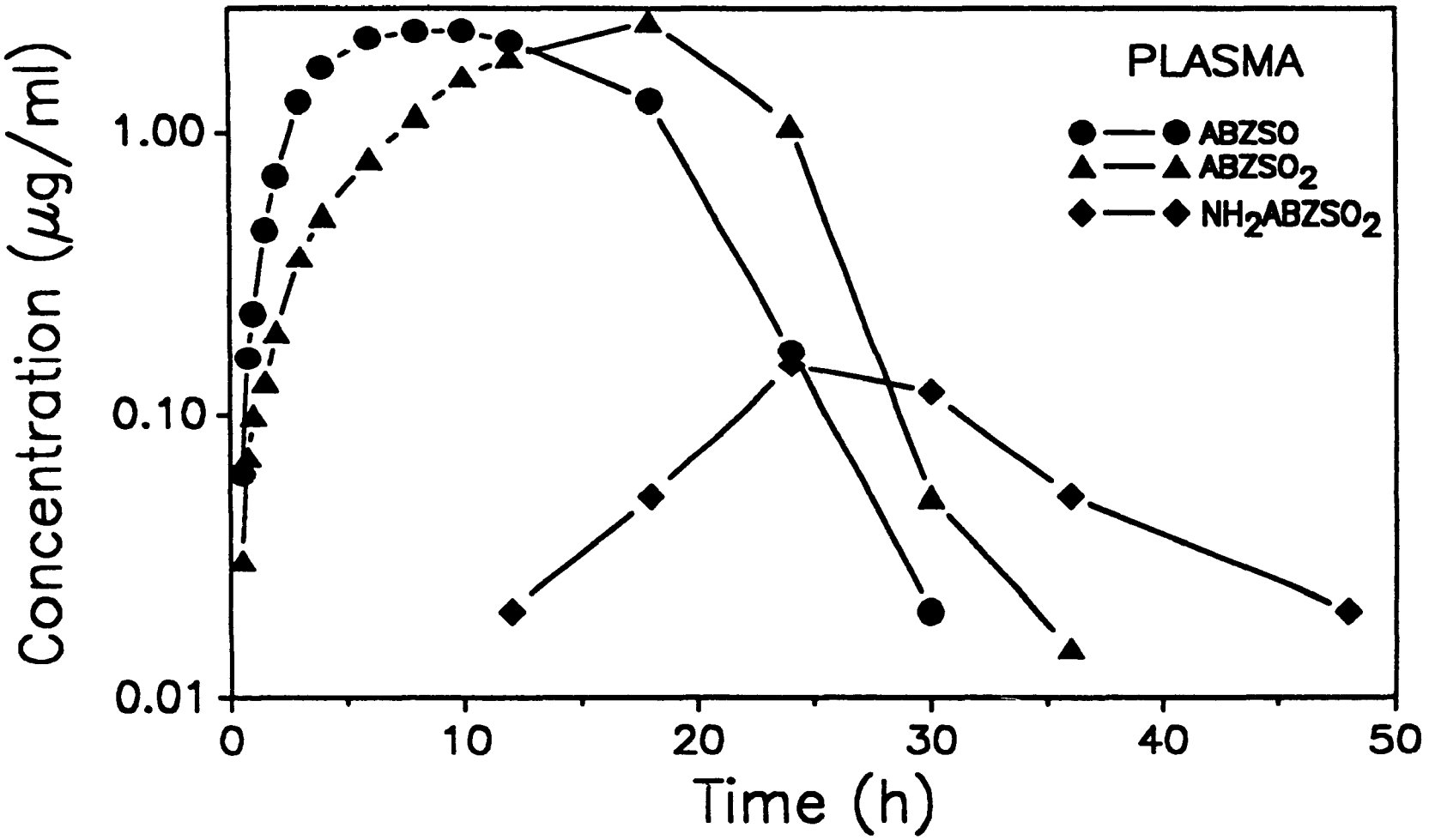


Figure 4.1: Mean plasma concentration (n=8) of ABZSO, $ABZSO_2$ and NH_2ABZSO_2 obtained after oral administration of NTB (20 mg/kg) to cattle.

Table 4.2: Plasma disposition kinetics data for ABZSO, $ABZSO_2$ and NH_2ABZSO_2 cbtained after oral administration of NTB (20 mg/kg) to cattle.

Parameter	Unit	ABZSO	$ABZSO_2$	NH_2ABZSO_2
Lag Time	(h)	1.65 ± 0.15	2.36 ± 0.16	12.90 ± 1.64
T ½ for	(h)	1.84 ± 0.12	1.87 ± 0.12	5.17 ± 0.61
C_{max}	(μg/ml)	2.50 ± 0.18	2.64 ± 0.21	0.17 ± 0.01
T_{max}	(h)	9.25 ± 0.84	17.25 ± 0.75	25.50 ± 1.50
AUC	(μg.h/ml)	35.72 ± 3.99	37.65 ± 3.78	2.61 ± 0.16
AUMC	($\mu g.h^2/ml$)	394.90 ± 55.6	586.20 ± 76.5	81.80 ± 5.96
T ½ ß	(h)	2.29 ± 0.27	1.99 ± 0.19	7.96 ± 0.65
MRT*	(h)	8.15 ± 0.47	12.48 ± 0.76	25.40 ± 0.83

Data are expressed as mean ± SEM (n=8)

*Value obtained by non-compartmental analysis of the data based on the statistical moment theory.

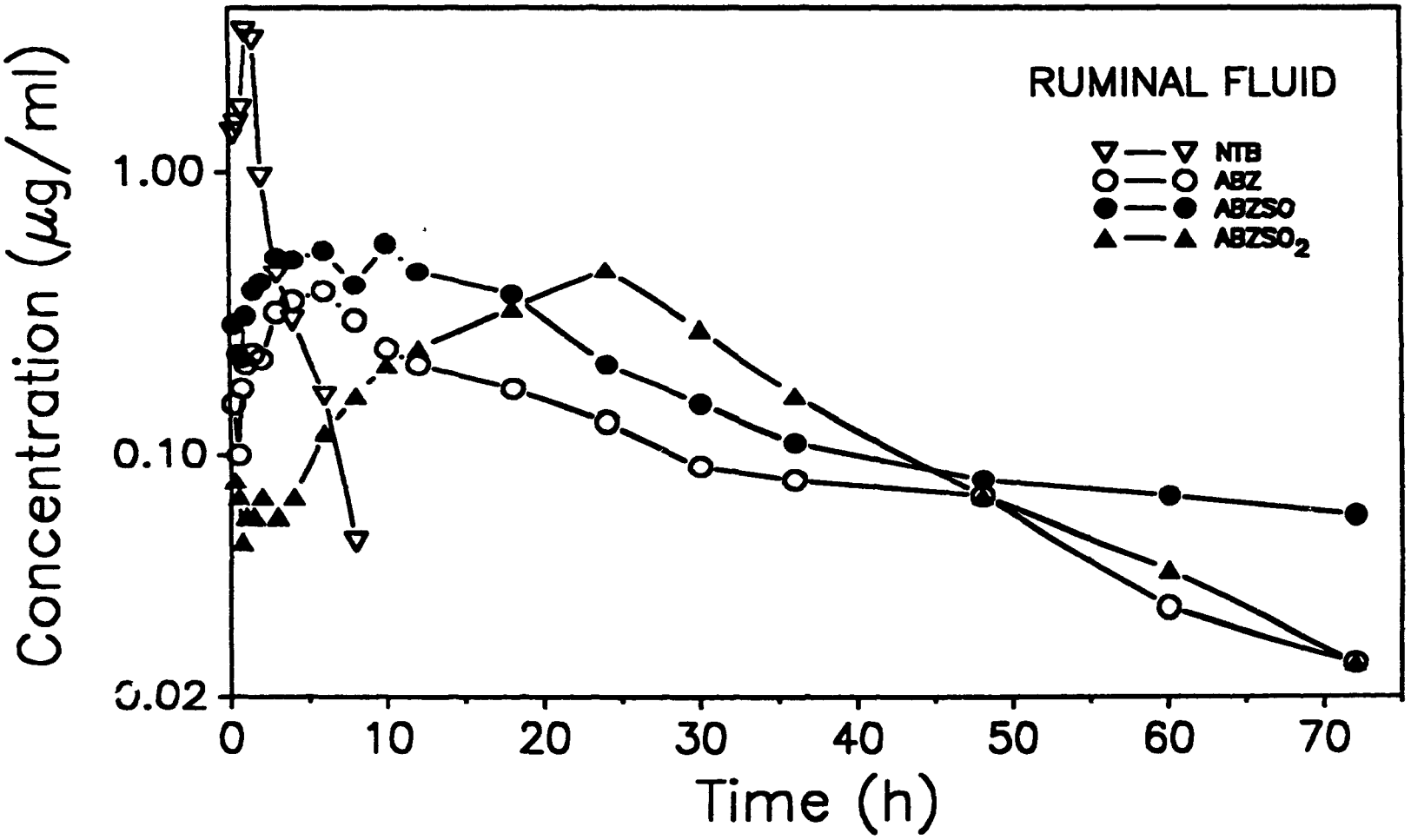


Figure 4.2: Mean concentration (n=8) of NTB and its ABZ metabolites in ruminal fluid obtained after oral administration of NTB (20 mg/kg) to cattle.

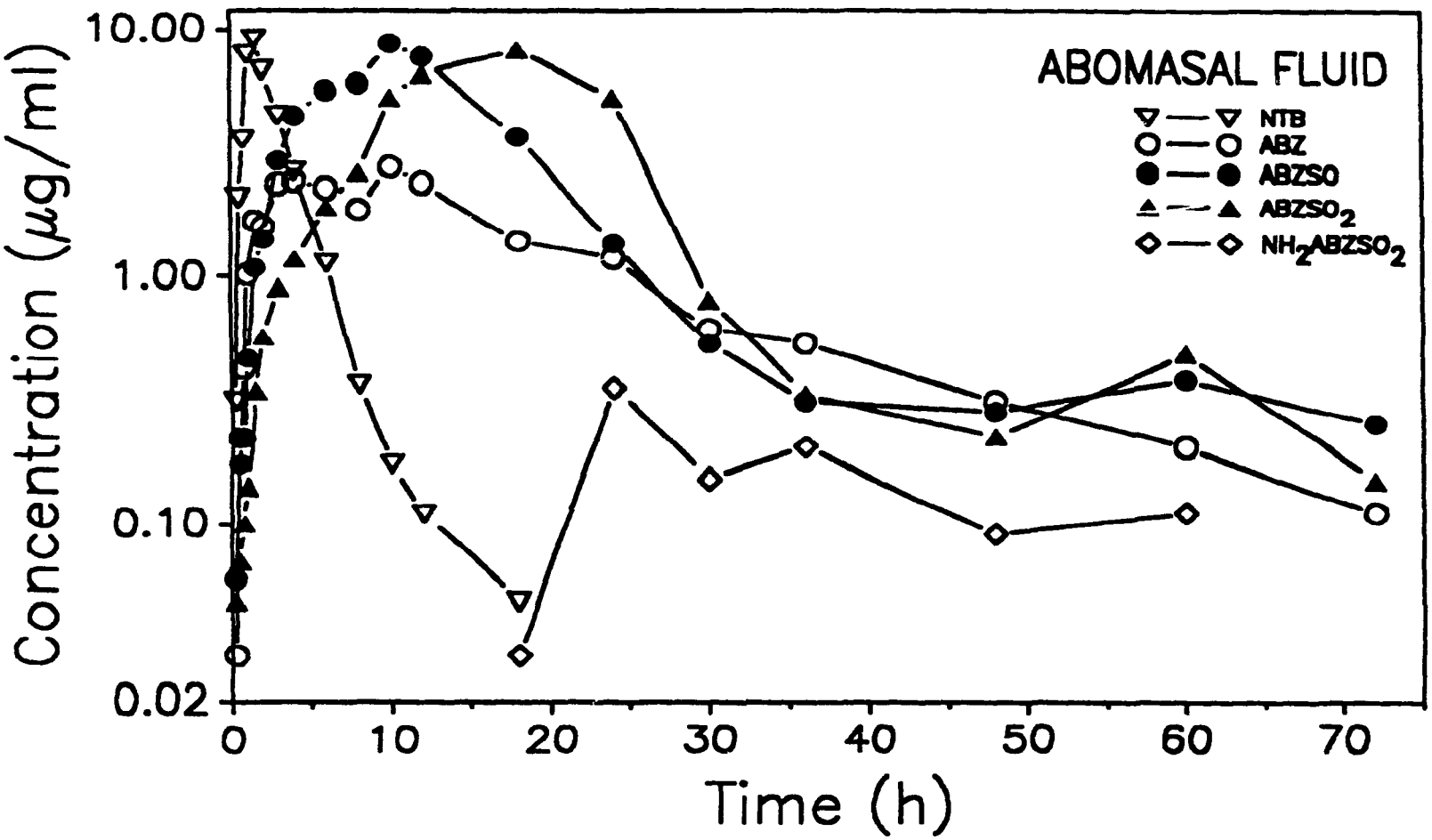


Figure 4.3: Mean concentration (n=8) of NTB and its ABZ metabolites in abomasal fluid obtained after oral administration of NTB (20 mg/kg) to cattle.

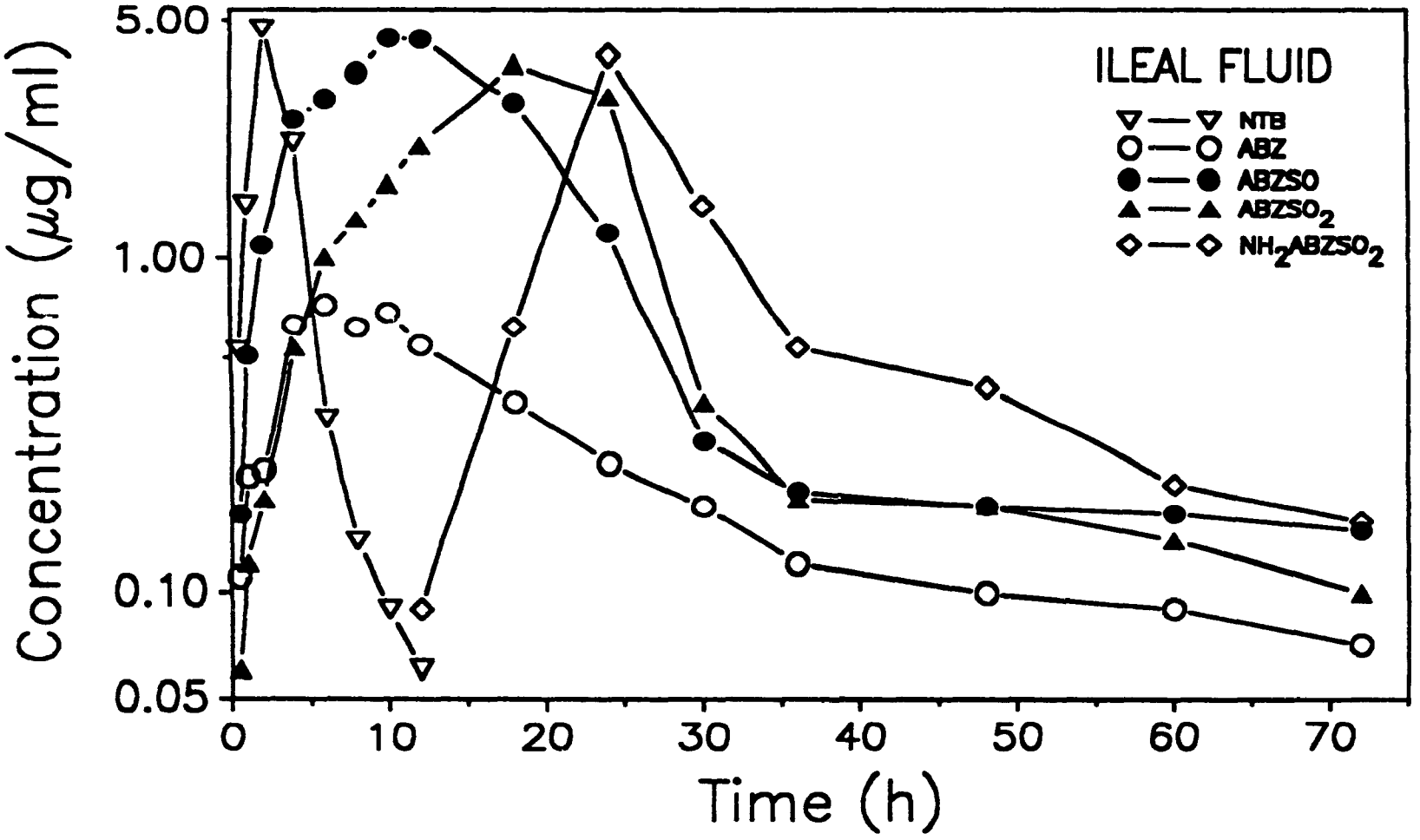


Figure 4.4: Mean concentration (n=6) of NTB and its ABZ metabolites in ileal fluid obtained after oral administration of NTB (20 mg/kg) to cattle.

Table 4.3: Pharmacokinetic parameters for NTB and its metabolites in different gastrointestinal compartments obtained after oral administration of NTB (20 mg/kg) to cattle.

Compartment/Parameter	NTB	ABZ	ABZSO	$ABZSO_2$	NH_2ABZSO_2
Rumen					
C_{max} (μg/ml)	3.60±0.82	0.44±0.06	0.76±0.14	0.50±0.005	NA
T_{max} (h)	0.97±0.18	4.50±0.46	6.25±0.96	22.0±2.30	NA
AUC_{0-72} (μg.h/ml)	4.70±1.16	7.63±1.28	13.83±1.87	11.37±0.90	NA
T ½ ß (h)	1.14±0.11	13.65±1.52	15.89±1.18	11.16±1.06	NA
Ratio AUC rumen/plasma	NA	NA	0.38	0.30	NA
Abomasum					
C_{max} (μg/ml)	10.02±2.91	3.33±0.49	8.86±0.84	8.70±1.10	0.38±0.21
T_{max} (h)	1.39±0.15	6.57±1.27	10.29±0.29	15.14±1.37	28.29±2.15
AUC_{0-72} (μg.h/ml)	25.41±7.94	64.06±6.06	128.3±13.20	145.0±16.71	1.83±0.21
T ½ ß (h)	1.36±0.23	13.15±0.70	33.97±7.31[‡]	53.20±21.23[‡]	17.88±3.47
Ratio AUC abom./plasma	NA	NA	3.59	3.85	0.70
Ileum					
C_{max} (μg/ml)	4.71±1.25	0.97±0.12	4.64±0.35	3.61±0.46	3.89±0.88
T_{max} (h)	2.00±0.00	7.00±1.13	10.0±0.73	19.0±1.84	24.0±0.00
AUC_{0-72} (μg.h/ml)	13.60±4.05	16.19±2.32	79.90±5.500	64.22±5.45	49.67±9.20
T ½ ß (h)	1.28±0.10	15.88±2.83	67.71±22.01[‡]	40.97±10.71[‡]	12.14±1.13
Ratio AUC ileum/plasma	NA	NA	2.23	1.71	19.03

Data expressed as mean ± SEM (n=8); data from ileum is with n=6.

NA=not applicable; [‡]= values obtained following a triexponential pharmacokinetic model

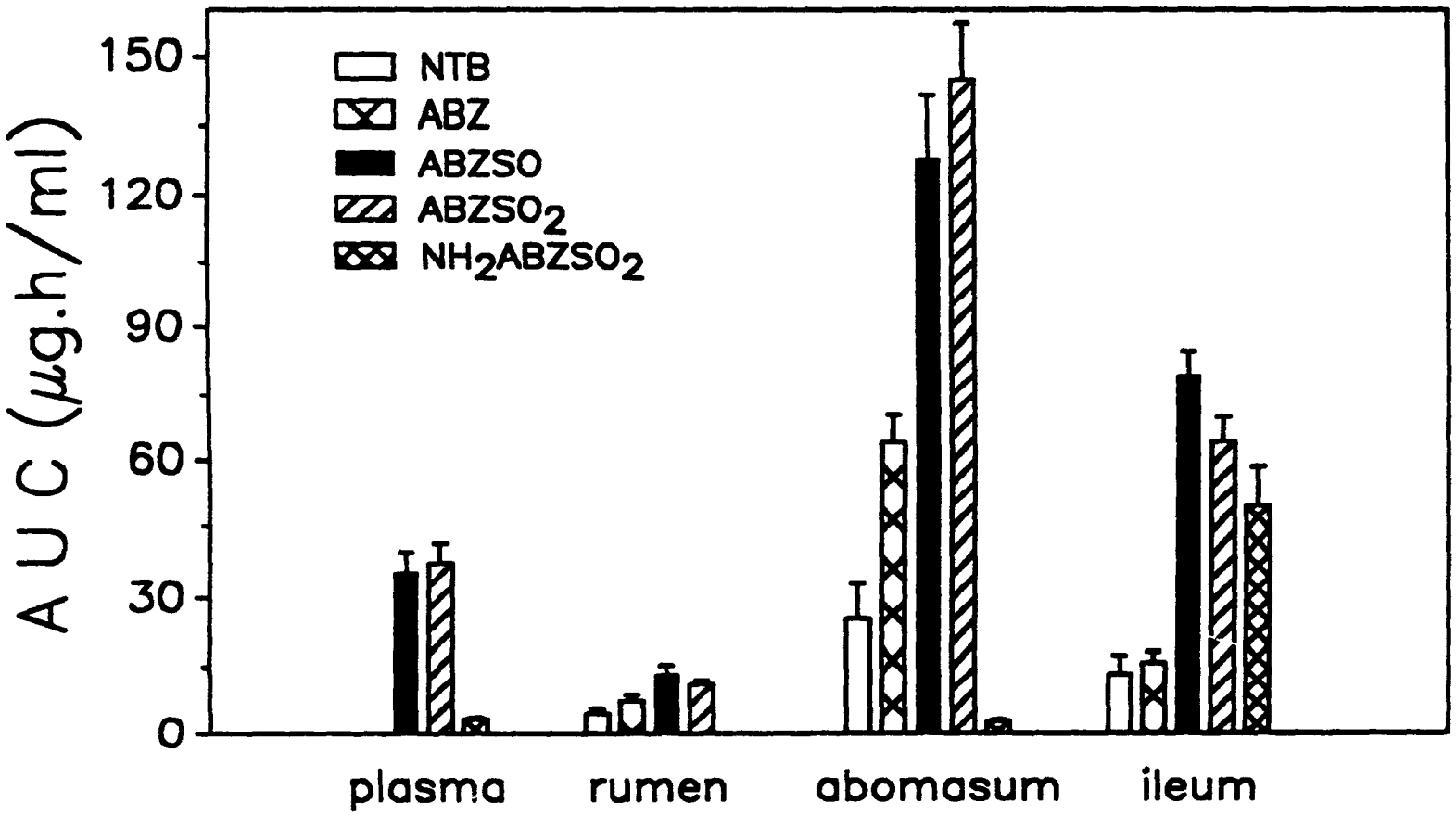


Figure 4.5: Comparative area under the concentration-time curves (mean ± SEM) for NTB and its metabolites in different compartments obtained after oral administration of NTB (20 mg/kg) to cattle.

Table 4.4: Statistical comparison (ANOVA plus Duncan's multiple range test) of the area under the concentration-time curves obtained for NTB and its metabolites in different compartments after NTB treatment in cattle.

		Plasma	Rumen	Abomasum	Ileum
	Rumen	NA	-	**	NS
NTB	Abomasum	NA	**	-	*
	Ileum	NA	NS	*	-
ABZ	Rumen	NA	-	**	**
	Abomasum	NA	**	-	NS
	Ileum	NA	**	**	-
	Plasma	-	NS	**	**
ABZSO	Rumen	NS	-	**	**
	Abomasum	**	**	-	**
	Ileum	**	**	**	-
	Plasma	-	NS	**	NS
$ABZSO_2$	Rumen	NS	-	**	**
	Abomasum	**	**	-	**
	Ileum	NS	**	**	-
	Plasma	-	NA	NS	**
NH_2ABZSO_2	Abomasum	NS	NA	-	**
	Ileum	**	NA	**	-

NA=not applicable; NS=not significant

*=significantly different at P< 0.05

**=significantly different at P< 0.01

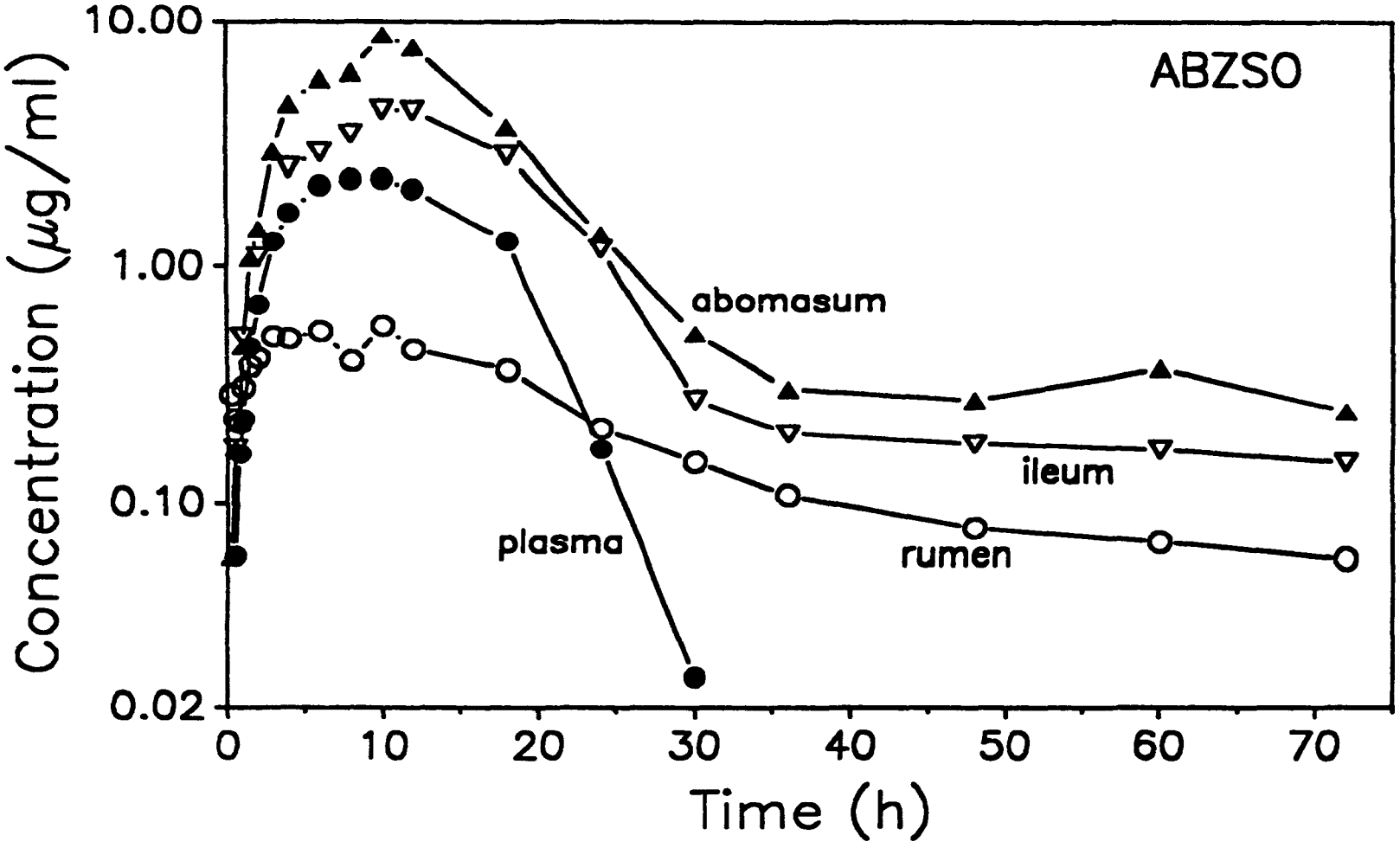


Figure 4.6: Comparative ABZSO profiles in different compartments obtained following the oral administration of NTB (20 mg/kg) to cattle.

4.4.: DISCUSSION

The rapid appearance of ABZ, ABZSO and $ABZSO_2$ in all GI compartments, as well as ABZSO and $ABZSO_2$ in plasma, and the fast removal of the parent drug (Figures 4.2, 4.3 and 4.4) seems to confirm that NTB is efficiently converted into ABZ in the GI tract.

As previously proposed (Hennessy *et al.*, 1989; Galtier *et al.*, 1991) a first pass liver microsomal oxidation may account for ABZ not being detected in jugular plasma. However in some in vitro metabolism studies reported in the following Chapters, I have found that to some extent the oxidation of ABZ occurs in the GI tract before absorption. This would be consistent with the early detection of ABZSO and $ABZSO_2$ both in the GI tract and plasma (Figures 4.1, 4.2, 4.3, and 4.4).

As reported in Chapter 3, ABZSO and $ABZSO_2$ showed a fast plasma disposition resulting in short elimination half-lives and MRT (Table 4.2). The $ABZSO_2$ T_{max} was delayed (17.25 ± 0.75 h) compared to that of ABZSO (9.25 ± 0.84) which also confirms the sequential steps of the ABZ oxidation after NTB cyclisation. Surprisingly, the ABZSO AUC in plasma (35.75 μg.h/ml) was two-fold higher than that found for this metabolite after oral administration of an NTB zwitterion suspension to cattle (18.55 μg.h/ml) (experiment reported in Chapter 3, section 3.3). In fact, the AUC $ABZSO_2$/ABZSO ratio was approximately 2.80 in that experiment, whereas a ratio of 1.05 was obtained in the present study with cattle treated with the same NTB dose rate. Since both experiments were carried out under similar conditions with animals of the same breed and age and fed on the same diet, it is unlikely that these differences are due to a differential oxidation pattern between animals in either experiment. It is, however, more likely that the improved plasma pharmacokinetic profile of ABZSO in the present study is related to a more efficient bacterial conversion of NTB. While the formulation used in

that previous experiment was a suspension of zwitterion NTB at 5% final concentration, the animals in the present study were treated with a more concentrated (40%) suspension of the same formulation which implies that a much lower final volume was given to the animals to achieve the 20 mg/kg dose rate. The oral administration of large volumes of liquid formulations to ruminants may result in spontaneous closure of the oesophageal groove, which has been observed for some anthelmintics in sheep (Prichard and Hennessy, 1981) and cattle (McEwan and Oakley, 1978). Such a phenomenon may facilitate a ruminal by-pass and may result in a smaller amount of NTB being exposed to the ruminal microflora following treatment with a 5% suspension. On the other hand, the administration of a smaller volume of a more concentrated formulation may assure a greater proportion of the total administered pro-drug reaching the rumen, facilitating more efficient bacterial uptake, nitro-reduction and cyclisation of NTB. This may result in an improved plasma profile for the ABZSO metabolite.

The NH_2ABZSO_2 metabolite has been identified and its pharmacokinetic behaviour characterized for the first time (Tables 4.2 and 4.3). Its appearance in plasma (Figure 4.1) and in abomasal and ileal fluids (Figures 4.3 and 4.4) follows the $ABZSO_2$ plasma peak. The hydrolysis of the $ABZSO_2$ methylcarbamate group, perhaps in the liver, forms this polar amino-derivative metabolite which is probably eliminated by bile and detected in high concentrations in the gut (Figures 4.4 and 4.5). The AUC and C_{max} values were significantly higher ($P< 0.01$) for ileal than for abomasal fluid or plasma.

The plasma profiles for ABZSO and $ABZSO_2$, although much lower, reflect the gastrointestinal profiles. Peak concentrations of ABZSO and $ABZSO_2$ were reached at 7-10 h (ABZSO) and at 15-22 h ($ABZSO_2$) post-treatment followed by a well defined concentration declining phase in both plasma and GI compartment concentration-time plots (Figures 4.1, 4.2, 4.3 and 4.4). However, whereas plasma concentration fell to

undetectable levels (30-36 h post-treatment), the profile of these metabolites in the rumen, abomasum and ileum showed an "extra" slow elimination phase that extended to 72 h post-treatment. This pharmacokinetic behaviour was particularly clear for the ABZSO (Figure 4.6) and $ABZSO_2$ metabolites in both the abomasum and ileum, where their concentration-time plots with this long elimination phase were best fitted using a triexponential equation, which resulted in significantly ($P< 0.01$) longer elimination half-lives for these metabolites in these two GI compartments compared to plasma (Table 4.3). This metabolite-distribution process may be driven by a plasma/GI tract pH gradient. A greater plasma/abomasum pH gradient compared with that of the rumen and ileum would produce a strong ionic trapping effect which would account for the significantly higher ($P< 0.01$) concentrations of ABZSO and $ABZSO_2$ found in the abomasum in comparison with plasma and other GI compartments (Figure 4.5; Tables 4.3 and 4.4).

