CANINE HEPATIC SLICES AS A MODEL FOR STUDYING DRUG TOXICITY AND METABOLISM

A Dissertation

by

MAYA MILLICENT SCOTT

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2005

Major Subject: Toxicology

CANINE HEPATIC SLICES AS A MODEL FOR STUDYING DRUG TOXICITY AND METABOLISM

A Dissertation

by

MAYA MILLICENT SCOTT

Submitted to Texas A&M University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Approved as to style and content by:

Dawn M. Boothe (Co-Chair of Committee)	Stephen H. Safe (Co-Chair of Committee)
Deborah T. Kochevar (Member)	Karen E. Russell (Member)
Timothy D. Phillips (Chair of Toxicology Faculty)	Glen A. Laine (Head of Department)

May 2005

Major Subject: Toxicology

ABSTRACT

Canine Hepatic Slices as a Model for Studying Drug Toxicity and Metabolism. (May 2005)
Maya Millicent Scott, B.S., University of Arkansas;
D.V.M., Oklahoma State University
Co-Chairs of Advisory Committee: Dr. Dawn M. Boothe
Dr. Stephen H. Safe

Tissue slices can be made from organs, such as liver, kidney, brain, and heart, and from various species including humans, dogs, non-human primates, rats and mice. It has been demonstrated that human and rat liver slices are viable for up to 2 days, and liver slices have been extensively used as an *in vitro* method to study hepatic drug metabolism and toxicity in humans. The objective of this study was to determine the utility of canine hepatic slices as an *in vitro* model for studying drug metabolism and hepatotoxicity in dogs. Canine hepatic slices were incubated in media containing various drugs to determine the hepatotoxicity of the agents and the ability of the slices to metabolize the drugs. The toxicity of phenobarbital, primidone, lidocaine and carprofen to canine hepatic slices was assessed by determining changes in supernatant concentrations of potassium ions and adenosine triphosphate (ATP); histologic lesions were determined as necrosis, extent of vacuolation and severity of vacuolation. Xenobiotic drug metabolizing enzymatic activity was investigated by determining the metabolism of lidocaine to monoethylglycinexylidide (MEGX), and administration of phenobarbital plus primidone was used as a positive control for hepatotoxicity in dogs. The function of drug-metabolizing enzymes was demonstrated by the successful metabolism of lidocaine to MEGX. Carprofen, a drug which causes idiosyncratic hepatic disease in dogs, did not show any hepatotoxicity at concentrations of 10, 50 and 100 µg/ml using potassium ion levels, ATP concentrations and histology as indicators of hepatotoxicity. Slices incubated in media without drug showed no toxicity over 24 hours based on potassium ion and ATP supernatant concentrations while significant increases in histologic lesions were noted at 8, 12 and 24 hours. Canine hepatic slices were a useful model for examining drug metabolism and toxicity for up to 24 hours.

Dedicated to
my mother, Julie, for always being there
my father, Freddie, for unending support
my brother, Darren, for making me smile
and my cat, Peanut, for just being

Just as Piglet always needs Pooh, I will always need you.

Piglet sidled up to Pooh from behind. "Pooh!" he whispered.
"Yes, Piglet?"
"Nothing," said Piglet, taking Pooh's paw. "I just wanted to be sure of you."
The House at Pooh Corner ~ A.A. Milne

ACKNOWLEDGMENTS

I would like to acknowledge the financial support of the U.S. Pharmacopeia Fellowship Program.

I would like to express gratitude to my family and friends for their support during my years at Texas A&M University. I would like to thank the members of my committee for their help with my research project. In addition, I acknowledge John Mackie and Ilona Petrikovics for their expertise. The following people deserve special acknowledgment—Julie Scott, Freddie Scott, Darren Scott, Sarah Jones, Scott Wilkie, Deborah Kochevar, Jeremy Perkins, J.T. Gasson, Paul Spencer, Nicole Ramlachan and Tiffany Finch—without their help and support, this dissertation would not have been possible. Thank you for making my years at A&M interesting and memorable. Though there were many obstacles, the final prize was worth all the effort. Finally, I would like to pay special thanks to my pets (past, present and future)—their unconditional love makes my life forever rich and complete.

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

FIGURE Page

FIGURE Page

FIGURE Page

FIGURE Page

FIGURE Page

LIST OF TABLES

TABLE Page

TABLE Page

TABLE Page

CHAPTER I
INTRODUCTION

Drug Metabolism

Drug metabolism, the process by which the body removes foreign and endogenous substances, is important not only for the detoxification of xenobiotics but also for the detoxification of endogenous substances. The study of the metabolism of compounds involves not only the reactions or pathways of their biotransformation but also their absorption, distribution, excretion, protein binding and membrane transport, all of which may vary with species (Williams, 1974). Drug metabolism is commonly called biotransformation, but biotransformation usually includes only the enzymatic transformation of endogenous and exogenous substrates. Watkins and Klaassen (1986) term biotransformation "as the sum of all chemical reactions that alter the structure, aqueous solubility and eventual disposition of non-nutritive [generally foreign] compounds." Meyer (1996) has a slightly different definition offering that biotransformation means "a lipid-soluble xenobiotic or endobiotic compound is enzymatically transformed into polar, water-soluble, and excretable metabolites." Although drug metabolism and biotransformation are similar, the other processes involved in metabolism—absorption, distribution and excretion—can influence biotransformation. Since metabolism encompasses more than just biotransformation of substances, it is important to note the difference between these terms when describing the enzymatic processes that occur within the body concerning foreign and endogenous compounds.

The processes by which substances enter the bloodstream, diffuse to tissues and cells and are removed from the body are called absorption, distribution and excretion (Rozman & Klaassen, 2001). Absorption is the process by which a substance crosses body membranes and enters the bloodstream. This process can occur through the skin, gastrointestinal tract or respiratory tract. Once a substance reaches systemic circulation, it is distributed throughout the body. The final amount of substance at each organ or tissue depends upon the ability of the substance to penetrate membranes and is also associated with its affinity for the organ or tissue (Rozman & Klaassen, 2001). The removal of substances from the body, excretion, occurs via several

---

This dissertation follows the style of *Journal of Veterinary Pharmacology and Therapeutics*.

routes—biliary, renal, pulmonary or dermal. Whether a substance is excreted directly or post-biotransformation depends on the physical properties, ionization and lipid solubility of the substance.

The process of drug biotransformation involves two stages—phase I and phase II. These processes occur mostly in the liver but may also occur in the kidney, intestinal tract or other organs. The liver is particularly adept at biotransformation because it is the main site of exchange for substances from the intestinal tract to the bloodstream. Phase I biotransformation is typically a detoxifying process, but in the case of some xenobiotics, an active metabolite is formed (Nebert & Dieter, 2000). This active metabolite may be beneficial or toxic. In the former situation, the metabolite may be the agent imparting therapeutic benefit. In the latter case, the metabolite may have toxic side-effects. Phase II biotransformation involves the addition of polar components to xenobiotics or endogenous compounds that have gone through phase I biotransformation. The addition of the polar compounds makes these substances more water soluble and this facilitates their removal through urinary or biliary excretion. Some compounds can undergo phase II biotransformation without previous phase I transformation. The reverse may also occur as some products of phase I biotransformation may be eliminated without further processing by phase II enzymes. Additionally, other compounds are eliminated from the body unchanged.

Phase I metabolism involves oxidation, reduction, and hydrolysis reactions. During phase I, hydroxyl (-OH), carboxyl (-COOH), amino ($-NH_2$) and, occasionally, sulfhydryl (-SH) groups are introduced into the molecule (Williams, 1974; Parkinson, 2001). Phase I metabolism usually only produces a small increase in water solubility of the substrate (Parkinson, 2001). The functional groups added in phase I are often the sites for phase II conjugation. The enzymes involved in phase II metabolism, such as UDP-glucuronosyltransferases, glutathione transferases, and sulfotransferases, conjugate various substrates and reactive intermediates to form water soluble derivatives which are subsequently excreted and thereby complete the detoxification process (Nebert & Dieter, 2000).

The most common enzymes involved in biotransformation are cytochrome P450 (phase I), UDP-glucuronosyltransferase (phase II), glutyltransferase (phase II), sulfotransferase (phase II), epoxide hydrolase (phase II), and acetyltransferase (phase II). Other enzymes that may play a role in phase I metabolism are dehydrogenases, oxidases, esterases, or reductases (Meyer, 1996). These enzymes are located either anchored in the membrane of the endoplasmic reticulum

(P450-dependent monooxygenases, epoxide hydrolase, glucuronosyltansferase) or located in the cytosol (acetyltransferase, sulfotransferase, xanthine oxidase) (Meyer, 1996; Parkinson, 2001).

Enzymes of Drug Biotransformation

Cytochrome P450 Monooxygenase

Cytochrome P450 monooxygenases have two parts, a hemoprotein and a flavoprotein (Meyer, 1996). Cytochrome P450 (CYP), the hemoprotein, is the binding site for substrates and oxygen (Meyer, 1996). NADPH-cytochrome P450 reductase, the flavoprotein, carries electrons from NADPH to the cytochrome P450 substrate complex, thus providing the electrons required for microsomal P450 activity (Meyer, 1996; Waxman, 1999). The cytochrome P450 enzymes are designated by "CYP" followed by an Arabic numeral representing the family. This numeral is followed by a letter indicating the subfamily and a second Arabic numeral representing the gene within the subfamily.

There are 17 distinct P450 gene families in mammals (Waxman, 1999). Four of these gene families (CYPs 1-4) code for liver-expressed enzymes that metabolize foreign compounds and endogenous lipophilic substrates (Waxman, 1999). The remaining families are not regularly involved in the metabolism of foreign compounds (Waxman, 1999). For humans, the prominent CYP enzymes involved in drug biotransformation are CYP3A4, CYP2D6, CYP2C9, CYP2C19, CYP1A2 and CYP2E1 (Meyer, 1996). Examples of substrates for each enzyme are caffeine and theophylline for CYP1A2, phenytoin and warfarin for CYP2C9, omeprazole and diazepam for CYP2C19, dextromethorphan and metoprolol for CYP2D6, ethanol and 4-nitrophenol for CYP2E1 and lidocaine and cyclosporine for CYP3A4 (Meyer, 1996). The various P450 isozymes can be induced or inhibited by various chemicals. Additionally, the agents capable of inducing or inhibiting may vary with species. In humans, ketoconazole is a CYP3A4 inhibitor, and rifampin is an inducer of CYP3A4 (Meyer, 1996; Parkinson, 2001).

The highest concentration of P450 enzymes active in xenobiotic biotransformation are located in the liver (Parkinson, 2001). When using enzyme concentration in rats as a basis of comparison for several species, the total cytochrome P450 concentration in the liver of cattle, sheep, guinea pigs and mice is approximately the same as that in rats (Watkins & Klaassen, 1986). The total hepatic CYP concentration in dogs, cats and rainbow trout is approximately 35% less than that found in rat liver (Watkins & Klaassen, 1986). Swine and quail have the

lowest CYP levels in the liver (approximately 50% less than in rats), and rabbits have about 41% more CYP than rats (Watkins & Klaassen, 1986).

Uridine diphosphate (UDP)-glucuronosyltransferase

UDP-glucuronosyltransferases catalyze the transfer of glucuronic acid from UDP-glucuronic acid to acceptor substrates. The site of glucuronidation is usually an electron-rich nucleophilic heteroatom (O, N or S) (Parkinson, 2001). The activity of these enzymes varies with species and is dependent upon the lipid environment of the endoplasmic reticulum membrane (Watkins & Klaassen, 1986).

Glucuronidation is a major phase II biotransformation pathway of most mammals (Williams, 1974; Parkinson, 2001). Domestic cats, lions, lynxes and civets are deficient in glucuronidation, but they are not completely devoid of the ability to form glucuronides; their ability to conjugate glucuronides depends on the isozymes and substrates involved (Williams, 1974; Caldwell, 1980; Parkinson, 2001). UDP-glucuronosyltransferases are found in the liver, kidney, spleen, intestine and other tissues, and glucuronide conjugates are normally eliminated in the urine and bile (Parkinson, 2001). Some compounds which undergo glucuronidation are acetaminophen, morphine, naproxen and amitryptyline (Parkinson, 2001).

N-acetyltransferase

N-acetylation is a major phase II biotransformation pathway for compounds which contain aromatic amines ($R\text{-}NH_2$) or hydrazine groups ($R\text{-}NH\text{-}NH_2$) (Watkins & Klaassen, 1986; Parkinson, 2001). N-acetyltransferase catalyzes acetyl group transfer from the cofactor acetyl-coenzyme A to an arylamine (Watkins & Klaassen, 1986). There are two steps in the N-acetylation process; first, the acetyl group is transferred from acetyl-coenzyme A to an active site cysteine residue within N-acetyltransferase, releasing coenzyme A (Parkinson, 2001). Second, the acetyl group is transferred from the acylated enzyme to the amino group of the compound, regenerating the enzyme (Parkinson, 2001).

N-acetyltransferases are found in the liver and other tissues of most mammals (Parkinson, 2001). When comparing N-acetyltransferase activity in the liver of several species, rabbits have the highest activity while dogs have very low activity (Watkins & Klaassen, 1986). The fox and guinea pig are also deficient in N-acetylation (Williams, 1974; Caldwell, 1980; Parkinson, 2001).

Glutathione S-transferase

Glutathione conjugation involves the addition of the tripeptide glutathione to xenobiotics (Parkinson, 2001). Glutathione is made of glycine, cysteine and glutamic acid (Parkinson, 2001). The substrates for glutathione S-transferase are hydrophobic, contain an electrophilic atom and react nonnenzymatically with glutathione (Parkinson, 2001). High concentrations of glutathione S-transferase are found in the liver, kidneys, lung and other tissues (Parkinson, 2001). These enzymes are located primarily in the cytoplasm with less than 5% located in the endoplasmic reticulum (Parkinson, 2001). The amount of glutathione conjugation varies among species; glutathione conjugation in cattle and sheep liver is approximately half of that of rats (Watkins & Klaassen, 1986).

Sulfotransferase

Sulfonate conjugation of xenobiotics is catalyzed by sulfotransferases and results in highly water soluble sulfuric acid esters (Parkinson, 2001). Sulfotransferases are cytosolic enzymes found in the liver, kidney and intestinal tract, as well as, other tissues (Parkinson, 2001). During sulfonate conjugation, sulfonate is transferred from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to the xenobiotics; PAPS is a cofactor for the reaction (Parkinson, 2001). Xenobiotics conjugated with sulfonate are usually excreted in the urine (Parkinson, 2001). The pig and opossum are deficient in sulfonation, but this deficiency is highly dependent upon the substrate (Caldwell, 1980).

Drug-induced Hepatotoxicity

As many drugs administered are lipophilic, their conversion to more water-soluble forms is necessary for their elimination (Watkins, 1990; Parkinson, 2001). The liver is the major location for xenobiotic biotransformation in mammals, and it is often a site of drug-induced toxicity. Some drugs are inherently hepatotoxic and a reduction in the ability to detoxify or eliminate these compounds may predispose a patient to hepatotoxicity (Watkins, 1990). Other drugs may cause hepatotoxicity as a result of formation of toxic metabolites via biotransformation.

Most adverse hepatic drug events (AHDEs) in companion animals are the result of direct hepatic injury although some reflect immunologic (allergic) responses (Bunch, 1993). Metabolism of xenobiotics by mixed function oxidases may lead to the formation of toxic

metabolites which can cause direct hepatic damage via formation of free radicals, electrophiles or activated oxygen species (Farber & Gerson, 1984; Kaplowitz *et al*., 1986). The drug or toxic metabolites may cause an immune response and immune-mediated injury by binding covalently to or altering liver proteins (Lee, 2003; Kaplowitz, 2004). Large adducts can serve as immune targets leading to formation of antibodies or cytolytic T-cell responses (Lee, 2003; Kaplowitz, 2004).

Interactions between host-related factors and the chemical features of drugs contribute to the development of AHDEs (Bunch, 1993). Age, hepatic blood flow, nutritional status and genetics are factors related to the development of AHDEs in both animals and humans (Bunch, 1993; Van Steenbergen *et al*., 1998). As humans age, blood flow in the liver decreases; therefore, hepatic metabolism of drugs may be altered for those drugs whose biotransformation is highly blood-flow dependent; hepatic elimination, first pass metabolism or hepatic clearance may decrease for some drugs in elderly patients (Bunch, 1993). Differences in hepatic biotransformation also exist among immature, adult and geriatric dogs; the changes in metabolic activity, however, vary with the compound (e.g. felbamate) (Tibbitts, 2003). Women have a higher incidence of drug-induced hepatotoxicity, but the reasons for the gender difference are not clear (Lee, 2003). Hepatic enzyme polymorphisms exist for humans and dogs; differing metabolism among breeds has been noted with propofol for beagles and greyhounds (Hay Kraus *et al*., 2000; Nebert & Dieter, 2000; Tibbitts, 2003). Poor nutrition can affect the quantity or quality of drug detoxifying enzymes which may alter xenobiotic metabolism (Bidlack *et al*., 1986). Certain foods and drugs can induce or inhibit hepatic enzymes. Hepatic enzyme induction or inhibition may contribute to the potential for a drug to cause hepatotoxicity by increasing formation of toxic metabolites or increasing exposure to the parent compound (Bunch, 1993; Lee, 2003).

Drug-induced liver damage can vary from hepatocyte swelling and rupture to cholestatsis without cell injury to mixed forms involving both the hepatocytes and bile canaliculi (Kaplowitz *et al*., 1986; Lee, 2003). The injury to the liver cells is specific to the intracellular organelles affected (Lee, 2003). The usual clinical expression of hepatic injury in animals is a hepatocellular or a mixed hepatocellular and cholestatic pattern of biochemical abnormalities and histopathologic findings (Bunch, 1993).

In Vivo Model

Several methods are used to study drug-induced hepatotoxicity. The goal of these models is to mimic or re-create the toxicity so that the cause can be elucidated. *In vivo* and *in vitro* models are used to examine toxicity at the molecular, cellular or organ level (Groneberg *et al*., 2002).

Dogs are common animal models for study of agents that cause toxicity in humans (Tibbitts, 2003). Dogs have many comparable physiologic processes as man, but there are also some differences which may not provide the best representation for all human situations (Tibbitts, 2003). Because of their similarity with humans, dogs are a valuable model for characterizing and predicting toxicity (Tibbitts, 2003).

The whole animal model is used to study toxicity as it occurs in nature. Because the whole animal is exposed to the xenobiotic, the organ of interest can be examined along with other system interactions. Only *in vivo* studies can be used to assess the effects of a substance on the whole animal. It is often difficult to replicate complex interactions *in vitro* since *in vitro* techniques only examine one cell type, tissue or organ.

Whole animal studies are limited by animal welfare and ethical concerns (Groneberg *et al*., 2002). The expense of maintaining numerous animals in a colony along with increased federal requirements for housing, exercise and socialization is a major limitation of *in vivo* studies (Azri *et al*., 1990; Groneberg *et al*., 2002). As with all models, gaps between the data collected from the species studied and the target population exist and can be an issue when interpreting the effects for comparison. With *in vivo* studies, it is difficult to delineate the mechanism of toxicity, and it is hard to distinguish primary and secondary toxic effects (Azri *et al*., 1990; Groneberg *et al*., 2002).

In Vitro Models

In vitro models can help alleviate some of the limitations of *in vivo* studies. With *in vitro* techniques, the number of experiments that could be done is increased while decreasing the number of animals used. Microsomes, cell suspensions, cell culture, tissue slices and ex vivo isolated perfused organs are methods used to study hepatic metabolism and toxicity.

Microsomes

Microsomes are vesicles derived from the endoplasmic reticulum. They are the most widely used subcellular fraction in the *in vitro* study of drug metabolism (Ekins *et al*., 2000). They are

prepared by differential centrifugation of homogenized tissue, have a reproducible nature, can be stored for long periods of time and have well-characterized incubation conditions (Ekins *et al*., 2000).

Microsomes contain cytochrome P450 enzymes, as well as, other enzymes involved in drug biotransformation, allowing for the study of phase I and phase II biotransformation (Cervenkova *et al*., 2001). They are also useful in studying drug-drug interactions (Ekins *et al*., 2000). Microsomes have limitations as the addition of cofactors is needed to maintain enzyme activity and some enzymes may be labile and be lost in preparation (Ekins *et al*., 2000; Cervenkova *et al*., 2001). Additionally, they only represent one organelle and cannot provide information about the entire intracellular compartment (Cervenkova *et al*., 2001). This is particularly true for phase I and some phase II enzymes which are cytosolic.

Isolated Cells and Cell Suspensions

Isolated cells and cell suspensions are used during development of new drugs and in metabolism and toxicity studies of xenobiotics (Cervenkova *et al*., 2001). They are used to predict *in vivo* drug clearance and represent a more physiological model than microsomes (Griffin & Houston, 2005). They are employed to assess cellular metabolism, cytotoxicity and genotoxicity (Groneberg *et al*., 2002).

Hepatocytes are prepared through a two-step collagenase process. The process disrupts the intracellular contacts and alters the transport capabilities of the cells (Azri *et al*., 1990; Cervenkova *et al*., 2001). The lack of cell-to-cell interactions is a major disadvantage to hepatocytes (Groneberg *et al*., 2002). The cells maintain phase I and II drug-metabolizing enzymes, have cell membrane receptors and do not need artificially high concentrations of cofactors (Groneberg *et al*., 2002; Griffin & Houston, 2005). Cells in suspension allow for rapid dispersal of the agent being tested aiding distribution and sampling (Griffin & Houston, 2005). Use of hepatocyte suspensions is limited as they only remain viable for four to six hours (Cervenkova *et al*., 2001; Griffin & Houston, 2005).

Primary Cell Culture

Primary cell culture is a frequently used *in vitro* cell model, which can be maintained for weeks. This *in vitro* model only represents selected (or specific) cell types (Bach *et al*., 1996; Cervenkova *et al*., 2001). Isolation is time consuming, and cells must be protected from

overgrowth, infection and contamination (Cervenkova *et al*., 2001). Cultured cells have cell-to-cell interactions, but the monolayers have a larger contact area with surroundings than cells have *in vivo* or in tissue slices (Cervenkova *et al*., 2001). This model is useful for assessing metabolism and cellular cytotoxicity.

Similar to isolated cells and cell suspensions, hepatocytes used for cell culture are obtained from a collagenase liver perfusion (Cervenkova *et al*., 2001). Hepatocytes are separated from other cells by differential centrifugation, and the viable cells are seeded onto collagen-coated culture dishes (Cervenkova *et al*., 2001). To maintain the health of cells, culture media must be changed every twenty-four hours (Cervenkova *et al*., 2001).

Cells in culture simplify the experimental system, and because cultured cells can be maintained for extended periods of time, experiments requiring long time periods can be performed (Cervenkova *et al*., 2001). As cells in culture dedifferentiate, this is not a reliable method for comparison of interspecies differences of metabolism (Azri *et al*., 1990; Bach *et al*., 1996; Cervenkova *et al*., 2001). For hepatocytes, there is usually only basal cytochrome P450 activity as a rapid decrease in cytochrome P450 activity occurs within 24 hours after formation of a monolayer (Cervenkova *et al*., 2001). The range of cytochrome P450 enzymes is often different in cell culture compared to fresh tissue (Cervenkova *et al*., 2001).

Isolated Perfused Organs

Isolated perfused organs are used to investigate drug and chemical-induced hepatotoxicity and are the closest model to *in vivo* conditions (Groneberg *et al*., 2002). Perfused organs are a transition between tissue slices and whole organisms (Cervenkova *et al*., 2001). Because they maintain organ physiology and morphology, they can be used to assess gross organ function, bile production and tissue histology (Groneberg *et al*., 2002).

The organ is excised from the donor animal and perfused with blood-free or autologous blood perfusates (Groneberg *et al*., 2002). With the isolated perfused organ model three-dimensional organ structure and all cell-to-cell interactions are preserved (Groneberg *et al*., 2002). Real-time bile collection and analysis can be performed, and hemodynamic parameters can be studied if blood is used as the perfusate (Groneberg *et al*., 2002). Isolated perfused livers can be used for *in vitro* toxicity testing, studying induction or inhibition of drug metabolizing enzymes of various xenobiotics, exploring biotransformation and generating metabolites (Kurihara *et al*., 1993).

Preservation of function and viability within physiological ranges is difficult with perfused organ models (Groneberg *et al*., 2002). Additionally, functional integrity is not maintained over a prolonged period (Groneberg *et al*., 2002). When using perfused livers, liver cells are subject to ischemia-reperfusion injury and hemolysis; this effect may alter the results of an experiment and is of concern when using this model (Groneberg *et al*., 2002). With the rat model, there are significant differences in organ size, function and geometry compared to humans, so porcine, canine or bovine livers are usually used to better simulate human *in vivo* conditions (Groneberg *et al*., 2002). Establishment of the isolated perfused organ model is expensive and ethical concerns about animal welfare have limited its use (Groneberg *et al*., 2002).

Tissue Slice Model

Early Development

Tissue slices can be made from various organs—e.g., liver, kidney, brain, lung, heart—and various species—e.g., man, dog, non-human primates, rat, mice (Bach *et al*., 1996). They are one of the oldest *in vitro* methods used to study metabolism (Bach *et al*, 1996). Use of tissue slices was initially reported in the 1920s (Warburg, 1923; Bach *et al*., 1996; Groneberg *et al*., 2002). Slices were prepared using "free-hand" techniques, and as time progressed, simple "slicers" were developed (Azri *et al*., 1990; Bach *et al*., 1996).

The early slicers made it difficult to obtain reproducible slices, and the quality of the slices restricted use to a few hours (Azri *et al*., 1990; Bach *et al*., 1996). The techniques were also complex making it difficult for a beginner to get replicable data (Bach *et al*., 1996). Inconsistency in slicing and poor incubation techniques also contributed to inconsistent results (Bach *et al*., 1996; Gandolfi *et al*., 1996). Because of the problems associated with tissue slices, their use declined in favor of other *in vitro* models.