The presence of both anthelmintically active metabolites, ABZ and ABZSO, in the abomasum and intestine for an extended period of time with markedly slow elimination phases, is of major relevance in terms of efficacy against GI parasites. Furthermore, ABZSO can be reduced back to ABZ by ruminal and intestinal flora (results shown in Chapter 5) and may act as a source of ABZ in the GI tract. This may explain the presence of ABZ for 72 h post-treatment in this experiment when the parent NTB compound has been completely removed from the digestive tract by 12 to 18 h post-administration.

In conclusion, this study has permitted the characterization of the compartmental distribution of NTB and its ABZ metabolites. The pattern of plasma-GI exchange for active and inactive metabolites and its implications on antiparasite efficacy have been determined. The pharmacokinetic characterization of the NH_2ABZSO_2 metabolite is a further contribution to the understanding of the metabolism and fate of NTB and ABZ-related anthelmintics in ruminants.

CHAPTER 5

METABOLISM OF BENZIMIDAZOLE ANTHELMINTICS BY RUMINAL AND INTESTINAL FLUIDS OF SHEEP AND CATTLE

5.1.: INTRODUCTION

From the pharmacokinetic characterizations described in previous Chapters it is evident that BZD and pro-BZD are extensively metabolized in the host. Their metabolic pattern and resultant pharmacokinetic behaviour are relevant to achieve high and sustained concentrations of pharmacologically active drug/metabolites at the parasite target.

The nitro-reduction and cyclization into ABZ in the host are crucial for the broad spectrum anthelmintic efficacy of NTB pro-drug. The GI microflora have been proposed as the main site for NTB conversion into its anthelmintically active ABZ metabolites (Delatour *et al.*, 1986). However, both route of administration and formulation have been shown to greatly influence the rate of NTB bioconversion and the resultant bioavailability and plasma disposition of its main metabolites in sheep and cattle (Chapters 2 and 3). Although bioequivalent following parenteral treatment, the zwitterion and trisamine salt formulations of NTB were not bioequivalent after oral administration to cattle. Differences in the rate of conversion of NTB to its metabolites may contribute to these differences between formulations.

Following the administration of NTB pro-drug in both sheep and cattle (Chapters 2, 3 and 4) and ABZ alone in the same species (Marriner and Bogan, 1980; Prichard *et al.*, 1985; Delatour *et al.*, 1990a), ABZSO and $ABZSO_2$ were the major metabolites found in plasma. However, marked differences in the plasma disposition kinetics of these metabolites between sheep and cattle have been described (Chapters 2 and 3) and attributed to differential metabolic patterns between the species

(Prichard *et al.*, 1985). The liver microsomal fraction is thought to be the main site for the sequential biotransformation of BZD thioether compounds (such as ABZ, FBZ, etc.) which leads to the production of more polar and less anthelmintically effective metabolites. However, the importance of the biotransformation of BZD and pro-BZD compounds in the GI tract should not be underestimated. In chapter 4, the characterization of the GI distribution of ABZ metabolites in cattle following NTB administration has been presented. These metabolites are reversibly exchanged between plasma and different GI compartments in a pH gradient-mediated distribution process. While ABZ was not found in plasma, ABZSO showed a fast plasma disposition, being detected for only 30-36 h post-treatment. However, these two anthelmintically active metabolites were present in different GI compartments for at least 72 h post-treatment. It is likely then that not only the plasma-GI tract exchange but also some metabolic interconversion in the digestive tract account for the presence of these two active metabolites for an extended period of time in the GI tract. This is of major relevance for efficacy against GI parasites.

Taking into consideration the extent of the exchange surface between plasma and digestive tract in ruminants and the large volume of the forestomach, any potential bioconversion of these compounds taking place in the GI tract could have a significant impact on both the pharmacokinetic behaviour and the availability of anthelmintically active metabolites at the sites where GI and tissue-dwelling parasites are located.

Since parasite uptake and binding to tubulin may differ greatly amongst different BZD metabolites, a complete understanding of the metabolic pattern of these compounds and their complex pharmacokinetic behaviours becomes crucial to optimize their broad-spectrum efficacy. The *in vitro* experiments reported in this Chapter were designed to investigate the ability of ruminal, abomasal and ileal fluids

of sheep and cattle to metabolize NTB pro-drug, ABZ and ABZ-related metabolites. The bioconversions of zwitterion and trisamine salt formulations of NTB by ruminal and ileal fluids were quantitatively compared. The metabolism of ABZ and ABZSO by digestive fluids obtained from both sheep and cattle was also studied on a comparative basis.

5.2.: MATERIALS AND METHODS

5.2.1. Chemicals

ABZ and oxibendazole (OBZ) were supplied by SmithKline Beecham Corp., West Chester, PA, USA. ABZSO, $ABZSO_2$ and the zwitterion suspension and trisamine salt solution of NTB were generous gifts from Schering Plough, Kenilworth, NJ, USA. All these compounds were between 96 and 99.5% pure and stock solutions were prepared in methanol. Fresh working solutions were prepared immediately before each incubation assay. HPLC grade solvents were obtained from Fisher Scientific, Ontario, Canada. Ammonium dihydrogen phosphate, sodium bicarbonate and ammonium acetate were purchased from Aldrich Chemical Co., Milwaukee, WI, USA.

5.2.2. Animals and collection of gastrointestinal fluids

Healthy Holstein Fresian calves (200-220 kg) and Finn Dorset cross-bred sheep (40-45 kg) were used as a source of ruminal, abomasal and ileal fluids. The animals were exclusively fed on high quality hay in the three months prior to slaughter, and water was permitted *ad libitum*. The animals were stunned by captive bolt and exsanguinated immediately. The abdomen was opened and the digestive organs were properly identified and removed. Ruminal fluid was obtained by making an incision in the dorsal sac of the rumen. The abomasum and distal region of the ileum were tied off, separate from the rest of the digestive tract and their contents drained into labelled containers. Aliquots of the collected fluids were kept at 37-38°C, transported from the abattoir to the laboratory and processed for incubation within two hours of their collection.

Samples were filtered through a hydrophilic gauze to remove solid material and the filtrate kept saturated with pure N_2 at 38°C until the incubation assays were carried out. The pH of the different fluids was determined for each individual animal used in the experiment and the range of values for both species was as follows: ruminal fluid = 6.4-6.7; abomasal fluid = 1.5-2.8; ileal fluid 7.4-7.6. Blank samples were prepared by boiling each fluid at 100°C for 60 min (Beretta *et al.*, 1987).

5.2.3. Incubation assays

One hundred microliters of one of the compounds under investigation were added to 1.9 ml of the filtrate of ruminal, abomasal or ileal fluid to a final drug concentration of 2 µg/ml of incubation mixture. Each incubation mixture was gently gassed with pure N_2 for 5 min at 38°C. Incubations were carried out in a thermostatic shaking water bath at 38°C under anaerobic conditions for 60 and 360 min (NTB incubations) and for 30, 60 and 360 min (ABZ metabolite incubations). Blank samples of each fluid (boiled) were prepared and incubated under the same conditions. Immediately after the incubation period, the samples were frozen at -20°C. Extractions and analyses were undertaken within 2 to 3 days post-incubation. Incubation assays were performed in duplicate and repeated several times with fluids obtained from different animals of both species. Incubations were always done using fresh digesta fluids and within 2 h of slaughter.

5.2.4. Analytical Procedures

An aliquot (1 ml) of each ruminal, abomasal and ileal fluid incubation mixture was spiked with 1 µg of OBZ (1000 µg/ml) as an internal standard. The different compounds and their formed metabolites were extracted by solvent-mediated extraction and subsequently by solid phase extraction using C_{18} Sep Pak Cartridges (Waters Associates, Milford, MA, USA). The solvent extraction procedure was as described in Chapter 4, section 4.2.3.1 for digesta samples.

Incubation samples were analyzed for NTB, ABZ, ABZSO and $ABZSO_2$ by HPLC. The HPLC equipment and analysis conditions were as previously reported (Chapter 2, section 2.2.3). Aliquots of sheep and cattle ruminal, abomasal and ileal fluids were spiked with known amounts of each analyte in a range of 0.01 to 5 μg/ml, extracted and then analyzed by HPLC (triplicate determinations) in order to establish the percentage of recovery and calibration curves for each fluid. The recovery values for the different analytes in the different fluids of both species ranged from 81 to 92%. Concentrations were calculated by comparison of unknown compounds and internal standard peak area, using Nelson Analytical software, model 2600 (Nelson Analytical Inc., CA, USA) on an IBM compatible AT computer. The sensitivity of the assay was: 0.040 μg/ml for NTB in all fluids; 0.010-0.020 μg/ml for ABZ and ABZSO in ruminal and abomasal fluids; 0.020-0.040 μg/ml for ABZ and ABZSO in ileal fluid and for $ABZSO_2$ in each fluid.

5.2.5. Data and statistical analyses

The results presented are mean values of at least four determinations. The products (metabolites) formed after each incubation are expressed as a percentage of the total recovered products. The ratio ABZ/ABZSO represents the mean value of the ratio between the amount of ABZ and ABZSO found in all the incubation assays carried out for a particular compound under particular conditions. Mean results were statistically compared using Student's *t*-test for either paired or unpaired data. A value of $P < 0.05$ was considered statistically significant.

5.3.: RESULTS

There was no conversion of NTB in the boiled ruminal, abomasal or ileal fluid from each species. NTB was not affected by incubation with fresh abomasal fluid either for 60 or 360 min.

Both ileal and ruminal fluids of sheep and cattle were able to convert NTB into ABZ metabolites. The comparison of the amounts of products formed following incubation of NTB, as either a zwitterion suspension or a trisamine salt solution, with sheep ruminal fluid for 60 min under anaerobic conditions is shown in Figure 5.1. ABZ, ABZSO and $ABZSO_2$ were the metabolites produced from both NTB formulations. However, a significantly greater amount of both ABZ ($P< 0.001$) and ABZSO ($P< 0.05$) was formed from the zwitterion compared with the trisamine formulation of NTB. Similarly, zwitterion NTB produced a significantly greater amount of ABZ metabolites than the trisamine salt of NTB after incubation with both cattle ruminal fluid (Table 5.1) and sheep and cattle ileal fluids (Table 5.2). The percentage of NTB consumption following incubation with sheep ruminal fluid for 360 min was significantly higher ($P< 0.05$) for the zwitterion (87.9 ± 1.76) than for the trisamine (65.6 ± 7.16) formulation. Figure 5.2 shows the comparison of the total amount of metabolites formed (ABZ, ABZSO and $ABZSO_2$) after incubation of the zwitterion (final pH 5.9), trisamine (final pH 8.0) and pH-modified zwitterion (final pH 8.0) formulations of NTB with sheep ruminal fluid for 60 and 360 min. The quantity of total products recovered was significantly higher for the zwitterion NTB than for both the trisamine and pH-modified zwitterion formulations after 60 ($P< 0.001$) and 360 ($P< 0.01$ and $P< 0.05$) min of incubation.

After 60 min of incubation, the amount of product recovered as ABZ was significantly lower in ileal ($P< 0.01$) than in ruminal fluid of sheep (Figure 5.3). However, the amount of ABZSO recovered was significantly greater ($P< 0.01$) for ileal fluid. These results are corroborated by the significantly higher ratio after 60 min incubation of ABZ/ABZSO obtained for ruminal fluid (3.47 and 2.11 for the zwitterion and trisamine, respectively) than for ileal fluid (0.29 and 0.16 for zwitterion and trisamine, respectively) with both NTB formulations. However, following a long incubation period

(6 h), these differences were not observed (ratios of ABZ/ABZSO of approximately 1.0).

The comparative bioconversion of NTB by ruminal and ileal fluids of sheep and cattle is summarized in Tables 5.1 and 5.2, respectively. After 60 min of incubation of both NTB formulations with sheep and cattle ruminal fluid, there were no differences in the total amount of NTB products formed (Table 5.1). However, the percentage of product recovered as ABZSO was higher in cattle (55.2 to 72.2%) than in sheep (27.5 to 34.4%) which accounted for the significantly lower ($P< 0.05$) ABZ/ABZSO ratio obtained for cattle ruminal fluid compared to sheep (Table 5.1). Although the incubation of NTB with sheep and cattle ileal fluids (360 min) showed the same trend, the production of ABZSO was higher when ileal fluid of cattle was incubated with trisamine NTB than with the zwitterion formulations. Again, the ABZ/ABZSO ratio was significantly lower ($P< 0.05$) for cattle (0.11) than for sheep (1.39) ileal fluid (Table 5.2).

No chemical modifications of ABZ, ABZSO or $ABZSO_2$ were observed upon incubation with abomasal fluid for 30, 60 and 360 min. While ABZ and ABZSO were extensively metabolized by both ruminal and ileal fluids of sheep and cattle, the $ABZSO_2$ metabolite was not affected either after short or after long incubation time with those fluids. ABZ was oxidized to ABZSO by both sheep fluids, but the production of ABZSO after 60 min of incubation was significantly higher ($P< 0.05$) for the ileal (44% of total recovered products) compared with the ruminal fluid (23.6% of total recovered products) of sheep. ABZSO was oxidized to $ABZSO_2$ at a very low rate by both digestive fluids. However, the most important metabolic change was the reduction of ABZSO back to ABZ (Figure 5.4).

Comparisons of the ability of sheep and cattle ruminal and ileal fluids to biotransform both ABZ and ABZSO are presented in Tables 5.3 and 5.4. The rate of ABZ oxidation was

significantly higher for cattle than for sheep ileal fluid. Furthermore, ABZSO was more efficiently reduced to ABZ by sheep than by cattle ruminal and ileal fluids following both 60 min (Figure 5.5) and 360 min (Table 5.4) of incubation. The ratio ABZ/ABZSO was significantly lower for cattle than for sheep ruminal and ileal fluids both after 60 and 360 min of incubation (Table 5.4).

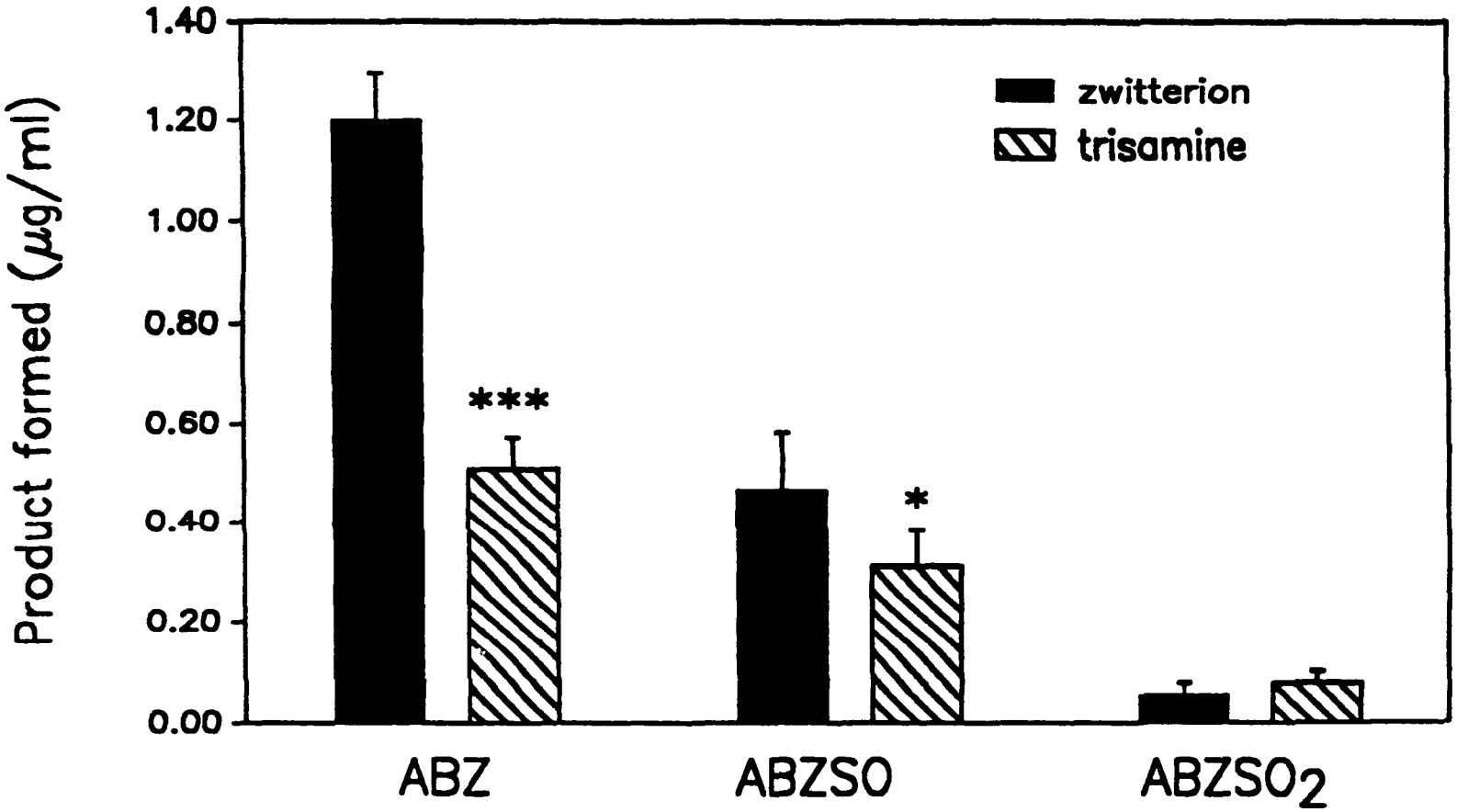


Figure 5.1: Conversion of NTB by sheep ruminal fluid. Amount of products formed (ABZ, ABZSO and $ABZSO_2$) following incubation of NTB, as either a zwitterion suspension or as a trisamine solution, with sheep ruminal fluid for 60 min under anaerobic conditions. Statistically different from the zwitterion NTB at $P < 0.05$ (*) and at $P < 0.001$ (***). Data are expressed as mean ± SEM (n = 6).

Table 5.1: Comparative biotransformation of NTB (as zwitterion and trisamine formulations) by sheep and cattle ruminal fluids following a 60 minutes incubation period under anaerobic conditions.

Formulation	Species	Total products# formed (μg/ml)	Products Formed (as % of total recovery) ABZ	ABZSO	$ABZSO_2$	Ratio## ABZ/ABZSO
Zwitterion	Sheep	1.70	70.2	27.5	2.28	3.47
	Cattle	1.78	44.8	55.2	0.00	0.97*
Trisamine	Sheep	0.86‡a	56.7	34.4	8.67	2.11
	Cattle	1.08‡b	27.8	72.2	0.00	0.37*

Values are expressed as means of at least 6 determinations.

\# = The mean value of the sum of ABZ, ABZSO and $ABZSO_2$ concentrations found in each assay.

= The mean value of the ratio between the amount of ABZ and ABZSO found in each individual assay.

* = Statistically different from sheep at $P< 0.05$

‡ = Statistically different from the value for the zwitterion formulation in sheep (a) and cattle (b) at $P< 0.05$.

Table 5.2: Comparative biotransformation of NTB (as zwitterion and trisamine formulations) by sheep and cattle ileal fluids following a 6 h incubation period under anaerobic conditions.

Formulation	Species	Total products# formed (μg/ml)	Products Formed (as % of total recovery) ABZ	ABZSO	$ABZSO_2$	Ratio## ABZ/ABZSO
Zwitterion	Sheep	2.93	51.2	46.8	2.00	1.29
	Cattle	3.66	34.2	59.0	6.80	0.68
Trisamine	Sheep	1.42‡a	55.6	42.3	2.10	1.39
	Cattle	1.43‡b	9.00	84.7	6.30	0.11*

Values are expressed as means of at least 6 determinations.

\# = The mean value of the sum of ABZ, ABZSO and $ABZSO_2$ concentrations found in each assay.

\#\# = The mean value of the ratio between the amount of ABZ and ABZSO found in each individual assay.

* = Statistically different from sheep at $P< 0.05$

‡ = Statistically different from the value for the zwitterion formulation in sheep (a) and cattle (b) at $P< 0.05$.

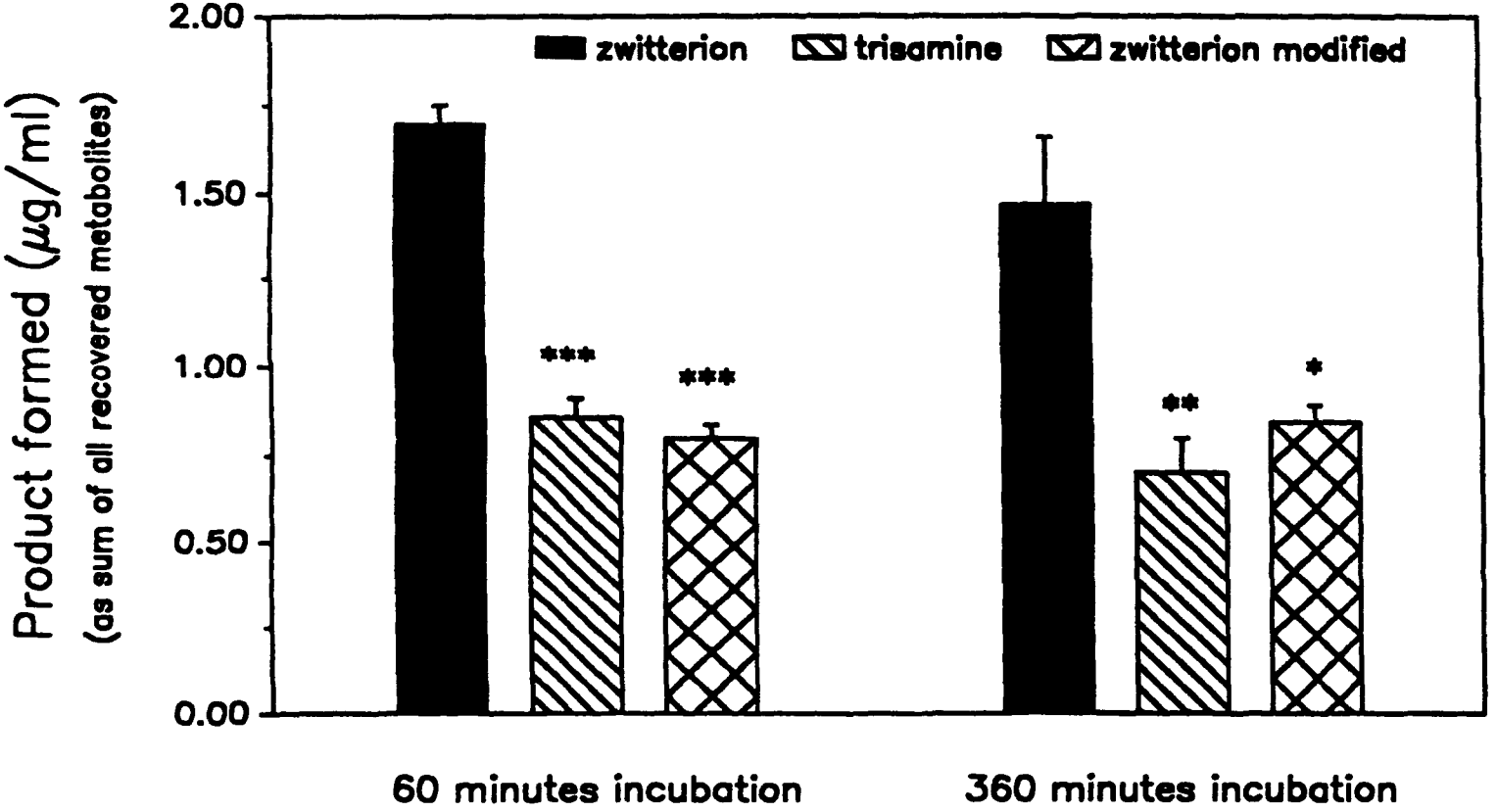


Figure 5.2: Product formed (expressed as the sum of ABZ, ABZSO and $ABZSO_2$ metabolites) following incubation of sheep ruminal fluid with NTB as zwitterion (final pH= 5.9), trisamine (final pH= 8.0) or pH-modified zwitterion (final pH = 8.0) formulations for 60 and 360 minutes, respectively, under anaerobic conditions. Statistically different from the zwitterion formulation at $P< 0.05$ (*), $P< 0.01$ (**) and $P< 0.001$ (***). Data are expressed as mean ± SEM (n= 4 to 6 determinations).

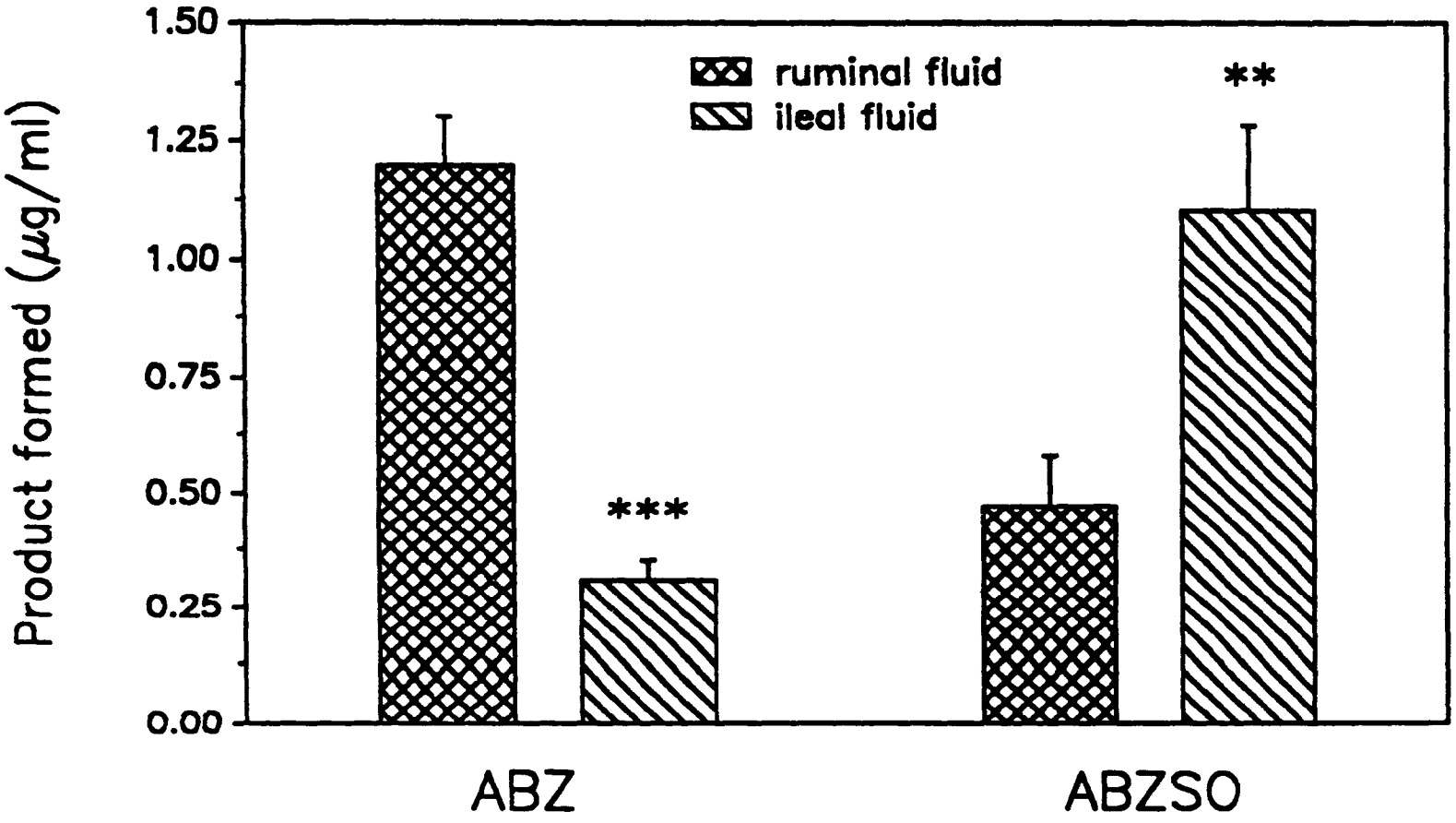


Figure 5.3: Comparative conversion of NTB zwitterion by sheep ruminal and ileal fluids following 60 min of incubation under anaerobic conditions. Statistically different from ruminal fluid at $P < 0.01$ (**) and at $P < 0.001$ (***). Values are mean ± SEM (n = 4).

Table 5.3: Comparative in vitro oxidation of ABZ by sheep and cattle ruminal and ileal fluids following a 6 h incubation period under anaerobic conditions.

Fluid	Species	Products Recovered (as % of total recovery)			Ratio ABZ/ABZSO
		ABZ	ABZSO	$ABZSO_2$	
Ruminal	Sheep	77.6	19.4	3.00	4.41
	Cattle	68.3	28.6	3.10	2.43
Ileal	Sheep	81.2	17.6	1.20	4.79
	Cattle	64.9	32.5	2.60	2.15*

Values are expressed as means of 4 to 6 determinations.

Incubations, sample extraction and HPLC determination were as described in Materials and Methods.

* = Statistically different from the value obtained for sheep ileal fluid at $P< 0.05$.

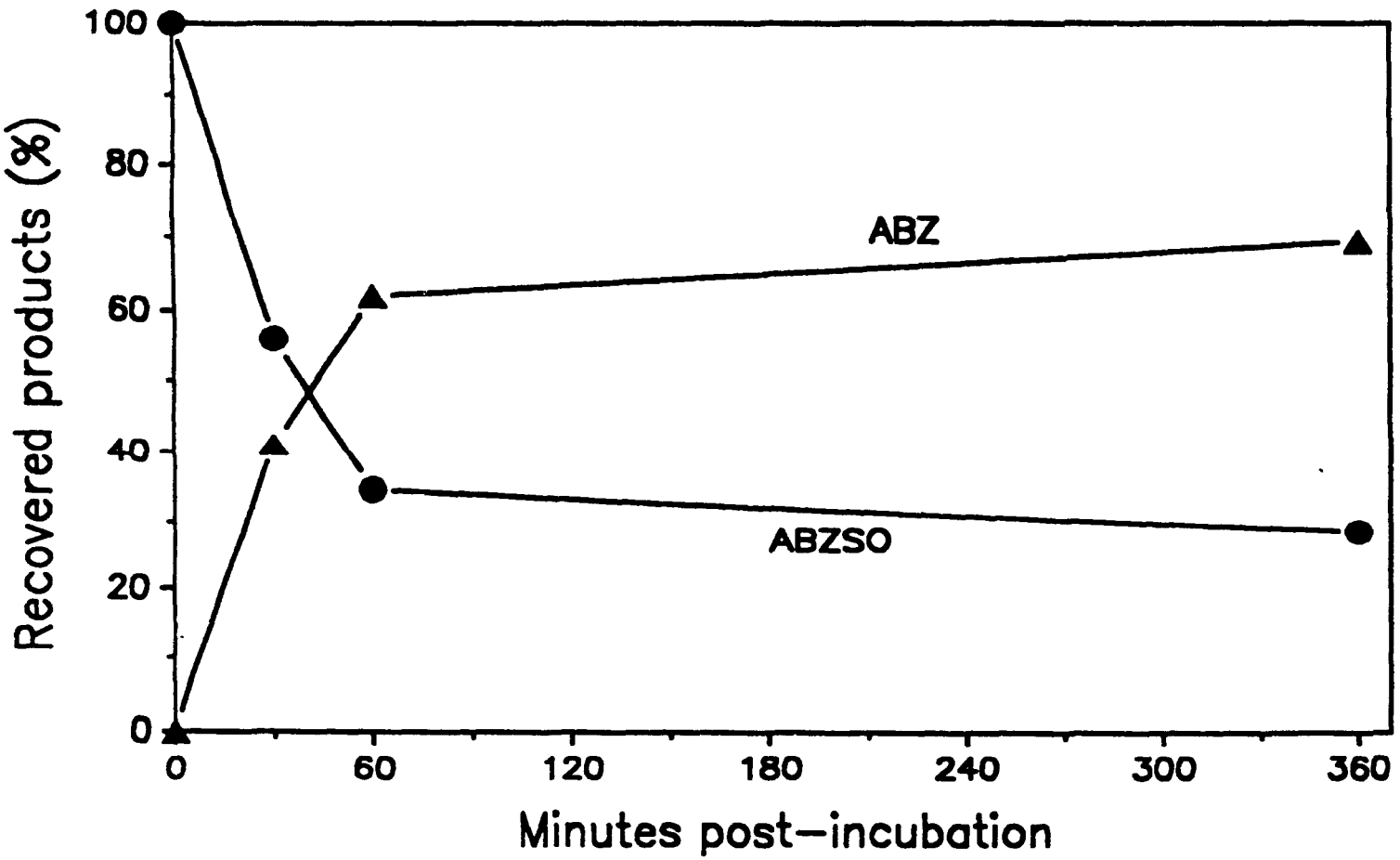


Figure 5.4: Reduction of ABZSO to ABZ following incubation of ABZSO (2 µg/ml) with sheep ruminal fluid under anaerobic conditions. Values for the $ABZSO_2$ metabolite were 0'= 0%, 30'= 2.8%, 60 = 4.1% and 360' = 3.9%, and are not included in this Figure.