In the mid 1980s changes in slicing instrumentation allowed for the production of thin, reproducible slices (Bach *et al*., 1996; Ekins *et al*., 2000). Precision-cut tissue slicer and improved incubation conditions brought about a resurgence in the use of liver slices in the late 1980s and early 1990s (Gandolfi *et al*., 1996; Ekins *et al*., 2000).

With precision-cut tissue slicers, slices are formed under physiological conditions and are of uniform thickness and diameter (Gandolfi *et al*., 1996). Eight millimeter diameter disks of tissue can be created with the optimal thickness of 200-250 microns (Bach *et al*., 1996; Gandolfi *et al*., 1996). The precision-cut slicing apparatuses used are either Krumdieck or Brendel-Vitron tissue

slicers. The basic slicing technique involves using a sharp coring tool to take a cylindrical core of tissue and placing it in the tissue holder of the slicer. A razor or microtome blade is moved across the core to produce the slices, and the slices are collected in a collecting device. During the process of slicing, the slices are kept in cold, oxygenated buffer or media.

With the use of surface culture techniques, tissue slices could be incubated for longer periods of time (Bach *et al*., 1996). Dynamic organ culture was developed to provide adequate gas and nutrient delivery to the slices during incubation (Azri *et al*., 1990; Gandolfi *et al*., 1996). A rotating incubator is used so that the slices are dipped in and out of the media to facilitate gas exchange to both sides of the slice (Gandolfi *et al*., 1996).

The changes made to the early liver slice techniques, producing slices of reliable quality with minimal trauma, have allowed slices to be used with increasing frequency in pharmaceutical, university and government laboratories (Fisher *et al*., 2001; Olinga *et al*., 2001). Slices are used as an *in vitro* method of examining organ toxicity and biotransformation. In this system, the cellular aspects of liver toxicology in a tissue-specific background can be studied (Groneberg *et al*., 2002).

Advantages

Liver slices are an intermediate between liver cells and isolated organs (Bach *et al*., 1996). A major advantage of hepatic slices compared to isolated hepatocytes is the lack of disruption of cell-to-cell contacts as occurs during the hepatocyte isolation procedure (Olinga *et al*., 2001). With liver slices the normal tissue architecture, cell heterogeneity and cell-cell interactions are maintained; the native cell types and integrity of the organ remain intact (Azri *et al*., 1990; Bach *et al*., 1996; Cervenkova *et al*., 2001; Lupp *et al*., 2001).

Liver slices are useful in the study of cytotoxicity, genotoxicity and xenobiotic biotransformation (Bach *et al*., 1996). Liver slices perform phase I and II biotransformations as are seen *in vivo* (Gandolfi *et al*., 1996). Liver slices exposed to xenobiotics appear to take up drugs as they would in an intact body; the rate of xenobiotic uptake, however, is influenced not only by the uptake rate of the cells but also the rate of penetration into the slice (Olinga *et al*., 2001). Freshly prepared rat liver slices retain high viability for up to 48 hours of incubation; phase I and II xenobiotic metabolizing enzyme activities are stable and functional, and cytochrome P450 expression is similar to that of normal liver (Lupp *et al*., 2001). Phase I hepatic enzymes can also be induced in fresh slices (Lupp *et al*., 2001). In humans, the tissue

slice system can be used for two to three days for studying hepatotoxicity (Groneberg *et al*., 2002).

Slices can be collected and prepared from several organs using the same type of media; several organs from same animal or human can be used, as well as, organs from treated or untreated subjects (Bach *et al*., 1996; Cervenkova *et al*., 2001). Because the methodology is comparable for all species and organs, comparison among species and organs is facilitated (Bach *et al*., 1996; Cervenkova *et al*., 2001). Collected slices can be stored in cold, oxygenated media for over one hour after slicing, and incubation and experimental conditions are easier compared to perfused organs (Gandolfi *et al*., 1996; Cervenkova *et al*., 2001).

Tissue slices are a viable alternative to *in vivo* studies as fewer animals are needed (Azri *et al*., 1990). This system maximizes the use of available tissue while allowing for the study of biotransformation, cell biology and toxicology (Bach *et al*., 1996). The biotransformation rates of various drugs using liver slices is more similar to that of perfused organs and *in vivo* than rates obtained with isolated hepatocytes (Cervenkova *et al*., 2001). Slices can be prepared from treated animals and humans or from organs with lesions (Bach *et al*., 1996). They are a valuable tool when whole cell metabolism is desired over short periods up to 4 hours and metabolite identification across species is desired (Ekins *et al*., 2000).

Limitations

In spite of the many advantages associated with tissue slices, this system also has its limitations. Generally, uptake or metabolism of xenobiotics is lower in liver slices than in isolated hepatocytes or hepatocyte suspensions (Ekins *et al*., 2000; Cervenkova *et al*., 2001). Slices have a shorter viability than cultured cells, and there are difficulties in maintaining the viability of slices for long-term culture (Ekins *et al*., 2000; Groneberg *et al*., 2002).

Although rat slices can be incubated for up to three to five days, the cytochrome P450 activity of the slices decreases during incubation (Cervenkova *et al*., 2001). Not only is cytochrome P450 enzyme activity decreased over time but cytochrome P450 content declines as well (Gandolfi *et al*., 1996; Ekins *et al*., 2000). Since slices are not whole organs, they cannot be used to analyze bile or portal flow (Groneberg *et al*., 2002).

During preparation and incubation, slices must be handled carefully as they are susceptible to compression and mechanical damage (Bach *et al*., 1996). Antifungal agents added to media to prevent contamination may affect cell membranes, and antimicrobials added to media may

interact with the chemical being studied (Bach *et al*., 1996). The availability of tissue and expense of the equipment and supplies needed for slicing and incubation are also potential drawbacks.

Long-term storage of unused prepared slices is an issue. Cryopreservation techniques have not been confirmed or standardized for all species, and viability can vary from 60 to 90% of the values of fresh slices depending on conditions and organs used (Bach *et al*., 1996). Additionally, not all cell types freeze the same (Bach *et al*., 1996). Recent studies have shown that cryopreserved liver slices retain phase I and II biotransformation ability but have decreased viability compared to fresh slices (Martignoni *et al*., 2004).

Results in Animal Models

Initial studies with precision-cut rat liver slices examined the culture conditions for maintenance of the slices, and it was determined that rat liver slices could be maintained for up to 20 hours with little loss in viability (Smith *et al*., 1986). It was noted that potassium (K^+) and ATP levels reached a plateau following a two to four hour recovery period (Smith *et al*., 1986). In the 1980s both precision-cut liver slices and other older slicing techniques were used. Using 0.4 millimeter thick liver slices prepared from a hand-held slicer, Powis *et al*. (1989) used liver slices and isolated hepatocytes of humans, dogs and rats to investigate the metabolism of biphenyl. This study was used to compare the metabolizing ability of isolated hepatocytes to liver slices, and it was concluded that liver slices were better than hepatocytes for comparing *in vitro* human metabolism among species (Powis *et al*., 1989).

By the early 1990s, techniques for cryopreservation of liver slices were being tested. As human tissue is difficult to obtain, Fisher *et al*. (1991) evaluated the effects of several cryopreservation methods on pig and human liver slices. They found that cryopreserved pig liver slices maintained 80 to 85% of the intracellular K^+ compared to fresh slices while cryopreserved human liver slices maintained 29 to 90% compared to fresh tissue. Fisher *et al*. (1996a) determined that dog kidney slices could be maintained in cold-storage for up to 10 days using intracellular K^+ content and protein synthesis as viability assays, but dog liver slices could only be cold-stored for 7 days based on intracellular K^+ concentrations and 4 days based on protein synthesis. Cryopreserved kidney slices and liver slices retained 60 to 70% viability following a four hour incubation (Fisher *et al*., 1996a). Martignoni *et al*. (2004) compared the phase I and II biotransformation capacity in cryopreserved liver slices among mice, rats, dogs,

monkeys and humans. They found that biotransformation ability was maintained but that viability was decreased (Martignoni *et al*., 2004). Vanhulle *et al*. (2003) noted that not only was viability in rat liver slices decreased following cryopreservation, but protein synthesis, lipid synthesis and drug conjugation were also rapidly lost following incubation after cryopresevation. Similar results were noted for human cryopreserved slices (Glockner *et al*., 1999).

Fresh liver slices are useful for evaluating xenobiotic biotransformation in humans and rats (Vickers, 1994; Oddy *et al*., 1997). Fresh rat liver slices have cytochrome P450 subtypes similar to that of normal liver though the expression is generally lower (Lupp *et al*., 2001). Lupp *et al*. (2001) noted that *in vitro* induction of phase I hepatic enzymes could be seen immunohistochemically in rat liver slices twenty-four hours after incubation with ß–naphthoflavone, phenobarbital and dexamethasone. Lupp *et al*. (2002) performed a similar study using cryopreserved rat liver slices; they reported similar results although the number of viable cells in the cryopreserved slices was lower. Ekins *et al*. (1996) compared the metabolism of several substances among rat, human and dog freshly isolated hepatocytes and 16 millimeter diameter liver slices. They found lower metabolism of substrates in rat liver slices compared to isolated hepatocytes and showed similar results with dog and human samples.

Liver slices are used in investigational pathology to assess hepatotoxic effects of substances (Gandolfi *et al*., 1995; Groneberg *et al*., 2002). The toxicity of several compounds was tested using rainbow trout liver slices (Fisher *et al*., 1996b). Liver slices can also be used to study drug uptake mechanisms; Olinga *et al*. (2001) studied the maintenance of uptake processes in rat and human liver slices. Liver slices exposed to xenobiotics appear to take up drugs as they would in an intact body; the rate of xenobiotic uptake, however, is influenced not only by the uptake rate of the cells but also the rate of penetration into the slice (Olinga *et al*., 2001).

Development of Dog Model

Studies using canine liver slices are limited and are related to using the dog as an *in vitro* model for humans or in comparison studies to determine whether the rat or dog better predict what occurs in human tissue (Fisher *et al*., 2001). As the availability of human tissue is often scarce, dog tissue slices are often used to develop and perfect techniques for use with human tissue slices.

The use of dog tissue slices for the purpose of studying drugs known to be toxic to dogs has not been reported. The aim of this study is to determine whether dog liver slices can be used as a

model for studying drug toxicity and metabolism. To determine the capacity of canine liver slices for the study of metabolism and toxicity, tissue slices were assessed for 1) viability and toxicity using potassium ion levels, ATP levels and histopathology, 2) metabolic and functional capacity by examining appearance of parent drug or metabolites in slice supernatant or the appearance of metabolites in slice media and 3) ability of compounds to induce or inhibit cytochrome P450 enzymes.

Liver slices should prove to be an effective mechanism for investigating hepatotoxicity and metabolism in dogs. The information gathered may be of further use to identify factors, such as gender, breed characteristics or drug interactions, which may increase a dog's risk of developing drug-induced hepatotoxicity.

Assessment of Slice Viability

Potassium (K^+) ion levels, adenosine triphosphate (ATP) cellular levels and histopathology are used as indicators of hepatotoxicity and tissue viability (Bach *et al*., 1996). Hepatic slice viability can be assessed via measurement of hepatic enzymes, intracellular ions (potassium), cellular energy content (ATP) and xenobiotic metabolism (Azri *et al*., 1990). The use of several viability parameters provides a better picture of tissue health than just one parameter.

Potassium

Intracellular potassium ion content is a sensitive indicator of cell membrane damage. Potassium ion concentrations are measured in the hepatic slice supernatant. Damaged cell membranes will cause potassium ions to leak out of the cell leading to decreased tissue concentrations. Intracellular potassium ion content reflects the function of Na^+-K^+-ATPase; the constant concentration of potassium ions in the cell is an indication that the plasma membrane is intact (Cervenkova *et al*., 2001). Alterations in intracellular ion content are indicative of cell death or injury and are used as a general index of viability (Azri *et al*., 1990).

Potassium ions are the primary and universal measure of slice viability; it is sensitive assay to monitor overall health of slices (Azri *et al*., 1990). The content of potassium ions in control slices should remain constant; changes in potassium ion concentrations in treated slices are an indication of cellular injury (Azri *et al*., 1990). Potassium ions are the most commonly used biochemical indicator of viability and toxicity; it is one of the first alterations observed and is often the most sensitive indicator (Gandolfi *et al*., 1996).

ATP

ATP content is measured in the hepatic slice supernatant and is an indication of the function of the ATP production chain. ATP provides the energy necessary for cellular processes, so a decrease in ATP content can indicate impairment in the production chain. ATP depletion is an indication of mitochondrial damage or impairment of mitochondrial function. Measurement of ATP concentration can be used as indication of slice viability (Azri *et al*., 1990; Cervenkova *et al*., 2001). Because of the high metabolic activity of the liver, it requires high-energy intermediates (ATP), functional mitochondria and oxygen (Gandolfi *et al*., 1996). Alterations in ATP can be used an indication of toxic insult (Gandolfi *et al*., 1996).

Histopathology

Histological examination is the method of choice for determining slice viability (Cervenkova *et al*., 2001). Correlation of biochemical and functional changes with histopathological changes provides a picture of overall slice health (Bach *et al*., 1996; Gandolfi *et al*., 1996). Because cellular architecture of liver is maintained in slices, changes in centrilobular or periportal hepatocytes can be monitored and cell-specific lesions can be seen (Bach *et al*., 1996; Gandolfi *et al*., 1996).

Histologically, the liver's response to toxic injury from xenobiotics can vary from lipid accumulation to cell death (Treinen-Moslen, 2001). Lipid accumulation in paraffin-embedded and solvent-extracted sections is noted as the presence of vacuoles displacing the nucleus to the periphery of the cell (Treinen-Moslen, 2001). Although fat accumulation is a common response of the liver to some hepatotoxins, frozen sections and special stains are needed to determine whether the vesicles are fat (Treinen-Moslen, 2001). Cell death may occur via necrosis or apoptosis. The features of necrosis are cell swelling, leakage, nuclear disintegration and presence of inflammatory cells (Treinen-Moslen, 2001). For apoptosis the features are cell shrinkage, nuclear fragmentation, apoptotic bodies and lack of inflammation (Treinen-Moslen, 2001). Liver cells may die in a focal, zonal or panacinar pattern. Focal cell death is represented by death of single or clusters of hepatocytes while zonal necrosis occurs in periportal or centrolobular areas (Treinen-Moslen, 2001). Panacinar necrosis is widespread hepatocyte death with a few or no surviving cells (Treinen-Moslen, 2001). Another necrotic pattern which can be seen following a hepatotoxic insult is bridging necrosis (Treinen-Moslen, 2001). This is a less

extensive form of necrosis than panacinar and is signified by zones of confluent cell lysis (Treinen-Moslen, 2001).

Maintenance of morphological integrity is one of the most important indicators of the viability of tissue slices; histology is a way of detecting target cell injury (Bach *et al*., 1996). Histological assessment is more difficult and resource-intensive than use of biochemical markers for determining toxicity (Bach *et al*., 1996). Fisher *et al*. (2001) noted that histological evaluation was a more sensitive indicator of slice viability than biochemical evaluation.

Determination of Slice Function and Metabolic Capacity

Several substances, such as cyclosporine A, carbamazepine, styrene and testosterone, have been used to determine the metabolic capacity and function of liver slices (Vickers *et al*., 1992; Vickers, 1994; Ekins *et al*., 1996; Martignoni *et al*., 2004). In this study, lidocaine, phenobarbital and primidone were used as marker drugs with drug disappearance in media or metabolic appearance in liver supernatant or incubation media serving as indicators of metabolism. Carprofen was used to assess the ability of a non-steroidal anti-inflammatory drug to cause toxicity to liver slices alone or following phenobarbital or cimetidine incubations. (The structures of drugs studied are in appendix II.)

Phenobarbital

Phenobarbital is an anticonvulsant used in the medical management of seizures in dogs. Phenobarbital is known to cause hepatotoxicity in dogs (Bunch, 1993; Dayrell-Hart *et al*., 1991; Podell, 1998; Müller *et al*., 2000), and the hepatotoxicity is usually associated with increased plasma concentrations of phenobarbital (Podell, 1998).

Phenobarbital was applied to slices to determine if it would cause dose-dependent changes in viability parameters indicating toxicity. Indications of toxicity at higher drug concentrations would mimic the *in vivo* toxicity. The presence of phenobarbital in the supernatant would also indicate that the drug was penetrating the hepatocytes.

Primidone

Primidone is an anticonvulsant used in the medical management of epilepsy in dogs. The use of primidone has fallen out of favor as it is associated with hepatotoxicity in dogs (Schwartz-Porsche *et al*., 1985; Podell, 1998). Primidone is metabolized to phenobarbital and

phenylethylmalonamide (Yeary, 1980). The measurement of phenobarbital concentrations in liver slices exposed to primidone was used to determine the metabolic ability of the slices and to determine if dose-dependent toxicity could be detected. Since both phenobarbital and primidone are associated with hepatotoxicity, and primidone is metabolized to phenobarbital, the results of the studies could be compared to determine any differential effects of the drugs.

Lidocaine

Lidocaine is a local anesthetic and is used in the treatment of ventricular arrhythmias in dogs. Lidocaine is metabolized to monoethylglycinexylidide (MEGX) and glycinexylidide (GX) in dogs (Keenaghan & Boyes, 1972; Wilcke *et al*., 1983). In humans, lidocaine is metabolized to MEGX by CYP3A4, and measurement of MEGX in blood following intravenous administration of lidocaine has been used as a sensitive indicator of oxidative drug metabolizing hepatic function (Tanaka & Breimer, 1997).

Slices exposed to lidocaine were used to determine the metabolic function of the slices. The measurement of lidocaine disappearance and appearance of MEGX and/or GX is an indication of the metabolic capacity.

Carprofen

Carprofen is a propionic acid non-steroidal anti-inflammatory drug (NSAID) approved for use in the medical management of canine osteoarthritis. Carprofen can cause a hepatocellular toxicosis in dogs which is believed to be idiosyncratic (MacPhail *et al*., 1998). The hepatic localization and hematologic changes from this toxicity are similar to those associated with other NSAIDs known to cause hepatic disease. The hepatocellular damage associated with carprofen varies from mild to severe, and the hepatic pathological changes can vary as well (MacPhail *et al*., 1998). Hepatocellular necrosis ranges from multifocal to extensive and is characterized by ballooning degeneration, lytic necrosis and apoptosis (MacPhail *et al*., 1998). Bridging necrosis, zones of confluent cell lysis, with parenchymal collapse is most common (MacPhail *et al*., 1998; Treinen-Moslen, 2001).

Since carprofen can cause a hepatotoxicity in dogs, it was used to determine if similar changes could be seen in canine liver slices exposed to the drug.

Inducers and Inhibitors

Cytochrome P450 (CYP) enzyme inducers and inhibitors were used to determine if they could alter the ability of carprofen to cause hepatotoxicity in canine liver slices. To induce and inhibit cytochrome P40 isoforms in hepatic slices, phenobarbital and cimetidine were used, respectively.

The cytochrome P450 2B subfamily is the most inducible by phenobarbital (Hojo *et al*., 2002). In dog liver microsomes, the phenobarbital inducible cytochrome P450 enzyme is CYP2B11 (Graham *et al*., 2002). Phenobarbital also induces CYP3A12 and CYP2C21 in dog liver hepatocytes and microsomes (Nishibe & Hirata, 1993; Eguchi *et al*., 1996). Phenobarbital has been used to induce cytochrome P450 isoforms in rat liver slices *in vitro* (Lupp *et al*., 2001; Lupp *et al*., 2002).

In microsomes of male rats, cimetidine inhibits CYP2C11 (Levine *et al*., 1998). In humans, substrates for the CYP2C subfamily include ibuprofen and flubiprofen, which are propionic acids like carprofen (Boelsterli *et al*., 1995).

The CYPs involved in carprofen metabolism in dogs are not known. Phenobarbital was selected as the inducer because it has been shown to induce CYP expression in rat liver slices *in vitro* (Lupp *et al*., 2001). Additionally, hepatic enzyme induction can lead to the formation of toxic metabolites that may cause hepatotoxicity (Nebert & Dieter, 2000). Cimetidine was selected as it inhibits isoforms of the CYP2C subfamily, and in humans the CYP2C subfamily has been shown to have a key role in the metabolism of NSAIDs (Leemann *et al*., 1993; Levine and Bellward, 1995; Levine *et al*., 1998; Boelsterli *et al*., 1995). Since inhibition studies using cimetidine have not been performed in liver slices, extrapolations from other *in vitro* models were made. The mechanism of carprofen-induced hepatotoxicity is unknown, and therefore, alterations in toxicity following induction or inhibition of drug metabolizing enzymes would support the role of metabolism in mediating hepatotoxicity by a metabolite.

CHAPTER II
MATERIALS AND METHODS

Tissue Collection, Incubation and Handling

Liver

The right liver lobe was harvested from euthanized dogs (n=10; male, hound cross) within fifteen minutes of death to maintain hepatic viability. Following removal, the liver was cut into quarters, placed in V-7, a cold preservation solution (Vitron, Inc., Tucson, AZ) (Fisher *et al*., 1996a), and kept cold in a refrigerator (4°C) until slice preparation (10 to 12 hours after collection). One dog was used for each day's experiment (Table 1).

Table 1. Study dates. Day one of each study performed is shown with the item(s) of interest tested for in the media or supernatant listed. The numbers in the dog column represent the identification number assigned to each liver.

Drug	Supernatant	Media	Date (Day 1)	Dog
Carprofen (repetition (rep.) 1) Carprofen (rep. 2) Carprofen C (rep. 3)	drug appearance	drug disappearance	2/25/03 10/14/03 10/14/03	1 4 4
Phenobarbital	drug appearance	drug disappearance	5/1/03	2
Lidocaine	drug or metabolite appearance	drug disappearance	5/1/03	2
Primidone	metabolite appearance	metabolite appearance	7/3/03	3
Diazepam (rep. 2)	drug or metabolite appearance	metabolite appearance or drug disappearance	10/14/03	4
Diazepam (rep. 1)	drug or metabolite appearance	metabolite appearance or drug disappearance	10/15/03	5
Diazepam and phenobarbital	drug or metabolite appearance	metabolite appearance or drug disappearance	10/15/03	5
Diazepam and cimetidine	drug or metabolite appearance	metabolite appearance or drug disappearance	10/15/03	5
Carprofen and phenobarbital (rep. 1, 2 & 3)	drug appearance	drug disappearance	11/10/03	6
Carprofen and cimetidine (rep. 1, 2 & 3)	drug appearance	drug disappearance	11/12/03	7
Carprofen and phenobarbital	drug appearance	drug disappearance	1/28/04	8

Slicing

Several cylindrical cores of tissue were made from each liver section using an 8 mm diameter coring tool (Vitron, Inc., Tucson, AZ). The cores were placed in a Brendel/Vitron Tissue Slicer (Figure 1) (Vitron, Inc., Tucson, AZ) and sliced to make disks of tissue 200 –250 µm thick. Throughout coring and slicing, a 95% oxygen and 5% carbon dioxide gas mixture was used to propel cold V-7 solution through the slicer to help maintain liver and slice integrity. Cores and slices were kept cold prior to, during and post-slicing. Following slicing, the tissue slices were loaded onto roller inserts (Type A) (Figure 1) (Vitron, Inc., Tucson, AZ) with a slice handling tool. One slice was loaded per roller insert. The roller inserts consisted of a Teflon cradle with a titanium wire mesh and Viton O-rings.

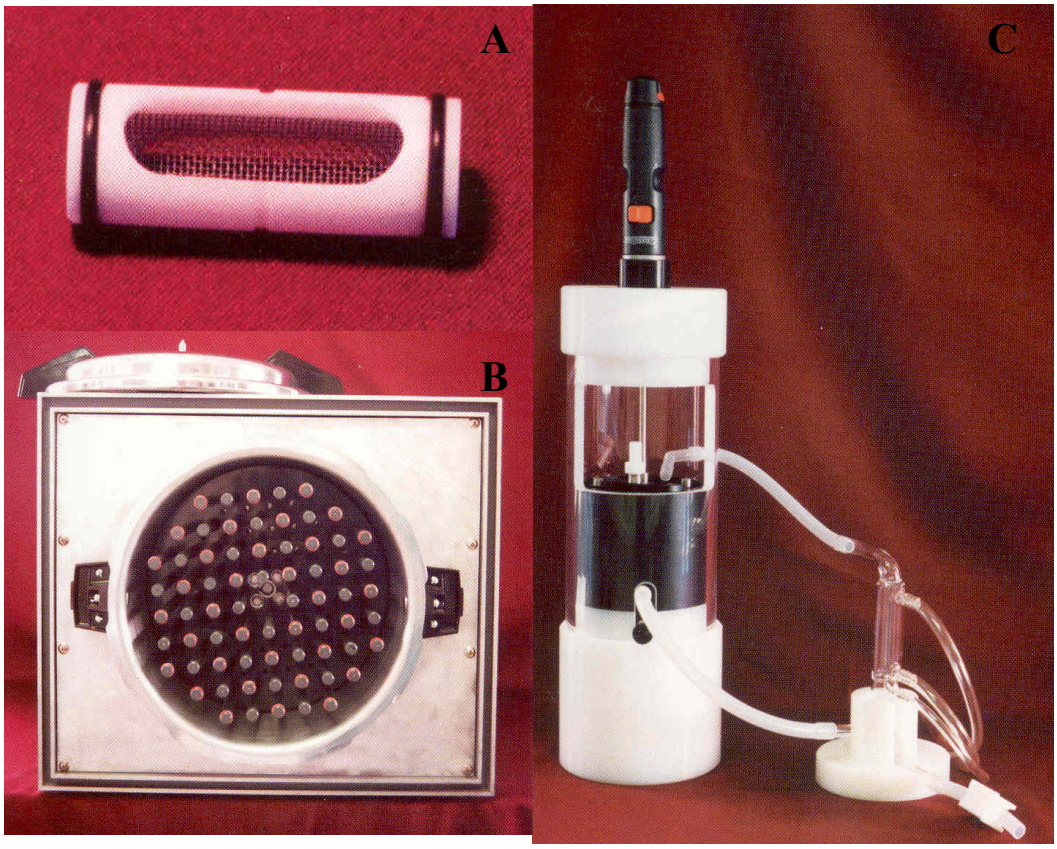

Fig. 1. Slicing and incubation instruments. A. Type A Roller Insert; B. Dynamic Organ Culture Incubator; C. Brendel/Vitron Tissue Slicer.