Table 5.4: Comparative in vitro bioconversion of ABZSO by sheep and cattle ruminal and ileal fluids.

Fluid	Species	Incubation Time (min)	Products Recovered (as % of total recovery)			Ratio ABZ/ABZSO
			ABZ	ABZSO	$ABZSO_2$	
Ruminal	Sheep	60	64.0	31.5	4.50	2.94
		360	67.7	30.1	2.20	1.80
	Cattle	60	41.1	56.5	2.40	0.76*
		360	15.1	79.9	5.03	0.21**
Ileal	Sheep	60	29.3	64.9	5.89	0.46
		360	62.4	36.3	1.36	1.76
	Cattle	60	11.9	83.1	5.00	0.02*
		360	2.78	95.7	1.52	0.03**

Values are expressed as means of 4 to 6 determinations.

Incubations, sample extraction and HPLC analysis were as described in Materials and Methods.

* = Statistically different from the value obtained for sheep after 60 min of incubation at $P< 0.05$.

** = Statistically different from the value obtained for sheep after 360 min of incubation at $P< 0.01$.

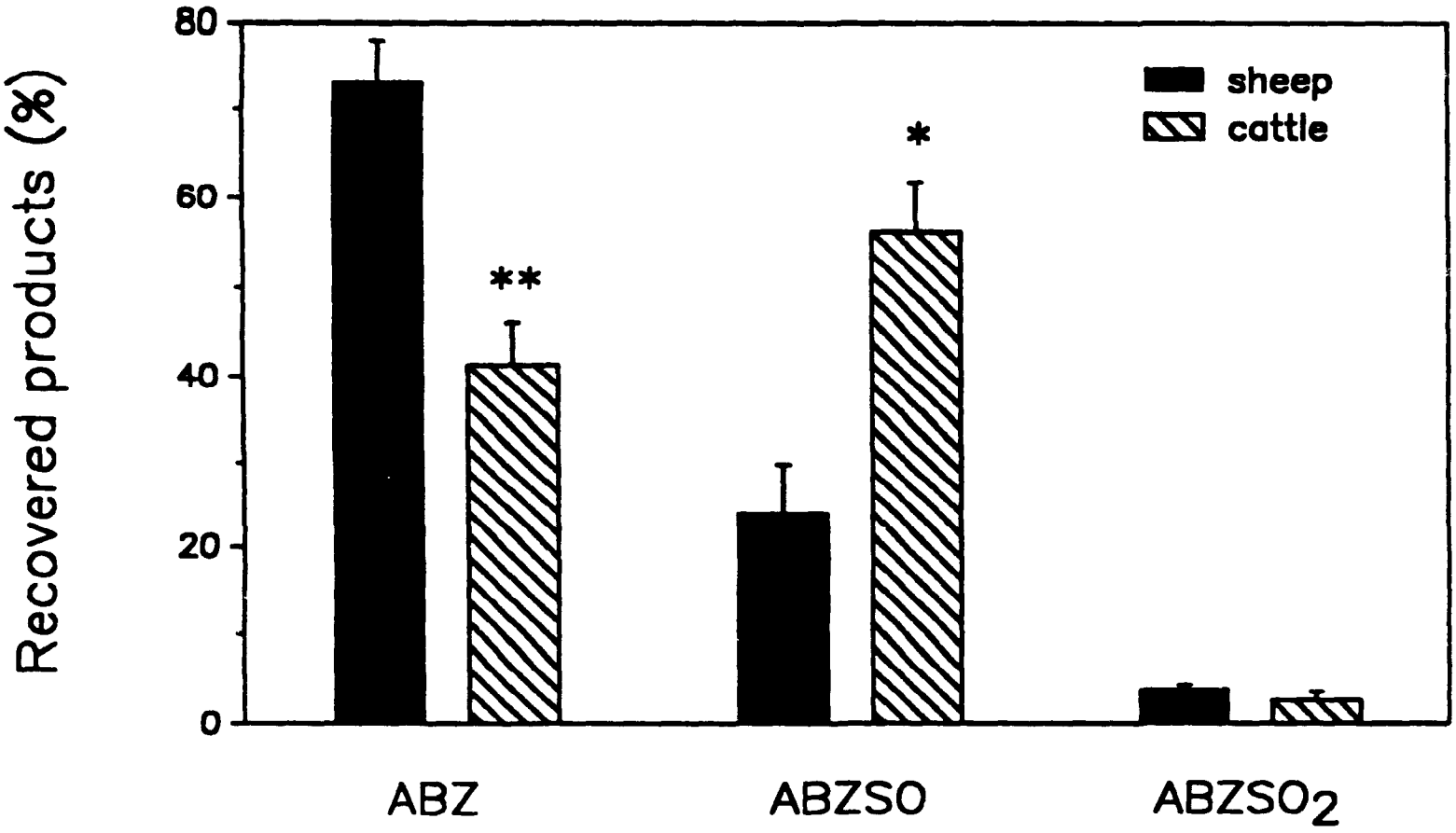


Figure 5.5: Comparative biotransformation of ABZSO by sheep and cattle ruminal fluids following a 60 min incubation period under anaerobic conditions.
Statistically different from sheep ruminal fluid at $P < 0.05$ (*) and at $P < 0.01$ (**). Values are presented as mean ± SEM (n= 6).

5.4.: DISCUSSION

The importance of GI microorganisms in the conversion of NTB pro-drug and in the metabolism of ABZ and ABZSO was demonstrated by the complete absence of metabolic activity in boiled samples of gastrointestinal fluids. Similarly, none of the molecules investigated was metabolically converted in the acidic pH of the abomasal fluid.

The results reported here demonstrated that ruminal and intestinal fluids are not only able to reduce and cyclise NTB pro-drug into ABZ, but also to oxidize ABZ to ABZSO and to a lesser extent to $ABZSO_2$. This is in contrast to the insignificant ruminal conversion of the pro-BZD febantel to FBZ and its metabolites (Beretta *et al.*, 1987). The efficient bacterial reduction of different nitro-compounds in the digestive tract of man and domestic animals has been reported (Rowland, 1986; Ladage *et al.*, 1989). Febantel (Beretta *et al.*, 1987) and thiophanate (Delatour *et al.*, 1988) are pro-BZD anthelmintics which are thought to be activated in the liver. However, as indicated by Delatour *et al.*, (1986) and corroborated in this thesis research (results in Chapters ahead), NTB is not transformed by liver microsomes; this evidence, and the extensive metabolism by ruminal and intestinal fluis reported here, indicate that the GI tract may be the only site where bioactivation of NTB takes place. This correlates well with the significantly lower profiles of ABZ metabolites obtained following parenteral administration of NTB in sheep and cattle, compared with those obtained after oral administration (Chapters 2 and 3). The efficiency of conversion of parenterally administered NTB may depend on the amount of pro-drug that reaches the GI tract in GI secretions or bile. Such a phenomenon is probably less efficient than oral administration in which the total dose of the pro-drug would be exposed to the digestive microflora.

In both sheep and cattle, regardless of incubation time or

digestive fluid used, the zwitterion formulation of NTB produced significantly greater amounts of both ABZ and ABZSO metabolites than the trisamine salt formulation. These *in vitro* results confirm the in vivo pharmacokinetic findings in cattle reported in Chapter 3, which showed that ABZSO and $ABZSO_2$ metabolites were two-fold more bioavailable after administration of a zwitterion suspension than after treatment with a trisamine solution of NTB. pH modification of the zwitterion formulation resulted in decreased NTB consumption and in lower production of its cyclised metabolites. This might indicate a lesser degree of ionization for the zwitterion formulation at ruminal pH compared with the strongly alkaline trisamine formulation, which may facilitate greater bacterial uptake and more efficient cyclization of NTB into ABZ. This and other potential explanations require further investigation. It is clear, however, that the differences between NTB formulations are based on the availability of the pro-drug to the GI microflora.

Comparison of the ability of sheep and cattle ruminal fluids to bioactivate NTB and to further metabolize its metabolites produced interesting results. Although there was no difference in the total recovered products, the amount of ABZSO produced by cattle ruminal fluid was significantly greater than that produced by sheep ruminal fluid (Table 5.1). This higher sulphoxidative activity of cattle ruminal fluid is well illustrated by the significantly lower ($P< 0.05$) ratio of ABZ/ABZSO obtained for this species compared with sheep. This ratio is a good indicator of the rate of sulphoxidation and of the relative concentrations of these two metabolites. Similarly, the ileal fluid of cattle showed a greater oxidative capacity than that of sheep, which resulted in a higher production of ABZSO and $ABZSO_2$ (Table 5.2).

Upon incubation with ruminal and ileal fluids of both species, ABZ was converted into its sulphoxide and this, in turn, further oxidized into $ABZSO_2$. The pattern of oxidation was

similar to that observed when NTB was the starting substrate in the incubation mixture. After a 60 min incubation period, sheep ileal fluid was more efficient at oxidizing ABZ than sheep ruminal fluid. The previously proposed first-pass liver microsomal oxidation of ABZ is quantitatively the principal site of ABZSO formation and accounts for the absence, or detection of only trace amounts of ABZ in jugular plasma following treatment with either NTB pro-drug or ABZ itself in sheep and cattle. The occurrence of an extrahepatic oxidation of ABZ has been evidenced by results showing that the total ABZ body clearance is two to three times greater than the reported hepatic blood flow in sheep (Galtier *et al.*, 1991). The results reported here demonstrate that ABZ oxidation is taking place in the GI tract, and that this may be the main site for extrahepatic ABZ metabolism. This would explain the rapid detection of ABZSO and $ABZSO_2$ in the digestive tract and plasma at 15-20 min post-treatment with orally administered NTB in cattle (reported in Chapter 4).

ABZSO was oxidized to a limited extent to $ABZSO_2$ by ruminal and intestinal fluids; the $ABZSO_2$ formed never reached more than 5 to 6% of the total recovered products. Perhaps the most relevant finding was the efficient reduction of ABZSO back to ABZ by ruminal and ileal fluids of both species. The reducing activity of sheep ruminal fluid was greater than that of sheep ileal fluid after 60 min of incubation. Comparison of ABZSO reduction activity between species was interesting. Both the ruminal and ileal fluids of sheep were significantly more efficient at converting ABZSO to ABZ than those of cattle. The reducing activity of cattle ruminal fluid was significantly lower than that of sheep following both short and long incubations with ABZSO under the experimental conditions of this study (Table 5.4).

Different studies have shown the GI tract as the principal site where the reduction of different sulphoxide-containing drugs takes place (Renwick *et al.*, 1986). Although it has

been previously suggested that the conversion of ABZ into ABZSO could be reversible in the liver (Gyurik *et al.*, 1981) results, reported in Chapter 9 of this thesis, demonstrated that neither sheep nor cattle liver microsomes are able to reduce ABZSO *in vitro*. This metabolic reduction may be of prime importance for the antiparasite efficacy of BZD thioethers and it probably only occurs in the GI tract. This *in vitro* evidence of ABZSO reduction correlates with the detection of ABZ in different GI compartments for 72 h post-NTB administration to cattle. This is interesting when one recalls that the parent NTB compound has been completely removed from the digestive tract by 12 to 18 h post-administration (Chapter 4). The reversible plasma-GI tract exchange facilitates a pH gradient-mediated concentration of ABZSO in the digestive compartments, which could act as a source of ABZ. Since ABZ has a greater affinity for parasite tubulin than ABZSO (Lubega and Prichard, 1991b), this bacteria-mediated reduction may have significant importance for efficacy against GI parasites.

Pronounced differences in the plasma disposition and in the bioavailability of ABZSO between sheep and cattle have been shown following the administration of either NTB pro-drug (Chapter 2 and 3) or ABZ itself (Delatour *et al.*, 1990a). The bioavailability of this active metabolite was significantly higher and its elimination half-life and mean time of residence were markedly longer in sheep compared to cattle. These differences seem to be related to a differential oxidation pattern between species. Since the rate of ABZ sulphoxidation by cattle liver microsomes is significantly lower than that obtained with sheep microsomes (results presented in Chapter 9), this differential liver metabolism pattern does not explain the *in vivo* pharmacokinetic differences between species. However, the more efficient reduction of ABZSO back to ABZ observed for GI fluids of sheep, may explain the substantially slowed disposition of this active ABZSO metabolite in sheep compared to cattle. The

greater reductive capacity of sheep GI fluids compared with that of cattle may also account for the higher $ABZSO_2$/ABZSO plasma ratio observed in cattle compared to sheep. Neither incubation with liver microsomes nor incubation with digestive fluids produced chemical modification of the $ABZSO_2$ metabolite. Since $ABZSO_2$ is an anthelmintically inactive metabolite (Lubega and Prichard, 1991b), these results confirm that the sequential oxidation of BZD thioethers represents a considerable reduction in antiparasite efficacy.

In conclusion, the bioconversion of NTB pro-drug by the ruminal and intestinal fluids of sheep and cattle was demonstrated and studied. The zwitterion formulation of NTB was more efficiently converted into its active metabolites than the trisamine salt of the same compound. Both ruminal and ileal fluids were able to biotransform ABZ and ABZSO. While oxidizing activity was greater in cattle, the reducing activity was greater, in the sheep ruminal and ileal fluids. These results contribute to the understanding of host metabolism of BZD and pro-BZD molecules and help explain the pharmacokinetic behaviour and the clinical efficacy of these anthelmintic compounds in ruminants.

CHAPTER 6

PHARMACOKINETIC INTERACTION BETWEEN PARENTERALLY ADMINISTERED NETOBIMIN AND OXIDATION INHIBITOR COMPOUNDS IN SHEEP

6.1.: INTRODUCTION

The flexibility of formulation and the broad-spectrum anthelmintic efficacy of NTB are important features. Its activity against *Fasciola hepatica, Dicrocoelium dendriticum* and *Thysanosoma actinoides* in sheep is of considerable interest. As stated earlier NTB contains a solubilising taurine moiety that allows parenteral administration which is an important practical advantage compared with insoluble BZD compounds. However, the results shown in Chapter 2 and 3 have unquestionably demonstrated that in both sheep and cattle the route of administration affects NTB cyclisation into its anthelmintically-active ABZ metabolites. In both species, the pharmacokinetic profiles of the main plasma metabolites were notably lower after parenteral administration of NTB than after oral/intraruminal treatment. These different pharmacokinetic patterns may account for differences in clinical efficacy.

The presence of a sulphur atom in the BZD substitution group at position 5- (i.e ABZ, FBZ) has a major effect on metabolism. Although in the previous Chapter it has been shown that the oxidation of ABZ at this sulphur atom can occur in the GI tract, a sequential liver microsomal oxidation is quantitatively the main biotransformation process for these thioether anthelmintics. Two distintic enzymatic pathways seem to be involved in the sequential ABZ microsomal biotransformation. It has been proposed that a flavin-containing monooxygenase (FMO) is responsible for the ABZ oxidation into ABZSO (sulphoxidation reaction) (Galtier *et al.*, 1986), while the cytochrome P-450 system would be involved in the second, slower and irreversible oxidative

step, by which ABZSO is converted into $ABZSO_2$ (sulphonation reaction) (Souhaili-El Amri *et al.*, 1988b). However in some species, the dual oxidation of ABZ into ABZSO by both the FMO and cytochrome P-450 systems may also occur (Souhaili-El Amri *et al.*, 1987).

These successive metabolic oxidations of BZD and pro-BZD thioethers in the host lead to more polar and less active metabolites. The oxidized sulphur atom present in both sulphoxides (S=O) and sulphones (O-S-O) tends to make them more polar than the parent thioether (ABZ). This greater polarity of the metabolites may result in a more difficult tissue distribution, facilitating a faster elimination. In terms of parasite uptake and binding to tubulin, the parent thioethers (ABZ, FBZ) are more potent than the sulphoxide metabolites, while the sulphones are inactive (Lubega and Prichard, 1991b). Therefore the oxidation of the parent thioether into its sulphoxide, and even more so into its sulphone, results in a considerable reduction in anthelmintic efficacy.

A number of compounds have been shown to be *in vitro* inhibitors of microsomal oxidation for drug substrates of this biotransformation reaction. Methimazole (MTZ) and other thioureas are anti-thyroid drugs, known to be substrates for the FAD^{+}-containing monooxygenase system. They inhibit the participation of this pathway in the microsomal oxidation of ABZ (Galtier *et al.*, 1986) and other xenobiotics (Tynes and Hodgson, 1983). Metyrapone (MTP) is an anti-steroid drug, which has been shown to be a potent *in vitro* inhibitor of hepatic microsomal monooxygenase activity (Tynes and Hodgson, 1983). Quinine (QNE) and other antimalarial drugs have also been shown to be *in vitro* microsomal oxidation-impairing compounds (Traficante *et al.*, 1979). An *in vivo* modulation of microsomal oxidation may result in a modified pharmacokinetic behaviour for BZD metabolites, which could lead to improved clinical efficacy as a result of higher levels of active

metabolites being presented to the parasite for longer periods of time. Such an effect could be important in order to improve the pharmacokinetic profiles of active metabolites and the resultant antiparasite activity of BZD and pro-BZD thiothers. Such a phenomenon may be relevant to increase the bioavailability of active metabolites following parenteral administration of NTB pro-drug. This study was undertaken to examine the potential pharmacokinetic interactions of different oxidation inhibitor compounds with parenterally administered NTB and its metabolites in sheep.

6.2.: MATERIALS AND METHODS

6.2.1. Experimental animals

Adult male Finn Dorset cross-bred sheep weighing 58-70 kg were used in this study. The sheep were housed in individual metabolism cages and given a daily pelleted sheep ration of 2000 grams. Water was offered *ad libitum*. The health of all animals was closely monitored prior to and throughout the experimental periods.

6.2.2. Drug administration

The study was conducted on a group of four sheep. The group received four different treatments consecutively with a four week wash-out period between each treatment. The treatments were given as follows:

Experiment 1. Animals were treated with a trisamine salt solution of NTB (250 mg/ml) (Schering Plough, NJ, USA) by subcutaneous (SC) injection into the shoulder area at 20 mg/kg.

Experiment 2. Animals received the same treatment as in Experiment 1 plus an intramuscular (IM) injection of methimazole (2-mercapto-1-methyl-imidazole, Aldrich Chemical Co., USA), in sterile saline solution (10 mg/ml), immediately after NTB administration at 1.5 mg/kg.

Experiment 3. Animals were treated as in Experiment 1 and immediately after with an aqueous solution of metyrapone (150 mg/ml) (2-methyl-1-2-di-3-pyridyl-1-propanone, Aldrich Chemical Co., USA) given SC at 20 mg/kg into the shoulder area on the opposite side to the NTB injection.

Experiment 4. Animals received the same treatment as in experiment 1, plus an intraruminal (IR) injection of quinine sulfate (Aldrich Chemical Co., USA) (suspension in propylene glycol/saline solution, 30/70) at 30 mg/kg given immediately after the NTB administration. Since the low water solubility of quinine sulfate did not allow formulation of a solution at the required concentration, the IR route was chosen for injection of the above mentioned suspension.

No adverse reactions were observed at the injection sites. The NTB administration was always given on the opposite side to that used in the previous treatment, and to a zone of the shoulder area not previously used as a site of injection. The dose rates for the oxidation inhibitor compounds used in these experiments were based on their recommended therapeutic doses.

6.2.3. Blood sample collection

In each experiment, blood samples (10 ml) were taken from the jugular vein into vacutainer tubes (Becton Dickinson, Canada) containing sodium heparin prior to treatment and at 0.5, 1, 2, 4, 6, 8, 10, 12, 18, 24, 30, 36, 48, 72, 96 and 120 hours post-administration. Plasma was separated by centrifugation at 2500 *g* for 15 minutes, placed into plastic vials and frozen at -20°C until analyzed.

6.2.4. Analytical procedures

Sample extraction procedures, HPLC equipment and analysis conditions, limits of detection and concentration

determinations were as described in Chapter 2, section 2.2.3. There was no interference of methimazole, metyrapone, quinine or any endogenous compound in the chromatographic determination of either NTB or its metabolites.

6.2.5. Pharmacokinetic and statistical analyses

The pharmacokinetic analysis of data for NTB and/or its metabolites after each treatment was done as reported in section 2.2.4. Pharmacokinetic parameters are expressed as means ± S.E.M. Student's *t*-test for paired observations was used to test the significance of differences between mean pharmacokinetic parameters obtained for the control group (NTB alone) and those for each additional treatment. A value of $P< 0.05$ was considered significant.

6.3.: RESULTS

The pharmacokinetic parameters for NTB parent drug obtained following its SC administration either alone or co-administered with MTZ, MTP or QNE are summarized in Table 6.1. The parent compound showed similar pharmacokinetic behaviour after the different treatments, being detected in plasma from 0.5 to 12 hours post-administration and resulting in a short T ½ ab (from 0.26 to 0.46 h) and early T_{max} (from 0.88 to 1.50 h post-treatment). Short T ½ ß and MRTs and low AUCs indicate a fast NTB disposition after SC administration. Although the overall NTB pharmacokinetic results were consistent for all treatments, AUC and Cl_b/F (NTB+MTP) and AUC, Vd_{area}/F and Cl_b/F (NTB+QNE) were significantly different from the control treatment. ABZ was not detectable in plasma at any time after any treatment in this trial.

The comparative mean plasma concentrations for ABZSO obtained after SC administration of NTB either alone or co-administered with MTZ, MTP or QNE are shown in Figure 6.1. The pharmacokinetic parameters for ABZSO obtained following these treatments are presented in Table 6.2. ABZSO was detected in

plasma for up to 30 hours post-administration of NTB alone. However, it was found in plasma for up to 36 hours following co-administration of NTB+QNE and up to 48 hours following either NTB+MTZ or NTB+MTP treatments. There were no differences in terms of ABZSO T ½ ß, MRT or T_{max} for any treatment compared with control NTB alone treatment. However, ABZSO AUC and C_{max} were significantly higher ($P< 0.01$ and $P< 0.05$, respectively) after the co-administration of NTB with either MTZ or MTP than after the administration of NTB alone. In addition, the ratios of AUC for ABZSO/NTB parent drug were significantly higher ($P< 0.001$) in the presence of MTZ and MTP.

$ABZSO_2$ was detected in plasma from 8-10 to 30 hours post-administration in the different treatments of this study. The results of the pharmacokinetic analysis for this metabolite obtained after SC administration of NTB, with and without oxidation inhibitor compounds, are shown in Table 6.3. There were no differences for $ABZSO_2$ T ½ ß, MRT and C_{max} between treatments. The $ABZSO_2$ AUC was significantly lower ($P< 0.05$) for the NTB+MTP and NTB+QNE treatments compared with the NTB alone treatment. In addition, both the NTB+MTZ and the NTB+QNE treatments resulted in significantly longer ($P <0.05$) $ABZSO_2$ T_{max}. The ratios of AUCs for $ABZSO/ABZSO_2$ were significantly higher following co-administration of NTB with MTZ, MTP or QNE compared with injection of NTB alone.

Table 6.1: Pharmacokinetic parameters for NTB parent compound obtained after its subcutaneous administration (20 mg/kg) either alone or with methimazole (MTZ) (IM, 1.5 mg/kg), metyrapone (MTP) (SC, 20 mg/kg), or quinine (QNE) (IR, 30 mg/kg) to sheep.

Parameter	NTB alone (n=4)	NTB + MTZ (n=4)	NTB + MTP (n=4)	NTB + QNE (n=4)
Kab (h^{-1})	2.73 ± 0.92	1.97 ± 0.58	2.50 ± 0.69	3.09 ± 0.76
T ½ ab (h)	0.36 ± 0.11	0.46 ± 0.12	0.37 ± 0.13	0.26 ± 0.05
ß (h^{-1})	0.240 ± 0.040	0.358 ± 0.029	0.337 ± 0.039	0.240 ± 0.020
T ½ ß (h)	3.13 ± 0.55	1.97 ± 0.15	2.13 ± 0.22	3.31 ± 0.65
AUC (µg.h/ml)	5.60 ± 0.22	5.19 ± 0.52	3.55 ± 0.26**	13.09 ± 2.07*
MRT (h)	4.10 ± 0.95	2.81 ± 0.21	2.81 ± 0.28	4.79 ± 0.82
Cmax (µg/ml)	1.11 ± 0.13	1.20 ± 0.08	1.17 ± 0.12	2.72 ± 0.48
Tmax (h)	0.88 ± 0.13	1.50 ± 0.29	1.25 ± 0.25	0.88 ± 0.13
Vd_{area}/F (l/kg)	15.68 ± 2.06	11.08 ± 0.38	17.46 ± 1.88	7.00 ± 0.32*
Cl_b/F (ml/h/kg)	3586 ± 134	3977 ± 441	5710 ± 370*	1680 ± 326*

* Significantly different from the control treatment (NTB alone) at $P< 0.05$

** Significantly different from the control treatment (NTB alone) at $P< 0.01$

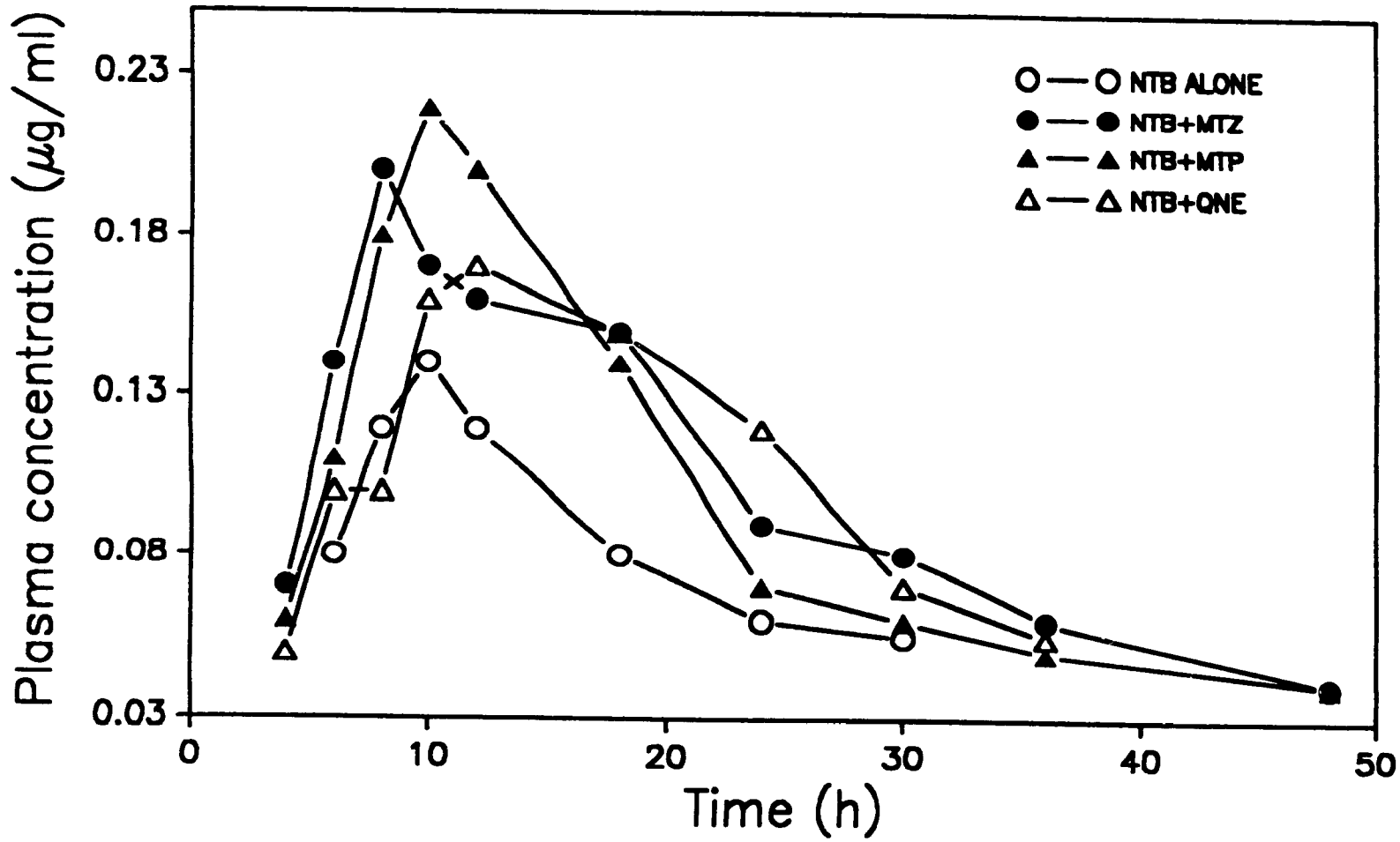


Figure 6.1: Mean plasma concentration (n=4) of ABZSO obtained following the subcutaneous administration of NTB (20 mg/kg) either alone or co-administered with MTZ (IM, 1.5 mg/kg), metyrapone (MTP) (SC, 20 mg/kg) or quinine (QNE) (IR, 30 mg/kg) in sheep.

Table 6.2: Pharmacokinetic parameters for ABZSO obtained after the subcutaneous administration of (NTB) (20 mg/kg) either alone or co-administered with methimazole (MTZ) (IM, 1.5 mg/kg), metyrapone (MTP) (SC, 20 mg/kg), or quinine (QNE) (IR, 30 mg/kg) to sheep.

Parameter	NTB alone (n=4)	NTB + MTZ (n=4)	NTB + MTP (n=4)	NTB + QNE (n=4)
ß (h^{-1})	0.057 ± 0.009	0.044 ± 0.004	0.043 ± 0.001	0.082 ± 0.020
T ½ ß (h)	12.95 ± 1.89	16.25 ± 1.39	16.78 ± 0.43	9.30 ± 1.39
AUC (µg.h/ml)	3.24 ± 0.22	5.29 ± 0.14**	5.13 ± 0.11**	4.14 ± 0.26
AUMC ($\mu g.h^2/ml$)	79.29 ± 11.75	148.2 ± 12.97	142.3 ± 4.96*	96.30 ± 10.50
MRT (h)	18.82 ± 2.67	21.10 ± 1.30	22.94 ± 1.24	16.51 ± 1.70
Cmax (µg/ml)	0.15 ± 0.01	0.21 ± 0.01*	0.24 ± 0.03*	0.20 ± 0.02
Tmax (h)	10.0 ± 0.82	8.00 ± 0.50	10.50 ± 0.50	13.0 ± 1.73
Ratio AUC ABZSO/NTB	0.59 ± 0.06	1.05 ± 0.09***	1.46 ± 0.08***	0.35 ± 0.08

* Significantly different from the control treatment (NTB alone) at P< 0.05

** Significantly different from the control treatment (NTB alone) at P< 0.01

*** Significantly different from the control treatment (NTB alone) at P< 0.001

Table 6.3: Pharmacokinetic parameters for $ABZSO_2$ obtained after the subcutaneous administration of NTB (20 mg/kg) either alone or co-administered with methimazole (MTZ) (IM, 1.5 mg/kg), metyrapone (MTP) (SC, 20 mg/kg), or quinine (QNE) (IR, 30 mg/kg) to sheep.

Parameter	NTB alone (n=4)	NTB + MTZ (n=4)	NTB + MTP (n=4)	NTB + QNE (n=4)
β (h^{-1})	0.057 ± 0.016	0.055 ± 0.006	0.074 ± 0.014	0.060 ± 0.005
T ½ β (h)	15.55 ± 4.25	12.92 ± 1.12	10.41 ± 1.89	11.66 ± 0.94
AUC (μg.h/ml)	4.30 ± 0.47	4.18 ± 0.62	2.51 ± 0.37*	2.98 ± 0.31*
AUMC ($\mu g.h^2$/ml)	136.8 ± 40.0	119.2 ± 23.11	60.21 ± 13.71	93.32 ± 10.30
MRT (h)	21.51 ± 5.89	19.57 ± 1.25	17.13 ± 2.63	25.28 ± 0.74
Cmax (μg/ml)	0.18 ± 0.03	0.16 ± 0.02	0.11 ± 0.01	0.11 ± 0.01
Tmax (h)	11.50 ± 0.50	16.50 ± 1.50**	13.00 ± 1.73	22.50 ± 1.50**
Ratio ABZSO/$ABZSO_2$	0.77 ± 0.04	1.39 ± 0.27*	2.21 ± 0.38**	1.44 ± 0.18**

* Significantly different from the control treatment (NTB alone) at $P < 0.05$

** Significantly different from the control treatment (NTB alone) at $P < 0.01$

6.4.: DISCUSSION

The co-administration of NTB with either MTZ, MTP or QNE did not affect the disposition kinetics of the parent compound. As previously reported the SC administration of NTB in sheep resulted in rapid absorption, early C_{max}, short T ½ ß and MRT, and a relatively high body clearance. Altogether, these results indicate a fast disposition of the parent compound when administered parenterally, which may account for a less efficient conversion into its cyclised ABZ metabolites compared with intraruminal or oral administration. As demonstrated in Chapter 5 the GI flora is responsible for the cyclisation of NTB, and parenterally administered NTB must be absorbed, distributed and, by secretion or bile elimination, reach the GI tract to be converted into ABZ metabolites. This is reflected in the delayed appearance of ABZSO and $ABZSO_2$ in plasma following the different SC treatments with NTB in this study.