Tissue Incubation Media

Waymouth's MB 752/1 liquid media with L-glutamine was purchased from Gibco Invitrogen Corporation (Carlsbud, CA; catalog no. 11220). One liter of the final media preparation contained 10% fetal bovine serum (BioWhittaker, Inc, Walkersville, MD; catalog no. 14501E), 50 mg of gentamicin (BioWhittaker, Inc, Walkersville, MD) and 10 ml antibiotic antimycotic solution (Sigma-Aldrich, St. Louis, MO; catalog no. A5955). The media was sterile filtered using a Millipore Stericup filtration system (Millipore Corporation, Bedford, MA; catalog no. SCGPU05RE). The media was kept protected from light at 2 to 8°C. Media for the study was prepared one to two days prior to use and was kept refrigerated and protected from light for up to one week. Unprepared Waymouth's media was stored until the labeled expiration date.

Incubation

Slices on roller inserts were placed into 20 ml scintillation vials (VWR Scientific Products, Willard, OH; catalog no. 66022-004) containing 1.7 ml media. The vials were allowed to reach room temperature before loading roller inserts. A cap, with a 1-2 mm hole to allow for gas exchange, was placed on each vial. Slices were incubated at 37°C in a Dynamic Organ Culture Incubator (Figure 1) (Vitron, Inc., Tucson, AZ). A gas atmosphere of 95% oxygen and 5% carbon dioxide was supplied to the incubator at a flow rate of 1 L/ min.

Slice and Media Handling Post-incubation

All slices were weighed (blotted weight recorded for potassium and ATP normalization), placed in a microcentrifuge tube containing one milliliter purified water and homogenized (Tissue-Tearor®, Spectrum Chemicals, Gardena, CA) for approximately five seconds. Homogenized slices were frozen then thawed and centrifuged at 16,110 g for 15 minutes. The supernatant was aliquoted and frozen until analysis. Media samples were frozen until assayed.

Tissue Viability

Potassium Analysis

A VITROS 250 Analyzer and VITROS K^+ Slides (Ortho-Clinical Diagnostics, Inc, Rochester, NY) were used to assay potassium content in hepatic slice supernatant by ion-selective electrodes. Samples were analyzed by the Clinical Pathology Lab at the Texas Veterinary Medical Center at Texas A&M University (College Station, TX).

ATP Analysis

An ATP determination kit from Molecular Probes (Eugene, OR; catalog no. A-22066) was used to measure ATP content in hepatic slice supernatant. The samples were analyzed according to kit directions on a FLUOstar Optima (BMG Labtechnologies, Inc, Durham, NC).

Histopathologic Analysis

Slices for histopathological analysis were fixed in five ml of 10% formalin for at least 24 hours. Tissues were embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin (H&E). The entire section of each tissue (approximately 50 mm^2) was examined by light microscopy. The extent and severity of microscopic changes were semiquantitatively graded as: score 0, normal; 1, minimal change; 2, mild change; 3, moderate change; 4, marked change; 5, severe change (Herbert *et al*., 2002). Grading of the extent of necrosis (including apoptosis) and the extent of vacuolation was based on the approximate percentage of hepatocytes affected (Table 2). Grading of the severity of vacuolation was based on visual assessment of the ratio of clear space to pink cytoplasm in the parenchyma. The slide observer was unaware of slice treatment.

Table 2. Lesion score scale. Histologic criteria for grading extent of necrosis and extent of vacuolation.

Lesion score	% hepatocytes affected
0 (normal)	<1
1 (minimal change)	1-10
2 (mild change)	11-20
3 (moderate change)	21-40
4 (marked change)	41-90
5 (severe change)	>90

Tissue Treatments

Incubation conditions were optimized for the hepatic slices, and the impact of the drug on slice viability was determined. Normal activity was assessed by the ability of slices to take up and metabolize drugs. Lidocaine, phenobarbital and primidone were used as marker drugs with drug disappearance or metabolic appearance in liver supernatant or incubation media serving as indicators of metabolism. Diazepam was used as an additional drug to measure hepatic slice

metabolism and to determine the ability to induce and inhibit cytochrome P450 drug metabolizing enzymes in slices. Carprofen was used to assess the ability of a non-steroidal anti-inflammatory drug to cause toxicity in the slices in normal, induced and inhibited conditions.

To induce and inhibit cytochrome P450 drug metabolizing enzymes in hepatic slices, phenobarbital and cimetidine were used, respectively. The cytochrome P450 2B subfamily is the most inducible by phenobarbital (Hojo *et al*., 2002). In dog liver microsomes, the phenobarbital inducible cytochrome P450 enzyme is CYP2B11 (Graham *et al*., 2002). Phenobarbital also induces CYP3A12 and CYP2C21 in dog liver hepatocytes and microsomes (Nishibe & Hirata, 1993; Eguchi *et al*., 1996). In male rats, cimetidine inhibits CYP2C11 (Levine *et al*., 1998). In humans, substrates for the CYP2C subfamily include ibuprofen and flubiprofen, which are propionic acids like carprofen (Boelsterli *et al*., 1995). To determine if phenobarbital induced CYP2B11, an enzyme-linked immunoassay (ELISA) was used to measure the concentration of the enzyme in the hepatic supernatant. An ELISA for CYP2C21 was used to determine cimetidine inhibition. The kits used specific antisera developed for dog liver microsomes.

Lidocaine

Slices were incubated in media containing 0, 3, 10 and 20 µg/ml of lidocaine. Following incubation for 1, 3, 6, 8, 12 and 24 hours, slices were removed and prepared for ATP, K^+ and high pressure liquid chromatography (HPLC) analysis for the presence of parent compound and metabolites monoethylglycinexylidide (MEGX) and glycinexylidide (GX). The media was also analyzed for parent compound and metabolites. Incubation media concentrations were approximately 0, 1, 3.3 and 6.5 times canine plasma concentrations measured following a therapeutic dose. A liquid-liquid extraction was used to extract the parent compound and metabolites for HPLC analysis.

Phenobarbital

Slices were incubated in media containing 0, 20, 45 and 75 µg/ml of phenobarbital. Incubation media concentrations were approximately 0, 1, 2.25 and 3.75 times canine plasma concentrations measured following a therapeutic dose. Following incubation for 1, 3, 6, 8, 12 and 24 hours, slices were removed and prepared for ATP, K^+ and fluorescence polarization immunoassay for the parent compound. The media was also analyzed for parent compound.

Primidone

Slices were incubated in media containing 0, 20, 45 and 75 µg/ml of primidone. Incubation media concentrations matched those of the phenobarbital experiment. Following incubation for 1, 3, 6, 8, 12 and 24 hours, slices were removed and prepared for ATP, K^+ and fluorescence polarization immunoassay for phenobarbital, an active metabolite of primidone. The media was also analyzed for the presence of phenobarbital.

Diazepam

Diazepam was applied to the slices at 0, 100, 250 and 500 ng/ml. The slices were incubated in the media for 0, 1, 4, 8, 12 and 24 hours. Incubation media concentrations were approximately 0, 1, 2.5 and 5 times canine plasma concentrations measured following a therapeutic dose. To determine the effects of hepatic drug metabolizing enzyme induction or inhibition on drug concentrations, the induction and inhibition methods described under cytochrome P450 induction and inhibition were used with diazepam as the drug of interest. (See Appendix IV for diazepam results.)

Carprofen

Slices were incubated in media containing increasing concentrations (0, 10, 25, 50, 75 or 100 µg/ml) of carprofen as the racemic mixture or its enantiomers. Incubation media concentrations were approximately 0, 1, 2.5, 5, 7.5 and 10 times canine plasma concentrations measured following a therapeutic dose. At 1, 6, 8, 12 and 24 hours or 0, 1, 4, 8, 12 and 24 hours incubation, the slices were removed from incubation and prepared for ATP, K^+, drug and histopathologic analysis. The drug concentrations in the media and the slice were determined by HPLC analysis.

Using the method described under cytochrome P450 induction, the ability of carprofen to induce toxicity was evaluated in the presence of a cytochrome P450 induced slice. The slice was exposed to the inducer in media, and the media was replaced by media containing the racemic mixture or enantiomers of carprofen. The method described under cytochrome P450 inhibition was used for determining the effect of a cytochrome P450 inhibitor on the ability of carprofen to cause toxicity.

Carprofen Enantiomers

An unsuccessful attempt was made to separate enantiomers by chiral derivatization. As separation did not occur, the same protocol that was used for the racemic mixture was repeated as would have been done with the individual enantiomers.

Additional Carprofen and Phenobarbital Incubation

Slices were incubated for 48 hours in 75 µg/ml phenobarbital or for 24 or 48 hours in 150 µg/ml phenobarbital. Following the 24 or 48 hour incubation, rollers were removed from the induction vials, blotted to remove excess media and placed in new vials containing media with 100 µg/ml carprofen. Slices were then incubated for an additional 4, 12 or 24 hours. At the end of the second incubation, slices and media were examined for concentration of carprofen.

Cytochrome P450 Induction

Slices were incubated for 24 hours in 75 µg/ml of phenobarbital. This concentration was chosen as it is the high end of the acceptable plasma concentrations for phenobarbital in dogs. Additionally, hepatic slices showed decreases in potassium and ATP content at this concentration. Concentrations used for *in vitro* induction studies with dog hepatocytes used 1 mM (233.24 µg/ml) or 2 mM (466.48 µg/ml) phenobarbital (Nishibe & Hirata, 1993).

Following the 24 hour incubation, rollers were removed from the induction vials, blotted to remove excess media and placed in new vials containing media with desired drug. Slices were then incubated for an additional 1, 4, 8, 12 or 24 hours. At the end of the second incubation, slices and media were handled as stated previously. Supernatant was evaluated for induction of cytochrome P450 2B11 by ELISA.

Cytochrome P450 Inhibition

Slices were incubated for 24 hours in 1000 µM of cimetidine. This concentration was based on *in vitro* studies using rat and human microsomes (Levine & Bellward, 1995; Furuta *et al*., 2001). Following the 24 hour incubation, rollers were removed from the inhibition vials blotted to remove excess media and placed in new vials containing media with desired drug. Slices were then incubated for an additional 1, 4, 8, 12 or 24 hours. At the end of the second incubation, slices and media were handled as stated previously. The supernatant was evaluated for inhibition of cytochrome P450 2C21 by enzyme-linked immunoassay (ELISA).

Tissue Analysis

Drug Analysis

For all high pressure liquid chromatography (HPLC) analyses, the following components, provided by Waters, Inc (Milford, MA), were used: Waters Millenium® software, Waters 474 Scanning Fluorescence Detector, Waters 2487 Dual Wavelength Absorbance Detector, Waters 996 Photodiode Array Detector, Waters 717plus Autosampler, and Waters 600 Controller.

Phenobarbital and Primidone Slice Analysis

A TDxFLx System with phenobarbital kit by Abbott Laboratories (Abbott Park, IL) was used to measure the amount of phenobarbital in the media and slices for the phenobarbital and primidone studies. The TDxFLx System uses fluorescence polarization immunoassay technology. The lower limit of quantification (LOQ) was 1.1 µg/ml. The upper LOQ was 80 µg/ml. The kit controls predicted in the acceptable range, and media controls were within 10% of expected concentrations.

Lidocaine Assay

Each 250 µl sample was combined with 300 µl of 2 M sodium hydroxide and 5 ml of methyl tert-butyl ether and vortexed for 10 seconds. The mixed samples were centrifuged at 1600 g at 5°C for 15 minutes. After centrifugation, the samples were placed in a freezer at -80° C for 15 minutes. The organic phase was transferred to another tube, combined with 250 µl of 17 mM phosphoric acid and vortexed for 30 seconds. Following centrifugation at 1600 g at 5°C for 15 minutes, the supernatant was removed and the remaining solution was analyzed by HPLC. A Luna 3 µ C-18(2) 150x4.6 mm column (Phenomenex, Torrance, CA) with Luna 4x3 mm guard column (Phenomenex, Torrance, CA). Lidocaine and its metabolites, MEGX and GX, were detected by photodiode array (210 nm-263 nm). The mobile phase contained 1800 ml of water, 400 µl of triethylamine, 2.4 ml of 85% phosphoric acid and 200 ml acetonitrile. For a flow rate of 0.7 ml/min, the retention times were 8.5 minutes, 9.8 minutes and 12.5 minutes for lidocaine, GX and MEGX, respectively. The lower and upper LOQs for lidocaine were 50 and 25000 ng/ml. For MEGX and GX, the lower and upper LOQs were 50 and 1000 ng/ml. Three media controls and three supernatant controls were used for each compound measured. The controls predicted within 10% of expected concentrations.

Diazepam Assay

Each 500 µl sample was prepared for HPLC analysis by adding 0.5 ml of distilled water and 20 µl of 5% acetic acid and vortexing the mixture. This mixture was applied to a BondElut C-18, 200 mg solid-phase extraction column (Varian, Inc., Harbor City, CA). The column was conditioned with one ml each acetonitrile and water. Following conditioning, the sample was applied to the cartridge, washed with one ml of water and eluted with one ml of methanol. The eluent was placed in the HPLC vial and analyzed. The HPLC system used consisted of a Prevail C-18 5 µm 250x4.6 mm column (Alltech Associates, Inc.) with a mobile phase of methanol:acetonitrile:water (10:40:50). The pH of the mobile phase was adjusted to 3.2 using acetic acid. The flow rate was 1 ml/min with an injection volume of 100 µl. The compounds were detected by UV at a wavelength of 232 nm. The retention times were 6.1 minutes for diazepam, 9.8 minutes for oxazepam, 14.3 minutes for temazepam and 17.2 minutes for nordiazepam. The lower and upper LOQs for diazepam were 40 and 500 ng/ml. For oxazepam and temazepam the lower and upper LOQs were 80 and 250 ng/ml. The lower and upper LOQs for nordiazepam were 40 and 250 ng/ml. Three supernatant and three media controls were used for each compound measured. The controls predicted within 10% except for the low control for temazepam which was within 15% of expected concentrations. (See Appendix IV for diazepam results.)

Carprofen Assay

Each 500 µl sample was prepared for HPLC analysis by mixing it with 500 µl of 1% phosphoric acid. This mixture was applied to a Strata C18E, 100 mg solid phase extraction cartridge (Phenomenex, Torrance, CA), which had been conditioned with 1 ml methanol and 1 ml 1% phosphoric acid. The cartridge was washed with 1 ml 1% phosphoric acid and eluted with 1 ml of ethanol. The eluent was placed in an HPLC vial and analyzed. The HPLC system used consisted of a CHIRALCEL® OD (4.6 x 250 mm) column (Chiral Technologies, Inc., Exton, PA) and OD guard column or cartridge (0.4 x 1 cm) with a mobile phase of hexane:ethanol:trifluoroacetic acid (90:10:0.15). The flow rate was 1 ml/min with an injection volume of 20 µl. The compound was detected by UV at a wavelength of 254 nm. The retention time was 12 –13 minutes. The lower and upper LOQs were 3 and 300 µg/ml. Three supernatant and three media controls predicted within 10% of expected concentrations.

Enantiomer Separation

An attempt was made to separate the enantiomers by chiral derivatization to form the diastereoisomers. The method used was based on Spahn *et al*. (1988) using R(+) and S(-)-α-methylbenzylamine (Spectrum Chemicals, Gardena, CA) as the derivatizing agent. Each α -methylbenzylamine (0.8g) was dissolved in 4 ml of acetone, and 1.8 g of racemic carprofen was dissolved in 36 ml acetone. The α -methylbenzylamine mixture was mixed with the carprofen mixture and allowed to stand at room temperature, protected from light, for three days. Four crystallizations were performed. The enantiomers were hydrolyzed with 0.1 M hydrochloric acid according to the article procedure. The enantiomers were analyzed by HPLC using a chiral column by the method described above under carprofen assay.

Cytochrome P450 Analysis

A BD Gentest Products canine-specific enzyme-linked immunoassay (ELISA) (BD Biosciences, Bedford, MA) was used to measure the amount of cytochromes P450 2B11 (catalog no. 458937) or 2C21 (catalog no. 458020) in the samples. Samples were analyzed at 450 nm. The antibodies and method were provided with the kit.

Statistical Analysis

Changes in potassium content, ATP content, drug concentrations and AUC were assessed as significant differences among treatments and across time using analysis of variance (ANOVA). Histopathologic changes indicative of hepatic damage (necrosis, vacuolation) were also assessed across time and among treatments by ANOVA. Cytochrome P450 concentration comparisons were made using paired t-tests. Statistical analyses were performed using SYSTAT®, Microsoft Excel® and GraphPad Prism®. A p-value (α) < 0.05 was considered statistically significant. Differences among ANOVA groups were determined using Tukey's test for multiple comparisons.

CHAPTER III
CANINE HEPATIC SLICE BEHAVIOR AND ASSESSMENT

Potassium, ATP and Histology

The behavior of canine hepatic slices, incubated in media without drug, was assessed across time, for up to twenty-four hours, and across experiment dates for potassium content (μmol/g-L), ATP content (nmol/g) and histologic lesion scores (necrosis, vacuolation extent and vacuolation severity). Mean potassium concentrations (Figure 2, Table 3) were compared using one-way ANOVA for time or experiment date. Significant differences were noted across time (df=7, F-ratio=28.51, p-value<0.0001) and among experiment dates (df=11, F-ratio=4.806, p-value<0.0001).

For time, mean zero hour concentrations of potassium were higher than those for all other time points (Figure 2A, Table 3). One, three and eight hour potassium concentrations were lower than those at 24 hours (Figure 2A, Table 3). For experiment date, mean potassium concentrations on 5/1/2003 were lower than those for 10/15/2003 (repetition (rep.) 1 and 2), 11/10/2003 (rep. 1 and 2) and 11/12/2003 (rep. 1 and 2) (Figure 2B, Table 3). Mean potassium concentrations on 7/3/2003 were lower than those on 10/15/2003 (rep. 2), 11/10/2003 (rep. 2) and 11/12/2003 (rep.1 and 2) (Figure 2B, Table 3).

Mean ATP concentrations across time and among experiment dates (Figure 3, Table 4) were compared using one-way ANOVA. Across time no significant differences were noted in the ATP concentrations (Figure 3A). Among experiment dates, however, differences were noted (df=11, F-ratio=5.561, p-value<0.0001) (Figure 3B). Mean ATP concentrations for 7/3/2003 were higher than those of 2/25/2003, 5/1/2003, 10/15/2003 (rep. 1), 11/10/2003 (rep. 2) and 11/12/2003 (rep. 1) (Figure 3B, Table 4). Additionally, ATP concentrations on 10/15/2003 (rep. 1) were lower than those of 10/14/2003 (rep. 1) and 11/12/2003 (rep. 2) (Figure 3B, Table 4).

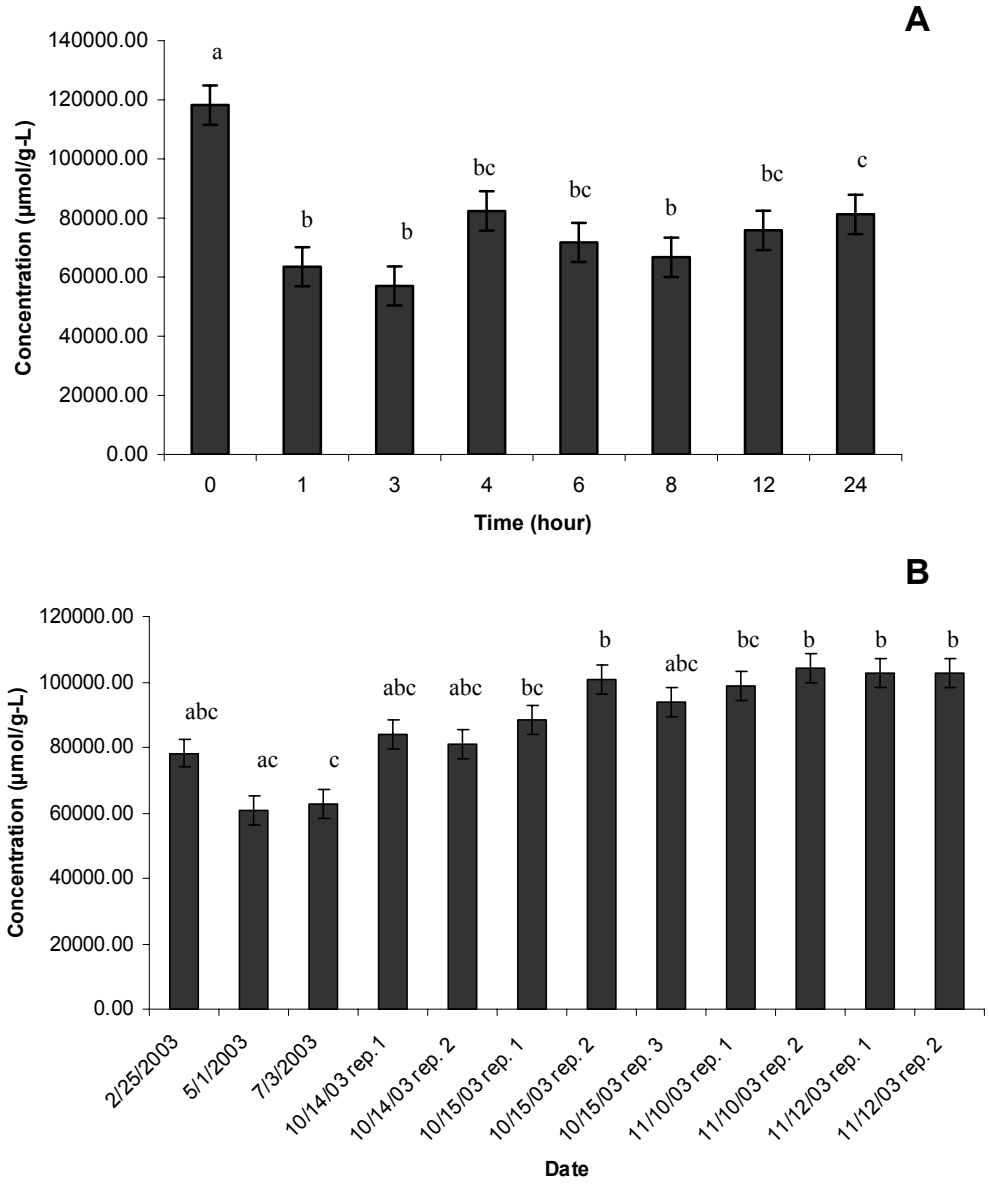

Fig. 2. Mean potassium concentrations for slices exposed to media with no drug. Potassium concentrations (mean±SE, µmol/g-L) of slices exposed to media without drug across 24 hours (A) or among experiment dates (B). Different repetitions on the same date are represented as rep. 1, 2 or 3. Homogeneous groups by Tukey's test for multiple comparisons ($p<0.05$) are shown as letters. Times or dates with the same letter are not significantly different. For time n=12, except hour 0, n=18; hour 3, n=4; hour 4 and 6, n=6; hour 24, n=24. For dates, n=4, except 2/25/2003, n=10; 5/1/2003, 7/3/2003, 10/14/2003 (rep.1 and 2) and 10/15/2003 (rep. 1), n=12.

Table 3. Potassium concentrations (μmol/g-L) for slices exposed to media with no drug. Means with standard deviations (std dev) represent variability across time, among day and between day. The mean values for time and date are plotted in Fig. 2.