Both NTB+MTZ and NTB+MTP treatments resulted in substantial changes in the pharmacokinetic profile of ABZSO in comparison with the NTB treatment alone. Increased ABZSO plasma concentrations resulted in significantly higher ($P< 0.01$) AUCs for the NTB+MTZ (63%) and NTB+MTP (58%) treatments compared with the control treatment. Also, ABZSO C_{max} was significantly higher ($P< 0.05$) for these treatments (Table 6.2). The overall AUMC was 87% (NTB+MTZ) and 79.5% (NTB+MTP) higher when compared to the administration of NTB alone. However, only the latter combination (NTB+MTP) reached statistical significance ($P< 0.05$). Although the differences in ABZSO T ½ ß and MRT between the combination and control treatments did not achieve statistical significance, they tended to be longer in the presence of MTZ and MTP. This is consistent with a slowed disposition of this active metabolite. The highly significant differences ($P< 0.001$) obtained for the ratio of AUC ABZSO/NTB parent compound are also indicators of an improved pharmacokinetic profile for the ABZSO metabolite for both the

NTB+MTZ and NTB+MTP treatments compared with the NTB alone treatment. Furthermore, a modified pattern of liver oxidation was reflected in the significantly higher ratio of AUC ABZSO/$ABZSO_2$ obtained after the co-administration of NTB with either MTZ or MTP.

While the FMO system has been shown to be responsible for microsomal sulphoxidation of ABZ into ABZSO (Galtier *et al.*, 1986), the cytochrome P-450 system, in a biphasic reaction, is thought to be involved in $ABZSO_2$ formation (Souhaili-El Amri *et al.*, 1988b). By selective blockage of oxygen binding sites, MTP is a potent inhibitor of cytochrome P-450 enzymatic activity (Tynes and Hodgson, 1983; Ivanetich *et al.*, 1982). It is likely that the observed changes in the pharmacokinetics of NTB metabolites in the presence of MTP are due to an *in vivo* interference by this compound in this pathway, as is evident by the significantly increased ABZSO AUC and significantly decreased $ABZSO_2$ AUC.

MTZ is a well known substrate for the FMO microsomal system (Tynes and Hodgson, 1983). It has also been shown that some MTZ-derived reactive metabolites could interfere with P-450 activity by binding to the drug binding site of this enzyme (Kedderis and Rickert, 1985). However, while the presence of MTP and QNE resulted in reduced AUCs for $ABZSO_2$, MTZ did not induce changes on the profile of this metabolite which may indicate that MTZ is primarily inhibiting the FMO pathway. Such an effect would be consistent with the postulated greater importance of this pathway compared to P-450 in sheep (Galtier *et al.*, 1986). Competition between ABZ and MTZ for the FMO system may result in slowed ABZ sulphoxidation, which could account for an extented time of ABZSO formation. Although ABZ does not reach detectable concentrations, such a phenomenon is sufficient to modify the plasma disposition kinetics of ABZSO and to increase the ratio of AUC ABZSO/$ABZSO_2$; the delayed $ABZSO_2$ T_{max} may also be a consequence of this retarded biotransformation process.

The co-administration of NTB with QNE did not alter the disposition kinetics of ABZSO. However, the significantly lower ($P < 0.05$) AUC and significantly longer T_{max} ($P < 0.01$) obtained for $ABZSO_2$ might indicate a QNE-mediated modification in the pattern of microsomal oxidation.

In conclusion, evidence has been presented that MTZ, MTP and QNE inhibit liver microsomal oxidation activity *in vivo*, causing significant modifications to the pharmacokinetics and bioavailability of NTB metabolites. The improved pharmacokinetic profile of ABZSO, the most relevant metabolite in terms of NTB clinical efficacy, obtained following the parenteral administration of NTB with MTZ or MTP may be highly important in terms of antiparasite activity. Since both MTZ and MTP are inexpensive safe compounds, their potential use with NTB and other BZD compounds should be further investigated.

CHAPTER 7

EFFECTS OF METHIMAZOLE ON THE DISPOSITION KINETICS OF NETOBIMIN METABOLITES IN CATTLE

7.1.: INTRODUCTION

The intrinsic action of the BZD molecule on the parasite is based on the disruption of basic cell functions for an extended period of time until the parasite's survival is threatened. The parasite may be able to survive in the short term, but if the impairment of essential functions is maintained for a sufficiently long period, the ability of the parasite to survive at its predilection site will be affected. There is now sufficient evidence that the extension of the residence time of anthelmintically active BZD moieties in the bloodstream plays a relevant role in the efficacy of these drugs.

The sequential liver microsomal oxidation which leads to less anthelmintically potent metabolites is relevant for the pharmacokinetic behaviour and clinical efficacy of BZD and pro-BZD thioether compounds. In Chapter 6, it has been shown that the co-administration of NTB pro-drug given parenterally and methimazole or metyrapone in sheep, resulted in an improved pharmacokinetic profile of the active ABZSO metabolite. Therefore, the manipulation of the metabolic and pharmacokinetic patterns for BZD and pro-BZD drugs may lead to improved clinical efficacy against GI and tissue-dwelling parasites; this could be particularly important for improving efficacy against parasites difficult to control in livestock, such as arrested *Ostertagia ostertagi* larval stage, immature stages of *Fasciola hepatica* or BZD-resistant strains which are only susceptible to BZD concentrations presented for long periods of time. The objective of this experimental work was to evaluate potential modification of the pharmacokinetic behaviour of NTB and its metabolites produced by the co-

administration of NTB subcutaneously and orally with methimazole (MTZ) (intramuscularly) in cattle.

7.2.: MATERIALS AND METHODS

7.2.1. Experimental animals

The study was conducted on eight Holstein Fresian bull calves, in parasite-free conditions, weighing from 110 to 130 kg. High quality hay and water were available to them ad libitum. The health of all animals was closely monitored prior to and throughout the experimental periods. A four week wash-out period was allowed before the same animals participated in the second phase of the study.

7.2.2. Drug administration

Animals were divided into two groups of four animals each and treatments were given as follows.

Experiment I

Group A: Animals were treated with trisamine salt solution of NTB (250 mg/ml)(Schering Plough, NJ, USA) by subcutaneous (SC) injection in the shoulder area at 12.5 mg/kg.

Group B: Animals were treated with trisamine salt solution of NTB (50 mg/ml) (Schering Plough, NJ, USA) by oral drenching at 20 mg/kg.

Experiment II

Group A: Animals received trisamine salt solution of NTB (250 mg/ml) by subcutaneous injection at 12.5 mg/kg together with a solution of methimazole (2-mercapto-1-methyl-imidazole, Aldrich Chemical Co., USA) (10 mg/ml in sterile physiological saline) given intramuscularly at 1.5 mg/kg, immediatedly after the NTB injection. Methimazole was given intramuscularly in this experimental study to ensure its systemic availability.

Group B: Animals received trisamine salt solution of NTB (50 mg/ml) by oral drench at 20 mg/kg immediately followed by an intramuscular injection of methimazole (10 mg/ml) at 1.5 mg/kg.

No adverse reactions were observed at the injection sites.

7.2.3. Blood sample collection

In each experiment, blood samples (10 ml) were taken from the jugular vein into Vacutainer tubes (Becton Dickinson, Canada) containing sodium heparin prior to the treatment and at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12, 16, 20, 24, 32, 48 and 72 h post-administration. Plasma was separated by centrifugation at 2500 *g* for 15 minutes, placed into plastic vials and frozen at -20°C until analyzed.

7.2.4. Analytical methods

Immediately after thawing, plasma samples (1.0 ml) were spiked with an internal standard (oxibendazole, 1 μg/10 μl methanol) and NTB and its metabolites extracted using disposable C_{18} SepPak cartridges (part # 51910, Waters Associates, MA, USA). Sample extraction procedures, HPLC equipment, analysis conditions and limits of detection were as previously reported. There was no interference of methimazole or any endogenous compounds in the chromatographic determination of either NTB or its metabolites.

7.2.5. Pharmacokinetic and statistical analyses

The plasma concentration versus time curves for NTB and/or its metabolites after each treatment were fitted with the PKCALC computer program. Pharmacokinetic parameters were obtained as previously indicated. Since the intravenous route was not used, the total body clearance (Cl_b) and volume of distribution (method of area) (Vd_{area}) for NTB parent drug represent their true values divided by the bioavailability (F) (Gibaldi and Perrier, 1982). Statistical moment theory was applied to calculate the mean residence time (MRT) for NTB and its metabolites (Perrier and Mayersohn, 1982).

Statistical comparison of mean pharmacokinetic parameters for NTB and its metabolites in the absence and presence of MTZ was performed using Student's *t*-test for paired observations. A value of $P< 0.05$ was considered significant. The pharmacokinetic parameters are reported as mean ± SEM.

7.3.: RESULTS

While NTB parent drug was detected only after subcutaneous treatment, ABZ was not found in plasma at any time after either subcutaneous or oral administrations. The appearance of detectable plasma concentrations of ABZSO and $ABZSO_2$ was delayed after the subcutaneous treatment (2-4 h) in comparison with the oral administration (0.5-0.75 h). The pharmacokinetic parameters for NTB parent drug obtained after the subcutaneous injection of NTB either alone or co-administered with MTZ are presented in Table 7.1. MTZ did not significantly affect the pharmacokinetic parameters for the NTB parent compound except that the C_{max} was significantly lower following the co-administration.

The pharmacokinetic analyses for ABZSO and $ABZSO_2$ obtained after the SC administration of NTB in the absence or presence of MTZ are shown in Table 7.2. ABZSO was detected in plasma between 4 and 20 h (NTB alone) and between 2 and 32 h (NTB+MTZ) post-administration. A significantly higher ($P< 0.01$) ABZSO C_{max} was obtained after the NTB alone treatment. However, the co-administration of NTB+MTZ resulted in a longer metabolite formation half-life than the SC treatment with NTB alone. The terminal slope (ß) and the $T_{½ ß}$ for ABZSO were significantly longer ($P< 0.001$) after the co-administration of NTB+MTZ (Table 7.2). Also ABZSO MRT was significantly longer ($P< 0.01$) after NTB+MTZ than after the NTB alone. Some pharmacokinetic parameters for ABZSO obtained following the SC administration of NTB either alone or co-administered with MTZ are compared in Figure 7.1. $ABZSO_2$ showed a similar pharmacokinetic profile after both SC

treatments.

Neither NTB parent drug nor ABZ was detected in plasma after either oral treatments with NTB. The mean plasma concentrations of ABZSO obtained after the oral administration of NTB alone or in combination with MTZ in calves are shown in Figure 7.2. In Table 7.3, the results of the pharmacokinetic anaylses for ABZSO and $ABZSO_2$ metabolites after the above mentioned oral NTB treatments are summarized. T ½ ß and MRT were significantly longer ($P< 0.05$) for the NTB+MTZ treatment than for the oral administration of NTB alone. The higher mean ABZSO AUC for the combined treatment was not significantly different from the treatment with NTB alone. However, the AUMC was significantly higher ($P< 0.05$) for the co-administration of NTB+MTZ compared with the oral NTB alone treatment. All the parameters obtained from the $ABZSO_2$ kinetic analysis showed no statistically significant difference between the treatments.

TABLE 7.1: Pharmacokinetic parameters for NTB parent compound obtained after its SC administration (12.5 mg/kg) either alone or with methimazole (MTZ) (1.5 mg/kg) to calves.

Parameter	NTB alone (n=4)	NTB + MTZ (n=4)
k ab (h^{-1})	5.203 ± 1.712	5.827 ± 1.205
T ½ ab (h)	0.18 ± 0.05	0.14 ± 0.03
ß (h^{-1})	0.325 ± 0.026	0.229 ± 0.015
T ½ ß (h)	2.17 ± 0.19	3.07 ± 0.21
AUC ($\mu g.h/ml$)	8.70 ± 2.14	4.81 ± 0.57
AUMC ($\mu g.h^{2}/ml$)	28.88 ± 7.74	22.08 ± 2.92
MRT (h)	3.04 ± 0.31	4.38 ± 0.27
C_{max} ($\mu g/ml$)	2.87 ± 0.19	1.02 ± 0.16**
T_{max} (h)	0.75 ± 0.10	0.75 ± 0.10
Vd_{area}/F (l/kg)	5.25 ± 1.09	11.96 ± 1.34
Cl_b/F (ml/h/kg)	1650 ± 295	2735 ± 384

Values are expressed as means ± SEM

** Statistically different from the NTB alone treatment at P< 0.01

Table 7.2: Pharmacokinetic parameters for ABZSO and $ABZSO_2$ obtained after SC administration of NTB (12.5 mg/kg) either alone or co-administered with methimazole (MTZ) (1.5 mg/kg) in calves.

Parameter	Unit	ABZSO		$ABZSO_2$	
		NTB alone (n=4)	NTB+MTZ (n=4)	NTB alone (n=4)	NTB+MTZ (n=4)
Kf	h^{-1}	0.362±0.022	0.230±0.036	0.292±0.017	0.275±0.001
T ½ for	h	1.93±0.10	3.23±0.49	2.39±0.29	2.52±0.09
ß	h^{-1}	0.267±0.009	0.083±0.002***	0.234±0.027	0.207±0.018
T ½ ß	h	2.62±0.10	8.43±0.24***	3.09±0.37	3.44±0.34
AUC	µg.h/ml	4.59±0.34	2.96±0.74	6.44±0.83	7.59±0.90
AUMC	$\mu g.h^2/ml$	48.20±1.94	51.51±14.52	76.02±9.74	100.5±17.80
MRT	h	7.84±0.44	13.31±0.88**	8.37±0.35	9.36±0.67
C_{max}	µg/ml	0.60±0.05	0.19±0.04**	0.69±0.09	0.70±0.05
T_{max}	h	10.0±0.82	9.50±0.50	11.50±0.50	11.50±0.50

Values are expressed as means ± SEM

** Significantly different from the control treatment (NTB alone) at P< 0.01
*** Significantly different from the control treatment (NTB alone) at P< 0.001

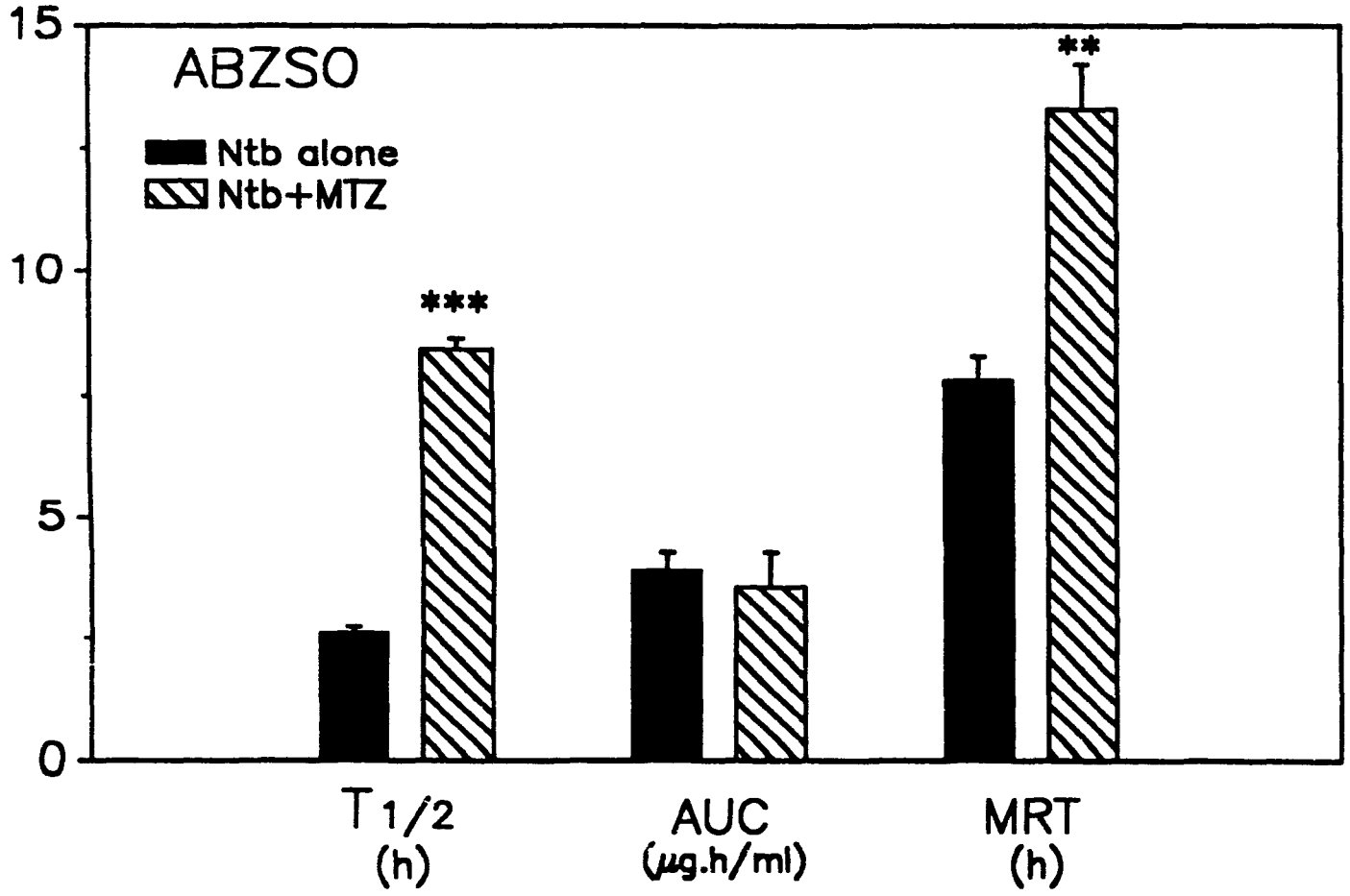


Figure 7.1: Comparative kinetics for ABZSO obtained after SC administration of NTB either alone or co-administered with methimazole (MTZ) in cattle. Parameters were statistically different at $P < 0.01$ (**) and at $P < 0.001$ (***).

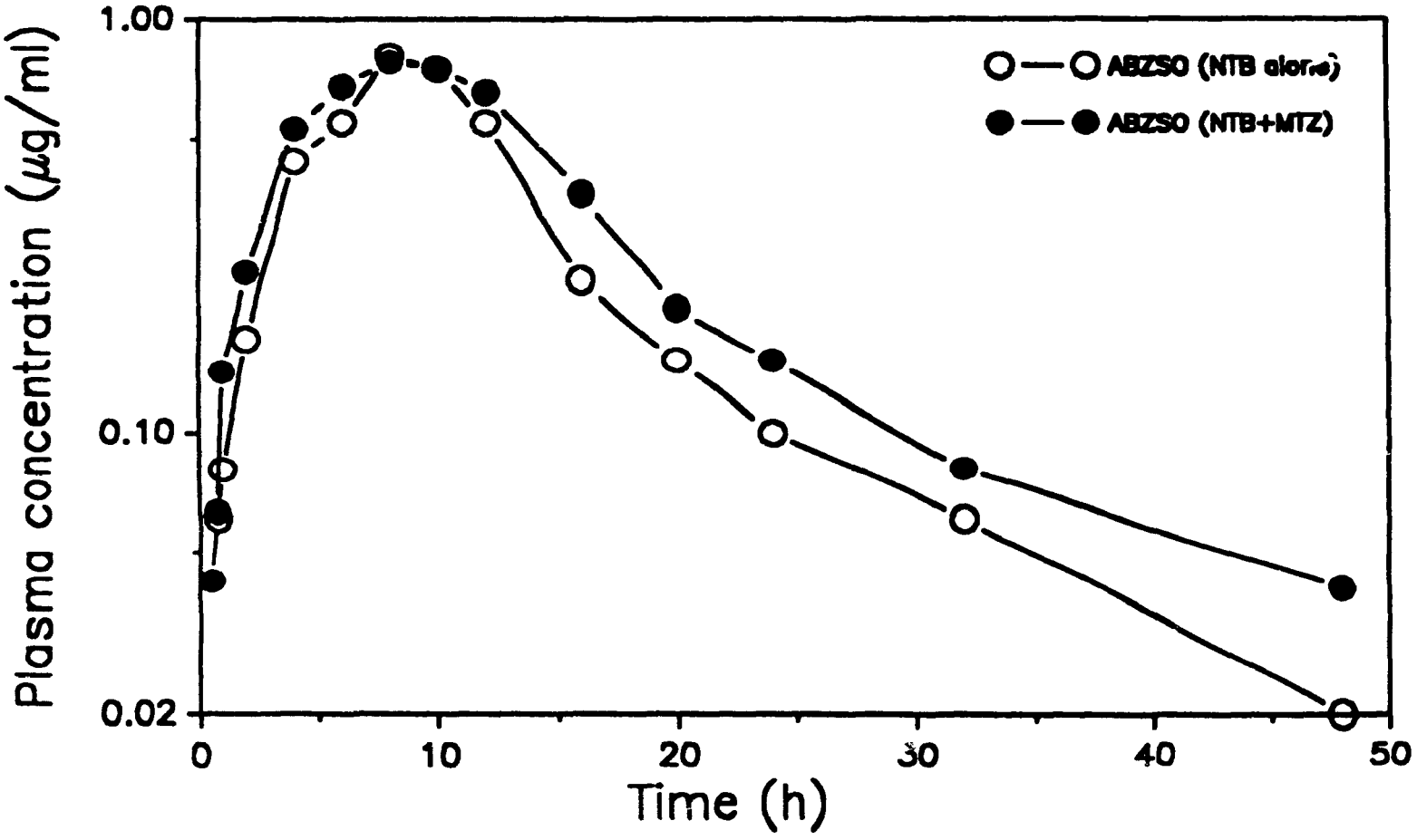


Figure 7.2: Mean plasma concentration (n=4) of ABZSO after oral administration of NTB (20 mg/kg) either alone or with methimazole (MTZ) (IM, 1.5 mg/kg) in calves.

Table 7.3: Pharmacokinetic parameters for ABZSO and $ABZSO_2$ obtained after oral administration of NTB (20 mg/kg) either alone or co-administered with methimazole (MTZ) (1.5 mg/kg) in calves.

Parameter	Unit	ABZSO		$ABZSO_2$	
		NTB alone (n=4)	NTB+MTZ (n=4)	NTB alone (n=4)	NTB+MTZ (n=4)
Kf	h^{-1}	0.270±0.034	0.318±0.048	0.256±0.020	0.291±0.033
T ½ for	h	2.71±0.41	2.40±0.50	2.75±0.21	2.47±0.25
ß	h^{-1}	0.126±0.016	0.085±0.008	0.152±0.021	0.269±0.037
T ½ ß	h	5.71±0.69	8.64±0.49*	4.94±0.95	2.70±0.30
AUC	µg.h/ml	9.83±1.16	12.46±1.72	24.41±1.26	28.93±3.04
AUMC	$\mu g.h^2/ml$	124.2±8.29	185.3±17.4*	335.3±29.24	418.2±59.0
MRT	h	9.36±0.70	11.60±0.24*	9.74±0.68	11.00±0.40
C_{max}	µg/ml	0.87±0.19	0.82±0.09	1.90±0.08	2.27±0.13
T_{max}	h	8.50±0.50	8.50±0.50	12.50±1.26	16.00±0.00

Values are expressed as means ± SEM

* Significantly different from the control treatment (NTB alone) at $P< 0.05$

7.4.: DISCUSSION

The co-administration with MTZ had little effect on the pharmacokinetic behaviour of NTB parent drug after its SC administration. The difference observed in C_{max} was consistent with a slightly higher rate of NTB metabolic conversion for the combined NTB+MTZ treatment, and it may not be related to a MTZ-mediated effect.

Important modifications in the disposition kinetics of ABZSO were obtained after the co-administration of NTB (SC) and MTZ, compared with the treatment with NTB alone. While there was no difference in terms of AUC, the overall elimination half-life of ABZSO was three times longer ($P< 0.001$) and the MRT was almost two-fold longer ($P< 0.01$) in the presence of MTZ (Figure 7.1).

The results of the oral NTB experiments also demonstrated that concomitant MTZ treatment affected the pharmacokinetic profile of ABZSO. The mean ABZSO AUC was 27% higher after the co-administration of NTB+MTZ, but this difference was not significant. This was probably due to the spurious results obtained in one animal of the group receiving NTB alone. Using the Q test for extraneous values (Dean and Dixon, 1951), the ABZSO AUC value for that animal in the control group could be rejected with 90% confidence. The elimination of this spurious observation resulted in an ABZSO AUC significantly higher ($P< 0.05$) for the oral NTB+MTZ treatment, when analyzed by unpaired *t*-test. Furthermore, AUMC, a parameter that reflects the area under the first moment in the plasma concentration versus time curve, was significantly higher ($P< 0.05$) for the oral NTB+MTZ treatment. This confirms an enhanced pharmacokinetic profile for ABZSO in the presence of MTZ. In addition, the concomitant administration of MTZ with oral NTB significantly increased the $T_{½}\ \beta$ (151%) and MRT (124%) for ABZSO.

In a free-model description, the smaller slope represents the rate-limiting step. Although the ratio k/ß was outside of the range proposed for the existence of the "flip flop" phenomenon (Notari, 1987), in our data the ß value could represent either the elimination rate or the metabolite formation rate. On the other hand, MRT is a non-compartmentally derived parameter, which represents an averaged time of permanence of all drug or metabolite molecules in the body, characterizing the distribution and elimination process. Thus, the significant differences obtained for $T_{½}$ ß and MRT are strong evidence of a MTZ-mediated alteration in the pharmacokinetic behaviour of ABZSO. These results are consistent with MTZ impairing the hepatic microsomal oxidation of ABZ metabolites.

In view of the fact that MTZ is known to be a substrate for the FMO (Tynes and Hodgson, 1983) and the fact that this system would be primarily involved in the sulphoxidation of ABZ to ABZSO (Galtier *et al.*, 1986), it is highly likely that the observed modifications in the ABZSO pharmacokinetics are due to competition between MTZ and ABZ in the FMO pathway. Thus, an inhibitory effect of MTZ at this microsomal enzymatic level may not dramatically affect the plasma concentrations (AUC, C_{max}), but may dramatically affect the disposition kinetics and residence time of ABZSO after both SC and oral administration of NTB in cattle.

The efficacy of an antiparasite drug depends on a toxic concentration being presented to the parasite for sufficient time to lead to irreversible damage. The systemic availability of ABZSO and its potential reduction to ABZ in the GI tract, indicate that this metabolite is the most relevant one in terms of NTB anthelmintic activity. Thus, even with low plasma concentrations, the prolonged residence time of the anthelmintically-active ABZSO, induced by MTZ, may be highly important in terms of clinical efficacy.

MTZ is an inexpensive and safe compound, regularly used in

human and veterinary medicine. In addition, high bioavailability and a relatively long half-life (Jansson *et al.*, 1985) may ensure that a single dose of MTZ will result in a prolonged residence time which would facilitate successful inhibition of ABZ oxidation. However, more research is necessary in order to adjust dosage levels, route of administration and to determine whether MTZ will enhance the clinical efficacy of NTB and other BZD compounds.

CHAPTER 8

METHIMAZOLE-MEDIATED MODULATION OF BENZIMIDAZOLE THIOETHER LIVER BIOTRANSFORMATION IN SHEEP

8.1.: INTRODUCTION

The biotransformation process tends to transform xenobiotics into less lipid soluble and more polar metabolites, which may facilitate faster elimination. BZD and pro-BZD anthelmintics are extensively metabolized in all studied mammalian species. Typically among different BZD compounds, the parent drug is short-lived and metabolic products predominate in plasma and all tissues and excreta of the host (Gottschall *et al.*, 1990), as well as in parasites recovered from BZD-treated animals (Fetterer and Rew, 1984). For the most anthelmintically potent BZD thioethers, the primary metabolites are products of oxidation at the level of the sulphur atom present in the group of substitution at position 5- of the BZD ring system. As remarked earlier, this phase I metabolism results in a reduction in antiparasite potency.

Results reported in the last two Chapters have shown that an *in vivo* induced impairing effect on the liver microsomal activity results in changes in the pattern of biotransformation for ABZ-related molecules. This *in vivo* modulation of the liver oxidation pattern leads to pronounced modifications of the pharmacokinetic behaviour of anthelmintically-active BZD metabolites, which may account for improved efficacy against GI and tissue-dwelling parasites difficult to control in human and veterinary medicine. The experiments described in this Chapter were designed to further understand the pharmacological basis of this "liver modulation" approach and to move forward in its potential practical application to improve parasite control.

8.2.: ENHANCEMENT OF PLASMA CONCENTRATIONS AND CHANGES ON DISPOSITION KINETICS OF ALBENDAZOLE METABOLITES FOLLOWING CO-ADMINISTRATION OF NETOBIMIN AND METHIMAZOLE

8.2.1.: OBJECTIVES

According to the results presented in Chapter 2, the intraruminal administration of NTB in sheep results in substantially improved pharmacokinetic profiles for both ABZSO and $ABZSO_2$, compared with the subcutaneous administration of the compound at the same dose rate. Furthermore, ABZ parent drug was only found in plasma following the intraruminal treatment in sheep. In the previously reported experiments on metabolism modulation (Chapter 6 and 7), it was not feasible to assess the influence of the oxidative impairing effect on the profiles of ABZ parent drug, because this did not reach detectable concentrations in plasma. The purpose of the present study was to determine potential modifications on the pharmacokinetics and bioavailability of ABZ metabolites following the co-administration of NTB pro-drug, given intraruminally (IR) at 20 mg/kg, and methimazole (MTZ) in adult sheep.

8.2.2.: MATERIALS AND METHODS

8.2.2.1. Experimental design

Adult male Finn Dorset cross-bred sheep weighing 50-62 kg were used in this study. The animals were housed in parasite-free conditions in individual metabolism cages and given a pelleted sheep ration of 2000 g per day. Water was offered *ad libitum*. The health of all animals was monitored prior to and throughout the experimental periods. The treatments were given as follows.

First Period:

Four animals were treated with a zwitterion suspension of NTB (150 mg/ml) (Sch-32481, Schering Plough, NJ, USA) by IR administration at 20 mg/kg.

Second period:

After a four week wash-out period, the same four animals received the zwitterion suspension of NTB (150 mg/ml) by IR administration at 20 mg/kg together with an aqueous solution of methimazole (10 mg/ml) (2-mercapto-1-methyl- imidazole, Aldrich Chemical Co., WI, USA) given intramuscularly at 1.5 mg/kg, immediately after the NTB injection.

Due to the spurious results obtained for one animal in the control group (NTB IR alone treatment, 2nd period), three extra animals were treated with the zwitterion suspension of NTB by IR injection at 20 mg/kg. Since the results for these three extra animals were not statistically different from the original control group (three animals), they were included in the overall results of this study.

Blood samples (10 ml) were taken from the jugular vein in vacutainer tubes with sodium heparin prior to the treatments and at 0.5, 1, 2, 4, 6, 8, 10, 12, 18, 24, 30, 36, 48, 72, 96 and 120 hours post-treatment. Plasma was separated by centrifugation at 2500 *g* for 15 minutes, placed into plastic vials and frozen at -20°C until analyzed.

8.2.2.2. Analytical methodology

The procedures for extraction and HPLC determination of NTB and its metabolites in plasma samples were as described in Chapter 2, section 2.2.3. Correlation coefficients for calibration curves, percentage of recovery and limits of detection for the different analytes were as previously reported in sheep plasma.

8.2.2.3. Pharmacokinetic and statistical analyses

The plasma concentration versus time curves for NTB and/or its metabolites after each treatment were fitted with the PKCALC computer program. The estimation of all the pharmacokinetic

parameters was as previously described (section 2.2.4). Student's *t*-test for unpaired observations was used to test the significance of differences between mean pharmacokinetic parameters obtained after each treatment. A value of $P< 0.05$ was considered significant. Pharmacokinetic parameters are expressed as mean ± SEM.

8.2.3.: RESULTS

Only the occasional detection of very low plasma concentrations of both NTB parent drug and ABZ, in some of the animals that received NTB intraruminally alone, precluded pharmacokinetic analysis in these animals. Because of this, only 3 animals could be included in the pharmacokinetic results for these two analytes in the control group. The presence of MTZ did not alter the pharmacokinetic behaviour of the parent compound, which was detected in plasma from 0.5 to 12 hours after both treatments. There were no statistical differences between treatments for any of the NTB pharmacokinetic parameters calculated.

The mean plasma concentrations of ABZ, ABZSO and $ABZSO_2$ obtained after the IR administration of NTB zwitterion suspension in the presence or absence of MTZ are plotted in Figures 8.1, 8.2 and 8.3, respectively. Pharmacokinetic parameters for ABZ after both treatments are shown in Table 8.1. The co-administration of MTZ with NTB significantly increased ($P< 0.05$) the T ½ ß, MRT, AUC and AUMC for ABZ.

The pharmacokinetic analyses for ABZSO and $ABZSO_2$ obtained after the IR administration of NTB with and without MTZ are summarized in Table 8.2. The presence of MTZ resulted in pronounced changes in the pharmacokinetic behaviour of ABZSO and $ABZSO_2$. Highly significant differences were obtained in terms of the slope of the elimination phase, T ½ ß, AUC, AUMC and MRT for both metabolites (Table 8.2). Also, metabolite formation half-life (T ½ for) and Tmax for $ABZSO_2$ were significantly longer when NTB was co-administered with MTZ.

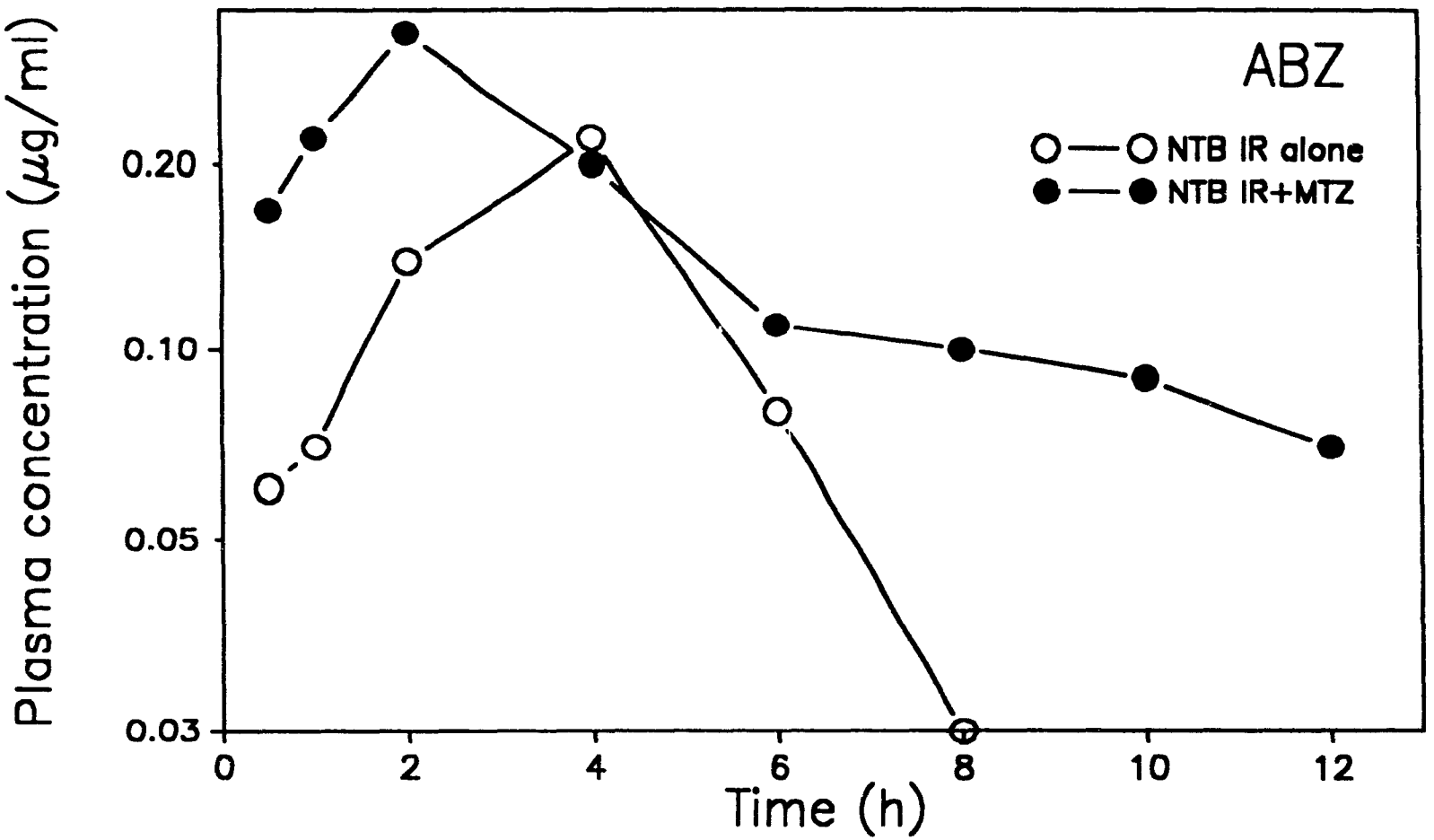


Figure 8.1: Mean plasma concentrations of ABZ obtained after intraruminal (IR) administration of NTB (20 mg/kg) either alone or co-administered with methimazole (MTZ) (IM, 1.5 mg/kg) to sheep.

Table 8.1: Pharmacokinetic parameters for ABZ obtained after intraruminal (IR) administration of NTB (20 mg/kg) either alone or with methimazole (MTZ) (IM, 1.5 mg/kg) to sheep.

Parameter	Unit	IR alone	IR + MTZ
ß	(h^{-1})	0.543 ± 0.124	0.202 ± 0.030*
T ½ ß	(h)	1.43 ± 0.35	3.79 ± 0.81*
AUC	($\mu g.h/ml$)	0.87 ± 0.17	2.07 ± 0.61*
AUMC	($\mu g.h^{2/}.ml$)	3.51 ± 0.51	7.58 ± 1.64*
MRT	(h)	2.76 ± 0.10	5.14 ± 1.05*
Cmax	($\mu g/ml$)	0.21 ± 0.05	0.33 ± 0.09
Tmax	(h)	3.33 ± 0.67	2.50 ± 0.50

Values are expressed as mean ± SEM with n=3 for IR alone treatment, and n=4 for IR+MTZ treatment

* Statistically different from the IR alone treatment at $P < 0.05$

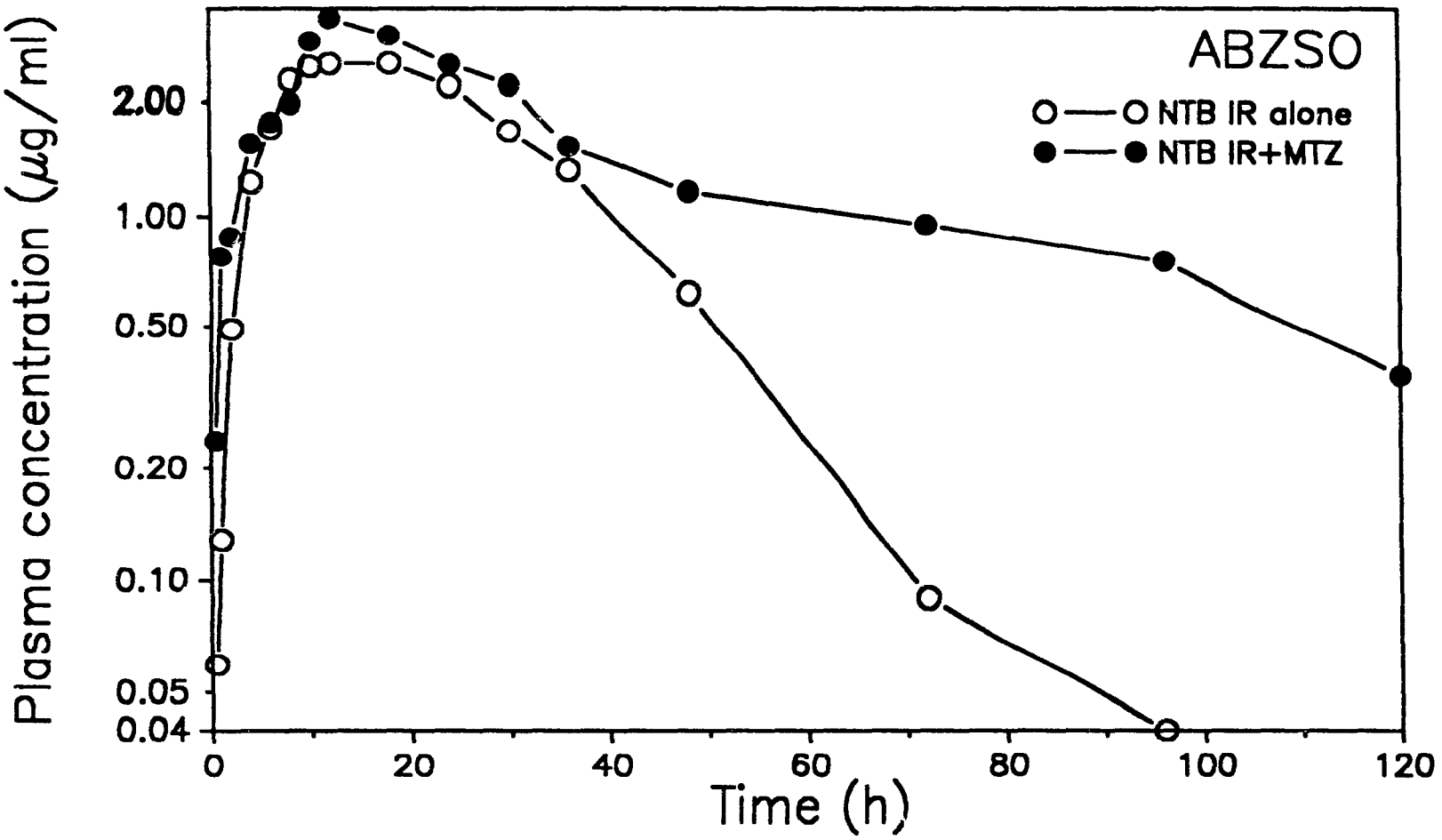


Figure 8.2: Mean plasma concentrations of ABZSO obtained after intraruminal (IR) administration of NTB (20 mg/kg) either alone or co-administered with methimazole (MTZ) (IM, 1.5 mg/kg) to sheep.

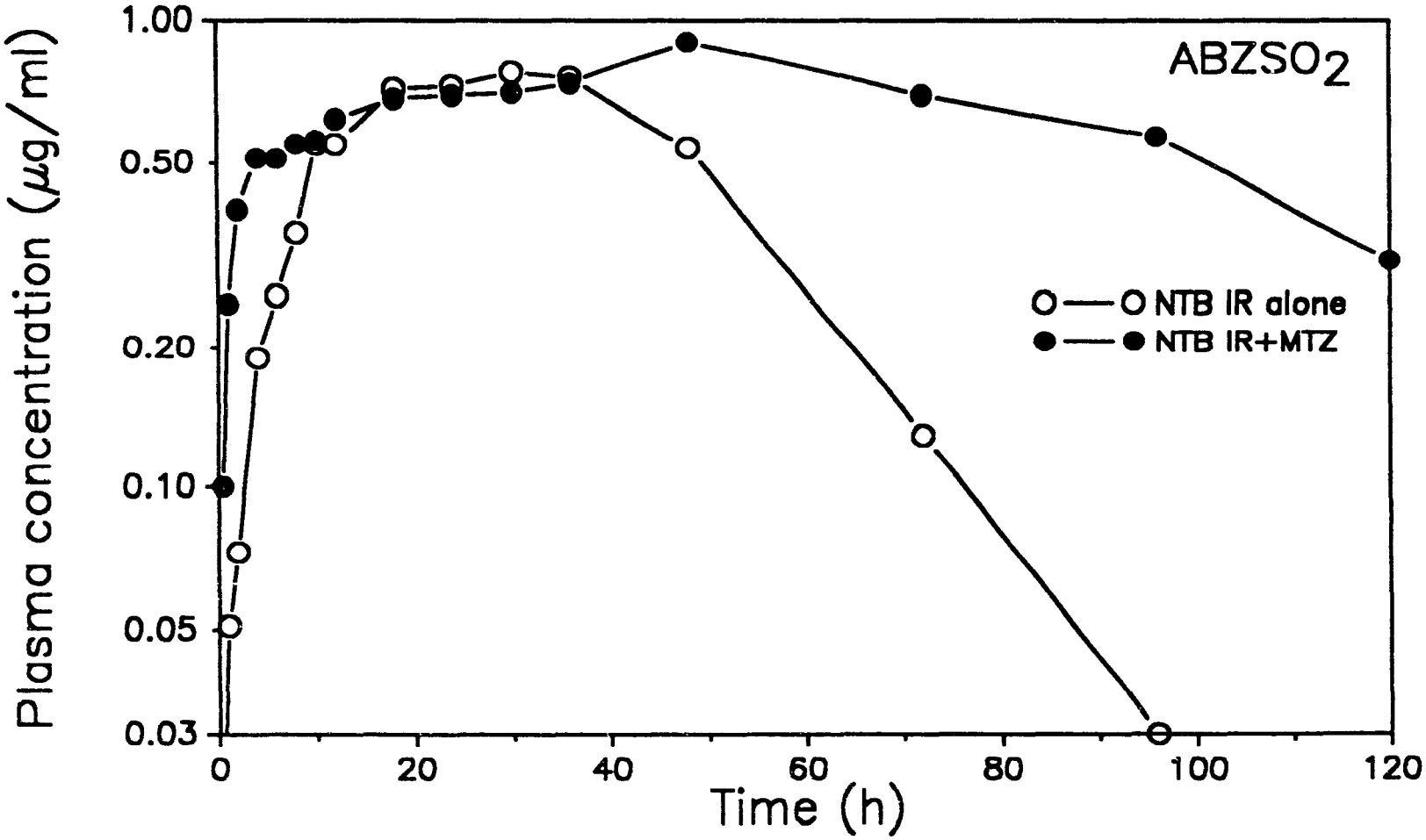


Figure 8.3: Mean plasma concentrations of $ABZSO_2$ obtained after intraruminal (IR) administration of NTB (20 mg/kg) either alone or co-administered with methimazole (MTZ) (IM, 1.5 mg/kg) to sheep.

Table 8.2: Pharmacokinetic parameters for ABZSO and $ABZSO_2$ obtained after intraruminal (IR) administration of NTB (20 mg/kg) either alone or with methimazole (MTZ) (IM, 1.5 mg/kg) to sheep.

Parameter	ABZSO		$ABZSO_2$	
	IR alone (n=6)	IR + MTZ (n=4)	IR alone (n=6)	IR + MTZ (n=4)
Kf (h^{-1})	0.124 ± 0.015	0.170 ± 0.040	0.087 ± 0.006	0.061 ± 0.019
T ½ for (h)	6.03 ± 0.81	4.83 ± 1.00	8.17 ± 0.62	14.21 ± 3.08*
ß (h^{-1})	0.071 ± 0.007	0.016 ± 0.003***	0.060 ± 0.005	0.017 ± 0.005***
T ½ ß (h)	10.26 ± 1.02	50.27 ± 10.67***	12.01 ± 0.95	52.38 ± 12.60***
AUC (µg.h/ml)	99.45 ± 15.67	193.92 ± 32.50*	37.89 ± 3.29	110.18 ± 8.79***
AUMC ($\mu g.h^2/ml$)	2749 ± 652	14328 ± 4407*	1352 ± 184	11961 ± 2804**
MRT (h)	17.63 ± 1.95	62.10 ± 11.58**	23.66 ± 1.66	84.37 ± 19.08**
C_{max} (µg/ml)	3.26 ± 0.52	3.91 ± 0.45	0.86 ± 0.08	0.95 ± 0.06
T_{max} (h)	16.33 ± 3.12	17.50 ± 4.50	25.00 ± 2.86	39.00 ± 5.74*

Values are expressed as mean ± SEM

* Significantly different from the IR alone treatment at $P < 0.05$
** Significantly different from the IR alone treatment at $P < 0.01$
*** Significantly different from the IR alone treatment at $P < 0.001$

8.3.: INFLUENCE OF THE ROUTE OF METHIMAZOLE ADMINISTRATION ON ITS BENZIMIDAZOLE METABOLISM-MODULATING EFFECTS

8.3.1.: OBJECTIVES

The present experimental study was conducted: a) to assess the pharmacokinetic profiles of NTB and its metabolites after oral administration of a zwitterion suspension of NTB to sheep at the recommended dose rate of 7.5 mg/kg, b) to determine whether or not the co-administration of MTZ induces changes to the pharmacokinetic behaviour of these metabolites, and c) to evaluate if the route of MTZ administration may influence the potential inhibitory effects of this compound on liver microsomal oxidation.

8.3.2.: MATERIALS AND METHODS

8.3.2.1. Experimental animals

Fifteen female 10 month old Finn Dorset crossbred sheep weighing 35-40 kg were used in this study. The animals were maintained on a mixed grain ration with hay and water *ad libitum*. The health of all animals was monitored prior to and throughout the experimental period.

8.3.2.2. Treatments

The animals were randomly divided into three groups of five animals each and the treatments given as follows:

Group I. Animals were treated with a zwitterion suspension of NTB (50 mg/ml) (Hapadex oral suspension, Schering Plough, NJ, U.S.A.) by oral drench at a dosing rate of 7.5 mg/kg.

Group II. Animals received the zwitterion suspension of NTB (50 mg/ml) by oral drench at 7.5 mg/kg plus an aqueous solution of methimazole (15 mg/ml) (2-mercapto-1-methyl-imidazole, Aldrich Chemical Co., WI, U.S.A.) given orally at 3 mg/kg immediately after NTB administration.

Group III. Animals were treated with the zwitterion suspension of NTB (50 mg/ml) by oral drench at 7.5 mg/kg

together with an intramuscular (IM) injection of methimazole (3 mg/kg) in sterile saline solution (25 mg/ml) given immediately after NTB administration.

Blood samples (10 ml) were taken from the jugular vein into heparinized vacutainer tubes (Becton Dickinson, Canada) prior to the treatments and at 0.5, 1, 2, 4, 6, 8, 10, 12, 18, 24, 30, 36, 48, 60 and 72 h post-administration. Plasma was separated by centrifugation at 3000 *g* for 15 min, placed into plastic vials and frozen at -20°C until analyzed.

Sample treatment, analytical procedures and pharmacokinetic analysis of the data were as described in Chapter 2, sections 2.2.3 and 2.2.4.

8.3.2.3. Statistical analysis

Pharmacokinetic parameters are presented as mean ± SEM. The mean pharmacokinetic parameters for each treatment were compared by analysis of variance. Where a significant F value was obtained, Duncan's multiple range test was performed to indicate order of significance. A value of $P< 0.05$ was considered statistically significant.

8.3.3.: RESULTS

Only trace amounts of NTB parent compound and ABZ were irregularly detected in plasma in the earliest samples of this trial after oral administration of NTB in sheep at 7.5 mg/kg.

The mean plasma concentrations of ABZSO obtained after oral administration of NTB zwitterion suspension at 7.5 mg/kg either alone or with MTZ, given orally or intramuscularly, to sheep are shown in Figure 8.4. Figure 8.5 shows the mean plasma concentrations of $ABZSO_2$ for the above mentioned treatments.

While detected between 0.5 and 60 h post-treatment in the control group (NTB oral alone), ABZSO was found in plasma from

0.5 up to 72 h post-administration when MTZ was co-administered with NTB, either orally or intramuscularly. The results of the pharmacokinetic analysis for ABZSO obtained after the oral administration of NTB in the absence or presence of MTZ are summarized in Table 8.3. The presence of MTZ caused important changes in the disposition kinetics of ABZSO. The terminal slope (β) was significantly smaller ($P< 0.01$) for the NTB+MTZ oral and NTB+MTZ IM treatments. ABZSO T ½ β increased from 7.27 ± 0.54 h (NTB alone) to 14.57 ± 2.86 h (NTB+MTZ oral) and to 11.39 ± 0.91 h (NTB+MTZ IM), but only reached the level of statistical significance ($P< 0.05$) for the NTB+MTZ oral treatment. Both ABZSO AUC and AUMC were significantly higher ($P< 0.05$) after the co-administration of MTZ (oral and IM) with NTB than after the administration of NTB alone. Also, the concomitant treatments with MTZ resulted in a longer MRT for ABZSO, which reached the level of statistical significance for the NTB+MTZ oral treatment. There was no difference between treatments, in terms of T ½ (for), Cmax and Tmax for ABZSO.

The mean pharmacokinetic parameters for $ABZSO_2$ obtained after each treatment are presented in Table 8.4. There were no major modifications in the $ABZSO_2$ pharmacokinetic profiles after the co-administration of MTZ with NTB compared with the NTB alone treatment. However, while detected in plasma in the earliest samples (0.5-1.0 h) after the NTB alone treatment, $ABZSO_2$ showed a delayed appearance in plasma (6-8 h post-treatment) after co-administration with MTZ given orally or intramuscularly. The $ABZSO_2$ lag time was significantly increased from 3.10 ± 0.19 h (NTB alone) to 8.88 ± 0.97 h (NTB+ MTZ oral) and to 9.03 ± 0.54 h (NTB+MTZ IM). Also, the $ABZSO_2$ Tmax was longer in the presence of MTZ, which achieved statistical significance for the NTB+MTZ IM treatment.

Table 8.3: Pharmacokinetic parameters for ABZSO following oral administration of NTB (7.5 mg/kg) either alone or co-administered with methimazole (MTZ) (3 mg/kg) orally and intramuscularly (IM) to sheep.

Parameter	NTB Oral alone (n=5)	NTB Oral + MTZ Oral (n=5	NTB Oral + MTZ IM (n= 5)
Kf (h^{-1})	0.176 ± 0.020	0.336 ± 0.110	0.245 ± 0.111
T ½ for (h)	4.16 ± 0.48	2.78 ± 0.58	4.43 ± 0.94
ß (h^{-1})	0.098 ± 0.009	0.055 ± 0.009**	0.062 ± 0.005**
T ½ ß (h)	7.27 ± 0.54	14.57 ± 2.86*	11.39 ± 0.91
AUC (µg.h/ml)	35.01 ± 3.66	54.35 ± 5.75*	56.36 ± 7.57*
AUMC (µg.h^2/ml)	666 ± 101	1271 ± 101*	1351 ± 248*
MRT (h)	12.66 ± 0.68	18.85 ± 2.35*	17.02 ± 0.90
Cmax (µg/ml)	1.68 ± 0.16	2.12 ± 0.19	1.89 ± 0.21
Tmax (h)	10.00 ± 2.19	8.80 ± 0.49	12.80 ± 2.24

Values are expressed as mean ± SEM.

* Significantly different from control treatment (NTB oral alone) at $P < 0.05$ when analyzed by ANOVA + Duncan's multiple range test

** Significantly different from control treatment (NTB oral alone) at $P< 0.01$ when analyzed by ANOVA + Duncan's multiple range test

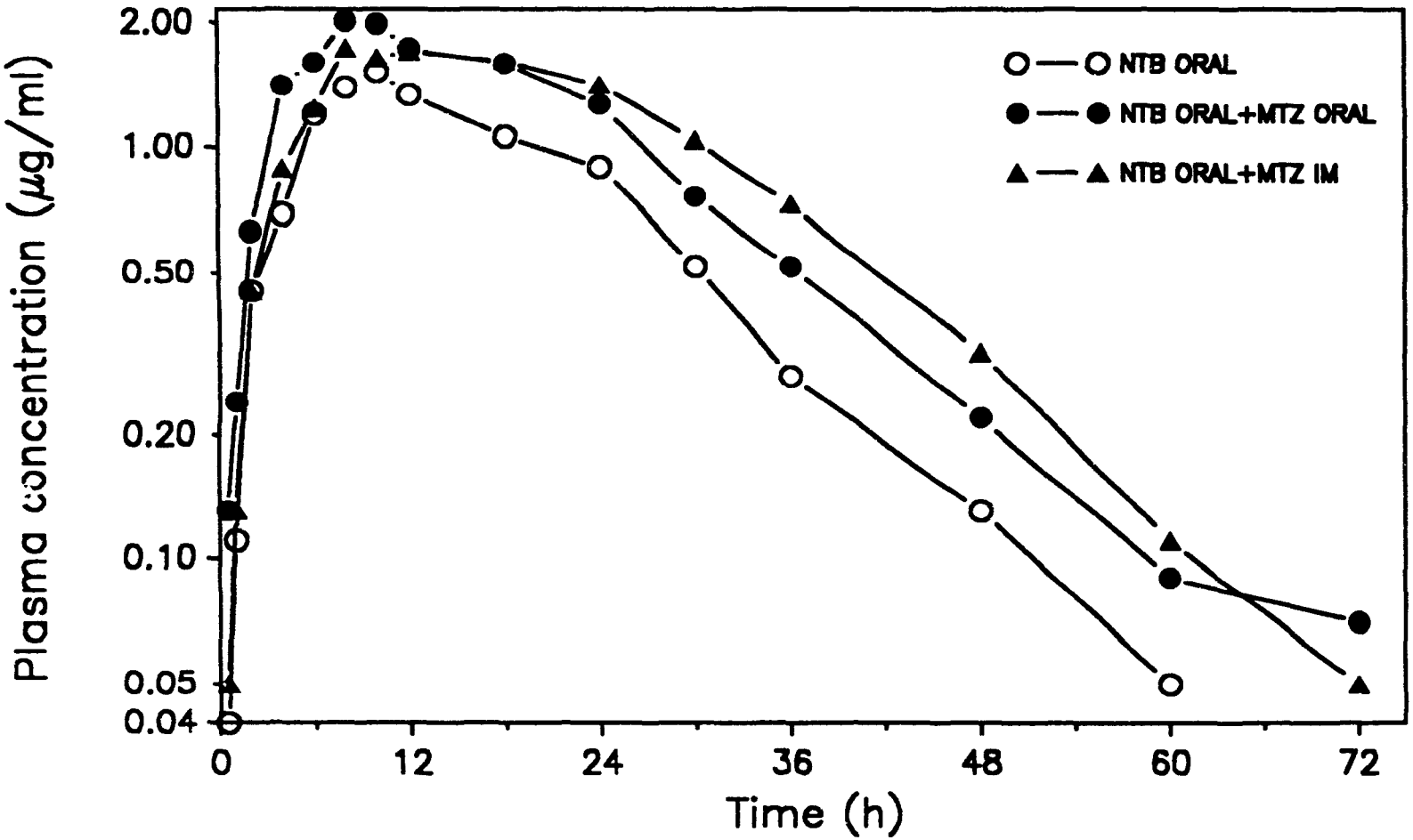


Figure 8.4: Mean plasma concentration of ABZSO (n=5) obtained after oral administration of NTB alone (7.5 mg/kg) or with methimazole (MTZ) (3 mg/kg) given orally or intramuscularly (IM) to sheep.

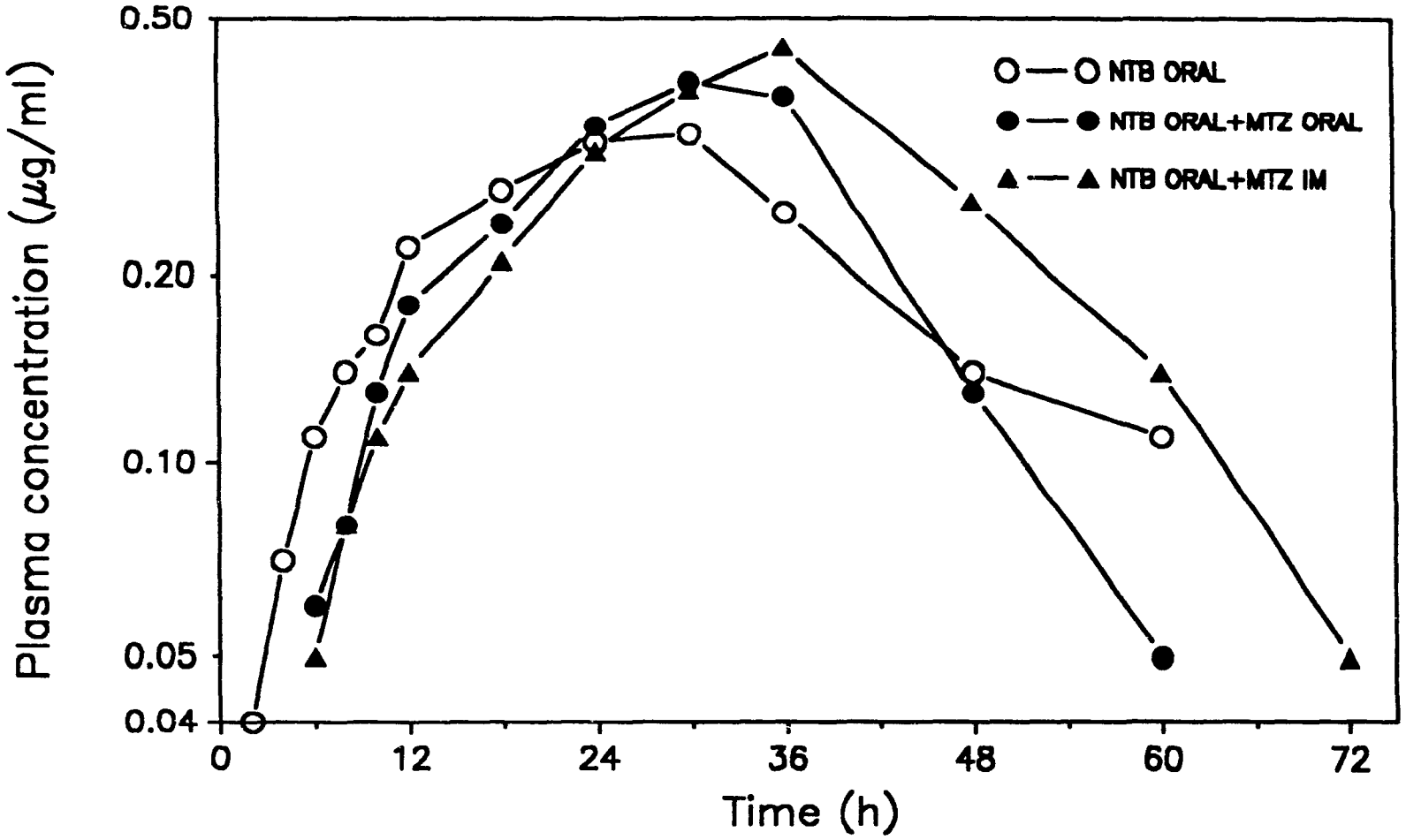


Figure 8.5: Mean plasma concentration of $ABZSO_2$ (n=5) obtained after oral administration of NTB alone (7.5 mg/kg) or with methimazole (MTZ) (3 mg/kg) given orally or intramuscularly (IM) to sheep.

Table 8.4: Pharmacokinetic parameters for $ABZSO_2$ following the oral administration of NTB (7.5 mg/kg) either alone or co-administered with methimazole (MTZ) (3 mg/kg) orally and intramuscularly (IM) to sheep.

Parameter	NTB oral alone (n= 5)	NTB oral + MTZ oral (n= 5)	NTB oral + MTZ IM (n= 5)
Kf (h^{-1})	0.108 ± 0.017	0.108 ± 0.013	0.097 ± 0.006
T ½ for (h)	6.94 ± 0.78	6.89 ± 1.04	7.29 ± 0.44
Lag time (h)	3.10 ± 0.19	8.88 ± 0.97**	9.03 ± 0.54**
ß (h^{-1})	0.067 ± 0.015	0.079 ± 0.013	0.071 ± 0.006
T ½ ß (h)	12.15 ± 2.20	10.10 ± 2.19	10.06 ± 0.88
AUC ($\mu g.h/ml$)	13.40 ± 2.57	13.16 ± 1.03	16.29 ± 1.50
AUMC ($\mu g.h^{2}/.ml$)	476 ± 134	443 ± 13	629 ± 96
MRT (h)	22.94 ± 2.49	24.28 ± 0.81	27.33 ± 2.02
Cmax ($\mu g/ml$)	0.36 ± 0.03	0.43 ± 0.05	0.52 ± 0.04*
Tmax (h)	27.60 ± 1.47	31.20 ± 2.24	37.20 ± 2.03*

* Significantly different from control treatment (oral NTB alone) at $P< 0.05$ when analyzed by ANOVA plus Duncan's range test

** Significantly different from control treatment (oral NTB alone) at $P< 0.01$ when analyzed by ANOVA plus Duncan's range test

8.4.: DISCUSSION

The occasional detection of trace amounts of NTB parent compound and ABZ in the earliest samples in the oral NTB treatments at 7.5 mg/kg, precluded any pharmacokinetic analysis for these two analytes. However, as expressed in Chapter 2, the parent NTB compound was found in plasma for only a short period following the IR treatment at 20 mg/kg. The concomitant administration of MTZ did not affect the pharmacokinetics of NTB parent drug after its IR administration to sheep. A fast absorption, early Cmax, short elimination half-life, relatively high body clearance and low AUC indicated a similar pharmacokinetic profile for the parent compound after both treatments.