Time (hour)	2/25/2003	5/1/2003	7/3/2003	10/14/2003	10/14/2003	10/15/2003	10/15/2003	10/15/2003	11/10/2003	11/10/2003	11/12/2003	11/12/2003	Mean	std dev
0				83623.69	125506.07	122676.58	122605.36	107279.69	97222.22	109890.11	126530.61	150537.63	118221.81	16260.00
				116104.87	123287.67	129870.13	101214.57	116363.64	102739.73	117241.38	139639.64	135658.91		
1	QNS	48076.92	56224.90	89494.16	70796.46	61855.67							63517.83	14746.84
	60200.67	60606.06	40293.04	87649.40	63348.42	60150.38								
3		45454.55	46931.41										57035.88	12573.83
		69090.91	66666.67											
4				82706.77	72072.07	83333.33							82375.89	7427.95
				85470.09	76923.08	93750.00								
6	88888.89	53763.44	64516.13										71739.84	14021.01
	85271.32	76595.74	61403.51											
8	68852.46	57692.31	64171.12	68273.09	68085.11	76530.61							66705.17	6885.96
	68840.58	62500.00	54347.83	70038.91	63063.06	78066.91								
12	99264.71	54474.71	63218.39	81180.81	80952.38	87155.96							75795.47	13929.12
	72727.27	55319.15	81871.35	75000.00	67010.31	91370.56								
24	83720.93	71713.15	76502.73	74380.17	84821.43	102766.80	90909.09	69869.00	92896.17	92682.93	62015.50	61728.40	81216.38	12029.37
	76305.22	74235.81	75000.00	95454.55	77586.21	74074.07	87719.30	81218.27	101604.28	97297.30	82191.78	62500.00		
Mean	78230.23	60793.56	62595.59	84114.71	81121.02	88466.75	100612.1	93682.65	98615.6	104277.9	102594.4	102606.2		
std dev	11406.34	10213.05	12040.72	12908.73	21301.69	21576.93	15752.66	21769.68	4494.216	11294.85	36552.27	47150.14		

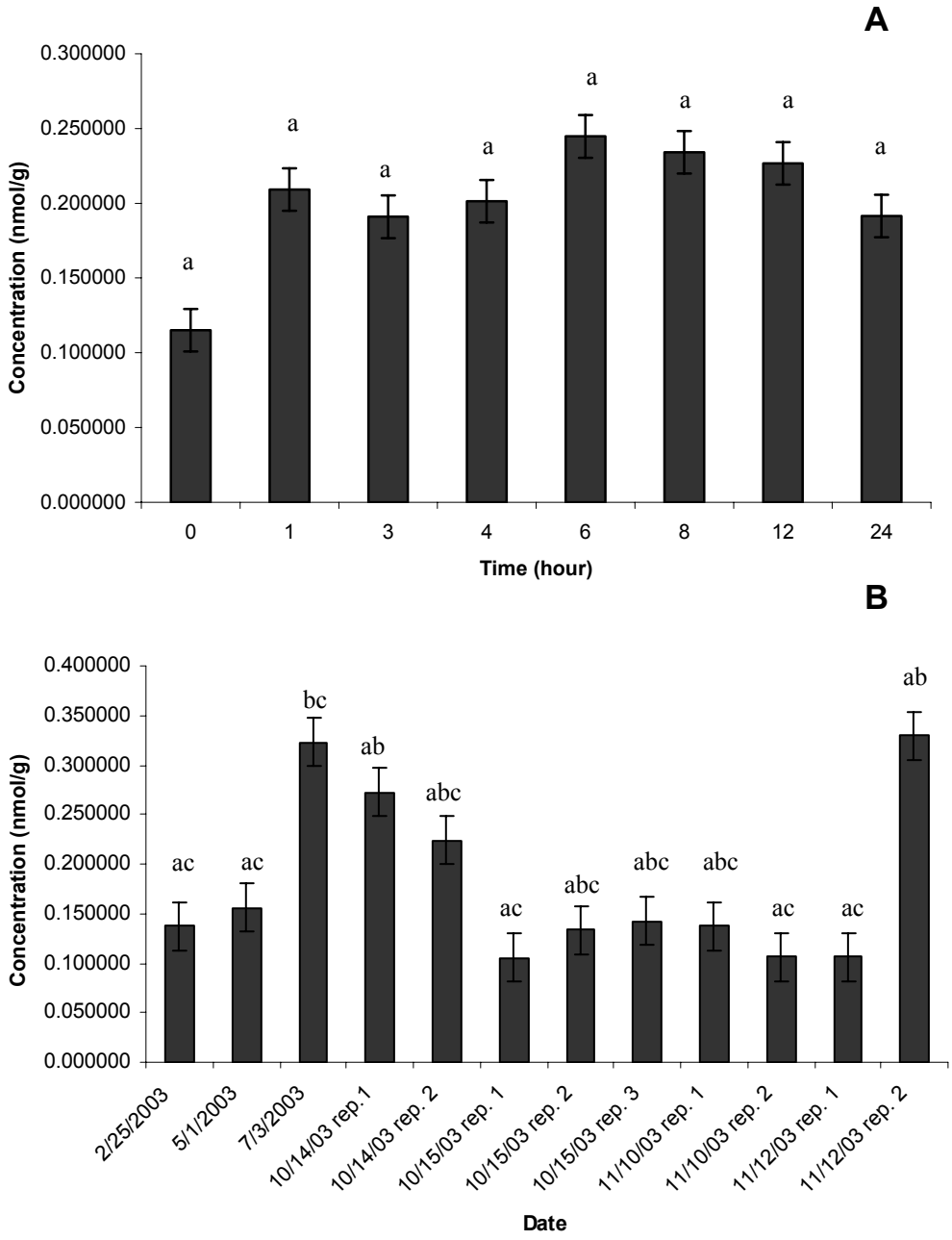

Fig. 3. Mean ATP concentrations for slices exposed to media with no drug. ATP concentrations (mean±SE , nmol/g) of slices exposed to media without drug across 24 hours (A) or among experiment dates (B). Different repetitions on the same date are represented as rep. 1, 2 or 3. Homogeneous groups by Tukey's test for multiple comparisons ($p<0.05$) are shown as letters. Times or dates with the same letter are not significantly different. For time n=12, except hour 0, n=18; hour 3, n=4; hour 4 and 6, n=6; hour 24, n=24. For dates, n=4, except 2/25/2003, n=10; 5/1/2003, 7/3/2003, 10/14/2003 (rep.1 and 2) and 10/15/2003 (rep. 1), n=12.

Table 4. ATP concentrations (nmol/g) for slices exposed to media with no drug. Means with standard deviations (std dev) represent variability across time, among days and between days. The mean values for time and date are plotted in Fig. 3.

Time (hour)	2/25/2003	5/1/2003	7/3/2003	10/14/2003	10/14/2003	10/15/2003	10/15/2003	10/15/2003	11/10/2003	11/10/2003	11/12/2003	11/12/2003	Mean	std dev
0				0.133563	0.082182	0.145604	0.127799	0.136371	0.209470	0.131331	0.108560	0.005837	0.115053	0.054142
				0.181772	0.094588	0.178024	0.149639	0.128633	0.070663	0.091572	0.091792	0.003558		
1	0.261167	0.161748	0.249336	0.388308	0.225251	0.051878							0.209271	0.121163
	0.023148	0.226146	0.237635	0.402588	0.230626	0.053424								
3		0.108910	0.238568										0.190976	0.106252
		0.098044	0.318382											
4				0.245519	0.252395	0.081670							0.201385	0.097244
				0.272121	0.283970	0.072635								
6	0.180345	0.188467	0.355537										0.244718	0.068725
	0.229858	0.215210	0.298894											
8	0.076692	0.120283	0.343441	0.427561	0.268529	0.101800							0.234136	0.130948
	0.171040	0.137252	0.332160	0.440005	0.280467	0.110401								
12	0.107492	0.137374	0.387272	0.252609	0.319044	0.137500							0.226771	0.109897
	0.122515	0.150300	0.390205	0.250094	0.342187	0.124659								
24	0.103992	0.158579	0.372342	0.131331	0.231777	0.093177	0.147107	0.139069	0.126450	0.120664	0.151701	0.713034	0.191397	0.161758
	0.097525	0.167968	0.348597	0.137486	0.069946	0.111598	0.110923	0.163441	0.143757	0.082875	0.073220	0.596978		
Mean	0.137378	0.155857	0.322697	0.271913	0.223414	0.105198	0.133867	0.141878	0.137585	0.106610	0.106318	0.329851		
std dev	0.072575	0.039596	0.055384	0.116678	0.092082	0.037803	0.018141	0.015040	0.057178	0.023080	0.033522	0.378435		

Histologic lesion scores for necrosis, vacuolation extent and vacuolation severity were examined by one-way ANOVA across time and among experiment dates (Figures 4-6). No significant group differences were noted among experiment dates for necrosis (Figure 4B, Table 5), vacuolation extent (Figure 5B, Table 6) and vacuolation severity (Figure 6B, Table 7).

Across time, however, significant differences were noted for all lesion score categories. Mean necrosis histologic lesion scores ($df=5$, F-ratio=23.07, p-value<0.0001) at 0 hour were lower than those at 8, 12 and 24 hours (Figure 4A, Table 5). At 1 hour, necrosis scores were lower than those at 8 and 24 hours, and scores at 4 and 12 hours were lower than those at 24 hours (Figure 4A, Table 5). Mean vacuolation extent histologic scores ($df=5$, F-ratio=4.956, p-value=0.0014) at 0 hour were lower than those at 8, 12 and 24 hours (Figure 5A, Table 6). Mean vacuolation severity histologic lesion scores ($df=5$, F-ratio=8.972, p-value<0.0001) at 0 hour were lower than those at 8, 12 and 24 hours (Figure 6A, Table 7). Scores at 24 hours were higher than those at 1 and 4 hours (Figure 6A, Table 7).

Potassium, ATP and Histology Summary

Mean potassium concentrations had little variability after an initial decrease after zero hour. The differences in mean potassium concentrations among experiments may be related to the differing number of samples comprising the mean. For 5/1/2003 and 7/3/203, twelve samples were averaged across twenty-four hours while for 10/15/2003 and 11/12/2003 only four samples per date were averaged. The higher potassium concentrations at 24 hours compared to one, three and eight hours may also be related to the number of samples averaged. Additionally, the presence of zero hour potassium values may have increased the mean values as zero hour potassium concentrations were higher than those for other times.

The average ATP concentrations across time were not statistically different from each other. The differences in ATP concentrations across dates may be related to the variability in health of the liver samples or inter-dog differences.

For all histologic lesion categories, no statistical differences were noted among experiment dates. Across time all lesion score categories had lower mean scores at zero hour compared to mean scores at 8, 12 and 24 hours. Differences in mean histologic lesion scores appear to be an effect of length of incubation and may indicate that hepatic slice histology is a more sensitive indicator of slice health than either potassium or ATP content.

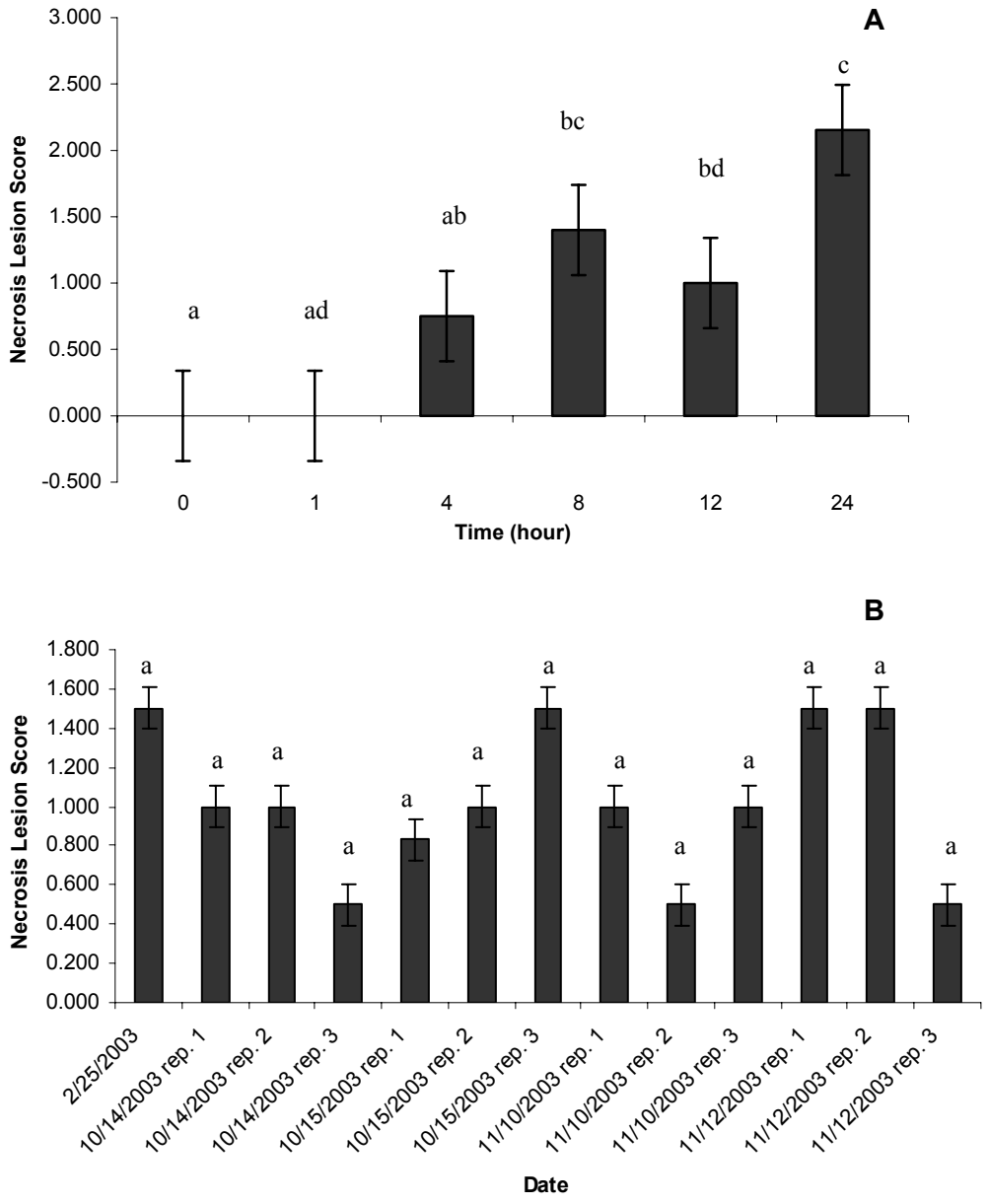

Fig. 4. Mean necrosis lesion scores for slices exposed to media with no drug. Necrosis lesion scores (mean±SE) of slices exposed to media without drug across 24 hours (A) or among experiment dates (B). Different repetitions on the same date are represented as rep. 1, 2 or 3. Homogeneous groups by Tukey's test for multiple comparisons ($p<0.05$) are shown as letters. Times or dates with the same letter are not significantly different. For time n=5, except hour 0, n=12; hour 4, n=4; hour 24, n=13. For dates, n=6, except 2/25/2003, n=4; 10/15/2003 (rep. 2 and 3), 11/10/2003 (rep. 1-3) and 11/12/2003 (rep. 1-3), n=2.

Table 5. Necrosis lesion scores for slices exposed to media with no drug. Necrosis lesion score means with standard deviations (std dev) represent variability across time, among days and between days. The mean values for time and date are plotted in Fig. 4.

Necrosis Lesion Scores															
Time (hour)	**2/25**	**10/14**	**10/14**	**10/14**	**10/15**	**10/15**	**10/15**	**11/10**	**11/10**	**11/10**	**11/12**	**11/12**	**11/12**	**Mean**	**std dev**
0		0	0	0	0	0	0	0	0	0	0	0	0	0.00	0.00
1	0	0	0	0	0									0.00	0.00
4		1	1	0	1									0.75	0.50
8	1	2	2	1	1									1.40	0.55
12	1	1	1	1	1									1.00	0.00
24	4	2	2	1	2	2	3	2	1	2	3	3	1	2.15	0.90
Mean	1.50	1.00	1.00	0.50	0.83	1.00	1.50	1.00	0.50	1.00	1.50	1.50	0.50		
std dev	1.73	0.89	0.89	0.55	0.75	1.41	2.12	1.41	0.71	1.41	2.12	2.12	0.71		

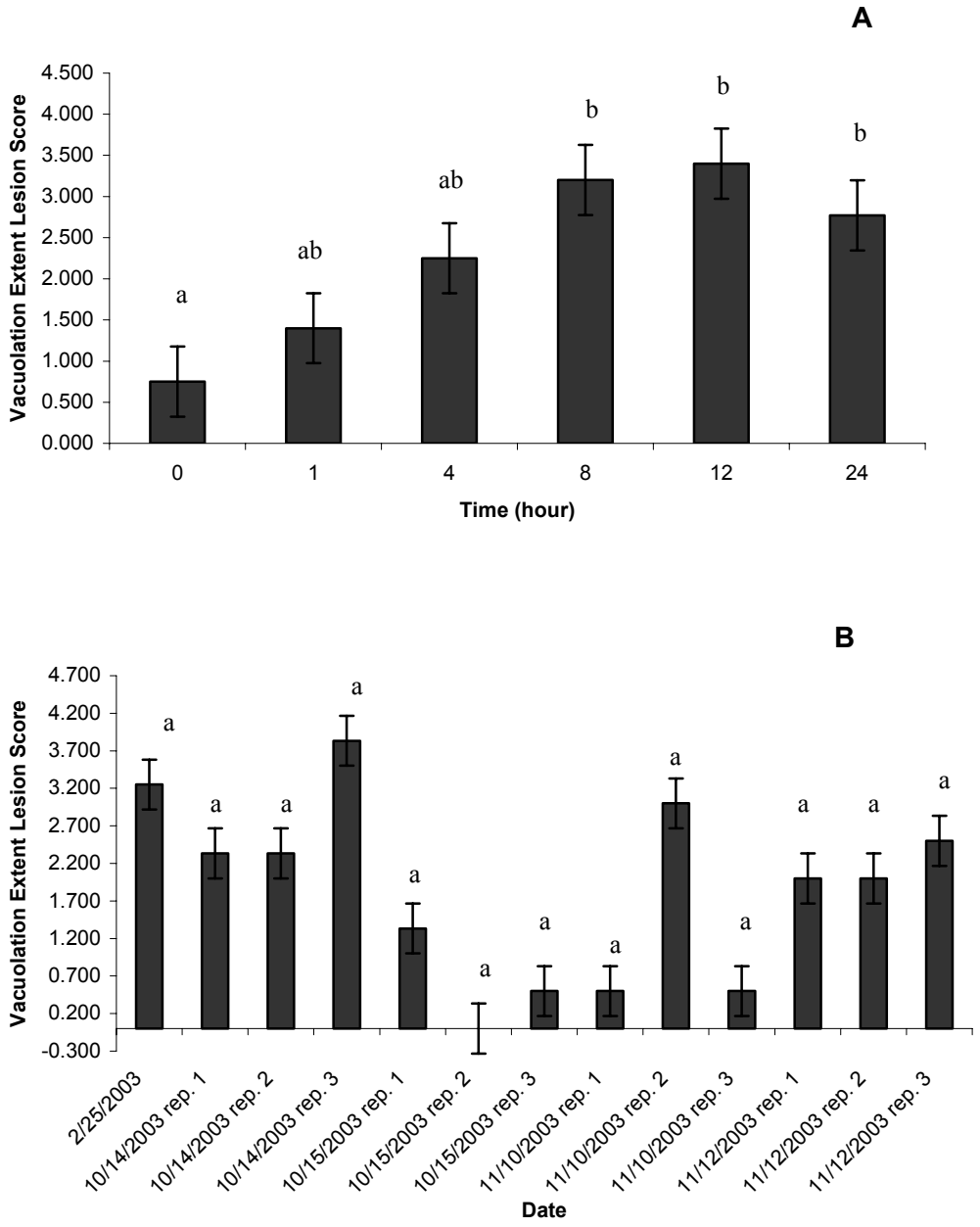

Fig. 5. Mean vacuolation extent lesion scores for slices exposed to media with no drug. Vacuolation extent lesion scores (mean±SE) of slices exposed to media without drug across 24 hours (A) or among experiment dates (B). Different repetitions on the same date are represented as rep. 1, 2 or 3. Homogeneous groups by Tukey's test for multiple comparisons ($p<0.05$) are shown as letters. Times or dates with the same letter are not significantly different. For time n=5, except hour 0, n=12; hour 4, n=4; hour 24, n=13. For dates, n=6, except 2/25/2003, n=4; 10/15/2003 (rep. 2 and 3), 11/10/2003 (rep. 1-3) and 11/12/2003 (rep. 1-3), n=2.

Table 6. Vacuolation extent lesion scores for slices exposed to media with no drug. Vacuolation extent lesion score means with standard deviations (std dev) represent variability across time, among days and between days. The mean values for time and date are plotted in Fig. 5.

Vacuolation Extent Lesion Scores															
Time (hour)	**2/25**	**10/14**	**10/14**	**10/14**	**10/15**	**10/15**	**10/15**	**11/10**	**11/10**	**11/10**	**11/12**	**11/12**	**11/12**	**Mean**	**std dev**
0		1	1	4	0	0	0	0	2	0	0	0	1	0.75	1.22
1	3	0	0	4	0									1.40	1.95
4		2	2	3	2									2.25	0.50
8	3	3	3	4	3									3.20	0.45
12	4	4	4	4	1									3.40	1.34
24	3	4	4	4	2	0	1	1	4	1	4	4	4	2.77	1.54
Mean	3.25	2.33	2.33	3.83	1.33	0.00	0.50	0.50	3.00	0.50	2.00	2.00	2.50		
std dev	0.50	1.63	1.63	0.41	1.21	0.00	0.71	0.71	1.41	0.71	2.83	2.83	2.12		

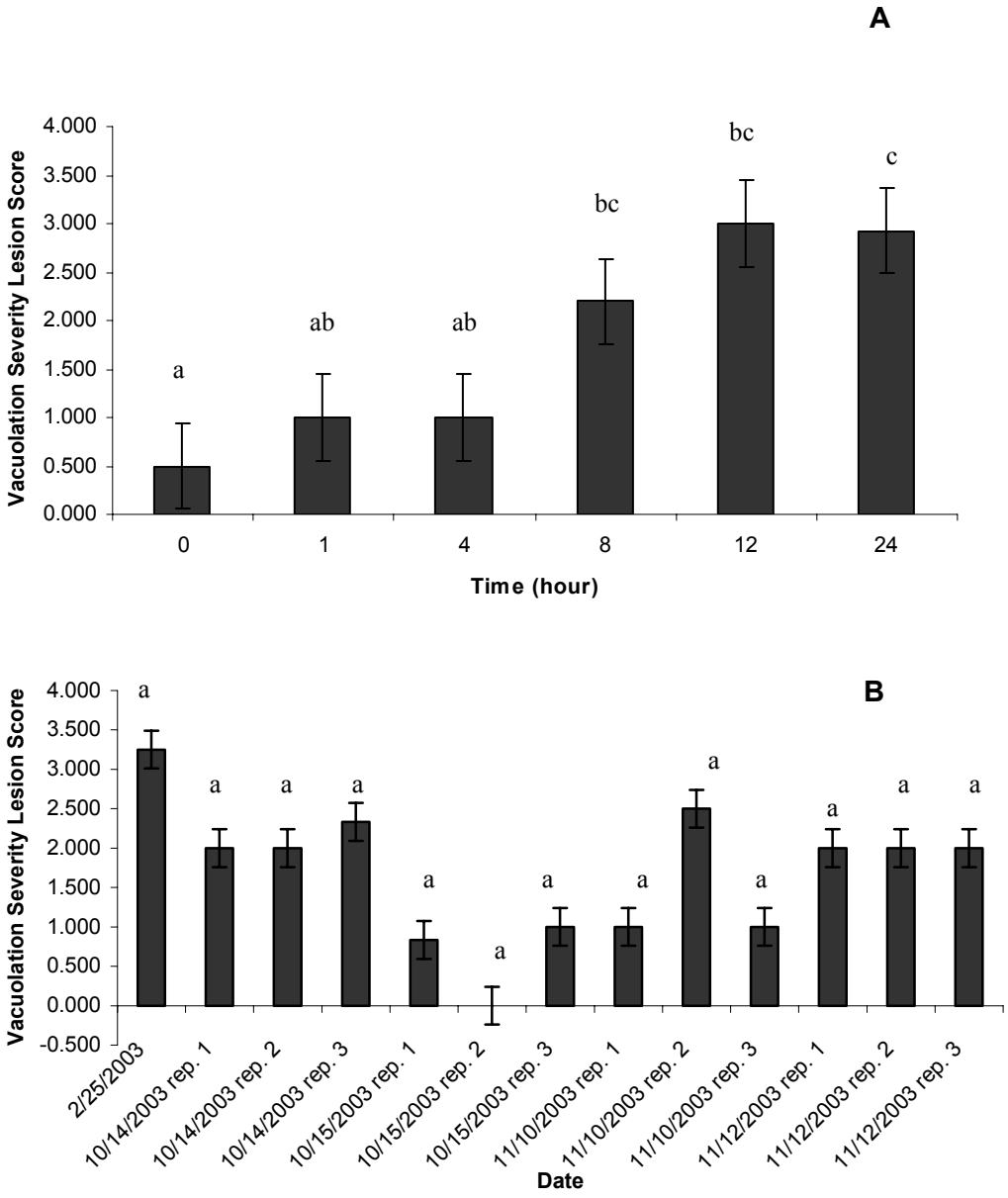

Fig. 6. Mean vacuolation severity lesion scores for slices exposed to media with no drug. Vacuolation severity lesion scores (mean±SE) of slices exposed to media without drug across 24 hours (A) or among experiment dates (B). Different repetitions on the same date are represented as rep. 1, 2 or 3. Homogeneous groups by Tukey's test for multiple comparisons ($p<0.05$) are shown as letters. Times or dates with the same letter are not significantly different. For time n=5, except hour 0, n=12; hour 4, n=4; hour 24, n=13. For dates, n=6, except 2/25/2003, n=4; 10/15/2003 (rep. 2 and 3), 11/10/2003 (rep. 1-3) and 11/12/2003 (rep. 1-3), n=2.

Table 7. Vacuolation severity lesion scores for slices exposed to media with no drug. Vacuolation severity lesion score means with standard deviations (std dev) represent variability across time, among days and between days. The mean values for time and date are plotted in Fig. 6.

Vacuolation Severity Lesion Scores															
Time (hour)	**2/25**	**10/14**	**10/14**	**10/14**	**10/15**	**10/15**	**10/15**	**11/10**	**11/10**	**11/10**	**11/12**	**11/12**	**11/12**	**Mean**	**std dev**
0		1	1	2	0	0	0	0	1	0	0	0	1	0.50	0.67
1	3	0	0	2	0									1.00	1.41
4		1	1	1	1									1.00	0.00
8	3	2	2	3	1									2.20	0.84
12	4	4	4	2	1									3.00	1.41
24	3	4	4	4	2	0	2	2	4	2	4	4	3	2.92	1.26
Mean	3.25	2.00	2.00	2.33	0.83	0.00	1.00	1.00	2.50	1.00	2.00	2.00	2.00		
std dev	0.50	1.67	1.67	1.03	0.75	0.00	1.41	1.41	2.12	1.41	2.83	2.83	1.41		

CHAPTER IV
CANINE HEPATIC SLICE RESPONSE TO PHENOBARBITAL, PRIMIDONE AND LIDOCAINE

Phenobarbital

Media and Supernatant Drug Concentrations

Hepatic slices were exposed to phenobarbital to determine the ability of slices to survive in the media and incubator conditions and to determine the hepatotoxic potential of phenobarbital in the liver slice system. Phenobarbital media and supernatant concentrations of slices incubated in media with no drug were below the limit of quantification. For media containing drug, the concentration of phenobarbital was consistent over 24 hours of incubation (Figure 7). Media concentrations of phenobarbital remained within ten percent of the original concentrations during incubation, and no significant changes in media phenobarbital concentrations were seen across time. All prepared media was within ten percent of expected concentrations. The area under the curve (AUC) (hour*µg/ml) of phenobarbital in media was calculated for each concentration (Table 8).