ABZ has not previously been detected in plasma after the administration of NTB orally and subcutaneously in cattle (results reported in Chapters 3 and 7) or after the oral administration of ABZ itself to sheep (Delatour *et al.*, 1990a; Galtier *et al.*, 1991) and cattle (Prichard *et al.*, 1985). Low ABZ plasma concentrations were detected in three animals of the control group of this trial that received NTB IR at 20 mg/kg, resulting in a low AUC and short T ½ ß. Both the lack of ABZ in plasma and the detection of very low levels and the early appearance of ABZSO in plasma may be attributed to a first-pass oxidation in the liver by which ABZ is rapidly converted into ABZSO after its GI absorption. The *in vitro* results presented in Chapter 5 indicate that ABZ could be, at least partly, oxidized (into ABZSO) in the digestive tract before absorption. However, the co-administration of NTB IR with MTZ resulted in a notably improved pharmacokinetic profile for ABZ. The significantly longer ($P< 0.05$) T ½ ß and MRT and significantly higher AUC (138%) for ABZ would indicate an MTZ interference in the enzymatic pathway responsible for the ABZ sulphoxidation.

As is apparent in the plot of ABZSO plasma concentrations

(Figure 8.2) and from the pharmacokinetic results in Table 8.2, the concomitant treatment of NTB IR (20 mg/kg) with MTZ caused dramatic changes in the disposition kinetics of ABZSO. Sulphoxide metabolites are important metabolites in terms of the clinical efficacy of BZD/pro-BZD thioethers. A markedly different ($P< 0.001$) terminal slope (ß) resulted in an elimination half-life four-fold longer ($P< 0.001$) for this active ABZSO metabolite following the co-administration with MTZ. While there was no difference in terms of Cmax and Tmax, the overall ABZSO MRT was 252% longer ($P< 0.01$) and the AUMC and AUC were significantly higher ($P< 0.05$) when MTZ was co-administered with NTB given intraruminally at the higher recommended dose rate (Table 8.2).

Similarly, significant modifications to the pharmacokinetic behaviour of the ABZSO metabolite were observed when MTZ, given either orally or intramuscularly at 3 mg/kg, was co-administered with oral NTB given at 7.5 mg/kg in sheep. There was a pronounced change in the elimination phase, which resulted in the terminal slope (ß) being significantly smaller ($P< 0.01$) for the NTB+MTZ (oral and IM) treatments as compared with the NTB alone treatment (Figure 8.4; Table 8.3). These modified slopes resulted in longer ABZSO elimination half-lives, which increased by 58% (NTB+MTZ IM) and 100% (NTB+MTZ oral) compared with the oral NTB control treatment. The enhanced plasma profile of ABZSO in the presence of MTZ, given by both routes, was evident in the significantly higher ($P< 0.05$) area under the zero (AUC) and first moment (AUMC) of the plasma concentration time curves obtained for these treatments. ABZSO AUC increased 55% (NTB+MTZ oral) and 61% (NTB+MTZ IM) compared with the oral NTB alone treatment. In addition, the non-compartmental pharmacokinetic analysis of the concentration-time data permitted incorporation of an additional parameter, the mean residence time, for the characterization of the comparative disposition of NTB metabolites after either treatment. The ABZSO MRT was longer for both NTB+MTZ co-administrations than for the NTB alone

treatment. However, this parameter only reached the level of statistical significance for the NTB+MTZ oral treatment.

The co-administration of NTB IR with MTZ also induced significant modifications to the pharmacokinetic behaviour of the $ABZSO_2$ metabolite, resulting in significantly longer T ½ β and MRT and significantly higher AUC and AUMC in comparison with the NTB treatment alone (Table 8.2). Although the overall pharmacokinetics of $ABZSO_2$ was unaffected by concurrent administration of oral NTB at 7.5 mg/kg and MTZ, the delayed appearance of this metabolite in plasma (Figure 8.5) resulted in significantly longer ($P< 0.01$) $ABZSO_2$ lag times when MTZ was both orally and intramuscularly co-administered with oral NTB. In addition, $ABZSO_2$ Tmax was delayed for these treatments, reaching the level of significance for the NTB+MTZ IM treatment (Table 8.4).

MTZ is a known substrate for the FMO (Tynes and Hodgson, 1983), which competitively inhibits the metabolism of other potential substrates of this enzymatic complex. The FMO system is probably of primary importance in the conversion of ABZ to ABZSO in sheep (Galtier *et al.*, 1986). Thus, it is likely that the observed pronounced modifications on the bioavailability and disposition kinetics of ABZ parent drug (IR treatment) and ABZSO (IR and oral treatment with NTB) are due to substrate competition between MTZ and ABZ for the FMO pathway. Thus, a reduced rate of oxidation could attenuate the liver first-pass phenomenon resulting in an improved plasma pharmacokinetic profile for ABZ. In addition, a delayed ABZ oxidation could result in an extended time of ABZSO formation which would explain the substantial modifications observed in the bioavailability and disposition kinetics of this active metabolite.

However, it has been also reported that the biotransformation of MTZ itself may produce some reactive metabolites which bind avidly to the cytochrome P-450 drug binding site, decreasing

its enzymatic activity (Kedderis and Rickert, 1985). Since the cytochrome P-450 system seems to be responsible, at least in some species, for liver microsomal oxidation of ABZSO to $ABZSO_2$ (Souhaili-El Amri *et al.*, 1988b), it is also likely that MTZ interferes at this step and thereby diminishes the activity of this mixed function oxidase system. Thus, a decreased metabolic rate for the conversion of ABZSO into $ABZSO_2$ could also account for the marked changes in ABZSO disposition kinetics in the presence of MTZ. Such an effect might be consistent with the improved pharmacokinetic profile of ABZSO and with the delayed detection of $ABZSO_2$ in plasma obtained in both concomitant NTB+MTZ treatments.

Altogether these pharmacokinetic modifications strongly indicate that MTZ reduced the rate of liver oxidation, which is reflected in an improved plasma profile and altered disposition kinetics for ABZSO. This is an active metabolite in terms of parasite uptake and binding to tubulin (Fetterer, 1986; Lubega and Prichard, 1991b).

MTZ is a safe compound, regularly used in human and veterinary medicine. High bioavailability (Jansson *et al.*, 1985) may ensure that a single dose of MTZ will result in successful inhibition of liver ABZ oxidation. The route of administration did not influence MTZ-induced changes on the pharmacokinetics of NTB/ABZ metabolites, which may indicate similar MTZ absorption and bioavailability from both oral and IM routes. Surprisingly, the MTZ-induced changes on ABZ metabolites pharmacokinetics did not increase by using a higher MTZ dose rate. Conversely, when MTZ was given at 1.5 mg/kg with IR NTB, administered at a dose rate of 20 mg/kg, the changes on the pharmacokinetic behaviour of ABZ-related molecules (Tables 8.1 and 8.2) were more pronounced than those observed when 3 mg/kg of MTZ were co-administered with oral NTB at the lower recommended dose of 7.5 mg/kg (Tables 8.3 and 8.4). It seems possible that provided there is a sufficient MTZ concentration to establish the metabolic competition, the impairment of

oxidation may depend on the amount of ABZ substrates reaching the liver.

The improved pharmacokinetic profile and residence time for the anthelmintically-active ABZ and ABZSO metabolites, obtained after the co-administration of MTZ with NTB may be highly important in terms of clinical efficacy. Although more research is necessary to establish the site(s) of MTZ interference and the concentration required for an optimal inhibition in different species, the findings reported in this Chapter demonstrate that the pharmacokinetic interaction between MTZ and NTB in sheep results in notable changes in the disposition and bioavailability of NTB metabolites. However, the implications of this interaction on human and veterinary parasite control need to be evaluated.

CHAPTER 9

COMPARATIVE SULPHOXIDATION OF ALBENDAZOLE BY SHEEP AND CATTLE LIVER MICROSOMES AND THE INHIBITORY EFFECT OF METHIMAZOLE[1]

9.1.: INTRODUCTION

The antiparasite efficacy of BZD and pro-BZD compounds not only depends on affinity for parasite tubulin but also on their ability to reach high and sustained concentrations at the site of parasite location. This, in turn, fully relies on the pharmacokinetic behaviour and metabolic pattern of these compounds in the host. The efficient biotransformation of BZD/pro-BZD thioethers into more polar BZD metabolites results in a reduction in antiparasite activity.

Following administration of either NTB in sheep and cattle (Chapters 2, 3 and 4) or ABZ itself in the same species (Marriner and Bogan, 1980; Prichard *et al.*, 1985; Delatour *et al.*, 1990a), ABZSO and $ABZSO_2$ were the major metabolites found in plasma. However, marked differences in the plasma profiles of these two metabolites between sheep and cattle have been observed after administration of ABZ, both as an oral drench and as a slow release device (Delatour *et al.*, 1990a). Similarly, the results of this thesis research show pronounced differences between species in the pharmacokinetics of the ABZSO and $ABZSO_2$ metabolites following administration of NTB pro-drug. These differences have been particularly evident in the plasma ratio of $ABZSO_2$/ABZSO and in the ABZSO disposition kinetics where cattle showed significantly shorter plasma elimination half-lives and residence times than sheep. These sheep/cattle differences have also been reported for other BZD thioether compounds (Prichard *et al.*, 1985).

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[1] These experiments were done in collaboration with Mr. Bakela Nare, B.Sc., Institute of Parasitology, McGill University.

The absence or the detection of only trace amounts of ABZ parent drug in jugular plasma has been attributed to a first-pass oxidation in the liver. *In vitro*, oxidation of ABZ into ABZSO has been shown for rat (Fargetton *et al.*,1986), pig (Souhaili-El Amri *et al.*, 1987), sheep (Galtier *et al.*, 1986) and human (Rolin *et al.*, 1989) liver microsomes. It has been proposed that the FMO system is responsible for the oxidation of ABZ into ABZSO (Fargetton *et al.*, 1986), while the cytochrome P-450 system would be involved in the second, biphasic and slower oxidative step by which ABZSO is further oxidized into $ABZSO_2$ (Souhaili-El Amri *et al.*, 1988b). However, a differential involvement of these pathways among the studied species seems to exist.

The metabolism of methimazole (MTZ) and other thiourea antithyroid drugs has been investigated using hepatic microsomes from several species (Poulsen *et al.*, 1979). MTZ was shown to be a high affinity substrate for purified pig liver microsomal FMO (Poulsen *et al.*, 1979), and to inhibit the *in vitro* microsomal oxidation of different xenobiotics (Tynes and Hodgson, 1983; Kedderis and Rickert, 1983).

In vivo, interference with the liver microsomal sulphoxidation process affects the pharmacokinetic behaviour of ABZ metabolites. This could lead to improved antiparasite efficacy as a result of higher concentration of anthelmintically active metabolites being presented to the parasite for a longer period of time. The findings described in previous Chapters conclusively demonstrate that the co-administration of NTB with MTZ results in pronounced changes on the plasma disposition kinetics of ABZ metabolites in both sheep and cattle. These changes have been particularly important for the active ABZSO metabolite, resulting in a marked increase in its bioavailability, elimination half-life and time of residence in comparison with treatments without MTZ. However, using the same MTZ dose rate and route of administration, the modifications in pharmacokinetic behaviour of the NTB/ABZ

metabolites were less pronounced in cattle than in sheep.

This study was designed to elucidate some of the mechanisms underlying the differential behaviour of ABZ and its metabolites in sheep and cattle. The comparative *in vitro* liver microsomal sulphoxidation of ABZ in these species has been established. The inhibitory effect of MTZ on the ability of microsomes from both species to convert ABZ to ABZSO has been investigated on a comparative basis. The microsomal enzyme systems involved in the sequential oxidation of ABZ are outlined and the possible site(s) of MTZ inhibition is discussed.

9.2.: MATERIALS AND METHODS

9.2.1. Chemicals

NADPH was purchased from Boehringer Mannheim (Québec, Canada) and analytical grade solvents were obtained from Fisher Scientific (Ontario, Canada). ABZ and oxibendazole were supplied by SmithKline Beecham Corp. (West Chester, PA). ABZSO, $ABZSO_2$ and NTB were gifts from Schering Plough (Kenilworth, NJ). MTZ was purchased from Aldrich Chemical Co. (Milwaukee, WI), and *n*-octylamine and thiourea from Sigma Chemical Co. (St. Louis, MO).

9.2.2. Animals and preparation of microsomes

Healthy Holstein Fresian calves (180-200 kg) and Finn Dorset cross-bred sheep (35-40 kg) were used as a source of liver material. The animals were stunned by captive bolt and exsanguinated immediately. The abdomen was opened and the liver removed and rinsed immediately with ice-cold 1.15% KCl solution. The liver samples were transported to the laboratory from the abattoir on ice and all subsequent procedures were performed at 0-4°C. For each animal species, liver samples (10 g) were cut into small pieces with scissors and washed several times in 1.15% KCl (to remove hemoglobin). Samples from different animals were pooled and homogenized in

an Potter Elvenjem homogenizer with a teflon pestle for 45 sec in 0.1 M potassium phosphate buffer pH 7.4. Samples were centrifuged at 10 000 *g* for 20 min and the resulting supernatant at 100 000 *g* for 60 min. The pellet (microsomal preparation) was suspended in 0.1 M potassium phosphate buffer and protein content immediately determined by the Lowry assay (Lowry *et al.*, 1951) using bovine serum albumin as a standard. Microsomes were stored at -70°C until used for assays.

9.2.3. Microsomal enzyme assays

9.2.3.1. Spectrophotometric determination.

Rates of NADPH oxidation in the presence of the various substrates (drugs) were determined using a DU-7 Beckman spectrophotometer. The incubation mixtures contained 0.1 M potassium phosphate buffer pH 7.4, 125 μM of NADPH, 0.5-1 mg of microsomal protein and 200 μM of each substrate in a total volume of 1 ml. The mixtures were preincubated for 2 min at 37°C and the decrease in NADPH concentration monitored at 340 nm over 5 min. Reported velocities represent substrate-stimulated NADPH consumption minus background.

9.2.3.2. HPLC determination of sulphoxidase activity

Metabolic assays were carried out in aerobic conditions in a shaking water bath for 60 min. Under typical reaction conditions (1 ml final volume), 250 μM of NADPH, potassium phosphate buffer pH 7.4, 4.33 mg of protein and 20 μM of ABZ were included. ABZSO, $ABZSO_2$ and NTB were used at 200 μM while various concentrations of methimazole (5-1000 μM) were used to test inhibition on sulphoxidation of 20 μg of ABZ. Incubations without microsomal tissue were used as controls. At the end of the incubation period, the reaction was stopped by boiling in a water bath for 2 min (Galtier *et al.*, 1986)) or by the addition of 50 μl of concentrated ammonium hydroxide (Short *et al.*, 1988a). Assays were performed in triplicate and repeated several times. Following termination of the reaction, samples were processed for HPLC analysis.

Sample extraction: Samples were spiked with 1 μg of oxibendazole (99.2% pure) as an internal standard. ABZ and/or its metabolites were extracted with C_{18} SepPak cartridges (Part # 51910, Waters Associates, Milford, MA). Cartridges were conditioned by washing with 5.0 ml of methanol followed by 5.0 ml of 0.017 M ammonium phosphate buffer pH 5.5. After application of the spiked sample, the cartridge was successively washed as described for plasma samples in Chapter 2. The analytes eluted in the last 2.5 ml of 100% methanol were concentrated to approximately 0.4-0.5 ml under a stream of nitrogen and refrigerated until analysis.

Drug/metabolite analysis: Samples were analyzed for ABZ and/or its metabolites by HPLC. Fifty microliters of each extracted sample were injected by an autosampler into an LKB Bromma HPLC system (LKB Bromma, Sweden) fitted with a Bondex 10 μm C_{18} reverse phase column (Phenomenex, CA). HPLC analysis conditions and retention times were as previously reported in section 2.2.3. Identification of each analyte was achieved by comparison with the retention times of pure reference standards (97-98.5% pure). These were also used to prepare standard solutions to establish calibration curves. The linear regression lines for the standard curves for the different analytes over a range of 0.02 to 5 μg/ml showed correlation coefficients between 0.975 and 0.993. Percentage recovery of metabolites from sheep and cattle microsomal incubation mixtures was established by spiking them with known amounts of each analyte in a range of 0.05-5 μg/ml. These results were used to correct final concentration data. Concentrations were calculated by comparison of unknown and internal standard peak area using the computer program Nelson Analytical, model 2600, version 3.1 (Nelson Analytical Inc., CA) on an AT computer. The sensitivity of the assay was 0.010 μg/ml for ABZ and ABZSO, 0.020 μg/ml for $ABZSO_2$ and 0.040 μg/ml for NTB. There was no interference by any of the compounds studied in the chromatographic determination of either ABZ or its metabolites.

9.2.3.3. Statistical analysis

Results from either NADPH consumption studies or HPLC quantitation of products formed were statistically compared by either paired or unpaired Student's *t*-test and a value of $P < 0.05$ was considered statistically significant.

9.3.: RESULTS

The sulphoxidation of ABZ to ABZSO over a 1 h period was observed in microsomal preparations supplied with different concentrations of NADPH at 37°C (Figure 9.1). Virtually no sulphoxidase activity was observed in the absence of NADPH. ABZSO production increased with an increase in the concentration of NADPH. All subsequent experiments in which ABZ and its metabolites were extracted and analyzed by HPLC were conducted in the presence of 250 μM of NADPH.

In both cattle and sheep-derived liver microsome fractions, the rate of NADPH consumption increased beyond background levels upon the addition of ABZ, MTZ or thiourea (Figure 9.2). Incubation of microsomes with ABZSO or $ABZSO_2$ resulted in NADPH consumption rates that were not significantly different from background values. Addition of NTB resulted in essentially no change in background levels of NADPH consumption in both cattle and sheep microsomes (data not shown). MTZ induced the highest increase in NADPH consumption when incubated with both cattle and sheep microsomes. However, with all the substrates shown in Figure 9.2, sheep-derived microsomes produced significantly higher levels of NADPH consumption rates compared to those from cattle. Thermal pretreatment (50°C for 90 sec) of the microsomes resulted in a significant loss in their ability to consume NADPH in the presence of ABZ, MTZ and thiourea.

Representative chromatograms of metabolite analysis by HPLC following incubation of ABZ with microsomes from sheep are shown in Figure 9.3. The retention times were 5.20, 13.5 and

17.4 min for ABZSO, oxibendazole (internal standard) and ABZ, respectively. ABZ was converted to ABZSO in the presence of NADPH (Figure 9.3B), but not in its absence (Figure 9.3A). In the presence of MTZ (25 μM), less ABZ was oxidized to ABZSO (Figure 9.3C).

The relative inhibitory effect of several concentrations of MTZ on the sulphoxidation of ABZ was studied by measuring the amount of ABZSO produced by microsomes from sheep (Table 9.1) and cattle (Table 9.2) liver. Up to 82% inhibition was obtained in sheep microsomes with 1 mM MTZ, whereas 10 μM did not have any effect. With cattle microsomes, on the other hand, 1 mM MTZ inhibited ABZ sulphoxidase activity by about 56% under the same assay conditions (Figure 9.4). The results in Tables 9.1 and 9.2 indicate that ABZ sulphoxidase activity in sheep is significantly higher than that in cattle-derived liver microsomes. For all of the concentrations of MTZ examined, the percentage inhibition of sulphoxidation reactions was significantly higher in sheep than in cattle liver microsomes (Figure 9.4).

The effects of various inhibitors of the P-450 and FMO enzyme systems on the sulphoxidation of ABZ were investigated using microsomes derived from sheep livers. *n*-octylamine, which binds to and inhibits the terminal oxidase, cytochrome P-450, inhibited the ABZ sulphoxidation reaction in a concentration-dependent manner (Table 9.3). Thermal pretreatment of sheep microsomes, which inactivates the FMO system, synergizes with *n*-octylamine to inhibit the sulphoxidation reaction. Table 9.3 further demonstrates that MTZ, thought to inhibit the microsomal FMO, and *n*-octylamine have additive inhibitory effects on the ability of sheep liver microsomes to convert ABZ to its sulphoxide metabolite.

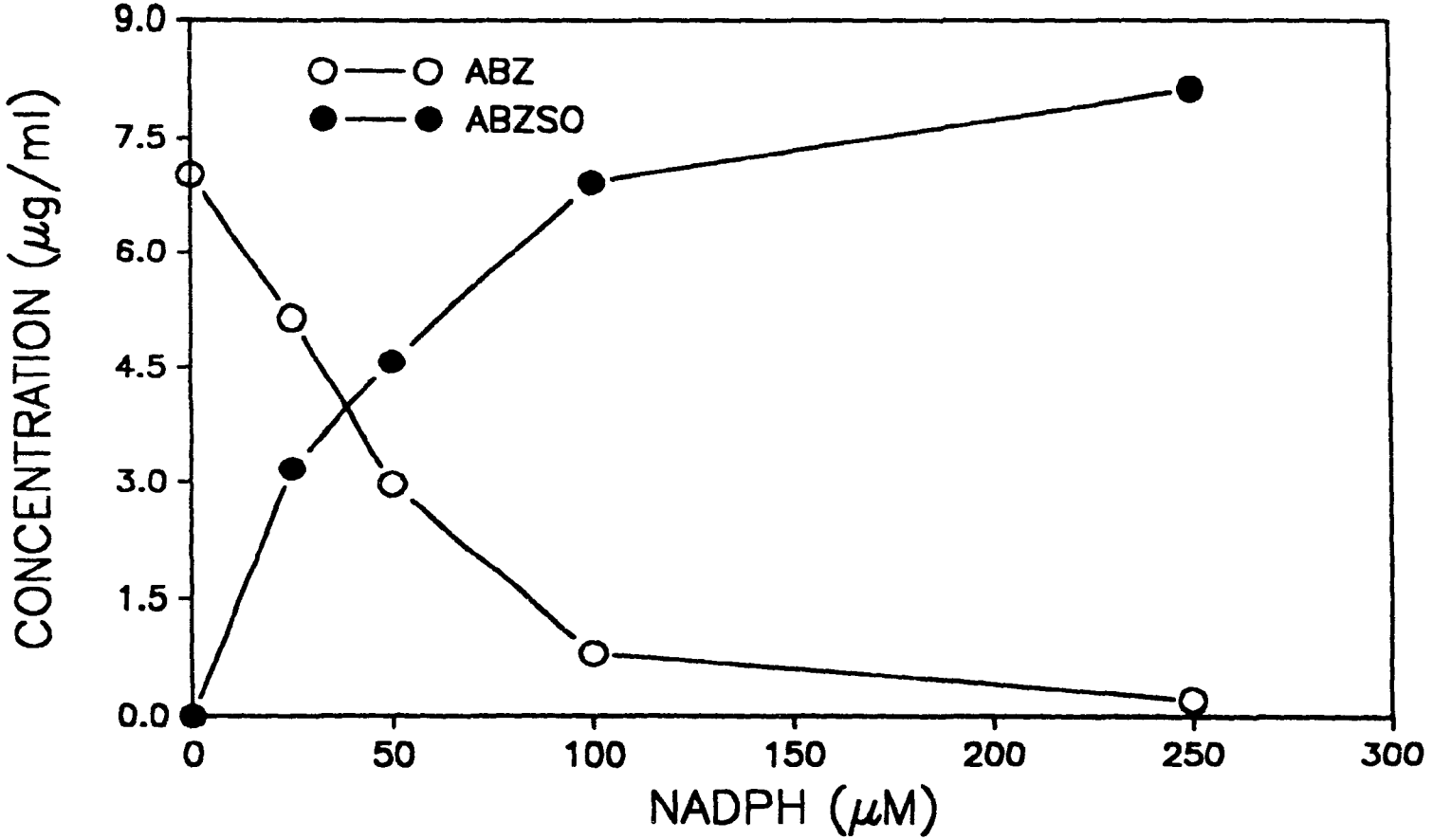

Figure 9.1: The effect of NADPH on the sulphoxidation of ABZ by microsomes from sheep liver. Incubations, metabolite extraction and analysis were carried out as described in section 9.2.3.2. Results represent the mean value (3 determinations) of the amount of ABZ remaining and that of ABZSO formed in the incubation medium during 60 min of incubation with different concentrations of NADPH.

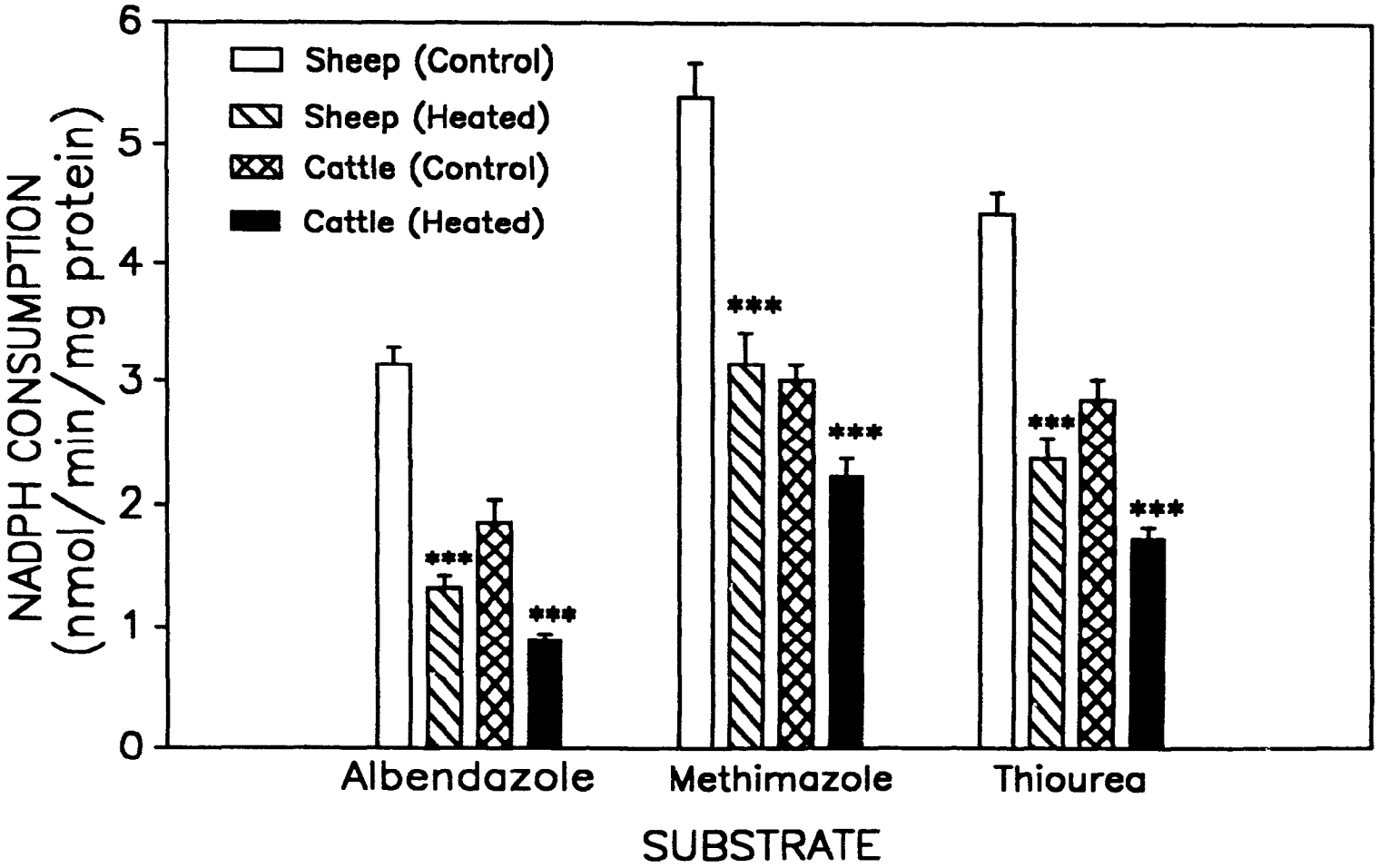

Figure 9.2: The effects of thermal pretreatment on substrate stimulated NADPH consumption by microsomes from sheep and cattle liver. Values are mean ± SEM of 5 determinations. Procedures were carried out as described in section 9.2.3.1. (***) Significantly different from control (without heat pretreatment) at P <0.001.

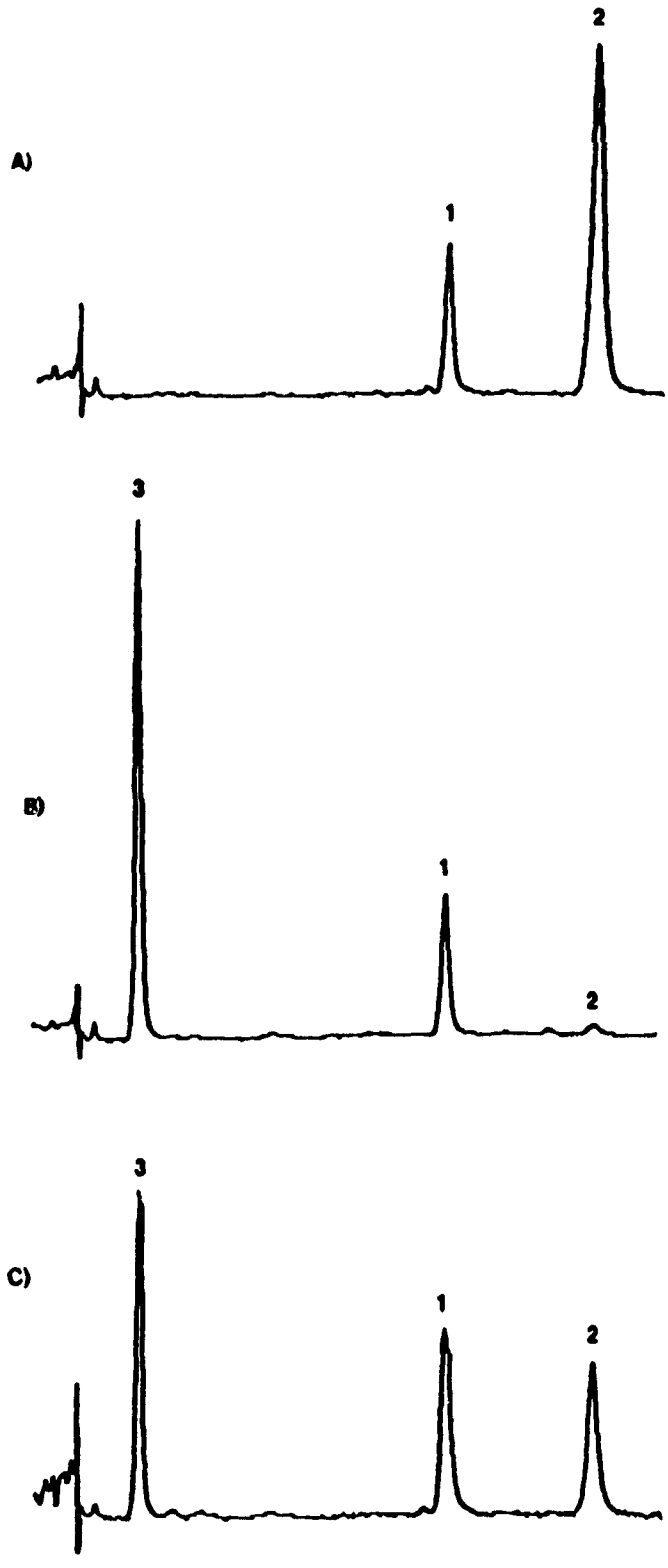

Figure 9.3: HPLC separation of ABZ (2) (17.4 min) and ABZSO (3) (5.20 min), using oxibendazole (1) (13.5 min) as internal standard, from sheep liver microsomal samples. ABZ (20 μM) was incubated with liver microsomes for 60 min at 37°C without (A) or with (B) NADPH or in the presence of 25 μM of methimazole and NADPH (C).

Table 9.1: Effects of methimazole on the sulphoxidation of ABZ to ABZSO by sheep liver microsomes.

Methimazole concentration (μM)	Sulphoxidase activity# (nmol/min/mg prot)	Ratio‡ ABZ/ABZSO
0 (control)	66.21 ± 4.58***	0.27
1000	11.70 ± 0.31	9.09
500	14.84 ± 0.56	7.39
300	21.09 ± 3.52	6.08
200	22.37 ± 6.16	5.40
100	23.74 ± 3.71	3.76
50	27.38 ± 3.81	2.74
25	31.74 ± 1.44	1.95
15	59.90 ± 2.80	0.38
10	68.79 ± 1.92	0.22

Data expressed as mean ± SEM (n= 6)

#: Expressed as nmoles of ABZSO formed per min of incubation per mg of microsomal protein.

‡: The averaged ratio between the amount of ABZ and ABZSO found in different assays.

***: Statistically different from cattle control values (Table 9.2) at $P< 0.001$.

Table 9.2: Effects of methimazole on the sulphoxidation of ABZ to ABZSO by cattle liver microsomes.

Methimazole concentration(μM)	**Sulphoxidase activity#** (nmol/min/mg prot.)	**Ratio‡ ABZ/ABZSO**
0 (control)	37.47 ± 1.56	0.08
1000	15.89 ± 0.44	1.13
500	18.02 ± 0.61	0.91
300	20.59 ± 0.69	0.82
200	23.49 ± 0.61	0.70
100	25.22 ± 1.18	0.66
50	29.79 ± 1.03	0.44
25	33.20 ± 2.48	0.21
10	39.50 ± 0.40	0.10

Data expressed as mean ± SEM (n= 6 to 8 determinations))

#: Expressed as nmoles of ABZSO formed per min of incubation per mg of microsomal protein.