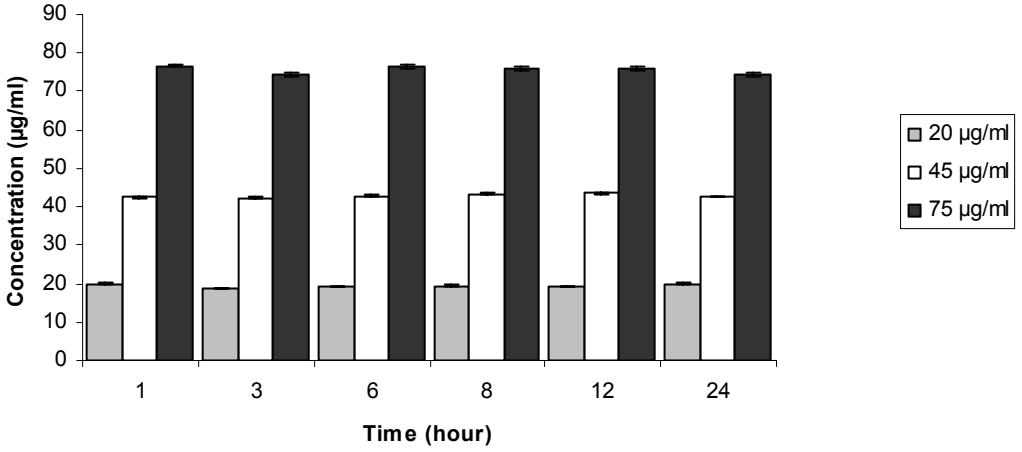

Fig. 7. Phenobarbital media concentrations. Phenobarbital media concentrations (µg/ml±SE, average) during 24 hours of incubation were within ± 10% of expected concentrations. No significant differences were noted across time for 20, 45 or 75 µg/ml media. Media concentrations are an average of two samples per concentration per time point.

Table 8. AUC of phenobarbital media. The AUC (hour*µg/ml) of phenobarbital in media following 24 hour hepatic slice incubation with media containing 0, 20, 45 or 75 µg/ml phenobarbital.

Phenobarbital Concentration (µg/ml)	Media AUC (hour*µg/ml)
0	below LOQ
20	445.90
45	989.50
75	1735.00

Quantifiable concentrations of phenobarbital were noted in the hepatic slice supernatant of slices incubated up to 24 hours in media containing 45 and 75 µg/ml phenobarbital (Figure 8). No statistically significant changes in hepatic slice supernatant concentrations (µg/ml) across time were noted. The AUC (hour*µg/ml) of phenobarbital in hepatic slice supernatant was calculated for each media concentration (Table 9).

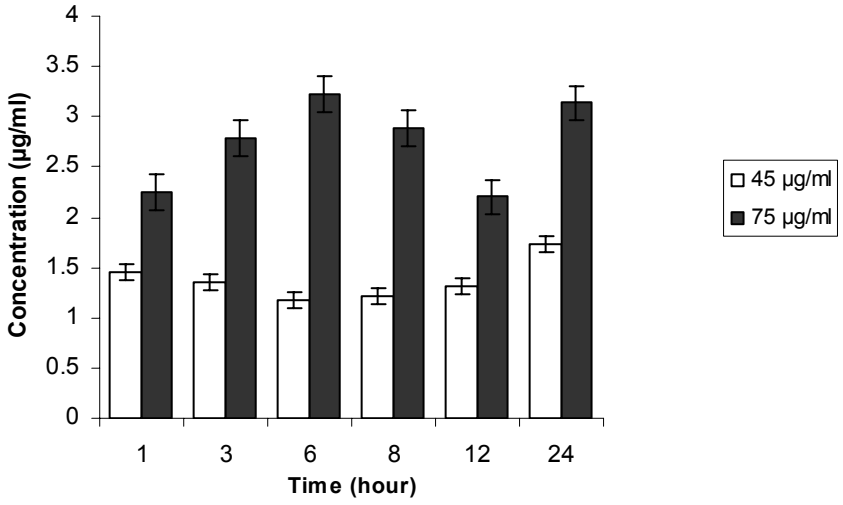

Fig. 8. Phenobarbital supernatant concentrations. Phenobarbital concentrations (µg/ml±SE, average) in hepatic slice supernatant during 24 hours of incubation. No statistically significant differences were noted across time for slices incubated in either 45 or 75 µg/ml media. Supernatant concentrations are an average of two samples per time point for each phenobarbital concentration.

Table 9. AUC of phenobarbital in hepatic slice supernatant. The AUC (hour*µg/ml) of phenobarbital in hepatic slice supernatant following 24 incubation in media containing 0, 20, 45 and 75 µg/ml phenobarbital.

Phenobarbital	Supernatant
Concentration (µg/ml)	AUC (hour*µg/ml)
0	below LOQ
20	below LOQ
45	32.22
75	62.35

Potassium

Potassium concentrations were measured in the hepatic slice supernatant to determine the viability of the slices during incubation. Figure 9 shows the concentrations of potassium (µmol/g-L) in hepatic slice supernatant following twenty-four hours of incubation with 0, 20, 45 and 75 µg/ml phenobarbital.

Two-way analysis of variance (ANOVA) was performed using time and concentration as factors. The interaction of the factors determined whether the main effects (time and concentration) were independent of each other. The time-concentration interaction (df=15, F-ratio=1.054, p-value =0.441) was not significant indicating that the factors were independent. ANOVA groups were based on the means of all samples at each time point (n=8) or the means of all times for each concentration (n=12). The two-way ANOVA results for potassium by time (Table 10, Figure 10) showed that there were two homogeneous groups (df = 5, F-ratio =4.021, p-value=0.009). Mean potassium concentrations at 6 and 24 hours were significantly higher than at 1 hour. No other times were significantly different. The two-way ANOVA results for concentration (df=3, F-ratio=3.538, p-value =0.030) revealed that mean potassium values for 75 µg/ml phenobarbital media were significantly lower than for media containing no phenobarbital (Table 11, Figure 11). The potassium content AUC (hour*µmol/g-L) was also determined for each concentration of phenobarbital (Table 12).

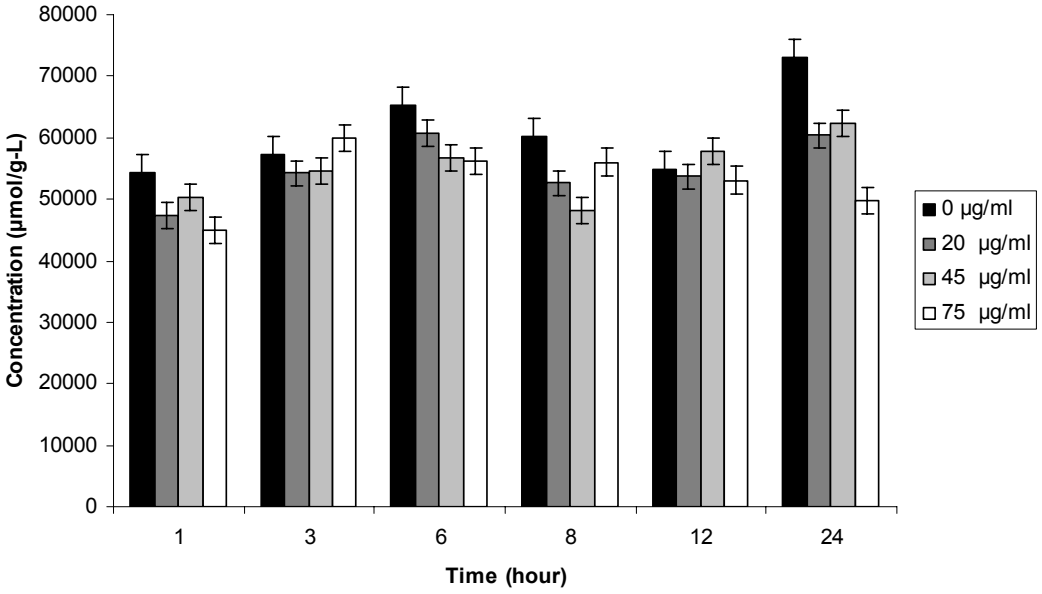

Fig. 9. Potassium supernatant levels after incubation of slices with phenobarbital. Potassium concentrations (µmol/g-L±SE, average) in hepatic slice supernatant during 24 hours of incubation in media containing phenobarbital at 0, 20, 45 and 75 µg/ml. Potassium concentrations are an average of two samples per time point for each concentration.

Table 10. Time analysis of potassium concentrations for slices incubated with phenobarbital. Means (µmol/g-L) of all potassium samples (n=8) at each time point. Homogeneous groups for potassium two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups		Means (µmol/g-L)
1	a		49272.14435
3	a	b	56455.60125
6		b	59730.50908
8	a	b	54234.82851
12	a	b	54867.40715
24		b	61344.60456

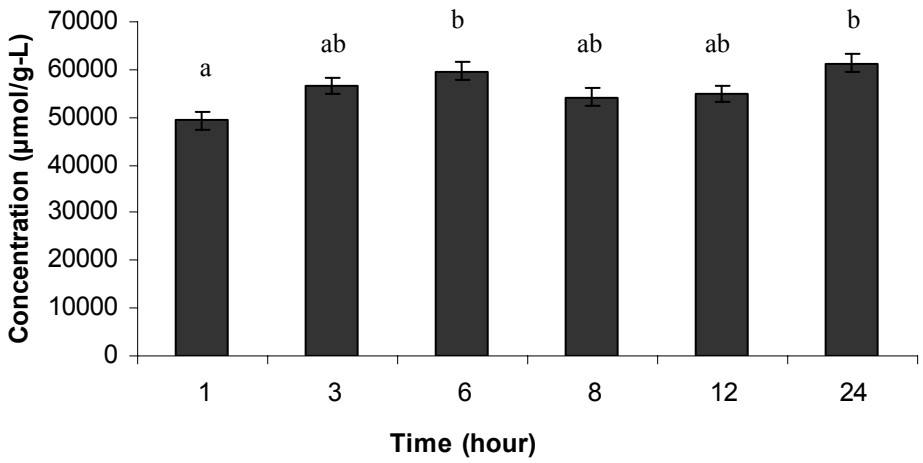

Fig. 10. Analysis across time of potassium concentrations for slices incubated with phenobarbital. Means (µmol/g-L±SE) of all potassium samples (n=8) at each time point. Homogeneous groups for potassium two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Table 11. Concentration analysis of potassium levels of slices incubated with phenobarbital. Means (µmol/g-L) of all potassium samples (n=12) at all times for each phenobarbital media concentration. Homogeneous groups for potassium two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Media Concentration (µg/ml)	Homogeneous groups	Means (µmol/g-L)
0	a	60793.56
20	a b	54821.81
45	a b	54989.80
75	b	53331.55

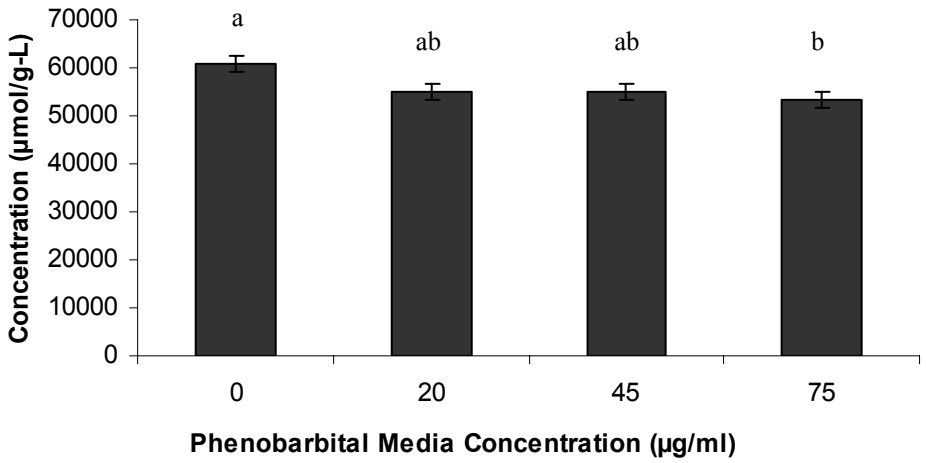

Fig. 11. Potassium analysis among media concentrations of slices incubated with phenobarbital. Means (µmol/g-L±SE) of potassium samples for all times (n=12) for each phenobarbital media concentration. Homogeneous groups for potassium two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Table 12. Potassium content AUC for slices incubated with phenobarbital. The AUC (hour*µmol/g-L) of potassium content for slices incubated in media containing 0, 20, 45 and 75 µg/ml phenobarbital.

Phenobarbital	Potassium Content
Concentration (µg/ml)	AUC (hour*µmol/g-L)
0	1.418E+06
20	1.284E+06
45	1.310E+06
75	1.226E+06

ATP

As another measure of hepatic slice viability, ATP concentrations were measured in hepatic slice supernatant. Concentrations of ATP (nmol/g) were measured following incubation in media containing varying concentrations of phenobarbital (Figure 12). The two-way ANOVA for ATP content revealed a significant interaction between the factors time and concentration (df=15, F-ratio=2.671, p-value =0.015). The effects of the two factors were not independent. ANOVA groups were based on the means of all samples at each time point (n=8) or the means of

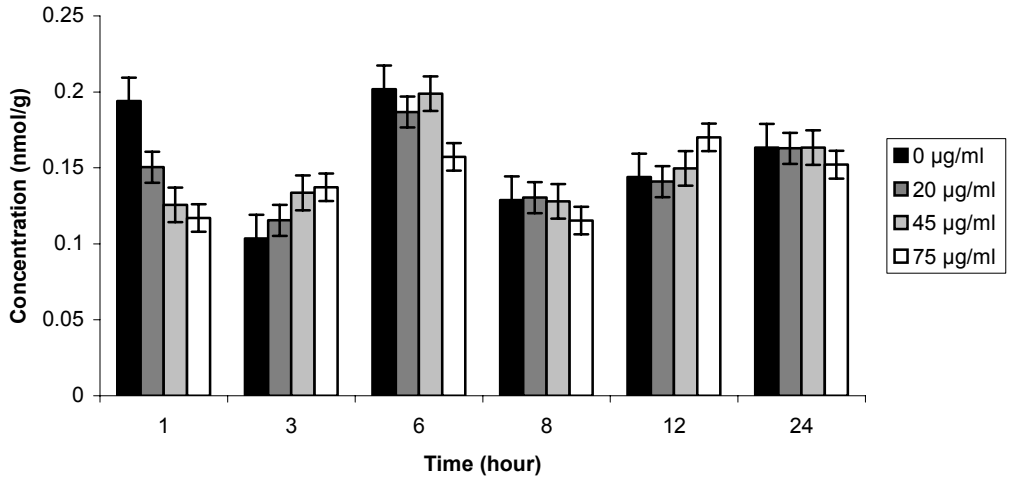

Fig. 12. ATP supernatant concentrations of slices incubated with phenobarbital. ATP concentrations (nmol/g±SE, average) in hepatic slice supernatant during 24 hours of incubation in media containing phenobarbital (0, 20, 45, 75 µg/ml). ATP concentrations are an average of two samples per time point per media concentration.

all times for each concentration (n=12). The two-way ANOVA of ATP content for time (Table 13, Figure 13) showed that there were three homogeneous groups (df = 5, F-ratio =12.641, p-value=0.000). Mean ATP concentrations at 1 and 24 hours were significantly higher than at 3 hours. Mean ATP values at 6 hours were significantly higher than at 1, 3, 8 and 12 hours.

Table 13. Time analysis of ATP concentrations for slices incubated with phenobarbital. Means (nmol/g) of all ATP samples (n=8) at each time point. Homogeneous groups for ATP two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups			Means (nmol/g)
1	a			0.146765
3		b		0.122435
6			c	0.186166
8	a	b		0.125638
12	a	b		0.151126
24	a		c	0.160356

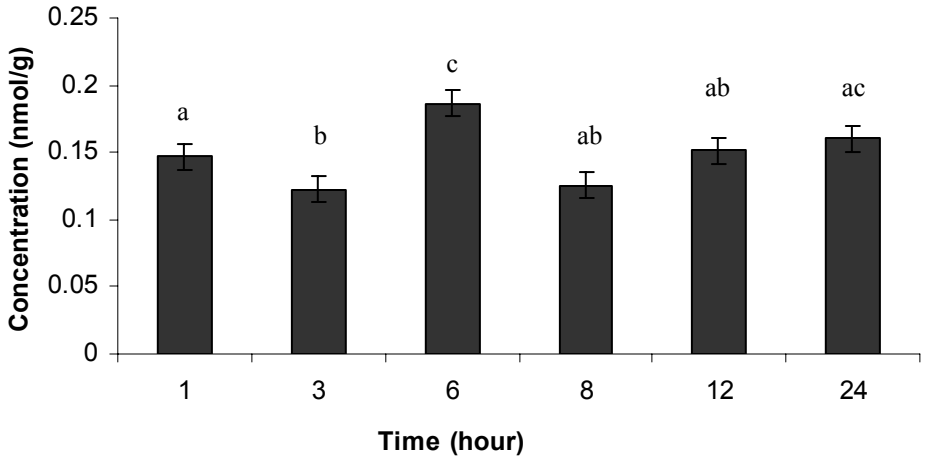

Fig. 13. Analysis across time of ATP concentrations for slices incubated with phenobarbital. Means (nmol/g±SE) of all ATP samples (n=8) at each time point. Homogeneous groups for ATP two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

The two-way ANOVA results for concentration (Table 14, Figure 14) revealed that a significant difference existed among the groups (df=3, F-ratio=167.701, p-value =0.000). The mean ATP values for 75 µg/ml phenobarbital media were significantly lower than for media containing 0, 20 or 45 µg/ml phenobarbital. The ATP content AUC (hour*nmol/g) was also determined for each concentration of phenobarbital (Table 15).

Table 14. Concentration analysis of ATP concentrations for slices incubated with phenobarbital. Means (nmol/g) of all ATP samples (n=12) at all times for each phenobarbital media concentration. Homogeneous groups for ATP two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Media Concentration (µg/ml)	Homogeneous groups		Means (nmol/g)
0	a		0.155857
20	a		0.147794
45	a		0.149845
75		b	0.141495

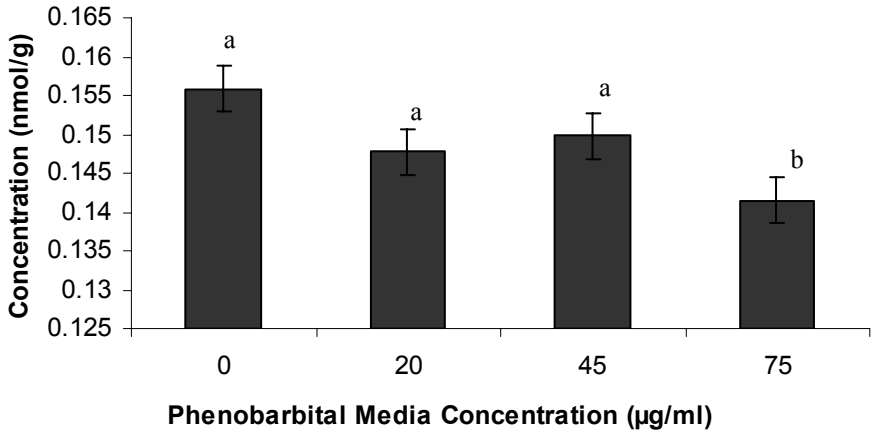

Fig. 14. ATP analysis among media concentrations of slices incubated with phenobarbital. Means (nmol/g±SE) of ATP samples for all times (n=12) for each phenobarbital media concentration. Homogeneous groups for ATP two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Table 15. ATP content AUC for slices incubated with phenobarbital. The AUC (hour*nmol/g) of ATP content for slices incubated in media containing 0, 20, 45 and 75 µg/ml phenobarbital.

Phenobarbital	ATP Content
Concentration (µg/ml)	AUC (hour*nmol/g)
0	3.47
20	3.40
45	3.52
75	3.47

Primidone

Media and Supernatant Drug Concentrations

Primidone was applied to the slices to determine their drug-metabolizing ability and to determine the hepatotoxic potential of primidone in the liver slice system. As phenobarbital is an active metabolite of primidone, phenobarbital was measured as an indication of the slices' ability to metabolize primidone in the media to phenobarbital (Yeary, 1980; Schwartz-Porsche *et al*., 1982). At the applied concentrations of primidone (0, 20, 45 and 75 µg/ml) and incubation for up to 24 hours, no quantifiable concentrations of phenobarbital were noted in either the media or supernatant. As shown in Tables 16 and 17, phenobarbital concentrations in the media and hepatic slice supernatant were detectable but below the limit of quantification, 1.1 µg/ml.

Table 16. Primidone metabolism in media. Phenobarbital concentrations in media of slices incubated in primidone media. The limit of quantification was 1.1 µg/ml. All values are shown as printed on the automated printout. Low indicates values were below lowest calibrator.

	Concentration of Primidone in Media				Time
	0 µg/ml	20 µg/ml	45 µg/ml	75 µg/ml	hour
Concentration of Phenobarbital in Media (µg/ml)	0.06	0.25	0.40	0.50	1
	0.11	0.21	0.30	0.40	
	0.23	0.28	0.43	0.52	3
	0.05	0.24	0.31	0.48	
	0.23	0.30	Low	Low	6
	0.06	0.24	Low	Low	
	Low	Low	Low	Low	8
	Low	Low	Low	Low	
	Low	Low	Low	Low	12
	Low	Low	Low	Low	
	Low	0.22	0.16	0.50	24
	0.08	0.19	0.23	0.36	

Table 17. Primidone metabolism in supernatant. Phenobarbital concentrations in hepatic slice supernatant of slices incubated in primidone media. The limit of quantification was 1.1 µg/ml. All values are shown as printed on the automated printout. Low indicates values were below lowest calibrator.

	Concentration of Primidone in Media				Time
	0 µg/ml	20 µg/ml	45 µg/ml	75 µg/ml	hour
Concentration of Phenobarbital in Supernatant (µg/ml)	0.17	0.03	0.05	0.11	1
	0.15	0.1	0.07	0.1	
	0.08	0.13	0.1	0.18	3
	0.11	0.16	0.14	0.05	
	0.09	0.04	Low	0.06	6
	Low	Low	0	0.04	
	0.02	0.02	Low	Low	8
	Low	0.09	Low	0.02	
	0.08	0	0.09	0.06	12
	Low	0.07	0.03	0.01	
	Low	0.1	0.02	Low	24
	Low	Low	Low	Low	

Potassium

Figure 15 shows the concentrations of potassium in hepatic supernatant across time following incubation in primidone. Two-way ANOVA performed on the data showed that the factors time

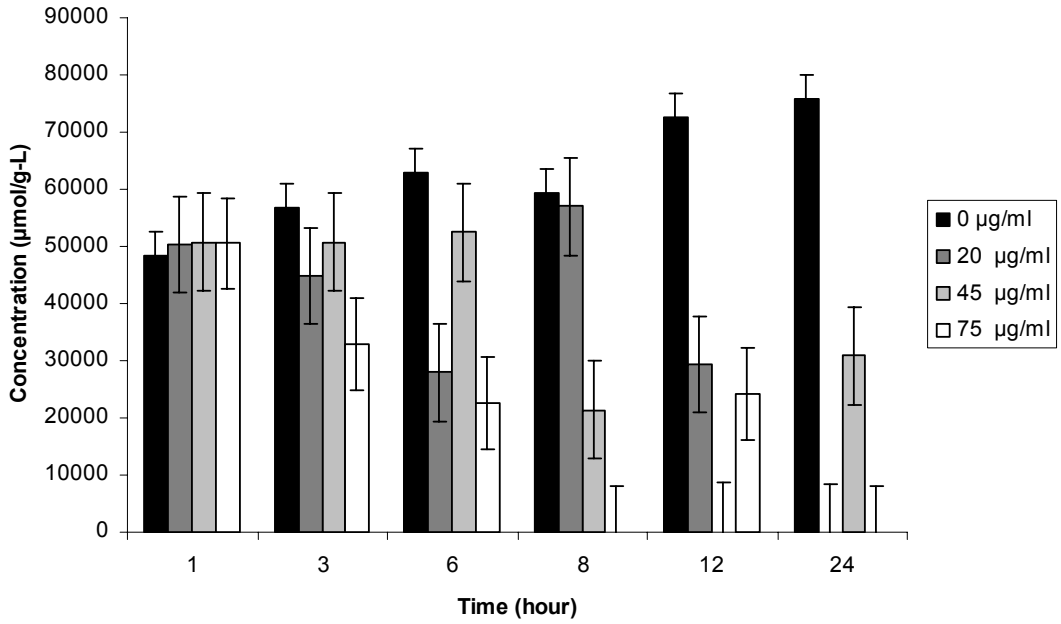

Fig. 15. Potassium supernatant concentrations for slices incubated with primidone. Potassium concentrations (µmol/g-L±SE, average) in hepatic slice supernatant during 24 hours of incubation in media containing 0, 20, 45 and 75 µg/ml primidone. Potassium concentrations are an average of two samples per time point per media concentration.

and concentration were independent (df=15, F-ratio=1.443, p-value =0.205). ANOVA groups were based on the means of all samples at each time point (n=8) or the means of all times for each concentration (n=12). The two-way ANOVA results for time showed that no means were significantly different (df = 5, F-ratio =1.371, p-value=0.270). The two-way ANOVA results for concentration (df=3, F-ratio=7.506, p-value =0.001) showed that two homogeneous groups were present (Table 18, Figure 16). The mean potassium values for media containing 20, 45 and 75 µg/ml primidone were significantly lower than for media without primidone (0 µg/ml). The potassium content AUC (hour*µmol/g-L) was also determined for each concentration of primidone (Table 19).

Table 18. Concentration analysis of potassium concentrations for slices incubated with primidone. Means (µmol/g-L) of all potassium samples (n=12) at all times for each primidone concentration. Homogeneous groups for potassium two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Media Concentration (µg/ml)	Homogeneous groups	Means (µmol/g-L)
0	a	62595.59
20	b	44371.76
45	b	43093.91
75	b	35028.63

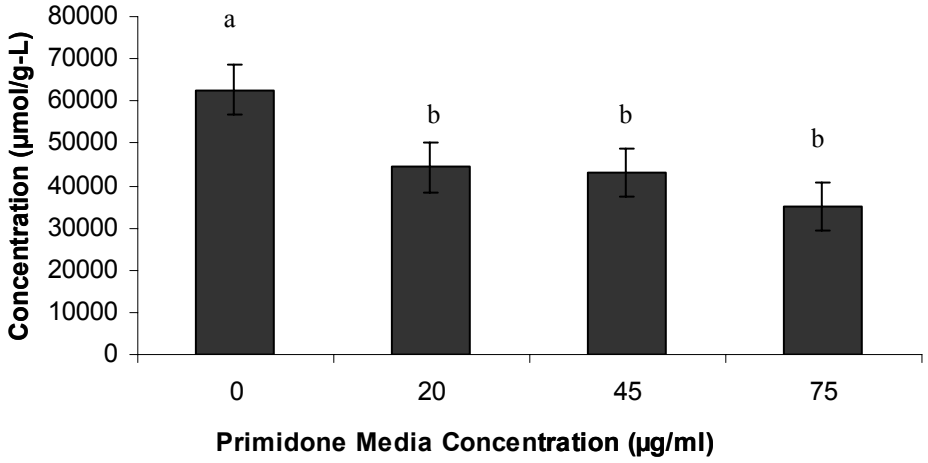

Fig. 16. Potassium analysis among media concentrations for slices incubated with primidone. Means (µmol/g-L±SE) of potassium samples for all times (n=12) for each primidone media concentration. Homogeneous groups for potassium two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Table 19. Potassium content AUC for slices incubated with primidone. The AUC (hour*µmol/g-L) of potassium content for slices incubated in media containing 0, 20, 45 and 75 µg/ml primidone.

Primidone	Potassium Content
Concentration (µg/ml)	AUC (hour*µmol/g-L)
0	1.560E+06
20	6.366E+05
45	5.583E+05
75	3.831E+05

ATP

Figure 17 depicts the ATP concentrations in hepatic slice supernatant following incubation in media containing primidone (0, 20, 45, 75 µg/ml). The two-way ANOVA for the interaction of time and concentration was not significant (df=15, F-ratio=1.684, p-value=0.123) indicating that the factors were independent of each other. ANOVA groups were based on the means of all

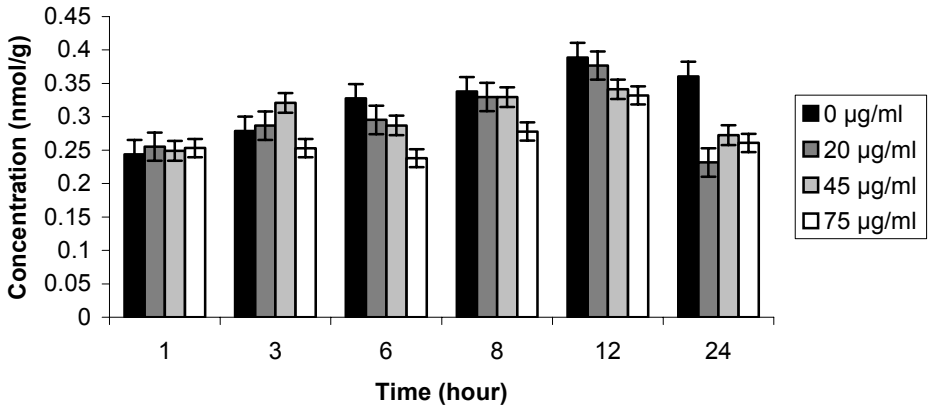

Fig. 17. ATP supernatant concentrations of slices incubated with primidone. ATP concentrations (nmol/g±SE, average) in hepatic slice supernatant during 24 hours of incubation in media containing primidone (0, 20, 45, 75 µg/ml). ATP concentrations are an average of two samples per time point per media concentration.

samples at each time point (n=8) or the means of all times for each concentration (n=12). Table 20 shows the homogeneous groups for the two-way ANOVA of ATP concentrations for time (df=5, F-ratio=12.353, p-value=0.000). The analysis revealed that at 12 hours mean ATP values were significantly higher than for 1, 3, 6 and 24 hours and that mean values at 8 hours were more than those for 1 hour (Table 20, Figure 18). For primidone media concentrations, the two-way

Table 20. Time analysis of ATP concentrations for slices incubated with primidone. Means (nmol/g) of all ATP samples (n=8) at each time point. Homogeneous groups for ATP two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups			Means (nmol/g)
1	a			0.250162
3	a	b		0.284632
6	a	b		0.286840
8		b	c	0.318584
12			c	0.359611
24	a	b		0.281255

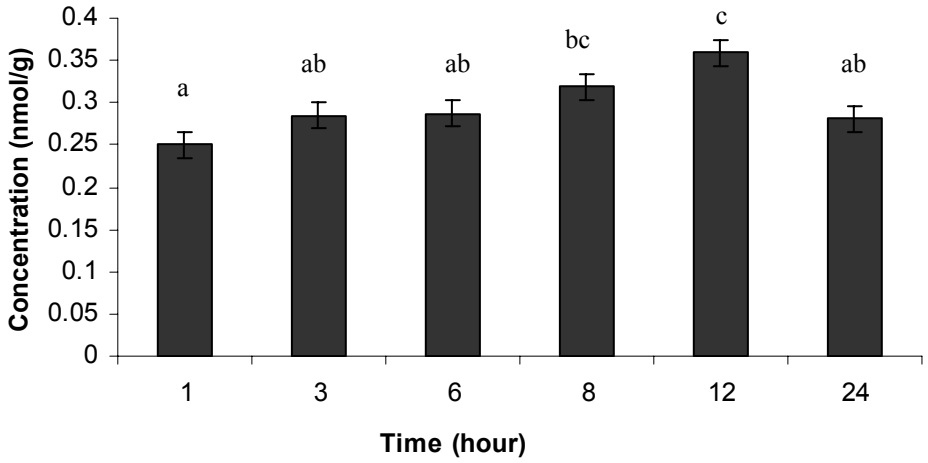

Fig. 18. Analysis across time of ATP concentrations for slices incubated with primidone. Means (nmol/g±SE) of all ATP samples (n=8) at each time point. Homogeneous groups for ATP two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

ANOVA showed that differences in the concentrations existed (df=3, F-ratio=6.330, p-value=0.003). Table 21 illustrates that ATP values in media containing no primidone were significantly higher than those for 75 µg/ml media (Figure 19). The ATP content AUC (hour*nmol/g) was also determined for each concentration of primidone (Table 22).

Table 21. Concentration analysis of ATP concentrations for slices incubated with primidone. Means (nmol/g) of all ATP samples (n=12) at all times for each primidone media concentration. Homogeneous groups for ATP two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Media Concentration (µg/ml)	Homogeneous groups	Means (nmol/g)
0	a	0.322697
20	a b	0.295795
45	a b	0.299865
75	b	0.269032

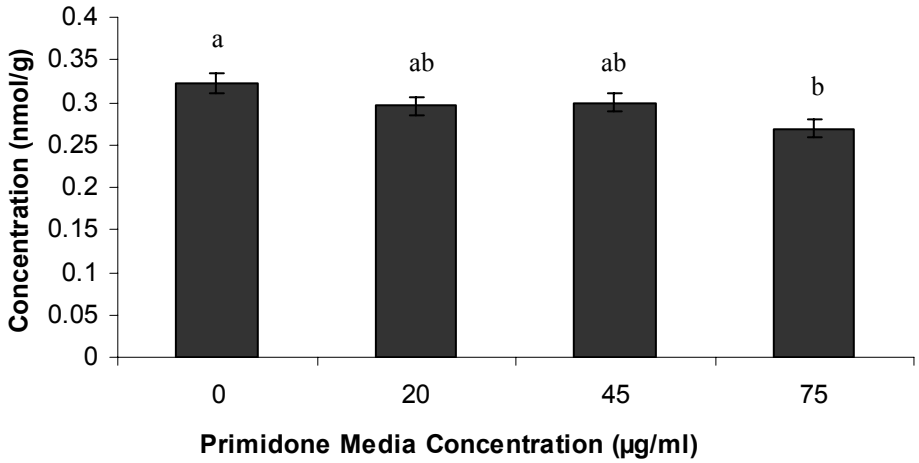

Fig. 19. ATP analysis among media concentrations of slices incubated with primidone. Means (nmol/g±SE) of ATP samples for all times (n=12) for each primidone media concentration. Homogeneous groups for ATP two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Table 22. ATP content AUC for slices incubated with primidone. The AUC (hour*nmol/g) of ATP content for slices incubated in media containing 0, 20, 45 and 75 µg/ml primidone.

Primidone	ATP Content
Concentration (µg/ml)	AUC (hour*nmol/g)
0	8.04
20	7.10
45	7.12
75	6.53

Lidocaine

Media and Supernatant Drug Concentrations

As another measure of hepatic slice drug-metabolizing ability, slices were incubated with media containing lidocaine. Two active metabolites of lidocaine are produced in dogs, monoethylglycinexylidide (MEGX) and glycinexylidide (GX) (Wilcke et al., 1983). Lidocaine and the metabolites, MEGX and GX, were measured over 24 hours incubation. Figures 20 through 22 illustrate the concentrations of lidocaine and MEGX in media. No quantifiable concentrations of GX were found in the media or hepatic slice supernatant.

For media containing 3 µg/ml lidocaine (Figure 20), the concentrations for lidocaine decreased over 24 hours while MEGX concentrations hovered around the lower limit of quantification (50 ng/ml). MEGX concentrations were above the LOQ at only 1 and 6 hours. Lidocaine concentrations at twelve hours were significantly lower than those at one hour (one-way ANOVA for time, p-value=0.0441) (Figure 20B).

Figure 21 shows the concentrations of lidocaine and MEGX in media with a beginning concentration of 10 µg/ml lidocaine. As with the 3 µg/ml media, the concentration of lidocaine decreases over time while the concentration of MEGX increases. One-way ANOVA for 10 µg/ml media revealed significant differences across time (p-value=0.0003). The lidocaine concentrations at one hour were higher than those at 6, 8, 12 and 24 hours (Figure 21B). Three hour concentrations were higher than 12 and 24 hour concentrations. Six and eight hour lidocaine concentrations were higher than those at 24 hours. Time differences were also noted for the one-way ANOVA of MEGX concentrations (p-value=0.0093). One hour concentrations were lower than those at 6, 8 and 12 hours (Figure 21C).

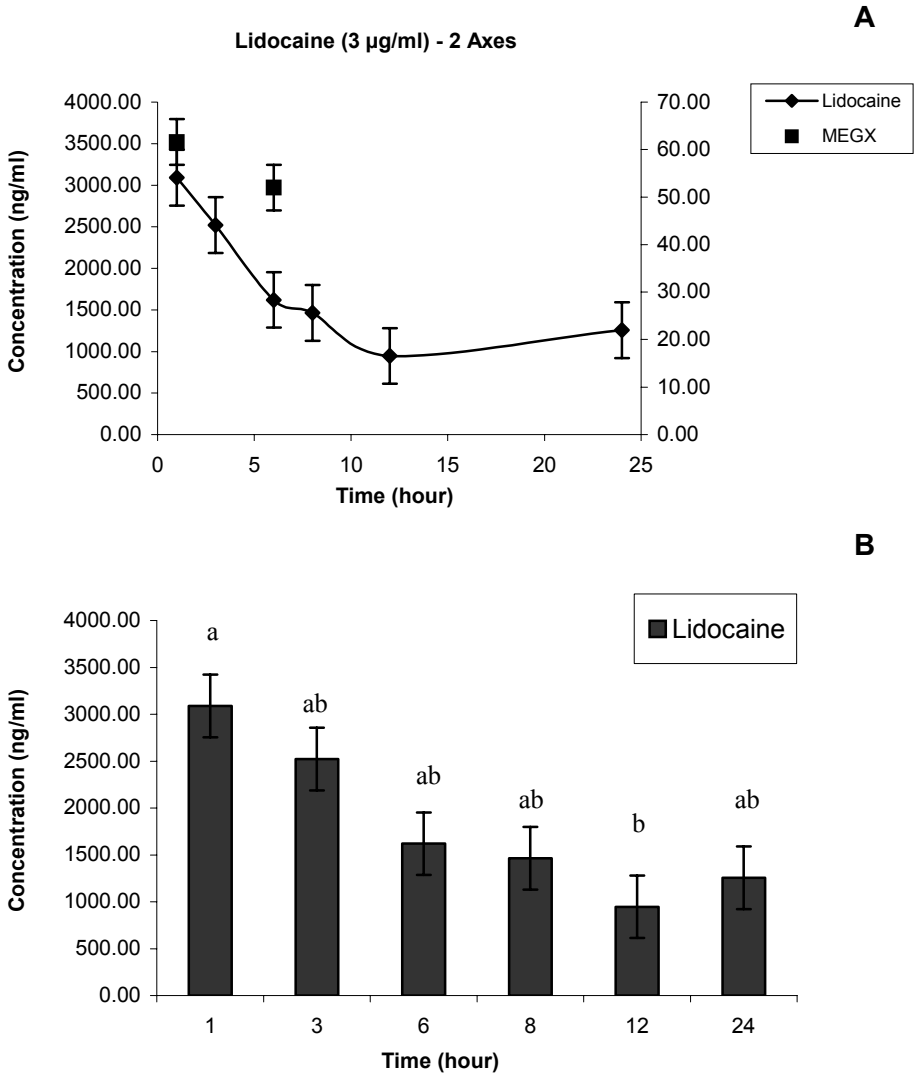

Fig. 20. Lidocaine (3µg/ml) and MEGX media concentrations. Lidocaine and MEGX concentrations (ng/ml±SE) in media following 24 hours of incubation in media containing 3 µg/ml lidocaine (A). Media concentrations are an average of two samples per time point. Homogenous groups (one-way ANOVA) across time for lidocaine concentrations (ng/ml±SE) are represented as letters (B). Times with the same letter are not significantly different. LOQ for lidocaine and MEGX is 50 ng/ml.

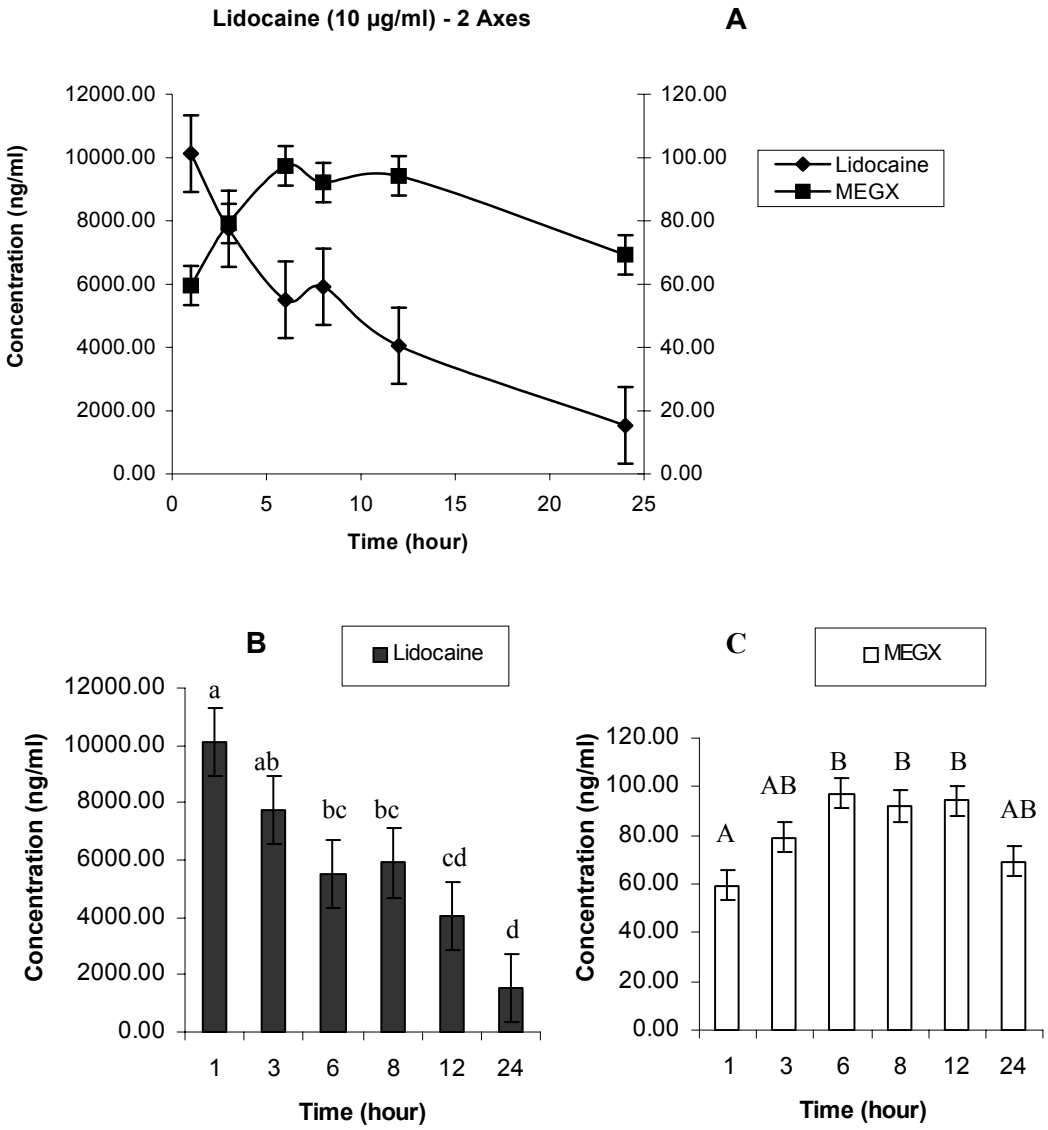

Fig. 21. Lidocaine (10 µg/ml) and MEGX media concentrations. Lidocaine and MEGX concentrations (ng/ml±SE) in media following 24 hours of incubation in media containing 10µg/ml lidocaine (A). Media concentrations are an average of two samples per time point. Homogenous groups (one-way ANOVA) across time for lidocaine (B) or MEGX (C) concentrations (ng/ml±SE) are represented as letters. Times with the same letter are not significantly different. LOQ for lidocaine and MEGX is 50 ng/ml.

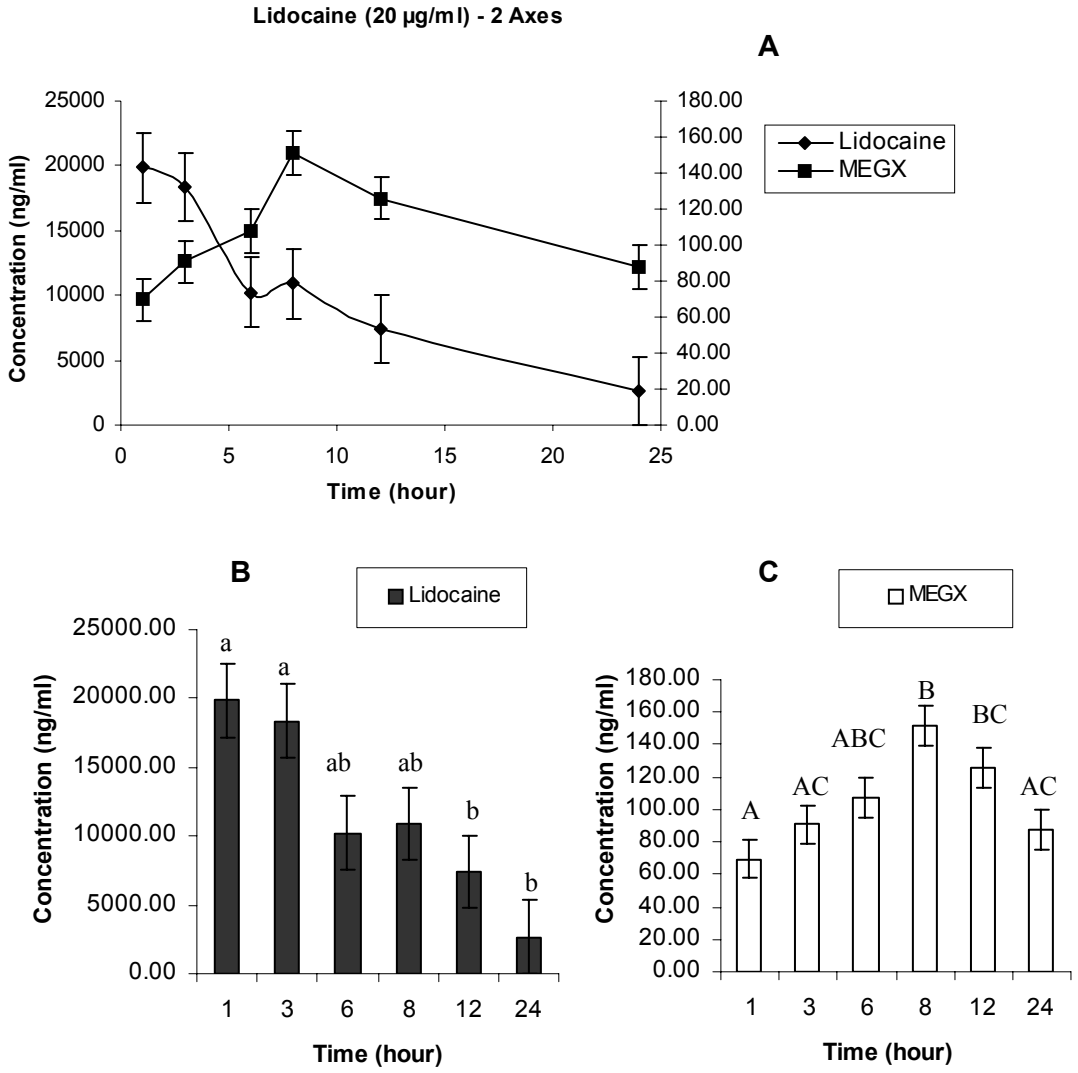

Fig. 22. Lidocaine (20 µg/ml) and MEGX media concentrations. Lidocaine and MEGX concentrations (ng/ml±SE) in media following 24 hours of incubation in media containing 20µg/ml lidocaine (A). Media concentrations are an average of two samples per time point. Homogenous groups (one-way ANOVA) across time for lidocaine (B) or MEGX (C) concentrations (ng/ml±SE) are represented as letters. Times with the same letter are not significantly different. LOQ for lidocaine and MEGX is 50 ng/ml.

A similar pattern of lidocaine concentrations decreasing over time while MEGX concentrations increased existed for 20 µg/ml lidocaine media (Figure 22). Significant differences across time were noted for both lidocaine (p-value=0.0027) and MEGX (p-value=0.0039) by one-way ANOVA. For lidocaine one and three hour concentrations were higher than those at 12 and 24 hours (Figure 22B). For MEGX one hour concentrations were

lower than those at 8 and 12 hours (Figure 22C). Additionally, eight hour concentrations were higher than 3 and 24 hour concentrations. The AUC (hour*ng/ml) of lidocaine in media was calculated for each concentration (0, 3, 10 or 20 µg/ml) (Table 23).

Lidocaine concentrations in the hepatic supernatant were not quantifiable at any of the media concentrations. MEGX concentrations in the supernatant were quantifiable for one time point for 20 µg/ml media (1 hour) and one time point for 10 µg/ml media (3 hour).

Table 23. AUC of lidocaine and MEGX in media. The AUC (hour*ng/ml) of lidocaine and MEGX in media following 24 hour hepatic slice incubation in media containing 0, 3, 10 or 20 µg/ml lidocaine.

Lidocaine	Media	
Concentration (µg/ml)	Lidocaine AUC (hour*ng/ml)	MEGX AUC (hour*ng/ml)
0	below LOQ	below LOQ
3	32947.00	283.98
10	102606.00	1946.10
20	199158.00	2554.80

Potassium

Figure 23 shows the potassium concentrations in hepatic slice supernatant following 24 hours incubation with lidocaine (0, 3, 10, 20 µg/ml). The two-way ANOVA of the interaction of time and concentration showed no significant differences (df=15, F-ratio=0.883, p-value=0.590). ANOVA groups were based on the means of all samples at each time point (n=8) or the means of all times for each concentration (n=12). For the analysis of concentration, no significant difference was noted (df=3, F-ratio=1.313, p-value=0.294). The two-way ANOVA for potassium values across time showed a significant difference (df=5, F-ratio=2.967, p-value=0.033). The homogeneous groups for time are displayed in Table 24. The mean potassium values for 1 hour were significantly lower than those for 24 hours (Figure 24). The potassium content AUC (hour*µmol/g-L) was also determined for each concentration of lidocaine (Table 25).