‡: The averaged ratio between the amount of ABZ and ABZSO found in different assays.

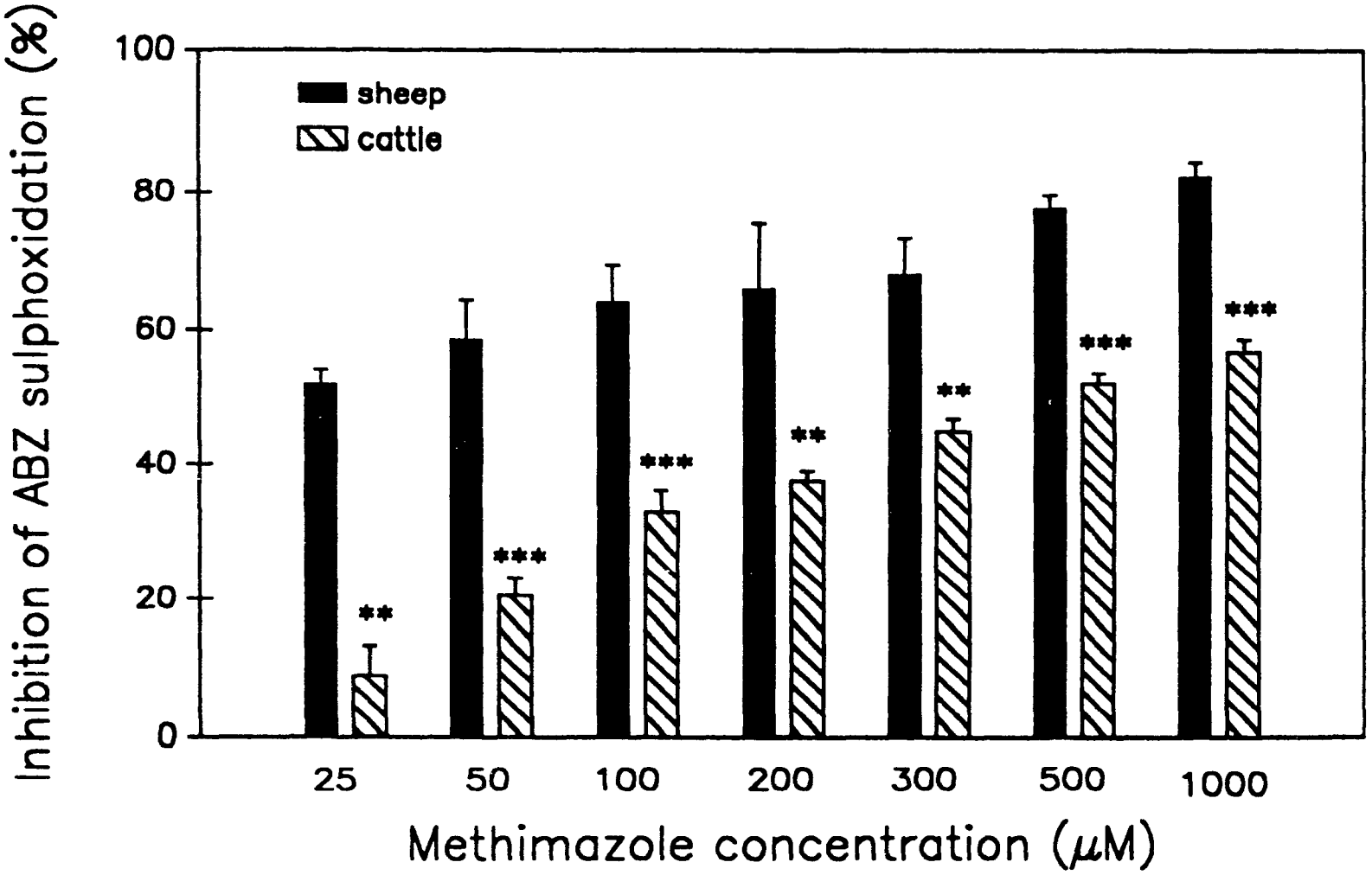

Figure 9.4: Comparative methimazole-mediated inhibition of ABZ microsomal sulphoxidation in sheep and cattle. Values are means ± SEM of 6-8 determinations. Results were calculated as the percentage of ABZSO formed in the presence of methimazole against control. Cattle values are statistically different from percent inhibition values in sheep at $P < 0.01$ (**) or $P < 0.001$ (***).

Table 9.3: In vitro inhibition of the sheep liver microsomal sulphoxidation of ABZ.

Treatment	Inhibition of ABZ sulphoxidation (%)‡
n-octylamine (1 mM)	11.6
n-octylamine (3 mM)	39.9
n-octylamine (3 mM) + heat pretreated microsomes	60.1
methimazole (300 μM)	68.3
n-octylamine (3 mM) + methimazole (300 μM)	91.3

‡: expressed as percentage of the amount of ABZSO formed against control.

Values are mean of at least 3 determinations.

9.4.: DISCUSSION

NTB pro-drug does not seem to be biotransformed by liver microsomal enzymes. NTB was not affected by incubation with the liver microsomal fraction of either sheep or cattle. This indicates, as proposed in Chapter 5, that the GI microflora are responsible for NTB reduction and cyclization into ABZ after both oral and parenteral administration.

As previously suggested (Galtier *et al.*, 1986), the conversion of ABZ into ABZSO by liver microsomes is an NADPH-dependent reaction. The production of ABZSO was correlated to the concentration of NADPH added to the incubation mixture (Figure 9.1), resulting in a decrease of the parent ABZ and a corresponding increase in its sulphoxide metabolite. The formation of ABZSO was completely abolished when ABZ was incubated with liver microsomal fraction in the absence of NADPH (Figure 9.3A).

The NADPH consumption experiments suggest that the FMO system is primarily involved in the sulphoxidation of ABZ into its pharmacologically active sulphoxide metabolite. Heat pretreatment of microsomal preparations has been shown to inactivate the FMO microsomal activity (Kinsler *et al.*, 1990). The thermal inactivation of the FMO system significantly reduced ($P< 0.001$) the NADPH consumption of MTZ and thiourea, two well characterized FMO substrates, as well as that of ABZ (Figure 9.2).

Interestingly, the oxidation of ABZ, MTZ and thiourea by sheep liver microsomes resulted in a significantly higher ($P< 0.001$) NADPH consumption than with cattle microsomes for the same substrates (Figure 9.2). This was consistent with the higher sulphoxidase activity, measured as amount of ABZSO formed, obtained for sheep than for cattle microsomes (Tables 9.1 and 9.2). These results indicate that sheep have a greater capacity to oxidize ABZ into ABZSO than cattle. However, such

a difference does not seem to correlate with some of the pharmacokinetic differences observed for BZD compounds between these species. It is likely that the observed sheep/cattle differential pharmacokinetic pattern is related to differences in the second oxidative step ($ABZSO_2$ formation), differences in the distribution/elimination processes of ABZ metabolites, or more likely, to the differences in the rate of GI reduction of ABZSO to ABZ presented in Chapter 5.

MTZ drastically inhibited the conversion of ABZ into ABZSO (Figure 9.3), which is well demonstrated by the change in the ABZ/ABZSO ratio found after incubation with different concentrations of MTZ in both species (Tables 9.1 and 9.2). The comparative percentages of inhibition of ABZ sulphoxidation between sheep and cattle showed interesting differences (Figure 9.4). The inhibitory effect of MTZ was significantly lower for the cattle microsomal oxidation compared to that of sheep at the same MTZ concentration. These results are consistent with the differential pattern of MTZ-induced changes on the pharmacokinetics of ABZ metabolites, observed between sheep and cattle *in vivo*. From the results shown in Chapters 6, 7 and 8, it is clear that the effects of the same MTZ dose rate on the disposition kinetics of the active ABZSO metabolite are much more pronounced in sheep than in cattle. It is evident that MTZ-mediated inhibition of ABZ microsomal oxidation, both *in vitro* and *in vivo*, is more efficient in sheep than in cattle. Consistently, MTZ is a better substrate for the sheep FMO system compared to cattle (Figure 9.2).

While the sulphoxidation of ABZ by rat (Fargetton *et al.*, 1986) and sheep (Galtier *et al.*, 1986) microsomes has been attributed to the FMO system, both the FMO and cytochrome P-450 systems seems to be involved in this sulphoxidation reaction in other species (Souhaili-El Amri *et al.*, 1987). These researchers, using pig liver microsomal fraction attributed between 37 and 48% of ABZ sulphoxidation to the

cytochrome P-450 system. Evidence for this was derived from the use of an antibody to NADPH-cytochrome c reductase to inhibit any P-450 isoenzyme that is involved in the sulphoxidation of ABZ. In addition, two different enantiomers of ABZSO have recently been identified in plasma by HPLC chiral separation, following administration of ABZ in different species (Delatour *et al.*, 1990b,c, 1991a,b). Variation in the proportions of these two ABZSO enantiomers between species has been related to relative differences in the involvement of the FMO and cytochrome P-450 systems in this first step of ABZ oxidation. Complementary participation of P-450 in this oxidation step would be consistent with a) inhibition of the sulphoxidase activity of sheep microsomes observed in the presence of *n*-octylamine, a typical cytochrome P-450 inhibitor (Table 9.3), and b) the synergistic inhibitory effect obtained with this compound and both heat pretreatment of microsomes and MTZ treatment (Table 3). The involvement of both microsomal pathways has been reported for the oxidation of thiobenzamidine and other sulphur-containing xenobiotics (Tynes and Hodgson, 1983). Depending on the nucleophilicity of the sulphur atom of the substrate (Souhaili-El Amri *et al.*, 1987), a balance between the participation of the FMO and cytochrome P-450 systems could be established, which in the case of ABZ may also be dependent on the animal species under investigation. Furthermore, it has been demonstrated that the FMO-dependent biotransformation of MTZ itself may produce some reactive metabolites which may bind to the active site of cytochrome P-450, blocking the entry of substrates (Kedderis and Rickert, 1985). It has been suggested that cytochrome P-450 is responsible for the oxidation of ABZSO into $ABZSO_2$ in a biphasic, slow and irreversible process (Souhaili-El Amri *et al.*, 1988b). As previously reported (Galtier *et al.* 1986)), $ABZSO_2$ reached HPLC detectable levels in the incubation mixture only when high concentrations of ABZSO (200 μM) were incubated for 3 h with twice as much microsomal protein as that used for ABZ incubations. However, the marked changes to the extension and

shape of the elimination phase for ABZSO and the delayed peak concentration for $ABZSO_2$, obtained in the presence of MTZ in both sheep and cattle *in vivo*, might be related to an indirect, FMO-mediated effect of MTZ on the cytochrome P-450 activity.

$ABZSO_2$ derivatives, such as NH_2ABZSO_2 (results presented in Chapter 4) and OH-$ABZSO_2$ (Hennessy *et al.*, 1989) have been detected in different fluids of ABZ or NTB-treated animals. However, in the present study, $ABZSO_2$ was unaffected by incubation with sheep or cattle liver microsomes. This suggests that the hydrolysis of the methyl-carbamate group of the $ABZSO_2$, an essential step in the formation of the metabolites mentioned above, may not require liver microsomal enzymes. Although ABZSO can be reduced back to ABZ by ruminal and intestinal microflora (Chapter 5), ABZ was not found after incubation of ABZSO with liver microsomal fractions; this suggests that the previously proposed reversibility of the ABZ sulphoxidation reaction (Gyurik *et al.*, 1981), which is relevant for antiparasite activity, may be valid only in the GI tract.

In conclusion, the comparative pattern of ABZ oxidation between sheep and cattle has been established. Sheep oxidized this BZD thioether at a higher rate than cattle. MTZ is a potent inhibitor of ABZ sulphoxidation. Higher MTZ concentrations are required in cattle than in sheep to produce a similar level of inhibition. This confirms the findings reported in this thesis on the MTZ-mediated modifications of the pharmacokinetic profiles of anthelmintically active ABZ metabolites *in vivo*. Taken together, these results contribute further to the understanding of the metabolism of BZD compounds, and to the potential practical application of this safe and inexpensive antithyroid compound, MTZ, in the improvement of efficacy for BZD anthelmintics in both human and veterinary medicine.

CHAPTER 10

GENERAL DISCUSSION

10.1.: PHARMACOKINETICS AND METABOLISM

The close relationship between pharmacokinetic behaviour and clinical efficacy for anthelmintic compounds is now well substantiated. The complex connections between route of administration, formulation, drug physicochemical properties and the resultant pharmacokinetic behaviour need to be understood to optimize the efficacy of BZD and pro-BZD anthelmintics; in addition, knowledge of differential drug pharmacologic behaviour among species is relevant for achieving optimal parasite control and avoiding selection for drug resistance.

The variation in efficacy and spectrum of activity among different compounds of the BZD family depends on a) the active drug/metabolite concentration to which the parasite is exposed, and b) the duration of parasite exposure to active drug concentrations. The ability of BZD/pro-BZD molecules to reach high and sustained concentrations at the site of parasite location relies on their pharmacokinetic and metabolic patterns in the host. The relevance of the absorbed drug, circulating drug/metabolite concentrations and tissue distribution pattern in worm control has been conclusively shown (Prichard and Hennessy, 1981; Prichard, 1985a). The plasma concentrations of anthelmintically active moieties reflect the pattern of exposure of worms in, or on, the GI mucosa, as well as those located in other tissues. Thus, the characterization of the plasma pharmacokinetic profiles and disposition kinetics of a parent drug and its metabolites, have been relevant to predict and optimize anthelmintic efficacy. To maximize the efficacy of BZD and pro-BZD compounds against parasites difficult to control in human and veterinary medicine, whilst preserving an adequate margin of

safety, a complete understanding of their pharmacokinetic and metabolic patterns in the host as well as of those factors affecting these processes, is necessary.

As a group, BZD anthelmintics have only limited water solubility and small differences in solubility may have a major influence on their dissolution and absorption rates, and on their resultant clinical efficacy. The lack of water solubility is an important limitation for the formulation of BZD anthelmintics. One of the most recent advances in the search for new BZD molecules has been the introduction of pro-BZD compounds. These are inactive, benzene substituted pro-drugs that are metabolically converted into anthelmintically active BZD molecules in the host (Townsend and Wise, 1990). NTB, the most recently developed guanidine-derivative pro-BZD anthelmintic, was the compound largely investigated in this thesis research project, as a model of both an anthelmintic pro-drug and a BZD thioether molecule. NTB parent drug is anthelmintically inactive (Delatour *et al.*, 1986), and its broad-spectrum efficacy exclusively depends on its rate of conversion into active ABZ metabolites, as well as on the bioavailability and disposition kinetics of these metabolites in the host. Unlike the majority of the BZD compounds, NTB as a salt is freely soluble in water, which allows a great flexibility for formulation and administration. It can be formulated for both oral and parenteral administration in ruminants. Nevertheless, results of this thesis research demonstrate that the route of administration substantially affects the rate of NTB nitro-reduction and cyclisation into its active ABZ metabolites, and the resultant disposition kinetics of these metabolites.

The plasma profiles of ABZ metabolites depend on the efficiency by which NTB pro-drug is nitro-reduced and cyclised in the GI tract of the host. This efficacy of conversion is markedly lower after parenteral administration of NTB compared with oral or intraruminal administration in both sheep and

cattle. This may account for differences in clinical efficacy between these two routes of administration. The rapid appearance of ABZ, ABZSO and $ABZSO_2$ in different GI compartments, the rapid removal of the parent NTB compound from these compartments, and the rapid detection of ABZSO and $ABZSO_2$ in plasma after oral administration of NTB, confirm that the parent drug is efficiently converted in the GI tract. This has been conclusively demonstrated by the *in vitro* metabolism experiments reported in this thesis. Unlike other pro-BZD such as FBT (Beretta *et al.*, 1987), NTB is not transformed by liver microsomal enzymes. This evidence, and the extensive biotransformation of NTB pro-drug by ruminal and intestinal fluids, indicates that the GI tract is the only site where the NTB bioactivation takes place. This correlates well with the significantly lower plasma profiles of ABZSO and $ABZSO_2$ obtained following parenteral administration of NTB in sheep and cattle, compared with those obtained after oral or intraruminal administration of the pro-drug. The efficiency of conversion of parenterally administered NTB depends on the amount of pro-drug that after absorption, reaches the digestive tract by plasma-GI tract distribution exchange or bile elimination. Such a phenomenon is less efficient than that occurring after the oral/intraruminal treatment where the total administered dose of the parent compound is exposed to the digestive microflora. This explains the delayed appearance of detectable plasma concentration of ABZ metabolites after the SC treatment in both species (4-8 h) in comparison with the oral/intraruminal treatment (0.25-0.5 h). Following the SC treatment in both species, the parent compound is rapidly absorbed, distributed and cleared from plasma within 12 h post-treatment, which results in a short elimination half-life and a relatively high body clearance. The rapid elimination of the hydrosoluble parent compound after SC administration, largely by urine, may also account for a less efficient conversion into its cyclised ABZ metabolites compared with the oral treatment, after which the absence, or occasional detection of trace amounts, of NTB and the high profile of its

metabolites in plasma indicate a more successful bioactivation. Consistently after SC treatment in sheep, 94.3-97.1% of the total recovered analytes in urine was unchanged parent drug, and only from 2.9 to 5.7% was measured as cyclised ABZ products; these results were reversed following the intraruminal administration of NTB at the same dose rate.

NTB contains a solubilizing taurine moiety that allows its formulation either as a water soluble trisamine salt or as an insoluble zwitterion suspension. It was interesting to undertake bioequivalence studies in an attempt to identify which formulation produced the most favourable bioavailability and disposition kinetics of active NTB metabolites. Although both formulations were bioequivalent after parenteral treatment, the zwitterion suspension was significantly more bioavailable (approximately two-fold) than the trisamine solution in terms of ABZSO and $ABZSO_2$ metabolites following oral administration to cattle. Agreeably, the amount of cyclised ABZ products formed following incubation of the zwitterion NTB formulation with ruminal and ileal fluids of both sheep and cattle, under anaerobic conditions, was significantly greater than that formed from the trisamine formulation. It is also possible that the differences in dissolution rate between the micronized suspension and solution may have delayed passage of the untransformed parent drug, allowing more time for bacterial reduction of the suspension in the rumen. Nevertheless, the lower pH of the zwitterion suspension (4.5-5.0), compared with the trisamine solution (8.0-8.8), may have facilitated more rapid bacterial reduction and cyclisation of NTB. pH modification of the zwitterion formulation (final pH= 8.0) resulted in decreased NTB consumption and in lower production of its cyclized metabolites compared to the regular zwitterion formulation. This might indicate a lesser degree of ionization for the zwitterion formulation at ruminal pH compared with the strongly alkaline trisamine formulation, which may facilitate

greater bacterial uptake and more efficient cyclization of NTB into ABZ.

ABZ was found in jugular plasma only after intraruminal administration of NTB to sheep at the highest recommended dose rate (20 mg/kg). As previously proposed (Hennessy *et al.*, 1989; Galtier *et al.*, 1991) a first-pass liver microsomal metabolism may account for the absence, or detection of only trace amounts of ABZ in jugular plasma following treatments with either NTB pro-drug (reported in this thesis) or ABZ itself in sheep and cattle (Prichard *et al.*, 1985; Hennessy *et al.*, 1989; Delatour *et al.*, 1990a). Although the liver microsomal fraction is the primary site for ABZ oxidation, results reported in this thesis demonstrate that both ruminal and intestinal fluids oxidize ABZ to ABZSO and the latter, to a lesser extent, to $ABZSO_2$ under anaerobic conditions; this indicates that ABZ oxidation may take place in the GI tract before absorption, as earlier suggested by Delatour *et al.* (1986). This would explain the rapid detection of ABZSO and $ABZSO_2$ in different GI compartments and plasma at 15-20 min post-treatment with orally administered NTB in cattle. The extent of the exchange surface between plasma and digestive tract in ruminants, and the large volumes of distribution described for ABZ and its metabolites (Galtier *et al.*, 1991), may account for the GI tract playing a major role in the pharmacokinetic behaviour of these anthelmintic molecules.

The peak plasma concentration of $ABZSO_2$ was delayed compared with that of ABZSO in both species, after administration of NTB either orally or parenterally. Similar results have been reported following ABZ treatment in sheep and cattle (Marriner and Bogan, 1980; Prichard *et al.*, 1985; Hennessy *et al.*, 1989). This confirms a relatively slower oxidation rate of ABZSO into $ABZSO_2$ in comparison with ABZ oxidation, and is consistent with the two distinct enzymatic pathways proposed for the liver microsomal biotransformation of ABZ. While the FMO system may be reponsible for the ABZ first step oxidation

(Galtier *et al.*, 1986; Fargetton *et al.*, 1986), the cytochrome P-450 system may be involved in the $ABZSO_2$ formation (Souhaili-El Amri *et al.*, 1988b). In addition, this latter sulphonation reaction could be the consequence of two enzyme systems, one characterized by low affinity and high capacity, the other by high affinity and low capacity.

The identification of the NH_2ABZSO_2 metabolite and its pharmacokinetic characterization for the first time are a further contribution to the understanding of the metabolism and fate of NTB and ABZ-related anthelmintics in ruminants. Low plasma concentrations of NH_2ABZSO_2 were detected between 12-18 h and up to 48 h post-NTB treatment in cattle. Its appearance in plasma, and in abomasal and ileal fluids follows the $ABZSO_2$ plasma peak. The hydrolysis of the $ABZSO_2$ methylcarbamate group forms this polar amino-derivative which is detected in high concentrations in the gut. Conjugated and unconjugated hydroxy-$ABZSO_2$ metabolites, but not NH_2ABZSO_2, have been found in bile of ABZ treated sheep (Hennessy *et al.*, 1989). Under the experimental conditions reported here, neither the incubation with liver microsomes nor the incubation with digestive fluids produced chemical modification of the $ABZSO_2$ metabolite. An amino-derivative metabolite of FBZ generated by hydrolysis of the methylcarbamate group and detected in plasma of FBZ-treated goats (Short *et al.*, 1987a) was not produced by liver microsomes of any species (Short *et al.*, 1988a). These results may indicate that hepatic esterases are not involved in the hydrolysis of BZD methylcarbamate groups to form these amino-metabolites. $ABZSO_2$ is an anthelminticaly inactive metabolite (Lubega and Prichard, 1991b) which does not seem to be converted into a more active molecule; probably, the $ABZSO_2$ hydroxy and amino derivatives are also anthelmintically inactive. Thus, these results are consistent with the notion that the sequential oxidation of BZD thioethers represents a considerable reduction in antiparasite efficacy.

Following oral administration of NTB to cattle, the plasma profiles for ABZSO and $ABZSO_2$, although much lower, reflect the gastrointestinal profiles. Peak concentrations of ABZSO and $ABZSO_2$ were reached at 7-10 h (ABZSO) and at 15-22 h ($ABZSO_2$) post-treatment followed by a well defined concentration declining phase in both plasma and GI compartment concentration-time plots. However, whereas plasma concentrations fell to undetectable levels (30-36 h post-treatment), the profile of these metabolites in the rumen, abomasum and ileum showed an "extra" slow elimination phase that extended to 72 h post-treatment. Such a phenomenon may indicate a reversible plasma-GI tract exchange. This metabolite-distribution process may be driven by a plasma/GI tract pH gradient. A greater plasma/abomasum pH gradient compared with that in the rumen and ileum would produce a strong ion trapping effect which would account for the significantly higher concentrations of ABZSO and $ABZSO_2$ found in the abomasum in comparison with plasma and other GI compartments. A schematic representation of the proposed metabolic and distribution processes occurring after oral or intraruminal administration of NTB pro-drug is shown in Figure 10.1.

The presence of both anthelmintically active metabolites, ABZ and ABZSO, in the abomasum and intestine for an extended period of time with markedly slow elimination phases, is of major relevance in terms of efficacy against GI parasites. However, since ABZ does not reach the peripheral bloodstream, ABZSO may be the only metabolite active against lungworms and tissue-dwelling parasites. ABZ has been shown to have a greater affinity for nematode tubulin than ABZSO (Lubega and Prichard, 1991b); however, the systemic availability of ABZSO and its higher and more sustained concentrations in the digestive tract could partially compensate for this lower affinity for the parasite-target molecule, making it still important in the overall antiparasite activity of NTB, ABZ and ABZSO anthelmintics. Unconjugated ABZSO has been shown to be

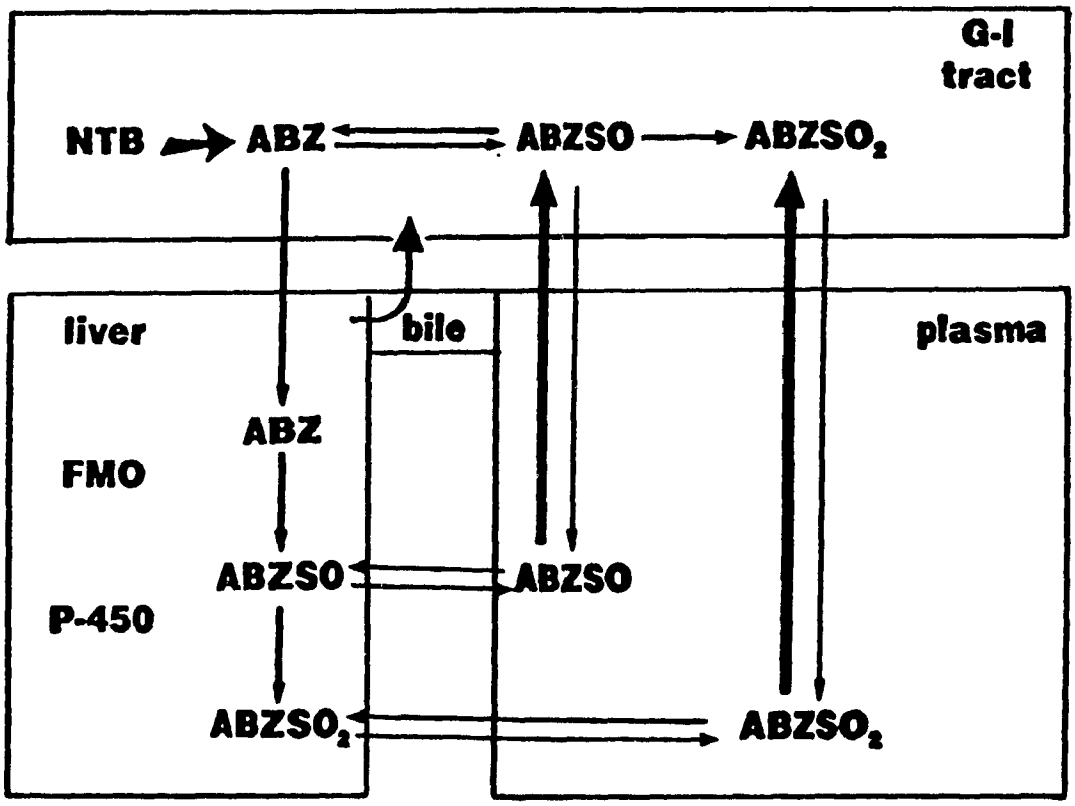

Figure 10.1: Proposed metabolic routes and compartmental distribution processes occurring after oral administration of NTB. FMO=flavin containing moncxygenase system; P-450=cytochrome P-450.

the major bile-eliminated metabolite after administration of ABZ to sheep (Hennessy *et al.*, 1989). As well, significant amounts of ABZSO and $ABZSO_2$, but not ABZ, have been found in adult *Fasciola hepatica* collected from the bile ducts of ABZ-treated sheep (Fetterer and Rew, 1984). Furthermore, ABZSO can be reduced back to ABZ by ruminal and intestinal flora and may act as a source of ABZ in the gastrointestinal tract. Although it has been previously suggested that the conversion of ABZ into ABZSO could be reversible in the liver (Gyurik *et al.*, 1981), results reported in this thesis demonstrated that neither sheep nor cattle liver microsomes were able to reduce ABZSO under the experimental conditions *in vitro*. Metabolic reduction may be of prime importance for the antiparasite efficacy of BZD thioethers and it probably only occurs in the GI tract. The *in vitro* evidence of ABZSO reduction by GI fluids, correlates with the detection of ABZ in different digestive compartments for 72 h post-NTB administration to cattle when the parent NTB compound has been completely removed from the digestive tract by 12 to 18 h post-administration. The reversible plasma-GI tract exchange facilitates a pH gradient-mediated concentration of ABZSO in the GI compartments, which could act as a source of ABZ. Since ABZ has a greater affinity for parasite tubulin than ABZSO (Lubega and Prichard, 1991b), this bacteria-mediated reduction may have significant importance for efficacy against GI parasites. As with ABZ, the thioether BZD anthelmintic FBZ is rapidly and extensively converted *in vivo* into its sulphoxide, OFZ (Short *et al.*, 1987a,b), which also has lower tubulin affinity than the parent thioether (Lubega and Prichard, 1991b). Reduction of OFZ to FBZ has been shown to occur in the ruminal fluid of sheep and cattle (Beretta *et al.*, 1987). Thus, the high antiparasite efficacy of ABZSO and OFZ against GI parasites, compared with tissue-dwelling parasites, may depend on this bacterial reduction of the sulphoxide to the more pharmacologically active thioethers. The efficacy of ABZSO against sheep and cattle nematodes *in vivo* is similar to that of NTB or ABZ (McKellar and Scott,

1990).

Regardless of the route of administration, formulation and delivery system used for the administration of both NTB pro-drug (experiments described here) and ABZ (Marriner and Bogan, 1980; Prichard *et al.*, 1985; Delatour *et al.*, 1990a), ABZSO and $ABZSO_2$ were the major metabolites recovered in plasma of sheep and cattle. However, pronounced differences in the plasma disposition and in the bioavailability of these metabolites between sheep and cattle have been shown. These species differences can be appreciated in Figure 10.2 which shows the comparative plasma profiles for the ABZSO and $ABZSO_2$ metabolites obtained after the administration of the same NTB formulation at 20 mg/kg to sheep (intraruminal) and cattle (oral). The ratio of AUC for $ABZSO_2$/ABZSO obtained after the different experiments in cattle (2.80-2.92) was higher than that obtained in sheep (0.35) treated with NTB at the same dose rate; these results are consistent with those obtained after a single dose treatment with ABZ in both species (Marriner and Bogan, 1980; Hennessy *et al.*, 1989; Delatour *et al.*, 1990a). This ratio is a good indicator of the net oxidation rates. After NTB treatment, the bioavailability of the active ABZSO metabolite was significantly higher and its elimination half-life and mean time of residence were markedly longer in sheep compared with cattle (Figure 10.3). These differences were initially attributed to a differential pattern of liver biotransformation between species, in which a greater capacity for BZD oxidation in cattle than in sheep could be expected. Nonetheless, findings described in this thesis demonstrate that the rate of ABZ sulphoxidation by sheep liver microsomes was significantly higher than that of cattle liver microsomes and there was no difference in the formation of $ABZSO_2$ between species. Surprisingly, the differential pattern of GI metabolism of these BZD compounds between species reported in Chapter 5, may explain the sheep/cattle differences in pharmacokinetic behaviour and in the required dose rates for optimal clinical efficacy. While

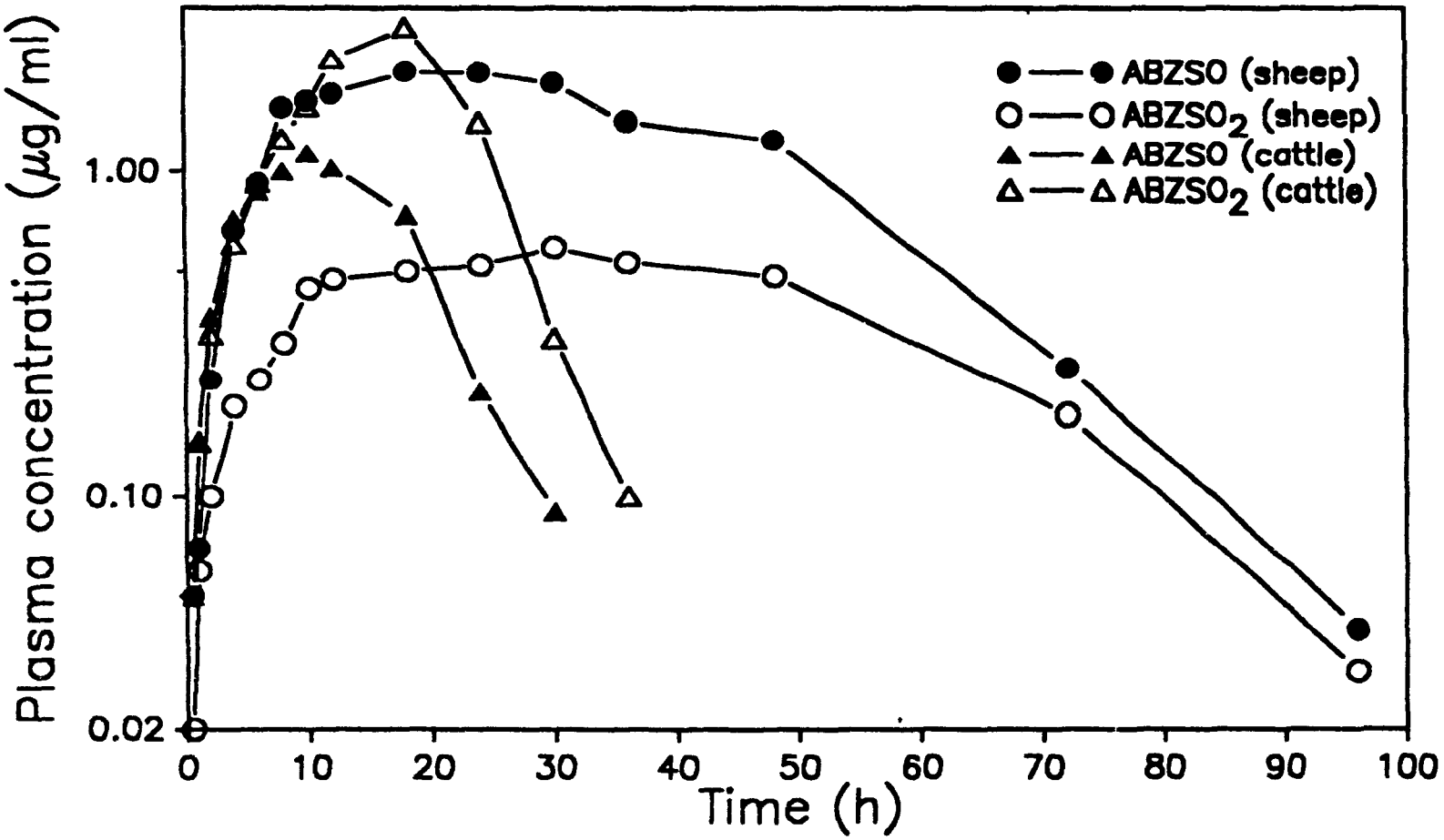

Figure 10.2: Comparative plasma profiles for ABZSO and $ABZSO_2$ obtained after administration of NTB (20 mg/kg) to sheep (IR) and cattle (oral).

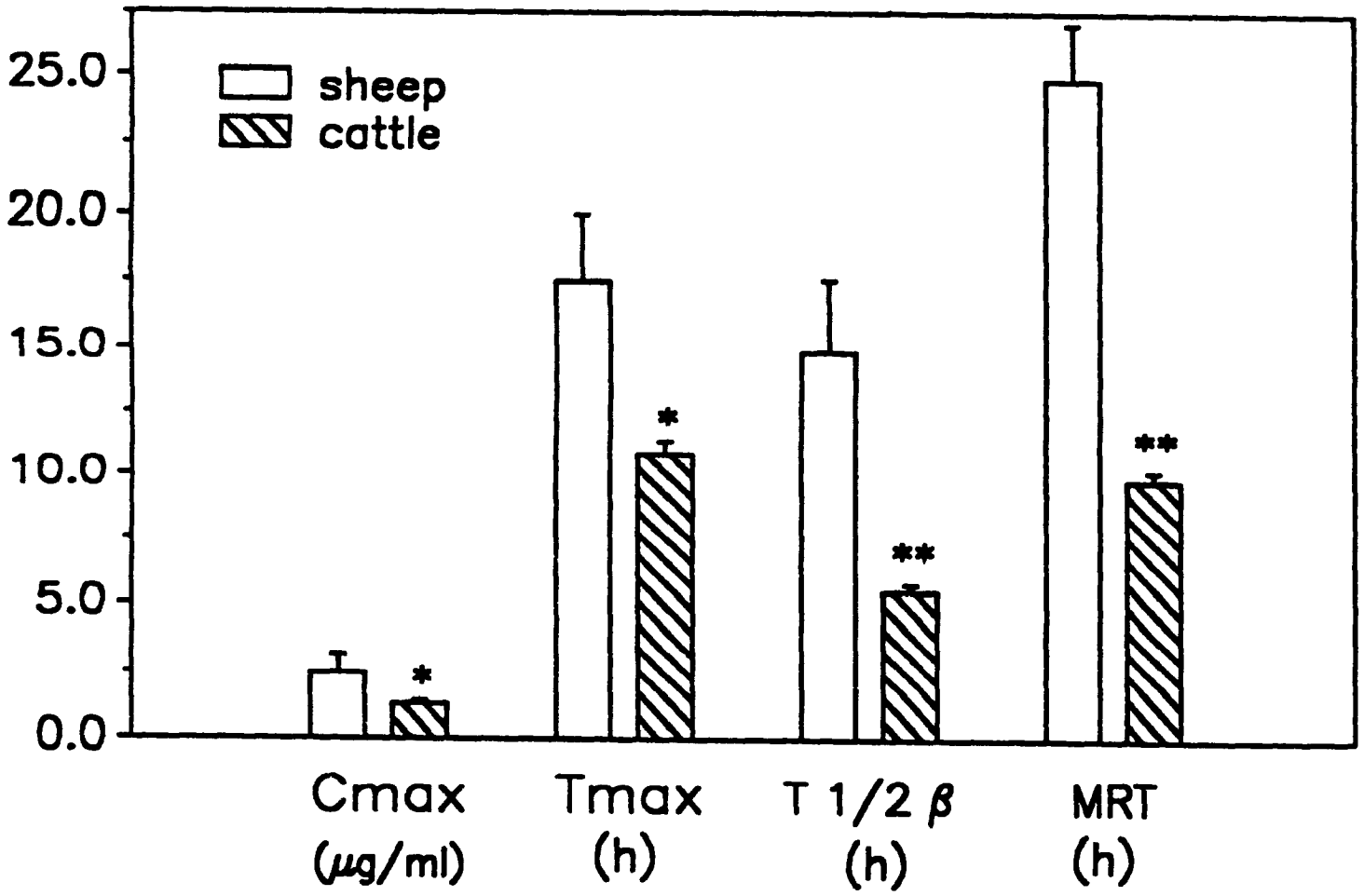

Figure 10.3: Comparative disposition kinetics for ABZSO in sheep and cattle obtained after administration of NTB (20 mg/kg). Statistically different from the value obtained for sheep at $P < 0.05$ (*) and $P < 0.01$ (**).

oxidizing activity was greater in GI fluids of cattle, the reducing activity (conversion of ABZSO back to ABZ) was prevalent in the ruminal and ileal fluids of sheep. This may account for the prolonged elimination half-life and extended presence of ABZSO in sheep plasma for 100 h post-NTB treatment, while in cattle ABZSO plasma removal is completed within 30-36 h post-treatment (Figure 10.2). The greater capacity of sheep GI fluids to reduce ABZSO back to ABZ, compared with that of cattle also accounts for the higher $ABZSO_2$/ABZSO plasma ratio observed in cattle compared to sheep following both NTB and ABZ treatments.

10.2.: MODULATION OF LIVER BIOTRANSFORMATION

The intrinsic anthelmintic action of the BZD molecule on the parasite is based on the disruption of basic cell functions, that require a sustained presence of the active drug at the site of parasite location. The extension of the residence time of anthelmintically active BZD moieties in the bloodstream is relevant for their efficacy, especially against parasites located in tissues to which the access of the active drug is difficult.

The sequential metabolic oxidations of BZD and pro-BZD thioethers in the host leads to a more polar and less active metabolites. The oxidized sulphur atom present in both sulphoxides (S=O) and sulphones (O-S-O) tends to make them more polar than the parent thioether (ABZ). The more polar metabolites are likely to be concentrated in the extracellular space, facilitating a faster elimination. In terms of parasite uptake and binding to tubulin, the parent thioethers (ABZ, FBZ) are more potent than the sulphoxides metabolites, while the sulphones are inactive (Fetterer, 1986; Lacey, 1990; Lubega and Prichard, 1991b). Therefore the oxidation of the parent thioether into its sulphoxide, and even more so into its sulphone, results in a considerable reduction in anthelmintic efficacy.

Although the oxidation of ABZ at the level of the sulphur atom can occur in the GI tract, a sequential liver microsomal oxidation is quantitatively the main biotransformation process for these thioether anthelmintics; this is a very efficient metabolic process and accounts for the absence, or detection of only trace amounts, of ABZ in the bloodstream. *In vivo* interference with the liver oxidation pattern resulted in pronounced modifications to the pharmacokinetic behaviour of anthelmintically-active BZD metabolites, which could lead to improved clinical efficacy as a result of higher levels of active metabolites being presented to the parasite for longer periods of time. Increased plasma concentrations of active moieties would increase the duration of the plasma-tissue recycling process, and the concentration of active drug traversing the GI mucosa to the habitats of the most important pathologic parasites in ruminants (largely in the abomasum and small intestine).

The co-administration of parenterally given NTB with different oxidation-impairing compounds in sheep resulted in a modified pattern of liver oxidation. While the presence of quinine did not alter the disposition of ABZSO, the co-administration of NTB with both metyrapone (MTP) and methimazole (MTZ) resulted in an important increase in the plasma profiles of this metabolite. Such an effect may be relevant to improving the bioavailability of active metabolites obtained after parenteral administration of NTB in sheep and cattle. The lower $ABZSO_2$/ABZSO ratios obtained for both NTB+MTP and NTB+MTZ treatments are also indicators of an altered liver oxidation pattern. While the FMO system has been shown to be responsible for microsomal sulphoxidation of ABZ into ABZSO (Galtier *et al.*, 1985), the cytochrome P-450 system, in a biphasic reaction, is thought to be involved in $ABZSO_2$ formation (Souhaili-El Amri *et al.*, 1988b). By selective blockage of oxygen binding sites, MTP is a potent inhibitor of cytochrome P-450 enzymatic activity (Tynes and Hodgson, 1983). It is likely that the observed changes to the pharmacokinetics

of NTB metabolites in the presence of MTP are due to an in vivo interference by this compound in this pathway, as is evident by the significantly increased ABZSO AUC and significantly decreased $ABZSO_2$ AUC.

Important modifications to the disposition kinetics of ABZSO were obtained after the co-administration of NTB, given both subcutaneously and orally, and MTZ in cattle, compared with the treatments with NTB alone. While the changes in AUC were not as pronounced as they were in sheep, the overall elimination half-lives of ABZSO were between 150 and 320% longer and the MRT was two-fold longer in the presence of MTZ. In view of the fact that MTZ is known to be a substrate for the FMO (Tynes and Hodgson, 1983) and the fact that this system would be primarily involved in the sulphoxidation of ABZ to ABZSO (Galtier *et al.*, 1986), it is highly likely that the observed modifications in the ABZSO pharmacokinetics are due to competition between MTZ and ABZ in the FMO pathway. Thus, an inhibitory effect of MTZ at this microsomal enzymatic level may not dramatically affect the plasma concentration (AUC, C_{max}), but may dramatically affect the disposition kinetics and residence time of ABZSO after both SC and oral administration of NTB in cattle.

In the experiments on modulation of NTB metabolism in cattle and sheep, treated either parenterally or orally at 7.5 mg/kg, it was not feasible to assess the influence of the oxidative impairing effect on the profiles of ABZ parent drug, because this did not reach detectable concentrations in plasma. ABZ parent drug was only found in plasma following the intraruminal treatment with NTB at 20 mg/kg in sheep. The co-administration of NTB IR at the highest dose rate with MTZ given IM at 1.5 mg/kg, resulted in a notably improved pharmacokinetic profile for ABZ. The significantly longer $T_{\frac{1}{2}\beta}$ and MRT, and significantly higher AUC (138%) for ABZ would indicate an MTZ interference in the enzymatic pathway responsible for the ABZ sulphoxidation. Similarly, this

concomitant treatment of NTB (IR) with MTZ (IM) in sheep caused dramatic changes on the disposition kinetics of the anthelmintically active ABZSO metabolite. A markedly different terminal slope (ß) resulted in an elimination half-life four-fold longer for this active ABZSO metabolite following the co-administration with MTZ. The overall MRT was 252% longer and AUMC and AUC were significantly higher when MTZ was co-administered with NTB given intraruminally at the higher recommended dose rate compared with the administration of NTB alone.

Significant modifications to the pharmacokinetic behaviour of the ABZSO metabolite were observed when MTZ, given either orally or intramuscularly at 3 mg/kg, was co-administered with oral NTB given at 7.5 mg/kg in sheep. In the presence of MTZ, given by both routes, there was a pronounced change in the elimination phase of ABZSO which resulted in a longer elimination half-life and significantly higher AUC and AUMC, compared with the oral administration of NTB alone.

MTZ, a substrate for the FMO, competitively inhibits the metabolism of other potential substrates of this enzymatic complex (Tynes and Hodgson, 1983). The FMO system is probably of primary importance in the conversion of ABZ to ABZSO in sheep (Galtier *et al.*, 1986). Thus, it is likely that the observed pronounced modifications to the bioavailability and disposition kinetics of ABZ parent drug (IR treatment) and ABZSO (IR and oral treatment with NTB) in sheep are due to substrate competition between MTZ and ABZ for the FMO pathway. Thus, a reduced rate of oxidation could attenuate the liver first-pass phenomenon resulting in an improved plasma pharmacokinetic profile for ABZ. Although ABZ did not reach detectable concentrations in some of the treatments, such a phenomenon was probably sufficient to modify the plasma disposition kinetics of ABZSO and to increase the ratio of AUC ABZSO/$ABZSO_2$. In addition, a delayed ABZ oxidation resulted in an extended time of ABZSO formation which would explain the

substantial modifications observed in the bioavailability and disposition kinetics of this active metabolite. Furthermore, the efficient reduction of ABZSO back to ABZ in the GI tract of sheep demonstrated in Chapter 5 may cooperate for these MTZ-induced pharmacokinetic changes. The changes observed on the pharmacokinetic behaviour of $ABZSO_2$ may also be a consequence of this retarded biotransformation process.

It has been also reported that the biotransformation of MTZ itself may produce some reactive metabolites which bind avidly to the cytochrome P-450 drug binding site, decreasing its enzymatic activity (Kedderis and Rickert, 1985). Since the cytochrome P-450 system seems to be responsible for liver microsomal oxidation of ABZSO to $ABZSO_2$ (Souhaili-El Amri *et al.*, 1988b), at least in some species, it is also likely that MTZ interferes at this enzymatic level and thereby diminishes the activity of this mixed function oxidase system. Thus, a decreased metabolic rate for the conversion of ABZSO into $ABZSO_2$ could also account for the marked changes in ABZSO disposition kinetics in the presence of MTZ. Such an effect might be consistent with the improved pharmacokinetic profile of ABZSO and with the delayed detection of $ABZSO_2$ in plasma obtained in the concomitant NTB+MTZ treatments in sheep.

In the pig, the cytochrome P-450 system has been shown to participate in the first step of ABZ oxidation into ABZSO (Souhaili-El Amri *et al.*, 1987). In other species such an involvement is unclear and it might be an alternative explanation for some of the MTZ-mediated changes to the metabolism and pharmacokinetics of ABZ. The involvement of both the FMO and cytochrome P-450 microsomal systems has been reported for the oxidation of thiobenzamidine and other sulphur-containing xenobiotics (Tynes and Hodgson, 1983). The results of the *in vitro* metabolism experiments clearly showed that MTZ primarily acts on the FMO system. MTZ drastically inhibited the FMO-mediated NADPH-dependent ABZ sulphoxidation by liver microsomes of sheep and cattle. However, in sheep

microsomes this MTZ-mediated inhibition was potentiated by *n*-octylamine, a typical cytochrome P-450 inhibitor, which might indicate a complementary participation of this system in the first step of ABZ oxidation. Depending on the nucleophilicity of the sulphur atom of the substrate (Souhaili-El Amri *et al.*, 1987), a balance between the participation of the FMO and cytochrome P-450 systems could be established, which in the case of ABZ may also be dependent on the animal species under investigation. In addition, two different enantiomers of ABZSO have recently been identified in plasma by HPLC chiral separation, following administration of ABZ to different species (Delatour *et al.*, 1990b,c, 1991a,b). Variation in the proportions of these two ABZSO enantiomers between species has been related to relative differences in the involvement of the FMO and cytochrome P-450 systems in this first step of ABZ oxidation. Although the modifications to the kinetics and bioavailability of ABZ metabolites induced by MTZ could be related to both, a primary inhibition of the FMO system and a complementary impairment on the P-450 activity, it is necessary to know more about the precise pathways involved in this biotransformation process in different species, before further speculation on the potential *in vivo* site(s) of MTZ inhibition can be made.

MTZ is a safe and inexpensive compound, regularly used in human and veterinary medicine. While other thionamide drugs are traditionally administered in several daily doses to achieve sustained antithyroid effect, only a single dose of MTZ is necessary to achieve the same pharmacological effect (Roti *et al.*, 1989). High bioavailability and extensive tissue distribution (Jansson *et al.*, 1985) may ensure that a single dose of MTZ will result in successful inhibition of liver ABZ oxidation. Surprisingly, the MTZ-induced changes on ABZ metabolites pharmacokinetics did not increase by using a higher MTZ dose rate. It seems likely that following a sufficient MTZ concentration to establish the metabolic competition, the impairment of oxidation depends on the amount

of ABZ substrate reaching the liver. Thus, the magnitude of the pharmacokinetic changes for ABZ metabolites were proportionally greater following the administration of NTB at 20 mg/kg because more ABZ substrate reach the liver to establish the competition with MTZ, in comparison to the oral treatment at 7.5 mg/kg. The route of administration did not influence MTZ-induced changes to the pharmacokinetics of NTB/ABZ metabolites, which may indicate similar MTZ absorption and bioavailability from both oral and IM routes. The potential use of MTZ by oral drench to improve BZD or pro-BZD clinical efficacy may lead to some practical advantages.

Undoubtedly, the presence of MTZ induces changes on the disposition kinetics of ABZ metabolites in both sheep and cattle. However, using the same MTZ dose rate and route of administration, the modifications to the pharmacokinetic behaviour of the NTB/ABZ metabolites, especially to ABZSO, were less pronounced in cattle than in sheep. Consistently, MTZ and ABZ were better substrates for the sheep FMO system compared with that of cattle. The inhibitory effect of MTZ on ABZ sulphoxidation was significantly lower for cattle microsomes compared to those of sheep at the same MTZ concentration. It is evident that MTZ-mediated inhibition of ABZ microsomal oxidation, both *in vitro* and *in vivo*, is more efficient in sheep than in cattle. These results must be taken into consideration to maximize the potentiating effect of this approach.

The improved pharmacokinetic profile and residence time for the anthelmintically-active metabolites, obtained in the concomitant treatment with MTZ in both sheep and cattle may be highly important in terms of efficacy for NTB, ABZ and other BZD thioether anthelmintics. Appendix I shows the results of a clinical efficacy trial in cattle, which was designed to assess the effects of this "metabolism modulation" approach on anthelmintic efficacy. The clinical efficacy against GI parasites of either NTB pro-drug or ABZ (oral treatments) with

and without co-administration with MTZ (given orally at 3 mg/kg), was compared in naturally-infected cattle. The concomitant treatment with MTZ increased the efficacy of both NTB (10 mg/kg) and ABZ (2.5 mg/kg) against arrested (EL_4) larvae of *Ostertagia ostertagi*, and against total adult GI nematodes. The potentiation effect of MTZ was conclusively shown by the fact that the efficacy of ABZ given at 2.5 mg/kg with MTZ, was equivalent to that obtained when ABZ was given alone at 10 mg/kg. These results clearly demonstrate the practical relevance of this pharmacokinetic interaction on parasite control.

The outcome of this research project is a major contribution to the pharmacology of anthelmintic drugs. The pharmacokinetic behaviour and pattern of biotransformation for NTB pro-drug and its ABZ metabolites have been extensively studied. The influence of drug formulation, route of administration and animal species on the pharmacokinetic patterns of these molecules has been determined. A further understanding of the liver microsomal and GI metabolism of BZD and pro-BZD anthelmintics has been achieved. Finally, it has been demonstrated for the first time that the *in vivo* modulation of the liver biotransformation process for BZD/pro-BZD thioethers, leads to pronounced changes on the bioavailability and disposition kinetics of anthelmintically-active BZD metabolites which result in an improved anthelmintic efficacy. The approach is a safe drug combination, which may have an important impact on parasite control. It may lead to improved clinical efficacy against the most difficult parasites to control in livestock, including BZD-resistant strains. Since MTZ is an inexpensive compound, a reduction in the cost of treatment by using lower dose rates of the active anthelmintic molecule may be also feasible. In addition, this could be an interesting approach to the design of new strategies for the control of filarial nematodes and different tissue-dwelling parasites in man and animals, unsolved problems in antiparasite therapy. These findings are now under

consideration for an industrial patent. Nonetheless, further research is required to optimize the practical use of the basic pharmacological approach.

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APPENDIX I

ASSESSMENT OF ANTHELMINTIC EFFICACY FOR THE CO-ADMINISTRATION OF NETOBIMIN AND ALBENDAZOLE WITH METHIMAZOLE IN CATTLE[2]

PURPOSE

To evaluate the anthelmintic efficacy of netobimin (NTB) and albendazole (ABZ) given either alone or co-administered with methimazole (MTZ), against gastrointestinal (GI) nematodes in naturally-infected cattle.

MATERIALS AND METHODS

Fourty-two beef male and female calves, weighing between 97 and 154 kg were used in this efficacy trial. The animals were naturally infected with nematodes parasites and they were randomly allocated to form six comparable groups of six calves each, based on body weights and pre-treatment egg counts. Treatments were given as follows:

Group 1: Animals received NTB (zwitterion suspension, 150 mg/ml) orally at 10 mg/kg.

Group 2: Animals received the same NTB treatment as Group 1, plus MTZ (75 mg/ml in saline solution) given orally at 3 mg/kg immediately after the NTB treatment.

Group 3: Animals were treated with ABZ (oral suspension, 113.6 mg/ml) orally at 2.5 mg/kg.

Group 4: Animals received the treatment indicated for Group 3, plus MTZ (75 mg/ml in saline solution) given orally at 3 mg/kg, immediately after the administration of ABZ.

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2 This study was carried out in collaboration with Dr. T.A. Yazwinski, Department of Animal & Poultry Sciences, University of Arkansas, and Schering-Plough Corporation, Animal Health Division, Kenilworth, NJ. I have only participated in the experimental design, analysis and interpretation of results.

Group 5: Animals were treated with ABZ (oral suspension, 113.6 mg/ml) orally at 10 mg/kg.

Group 6: Animals in this group were considered as control, and they were treated with MTZ (75 mg/ml in saline solution) at 3 mg/kg by oral drench.

Two pre-treatment (on the day of adquisition and on the day of treatment) and one post-treatment (on the day of necropsy) faecal samples were collected from each calf. The number of eggs per gram of faeces (epg) was determined using Wisconsin technique. Equal number of calves were necropsied from each experimental group on days 7-10 after treatment. Necropsy, recovery of larval and adult stages, worm counts and identification of GI nematodes were done according to procedures described within the "Guidelines for the Evaluation of Bovine Anthelmintics", F.D.A., Bureau of Veterinary Medicine.

RESULTS

Wilcoxon rank sum test was used to establish the level of significance among different treatments. A value of $P< 0.05$ was considered statistically different. All the treated groups resulted in worm counts significantly lower than the control group for the different species and genera recovered. Group 4 (ABZ 2.5 mg/kg+MTZ) resulted in significantly lower ($P< 0.05$) counts for arrested *Ostertagia ostertagi* (EL_4), total *Ostertagia spp.*, and total GI worms burden, compared with Group 3 (ABZ 2.5 mg/kg alone). The mean counts ± SEM for EL_4, total *Ostertagia spp.*, and total GI worm burden for the diferent groups are shown in Table I.1. Standard errors are large, however, this is usual with natural infections of nematode in cattle with a group size of six animals. Table I.2 summarized the percentages of efficacy for the different treatments compared with the control group.

Table I.1: Mean nematode counts for control and NTB/ABZ (with and without MTZ) treated groups.

	WORM BURDENS		
Treatment	**EL_4**	**Total Oster.spp.**	**Total GI Nematodes**
Group 1 NTB 10mg/kg	15381 ± 10170	18214 ± 11990	18221 ±11991
Group 2 NTB 10 mg/kg+MTZ	7586 ± 5875	10451 ± 7671	10586 ± 7643
Group 3 ABZ 2.5 mg/kg	10461 ± 5477	13428 ± 6227	15421 ± 5840
Group 4 ABZ 2.5 mg/kg+MTZ	2675 ± 2441*	4706 ± 4088*	5143 ± 4001*
Group 5 ABZ 10 mg/kg	4583 ± 2857	5490 ± 3320	5534 ± 3309
Group 6 (control)	21767 ± 6611	29366 ± 7498	41868 ± 9947

Data are presented as mean values ± SEM with n= 6

All the values were statistically different from the values obtained for the control group when analyzed by Wilcoxon rank test

*: Significantly different from the value obtained for Group 3 at $P< 0.05$

Table I.2: Percentage of anthelmintic efficacy against gastrointestinal nematodes obtained after administration of NTB and ABZ either alone or co-administered with MTZ in cattle.

Treatment	EL_4	Total Oster. spp.	Total GI Nematodes
Group 1 NTB 10mg/kg	29%	38%	56%
Group 2 NTB 10 mg/kg+MTZ	65%	64%	75%
Group 3 ABZ 2.5 mg/kg	52%	54%	63%
Group 4 ABZ 2.5 mg/kg+MTZ	88%	84%	88%
Group 5 ABZ 10 mg/kg	79%	81%	87%

% efficacy= post-treatment mean worm burden of treated groups compared to post-treatment mean worm burden of control group.

APPENDIX II

ABBREVIATIONS

Drugs and metabolites

BZD: benzimidazoles

pro-BZD: pro-benzimidazoles

NTB: netobimin

ABZ: albendazole

ABZSO: albendazole sulphoxide

$ABZSO_2$: albendazole sulphone

NH_2ABZSO_2: amino albendazole sulphone

OHABZSO: hydroxy albendazole sulphoxide

$OHABZSO_2$: hydroxy albendazole sulphone

FBZ: fenbendazole

OFZ: oxfendazole (fenbendazole sulphoxide)

$FBZSO_2$: fenbendazole sulphone

NH_2FBZ: amino fenbendazole

FBT: febantel

TPT: thiophanate

TBZ: thiabendazole

CBZ: cambendazole

PBZ: parbendazole

MBZ: mebendazole

FLBZ: flubendazole

CIBZ: ciclobendazole

OBZ: oxibendazole

LBZ: luxabendazole

TCBZ: triclabendazole

MTP: metyrapone

QNE: quinine

MTZ: methimazole

Pharmacokinetic parameters (units)

Cp: plasma concentration (μg/ml)

B: concentration at time zero extrapolated from the elimination phase (μg/ml)

e: base of the natural logarithm
Kab: absorption rate constant (h^{-1})
Kf: metabolite formation rate constant (h^{-1})
ß: elimination rate constant (h^{-1})
T ½ ab: absorption half-life (h)
T ½ for: metabolite formation half-life (h)
T ½ ß: elimination half-life (h)
Cmax: maximum plasma concentration (µg/ml)
Tmax: time of the maximum plasma concentration (h)
AUC: area under the concentration-time curve (µg.h/ml)
AUMC: area under the first moment of the concentration-time curve ($\mu g.h^2/ml$)
F: bioavailability
Cl_b: total body clearance (ml/h/kg)
Vd_{area}: volume of distribution (method of the area) (l/kg)
MRT: mean residence time (h)

Miscellaneous

HPLC: high performance liquid chromatography
trisamine: trisamine salt formulation of netobimin
zwitterion: zwitterion formulation of netobimin
d.l.: detection limit
% rec.: percentage of recovery
r: correlation coefficient
ANOVA: analysis of variance
x: arithmetic mean
SEM: standard error of the mean
NA: not applicable
NS: not significantly different
mg/kg: milligrams per kilogram of body weight
GI: gastrointestinal
IR: intraruminal
SC: subcutaneous
IM: intramuscular
FMO: flavin-containing monooxigenase system
P-450: cytochrome P-450 system
NADPH: reduced nicotinamide-adenine dinucleotide phosphate

APPENDIX III

PUBLICATIONS, CONFERENCE PRESENTATIONS AND AWARD ARISING FROM THIS THESIS RESEARCH

Publications

Lanusse, C., Ranjan, S., Prichard, R. (1990). Comparison of pharmacokinetic variables for two injectable formulations of netobimin administered to calves. *American Journal of Veterinary Research*, **51** (9), 1459-1463.

Lanusse, C., Prichard, R. (1990). Pharmacokinetic behaviour of netobimin and its metabolites in sheep. *Journal of Veterinary Pharmacology and Therapeutics*, **13** (2), 170-178.

Lanusse, C., Trudeau, C., Ranjan, S., Prichard, R. (1991). Pharmacokinetic profiles of netobimin metabolites after oral administration of zwitterion and trisamine formulations of netobimin to cattle. *Journal of Veterinary Pharmacology and Therapeutics*, **14** (1), 101-108.

Lanusse, C., Prichard, R. Effects of methimazole on the kinetics of netobimin metabolites in cattle. *Xenobiotica*, in press.

Lanusse, C., Prichard, R. Enhancement of plasma concentrations of albendazole sulphoxide in sheep following co-administration of parenteral netobimin and liver oxidase inhibitors. *Research in Veterinary Science*, in press.

Lanusse, C., Prichard, R. Methimazole increases plasma concentrations of albendazole metabolites of netobimin in sheep. *Biopharmaceutics and Drug Disposition*, in press.

Lanusse, C., Nare, B., Prichard, R. Comparative sulphoxidation of albendazole by sheep and cattle liver microsomes. *Drug Metabolism and Disposition*, submitted.

Lanusse, C., Nare, B., Gascon, L., Prichard, R. Bioconversion of netobimin pro-drug by gastrointestinal fluids of ruminants. *European Journal of Drug Metabolism and Pharmacokinetics*, submitted.

Lanusse, C., Gascon, L., Prichard, R. Methimazole-mediated modulation of netobimin biotransformation in sheep: a pharmacokinetic assessment. *Journal of Veterinary Pharmacology and Therapeutics*, in press.

Lanusse, C., Nare, B., Gascon, L., Prichard, R. Metabolism of albendazole and albendazole sulphoxide by ruminal and ileal fluids of sheep and cattle. *Xenobiotica*, submitted.

Lanusse, C., Gascon, L., Prichard, R. Gastrointestinal distribution of albendazole metabolites following netobimin administration to cattle: relationship with plasma disposition kinetics. *Journal of Veterinary Pharmacology and Therapeutics*, submitted.

Conference presentations

Lanusse, C., Prichard, R. (1989). Pharmacokinetic profiles of albendazole metabolites after IR and SC administration of netobimin in sheep. *Annual Meeting of the American Association of Veterinary Parasitologists*, Orlando, Florida, USA.

Lanusse, C., Prichard, R. (1989). Biotransformation of netobimin in sheep. *Annual Meeting of the Canadian Veterinary Medical Association*, Ottawa, Canada.

Lanusse, C., Gascon, L., Trudeau, C., Ranjan, S., Prichard, R. (1990). Cinétique plasmatique du netobimin et ses principaux metabolites chez les ruminants. *58e Congrès de l'Association Canadienne Française pour l'Avancement des Sciences*, Québec, Canada.

Lanusse, C., Ranjan, S., Trudeau, C., Prichard, R. (1990). Influence of formulation and route of administration on the pharmacokinetics of netobimin and its metabolites in cattle. *Annual Meeting of the American Association of Veterinary Parasitologists*, San Antonio, Texas, USA.

Lanusse, C., Prichard, R. (1990). Comparative disposition and bioavailability of albendazole metabolites after netobimin administration in ruminants. *VII International Congress of Parasitology*, Paris, France.

Lanusse, C., Gascon, L., Trudeau, C., Prichard, R. (1991). Characterization of the plasma-gastrointestinal exchange for albendazole metabolites after oral administration of netobimin to cattle. *Annual Meeting of the American Association of Veterinary Parasitologists*, Seattle, Washington, USA.

Nare, B., **Lanusse, C.**, Gascon, L., Prichard, R. (1991). Relevance of gastrointestinal metabolism on the efficacy of benzimidazole anthelmintics. *American Society for Parasitology*, Wisconsin, USA.

Lanusse, C., Gascon, L., Prichard, R. (1991). Plasma disposition and compartmental distribution of albendazole metabolites after netobimin administration to cattle. 5th *Congress of the European Association for Veterinary Pharmacology and Toxicology*, Copenhague, Denmark.

Award

The 1991 Hoechst-Roussel Agri-Vet Company Graduate Student Award of the American Association of Veterinary Parasitologists for overall research contribution during graduate studies in the field of Veterinary Parasitology.