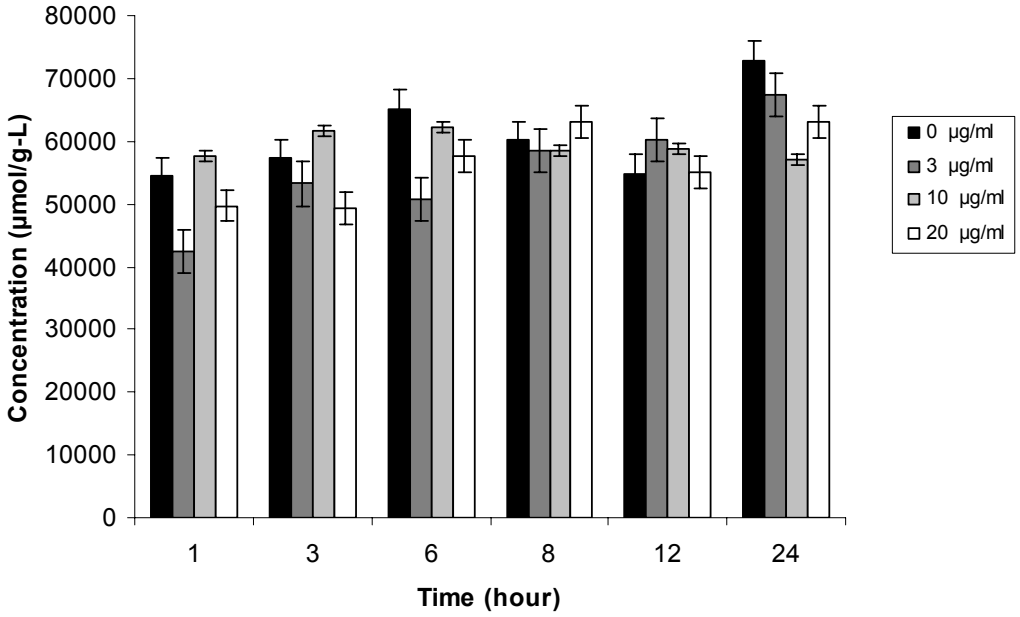

Fig. 23. Potassium supernatant concentrations of slices incubated with lidocaine. Potassium concentrations (µmol/g-L±SE, average) in hepatic slice supernatant during 24 hours of incubation in media containing 0, 3, 10 and 20 µg/ml lidocaine. Potassium concentrations are an average of two samples per time point per media concentration.

Table 24. Time analysis of potassium concentrations for slices incubated with lidocaine. Means (µmol/g-L) of all potassium samples (n=8) at each time point. Homogeneous groups for potassium two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups		Means (µmol/g-L)
1	a		51006.225
3	a	b	55351.079
6	a	b	58950.573
8	a	b	60018.558
12	a	b	57257.081
24		b	65153.067

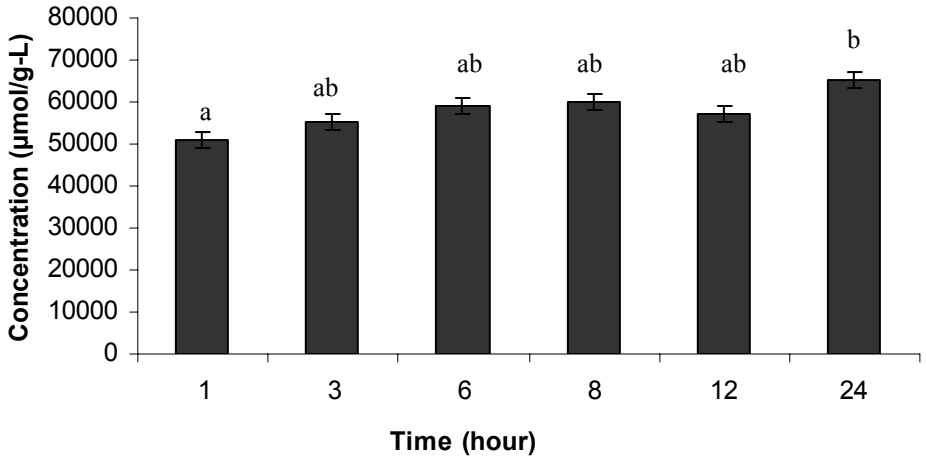

Fig. 24. Analysis across time of potassium concentrations for slices incubated with lidocaine. Means (µmol/g-L±SE) of all potassium samples (n=8) at each time point. Homogeneous groups for potassium two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Table 25. Potassium content AUC for slices incubated with lidocaine. The AUC (hour*µmol/g-L) of potassium content for slices incubated in media containing 0, 3, 10 and 20 µg/ml lidocaine.

Lidocaine	Potassium Content
Concentration (µg/ml)	AUC (hour*µmol/g-L)
0	1.418E+06
3	1.364E+06
10	1.356E+06
20	1.326E+06

ATP

Figure 25 depicts the concentrations of ATP in hepatic slice supernatant following exposure to media containing lidocaine. The two-way ANOVA for supernatant ATP values following incubation with lidocaine showed a significant interaction for the time and concentration factors (df=15, F-ratio=11.778, p-value=0.000). For the main effects of time (df=5, F-ratio=13.397, p-value=0.000) and concentration (df=3, F-ratio=5.672, p-value=0.004), a significant difference

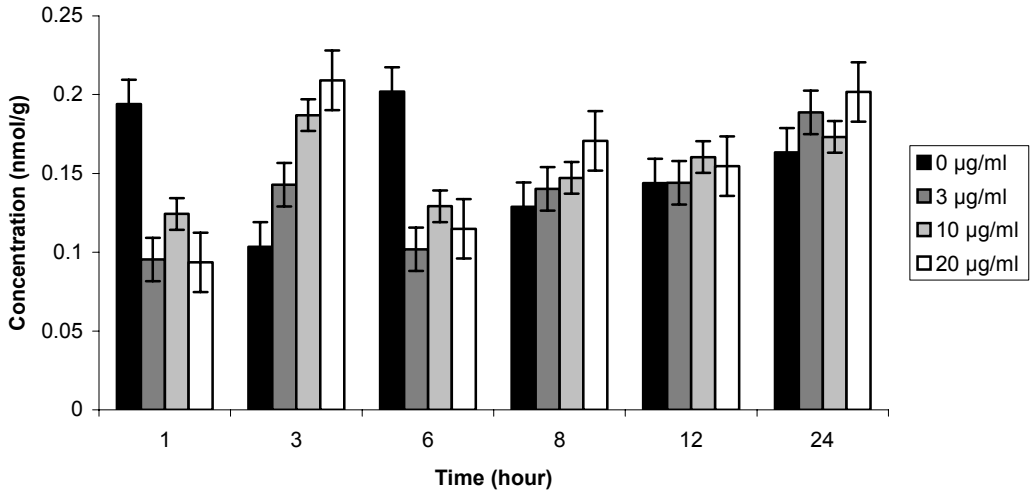

Fig. 25. ATP supernatant concentrations of slices incubated with lidocaine. ATP concentrations (nmol/g±SE, average) in hepatic slice supernatant during 24 hours of incubation in media containing 0, 3, 10 and 20 µg/ml lidocaine. ATP concentrations are an average of two samples per time point per media concentration.

was noted. ANOVA groups were based on the means of all samples at each time point (n=8) or the means of all times for each concentration (n=12). Table 26 depicts the homogeneous groups for time. The mean ATP values for 24 hours were significantly higher than those for 1, 6, 8 and 12 hours (Figure 26). Additionally, mean values for 12 hours were significantly higher than for 1 hour, and mean concentrations at 3 hours were higher than those at 1 and 6 hours. Table 27 shows the homogeneous groups for concentration. The mean ATP concentrations for 3 µg/ml lidocaine media were lower than those for all other concentrations (Figure 27). The ATP content AUC (hour*nmol/g) was also determined for each concentration of lidocaine (Table 28).

Table 26. Time analysis of ATP concentrations for slices incubated with lidocaine. Means (nmol/g) of all ATP samples (n=8) at each time point. Homogeneous groups for ATP two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (h)	Homogeneous groups			Means (nmol/g)
1	a			0.126838
3		b	c	0.160602
6	a			0.136932
8	a	b		0.146688
12		b		0.150712
24			c	0.181700

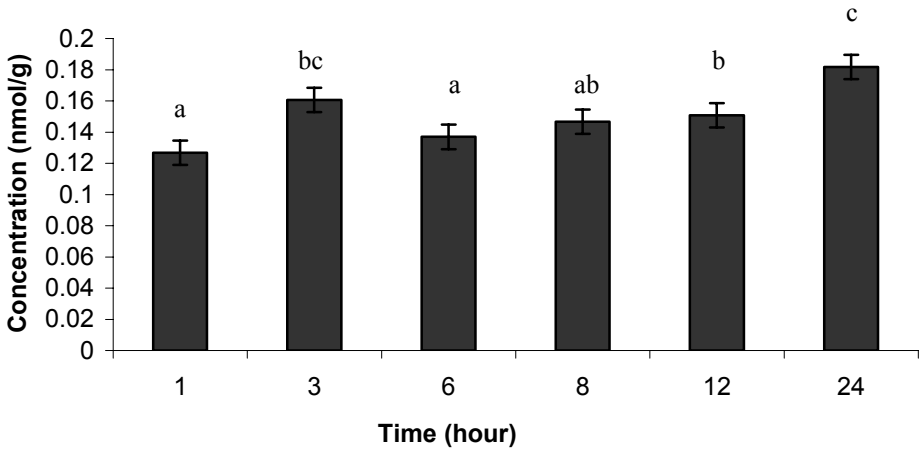

Fig. 26. Analysis across time of ATP concentrations for slices incubated with lidocaine. Means (nmol/g±SE) of all ATP samples (n=8) at each time point. Homogeneous groups for ATP two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Table 27. Concentration analysis of ATP concentrations for slices incubated with lidocaine. Means (nmol/g) of all ATP samples (n=12) at all times for each lidocaine media concentration. Homogeneous groups for ATP two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Concentration (μg/ml)	Homogeneous groups	Means (nmol/g)
0	a	0.155857
3	b	0.135506
10	a	0.153529
20	a	0.157423

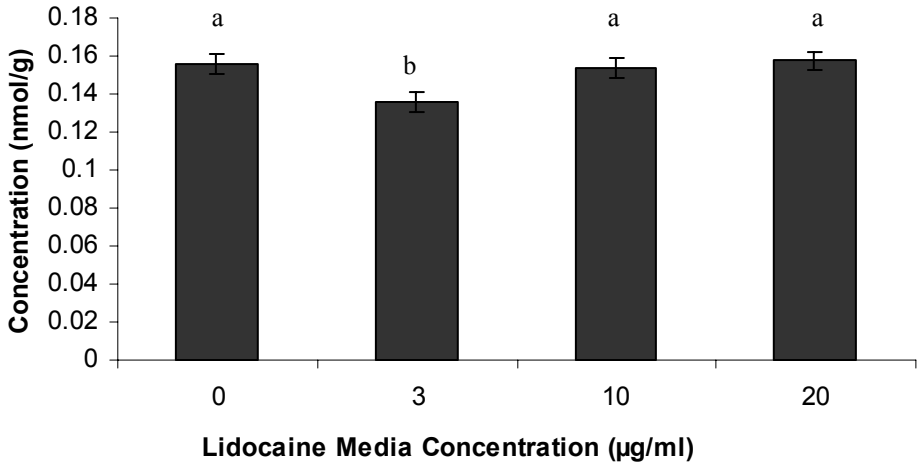

Fig. 27. Analysis among media concentrations of ATP concentrations for slices incubated with lidocaine. Means (nmol/g±SE) of ATP samples for all times (n=12) for each lidocaine media concentration. Homogeneous groups for ATP two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Table 28. ATP content AUC for slices incubated with lidocaine. The AUC (hour*nmol/g) of ATP content for slices incubated in media containing 0, 3, 10 and 20 µg/ml lidocaine.

Lidocaine Concentration (µg/ml)	ATP Content AUC (hour*nmol/g)
0	3.47
3	3.41
10	3.68
20	3.86

CHAPTER V

CANINE HEPATIC SLICE RESPONSE TO CARPROFEN, CARPROFEN WITH PHENOBARBITAL AND CARPROFEN WITH CIMETIDINE

Carprofen Media and Supernatant Drug Concentrations

Canine hepatic slices were incubated in media containing 0, 10, 50 and 100 µg/ml carprofen. Prior to incubation, media concentrations were within ten percent of expected concentrations. A chiral column was used to separate the carprofen enantiomers, but the R or S enantiomer designation corresponding to each peak could not be determined. As such, the peak with the shorter retention time was designated peak A, and the one with the longer retention time was designated peak B. Three replications of each experiment were performed using the racemic mixture. For all experiments, the carprofen concentrations in media containing 0 µg/ml carprofen were below the lower limit of quantification; the supernatant concentrations of carprofen for slices incubated in 0 µg/ml media were also below the lower limit of quantification.

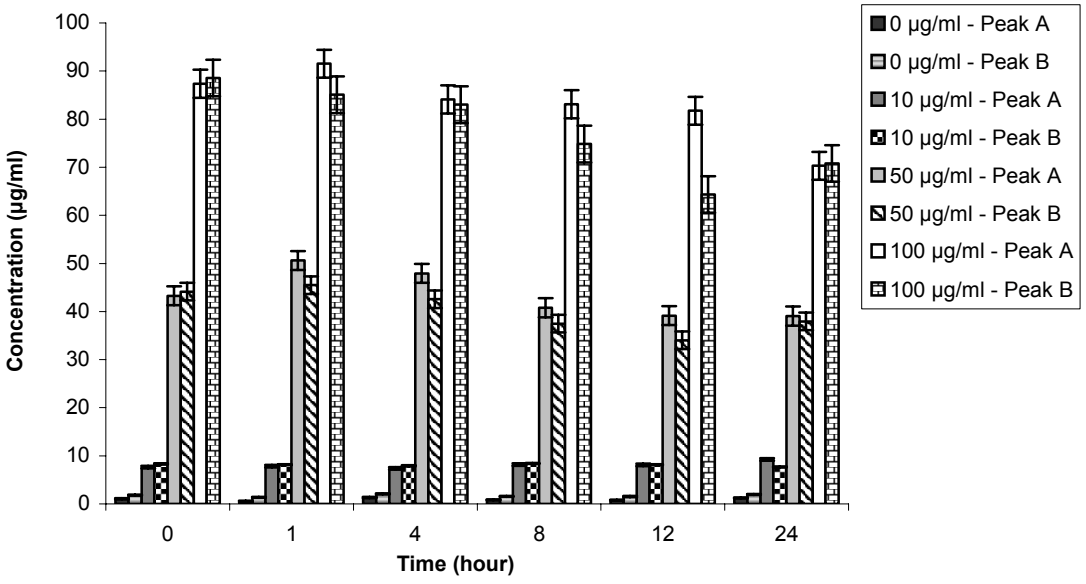

Fig. 28. Mean media concentrations of carprofen. Concentrations (µg/ml±SE, mean) of carprofen enantiomers (A or B) in media following incubation for 0, 1, 4, 8, 12 and 24 hours. Values are means of six (0 and 4 hour) or eight (1, 8, 12 and 24 hour) samples. Carprofen concentrations of 0 µg/ml media were below the LOQ.

The mean values of carprofen enantiomers (μg/ml) in media (Figure 28) were measured over twenty-four hours of incubation. The area under the curve (AUC) (hour*μg/ml) for each peak (A or B) was determined. AUC values for peaks were compared using paired t-tests. For 10, 50 and 100 μg/ml media, no differences were noted between the AUC for peak A or peak B (Table 29, Figure 29).

Table 29. AUC of carprofen in media. Mean AUC (hour*μg/ml, n=3) of carprofen in media (0, 10, 50 or 100 μg/ml) following 24 hour hepatic slice incubation.

Carprofen		Media
Concentration (μg/ml)	Peak	AUC (hour*μg/ml)
0	A	below LOQ
	B	below LOQ
10	A	202.43
	B	195.97
50	A	988.40
	B	909.73
100	A	1931.67
	B	1764.67

Carprofen (without 0 hour)		Media
Concentration (μg/ml)	Peak	AUC (hour*μg/ml)
0	A	below LOQ
	B	below LOQ
10	A	197.33
	B	190.43
50	A	956.80
	B	879.83
100	A	1871.67
	B	1707.00

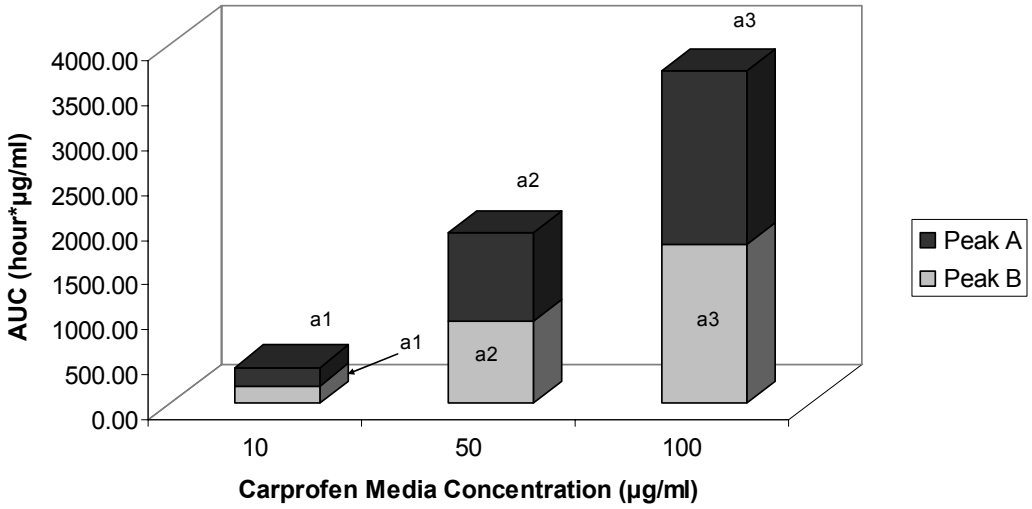

Fig. 29. AUC of carprofen in media. The AUC (hour*µg/ml, mean, n=3) of carprofen in media (10, 50 or 100 µg/ml) following 24 hour hepatic slice incubation. For each media concentration, peaks were compared for significant differences. Within each carprofen media concentration, peaks with the same letter are not significantly different.

For the mean carprofen concentrations in hepatic slice supernatant (ng/ml) (Figure 30), supernatant concentrations for slices incubated in 0 and 10 µg/ml carprofen were below the lower limit of quantification. The carprofen supernatant AUC (hour*ng/ml) of peak B was higher than that of peak A for 50 (p-value=0.004; without 0 hour p-value=0.0041) and 100 µg/ml media (p-value=0.0028; without 0 hour p-value=0.0027) (Table 30, Figure 31).

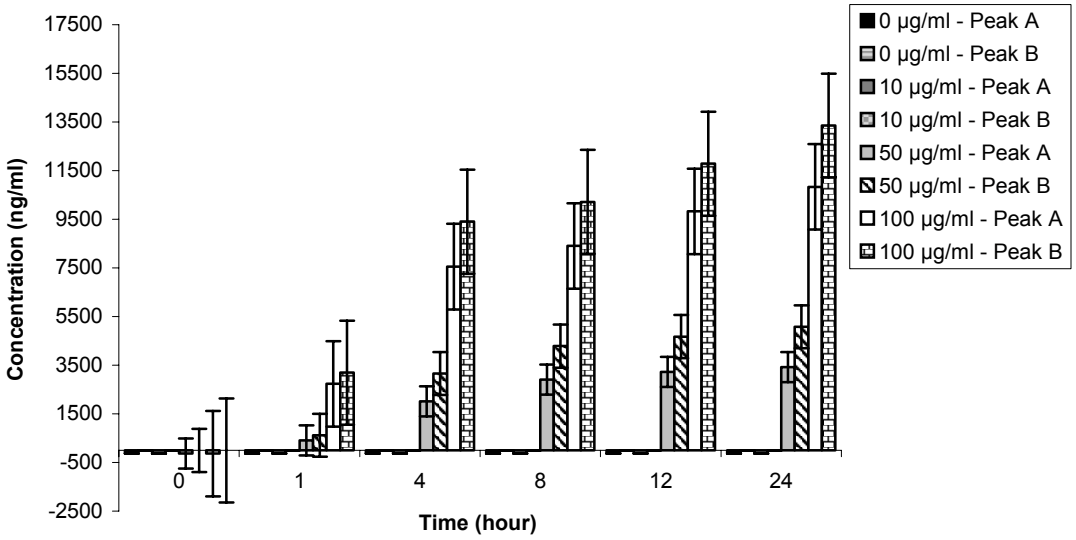

Fig. 30. Mean supernatant concentrations of carprofen. Concentrations (ng/ml±SE, mean) of carprofen enantiomers (A or B) in hepatic slice supernatant following incubation for 0, 1, 4, 8, 12 and 24 hours. The values are means of four (0 and 4 hour) or six (1, 8, 12 and 24 hour) samples. Carprofen supernatant concentrations for slices incubated in 0 and 10 µg/ml media were below the LOQ.

Table 30. AUC of carprofen in hepatic slice supernatant. Mean AUC (hour*ng/ml, n=3) of carprofen in supernatant of hepatic slices incubated for 24 hours in carprofen media (0, 10, 50 or 100 µg/ml).

Carprofen		Supernatant
Concentration (µg/ml)	Peak	AUC (hour*ng/ml)
0	A	below LOQ
	B	below LOQ
10	A	below LOQ
	B	below LOQ
50	A	65307.33
	B	96043.33
100	A	206537.00
	B	250988.67

Carprofen (without 0 hour)		Supernatant
Concentration (µg/ml)	Peak	AUC (hour*ng/ml)
0	A	below LOQ
	B	below LOQ
10	A	below LOQ
	B	below LOQ
50	A	65252.33
	B	95928.67
100	A	205801.33
	B	250131.33

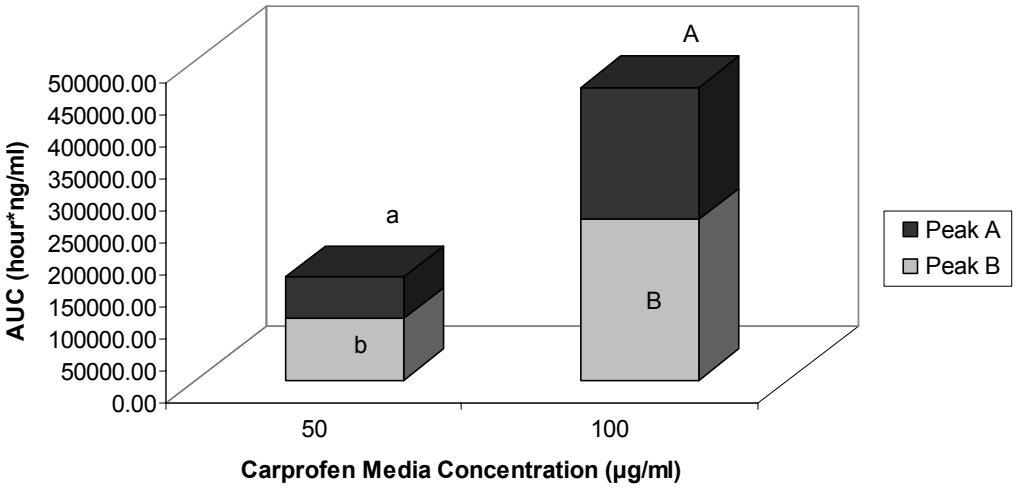

Fig. 31. AUC of carprofen in supernatant. The AUC (hour*ng/ml, mean, n=3) of carprofen in supernatant of hepatic slices incubated in carprofen media (50 or 100 µg/ml) for 24 hours. For each media concentration, peaks were compared for significant differences. Within each carprofen media concentration, peaks with the same letter are not significantly different. For both media concentrations, Peak B AUC is greater than that of Peak A ($p<0.005$).

Carprofen Potassium

Potassium concentrations (µmol/g-L) in the hepatic slice supernatant were used as an indication of toxicity; mean potassium concentrations were determined for hepatic slices exposed to carprofen media over twenty-four hours (Figure 32). Significant differences were noted across time (df=5, F-ratio=29.751, p-value=0.000) and among media concentrations (df=3, F-ratio=2.730, p-value=0.048). ANOVA groups were based on the means of all samples at each time point (n=24 except for 0 and 4 hour where n=16) or the means of all times for each concentration (n=32). For time, mean potassium concentrations at 0 hour were higher than those at all other time points. The mean potassium concentrations at 1 hour were lower than those at 4 and 24 hours. Among the media concentrations, potassium concentrations for 0 µg/ml media were higher than those for 50 µg/ml media. There were three homogeneous groups for time (Table 31, Figure 33) and two homogeneous groups for concentration (Table 32, Figure 34). The potassium content AUC (hour*µmol/g-L) was also determined for each concentration of carprofen (Table 33).

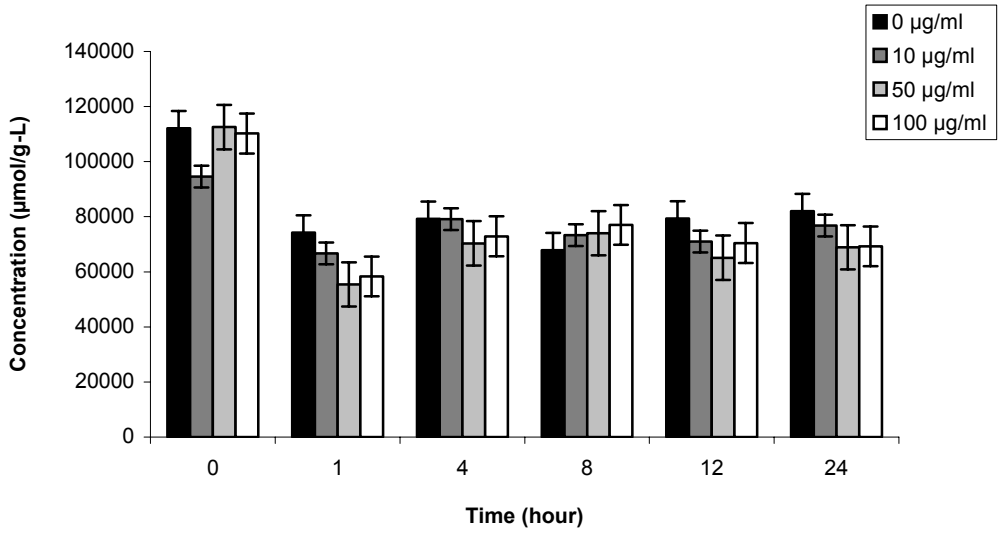

Fig. 32. Potassium concentrations in slices incubated with carprofen. Potassium concentrations (µmol/g-L±SE, mean) in hepatic slice supernatant during 24 hours of incubation in media containing 0, 10, 50 and 100 µg/ml carprofen. Potassium concentrations are a mean of four (0 and 4 hour) or six (1, 8, 12 and 24 hour) samples per time point for each concentration.

Table 31. Time analysis of potassium concentrations for slices incubated with carprofen. Comparison of mean potassium concentrations (µmol/g-L) of all potassium samples (n=24 except for 0 and 4 hour where n=16) at each time point for all carprofen concentrations. Homogeneous groups for potassium two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups	Means (µmol/g-L)
0	a	107359.1770
1	b	63688.0984
4	c	75413.7698
8	b c	73059.2283
12	b c	71471.0740
24	c	74233.1442

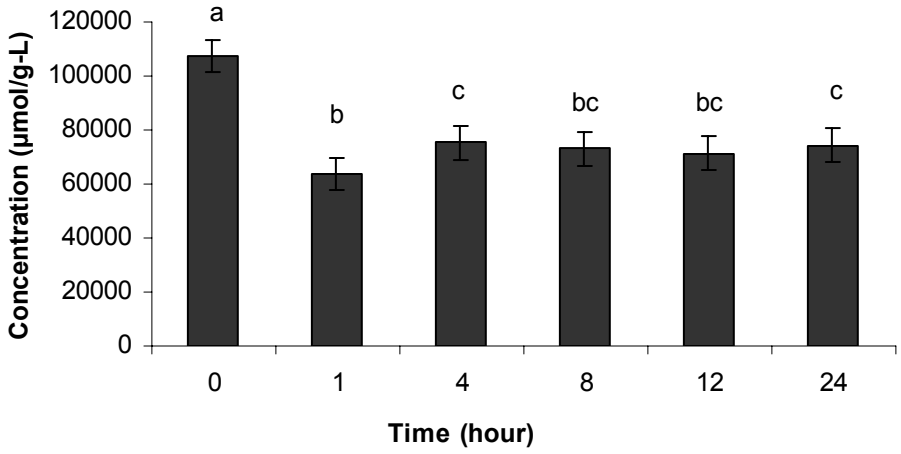

Fig. 33. Analysis across time of potassium levels after treatment of slices with carprofen. Comparison of mean potassium concentrations (µmol/g-L±SE) of all potassium samples (n=24 except for 0 and 4 hour where n=16) at each time point for all carprofen concentrations. Homogeneous groups for potassium two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Table 32. Concentration analysis of potassium levels after treatment of slices with carprofen. Comparison of mean potassium concentrations (µmol/g-L, n=32) at all times for each carprofen media concentration. Homogeneous groups for potassium two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Concentration (µg/ml)	Homogeneous groups	Means (µmol/g-L)
0	a	82496.8218
10	a b	76914.2584
50	b	74385.5688
100	a b	76353.0120

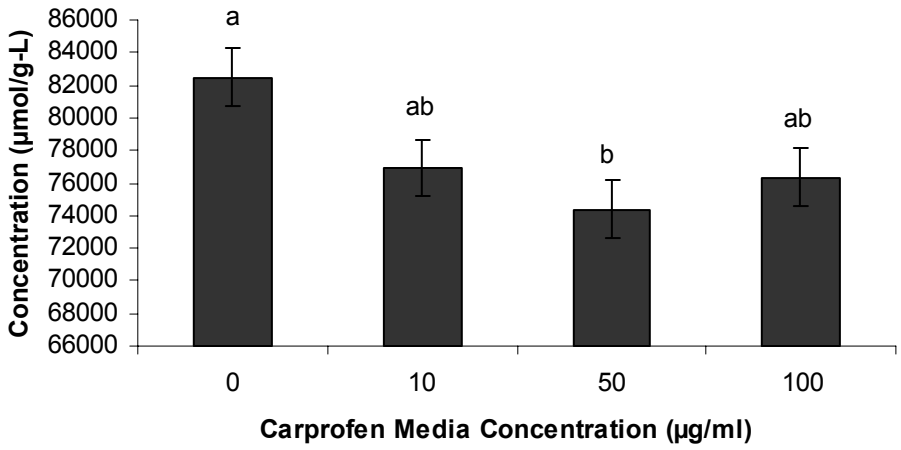

Fig. 34. Analysis among media concentrations of potassium levels after treatment of slices with carprofen. Comparison of mean potassium concentrations (µmol/g-L±SE, n=32) at all times for each carprofen media concentration. Homogeneous groups for potassium two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Table 33. Potassium content AUC for slices incubated with carprofen. Mean AUC (hour*µmol/g-L, n=3) of potassium content for slices incubated in media containing 0, 10, 50 and 100 µg/ml carprofen.

Carprofen	Potassium Content
Concentration (µg/ml)	AUC (hour*µmol/g-L)
0	1.881E+06
10	1.779E+06
50	1.644E+06
100	1.714E+06

Carprofen (without 0 hour)	Potassium Content
Concentration (µg/ml)	AUC (hour*µmol/g-L)
0	1.788E+06
10	1.699E+06
50	1.560E+06
100	1.630E+06

Carprofen ATP

ATP was used as another measure of slice viability. The mean ATP supernatant concentrations (nmol/g) for slices exposed to carprofen media were determined over twenty-four hours of incubation (Figure 35). Significant differences across time were noted in the two-way ANOVA of time and carprofen concentration. ANOVA groups were based on the means of all samples at each time point (n=24 except for 0 and 4 hour where n=16) or the means of all times for each concentration (n=32). For time (df=5, F-ratio=20.682, p-value=0.000), mean ATP

concentrations at 0 hour were less than those at 1, 4, 8, and 12 hours. ATP concentrations at 4 and 8 hours were higher than those at 12 and 24 hours. Twenty-four hour concentrations were less than those at 1and 12 hours. There were three homogeneous groups for time (Table 34, Figure 36). The ATP content AUC (hour*nmol/g) was also determined for each concentration of carprofen (Table 35).

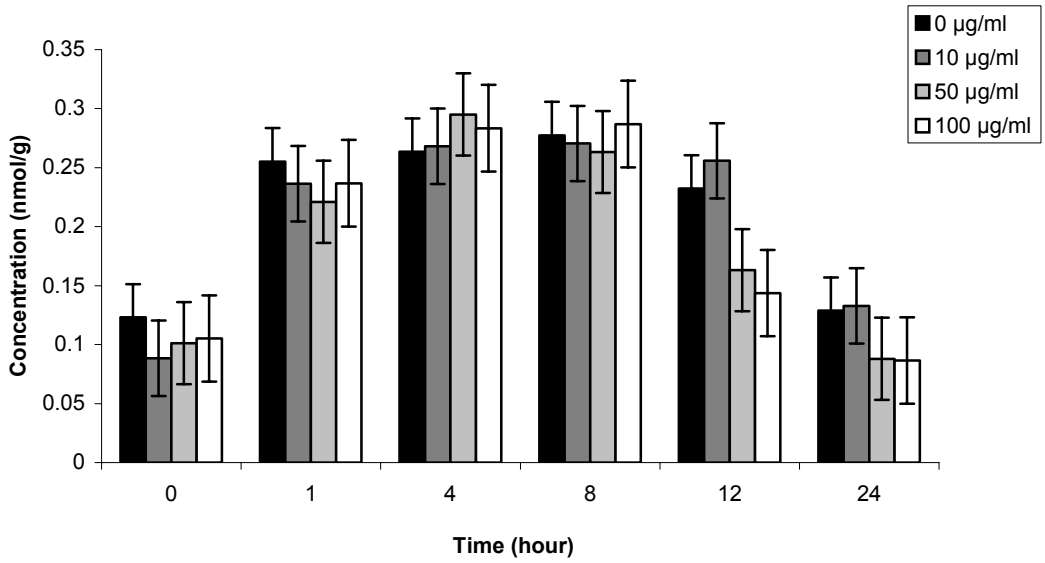

Fig. 35. ATP levels after treatment of slices with carprofen. ATP concentrations (nmol/g±SE, mean) in hepatic slice supernatant during 24 hours of incubation in media containing 0, 10, 50 and 100 µg/ml carprofen. ATP concentrations are a mean of four (0 and 4 hour) or six (1, 8, 12 and 24 hour) samples per time point for each concentration.

Table 34. Time analysis of ATP concentrations for slices incubated with carprofen. Comparison of mean ATP concentrations (nmol/g) of all ATP samples (n=24 except for 0 and 4 hour where n=16) at each time point for all carprofen concentrations. Homogeneous groups for ATP two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups	Means (nmol/g)
0	a	0.104476
1	b	0.237355
4	b	0.277526
8	b	0.274501
12	c	0.198707
24	a	0.108988

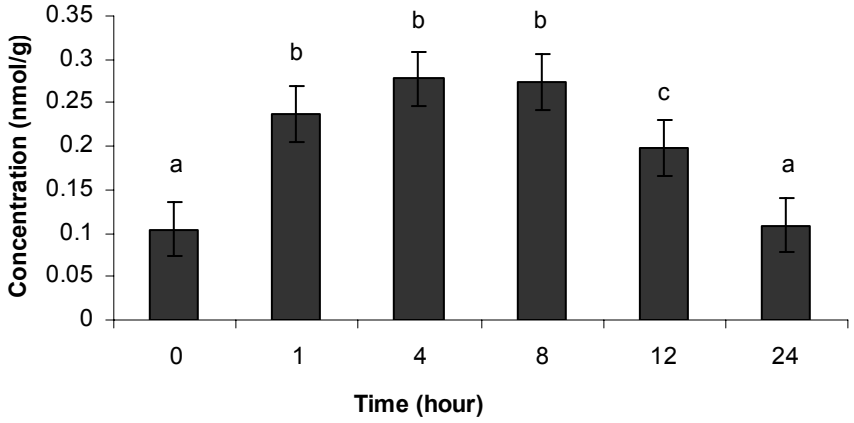

Fig. 36. Analysis across time of ATP levels after treatment of slices with carprofen. Comparison of mean ATP concentrations (nmol/g±SE) of all ATP samples (n=24 except for 0 and 4 hour where n=16) at each time point for all carprofen concentrations. Homogeneous groups for ATP two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Table 35. ATP content AUC for slices incubated with carprofen. Mean AUC (hour*nmol/g, n=3) of ATP content for slices incubated in media containing 0, 10, 50 and 100 µg/ml carprofen.

Carprofen	ATP Content
Concentration (µg/ml)	AUC (hour*nmol/g)
0	5.23
10	5.38
50	4.41
100	4.33

Carprofen (without 0 hour)	ATP Content
Concentration (µg/ml)	AUC (hour*nmol/g)
0	5.05
10	5.22
50	4.25
100	4.16

Carprofen Histology

Hepatic slices were examined for extent of necrosis and extent and severity of vacuolation as indicators of toxicity (Table 36). Slices exposed to carprofen were evaluated for necrosis (Figure 37), vacuolation extent (Figure 38) and vacuolation severity (Figure 39) on a scale from 0 to 5.

Table 36. Hepatic slice lesion scores for slices incubated with carprofen. Lesion scores of hepatic slices incubated in carprofen for up to twenty-four hours.

		Lesion scores*								
Media Concentration	**Time (hour)**	**Necrosis : extent**			**Vacuolation : extent**			**Vacuolation : severity**		
0 µg/ml	0		0	0		1	4		1	2
0 µg/ml	1	0	0	0	3	0	4	3	0	2
0 µg/ml	4		1	0		2	3		1	1
0 µg/ml	6	1			3			3		
0 µg/ml	8	1	2	1	3	3	4	3	2	3
0 µg/ml	12	1	1	1	4	4	4	4	4	2
0 µg/ml	24	4	2	1	3	4	4	3	4	4
10 µg/ml	0		0	0		2	2		2	2
10 µg/ml	1	0	1	0	3	1	1	3	1	1
10 µg/ml	4		4	1		2	1		1	1
10 µg/ml	6	2			3			3		
10 µg/ml	8	2	2	1	3	3	3	3	2	2
10 µg/ml	12	3	2	2	3	2	3	3	1	3
10 µg/ml	24	3	3	3	4	4	4	4	4	4
50 µg/ml	0		0	0		4	4		2	2
50 µg/ml	1	0	0	0	4	3	4	3	2	2
50 µg/ml	4		0	0		3	3		1	1
50 µg/ml	6	2			4			3		
50 µg/ml	8	2	2	1	3	3	3	2	2	2
50 µg/ml	12	3[a]	1	2[a]	3[a]	4	3[a]	2[a]	3	2[a]
50 µg/ml	24	2	2	1	3	3	4	3	2	4
100 µg/ml	0		0	0		4	3		2	2
100 µg/ml	1	0	0	0	4	3	3	3	2	2
100 µg/ml	4		0	0		3	3		1	1
100 µg/ml	6	2			4			4		
100 µg/ml	8	2	2[a]	3	2	3[a]	3	1	2[a]	2
100 µg/ml	12	4[a]	1	2[a]	2[a]	3	4[a]	2[a]	2	2[a]
100 µg/ml	24	2	3[a]	2[a]	1	4[a]	1[a]	2	3[a]	1[a]

* Lesion scoring: (outermost zone ~10 hepatocytes wide was disregarded)

0	< 1% hepatocytes affected	3	21-40%
1	1-10%	4	41-90%
2	11-20%	5	>90%

a Slides showing marked difference in lesion extent and/or severity as move across the slide

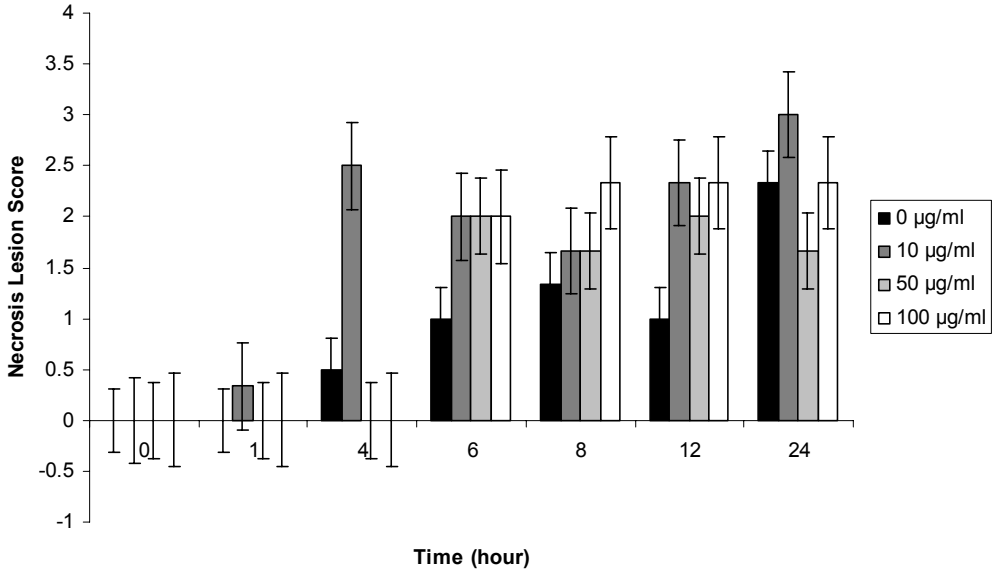

Fig. 37. Necrosis hepatic slice lesion score for slices incubated with carprofen. Necrosis lesion scores (±SE) for slices incubated for up to 24 hours in 0, 10, 50 or 100 µg/ml carprofen media. Scores are one (6 hour), an average of two (0 and 4 hour) or a mean of three (1, 8, 12 and 24 hours) samples per time point.

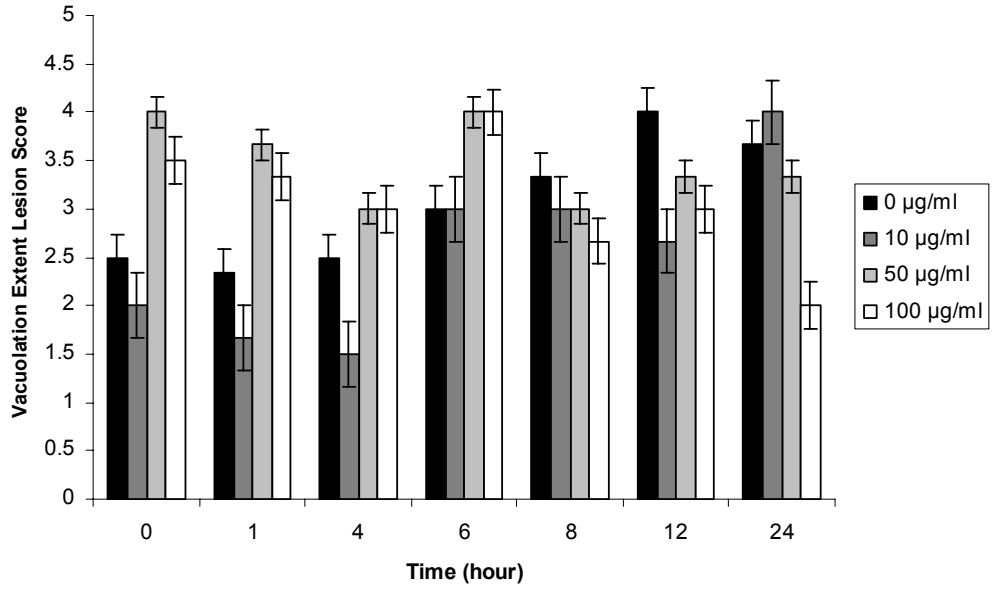

Fig. 38. Vacuolation extent hepatic slice lesion score for slices incubated with carprofen. Vacuolation extent lesion scores (±SE) for slices incubated for up to 24 hours in 0, 10, 50 or 100 µg/ml carprofen media. Scores are one (6 hour), an average of two (0 and 4 hour) or a mean of three (1, 8, 12 and 24 hours) samples per time point.

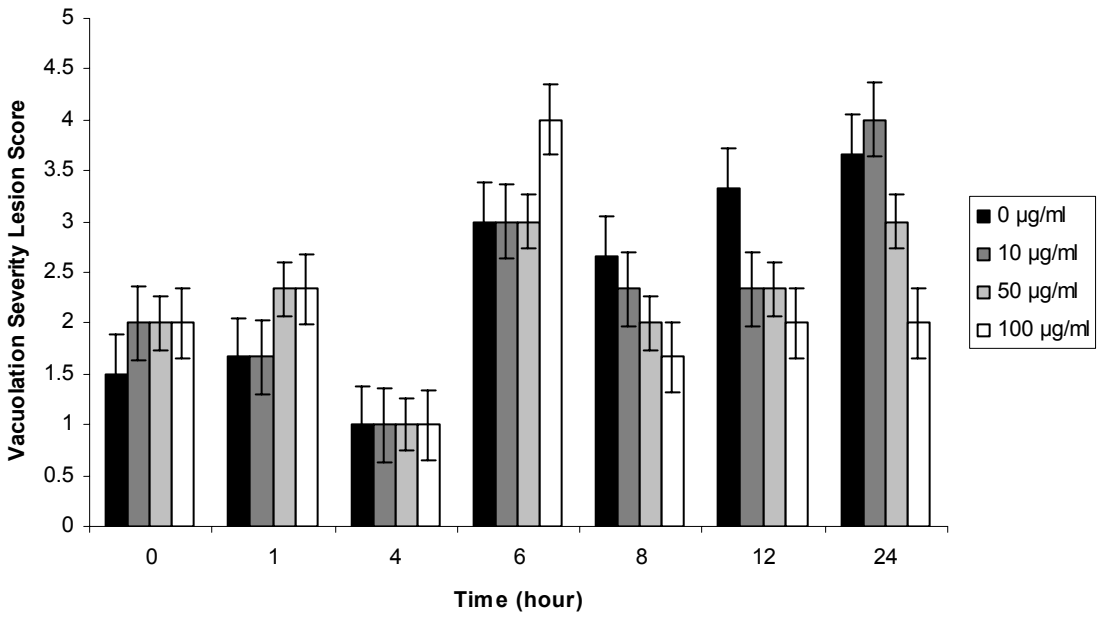

Fig. 39. Vacuolation severity hepatic slice lesion score for slices incubated with carprofen. Vacuolation severity lesion scores for slices incubated for up to 24 hours in 0, 10, 50 or 100 µg/ml carprofen media. Scores are one (6 hour), an average of two (0 and 4 hour) or a mean of three (1, 8, 12 and 24 hours) samples per time point.

When the lesion scores for carprofen media were analyzed for time and concentration in a two-way ANOVA for necrosis, vacuolation extent or vacuolation severity, some significant differences were noted. ANOVA groups were based on the means of all samples at each time point (n=12 except for 0 and 4 hour where n=8) or the means of all times for each concentration (n=16). For necrosis, two-way ANOVA revealed a significant change across time (df=5, F-ratio=28.801, p-value=0.000). Necrosis scores at 0, 1 and 4 hours were less than those at 8, 12 and 24 hours (Table 37, Figure 40). Vacuolation extent scores showed a significant difference across media concentrations (df=3, F-ratio=2.904, p-value=0.046); mean scores for 50 µg/ml media were higher than those for 10 µg/ml media (Table 38, Figure 41). Two-way ANOVA of vacuolation severity denoted differences across time (df=5, F-ratio=9.216, p-value=0.000) (Table 39, Figure 42); mean scores at 24 hours were higher than those at 0, 1, 4 and 8 hours. Vacuolation severity scores at four hours were lower than those at 8 and 12 hours.

Table 37. Time analysis of mean necrosis lesion scores for slices incubated with carprofen. Comparison of mean necrosis lesion scores (n=12 except for 0 and 4 hour where n=8) across time for slices incubated in all concentrations of carprofen. Homogeneous groups for necrosis two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups	Mean Lesion Score
0	a	0.00
1	a	0.08
4	a	0.75
8	b	1.75
12	b	1.92
24	b	2.33

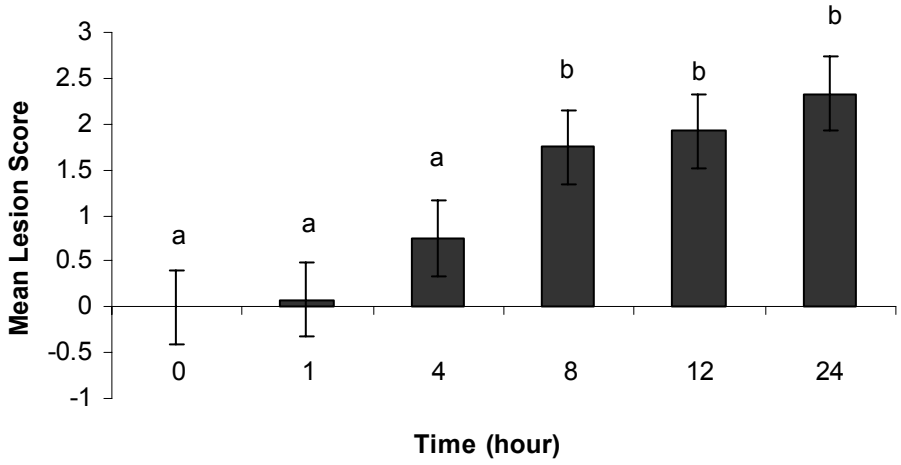

Fig. 40. Time analysis for mean necrosis lesion scores for slices incubated with carprofen. Comparison of mean necrosis lesion scores (±SE, n=12 except for 0 and 4 hour where n=8) across time for slices incubated in all concentrations of carprofen. Homogeneous groups for necrosis two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Table 38. Concentration analysis of mean vacuolation extent lesion scores for slices incubated with carprofen. Comparison of mean vacuolation extent lesion scores (n=16) among carprofen concentrations for all time points. Homogeneous groups for vacuolation extent two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Concentration (μg/ml)	Homogeneous groups	Mean Lesion Score
0	a b	3.125
10	a	2.563
50	b	3.375
100	a b	2.875

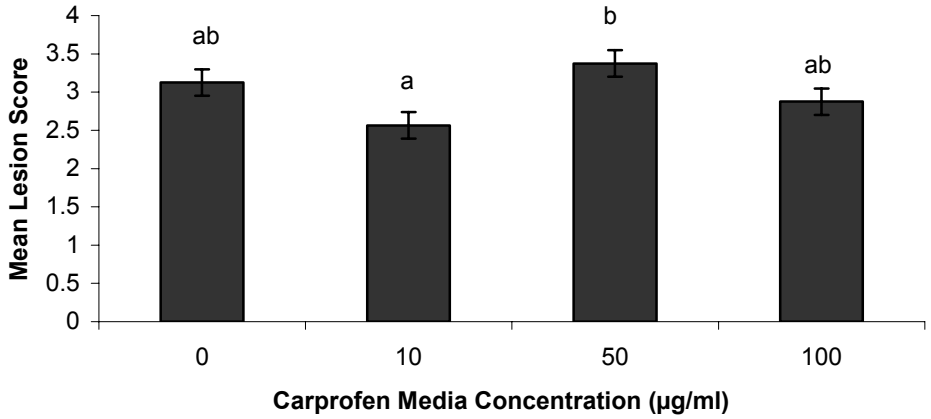

Fig. 41. Concentration analysis for mean vacuolation extent lesion scores for slices incubated with carprofen. Comparison of mean vacuolation extent lesion scores (±SE, n=16) among carprofen concentrations for all time points. Homogeneous groups for vacuolation extent two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Table 39. Time analysis of mean vacuolation severity lesion scores for slices incubated with carprofen. Comparison of mean vacuolation severity lesion scores (n=12 except for 0 and 4 hour where n=8) across time for slices incubated in all concentrations of carprofen. Homogeneous groups for vacuolation severity two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups			Mean Lesion Score
0	a	b		1.88
1	a	b		2.00
4	a			1.00
8		b		2.17
12		b	c	2.50
24			c	3.17

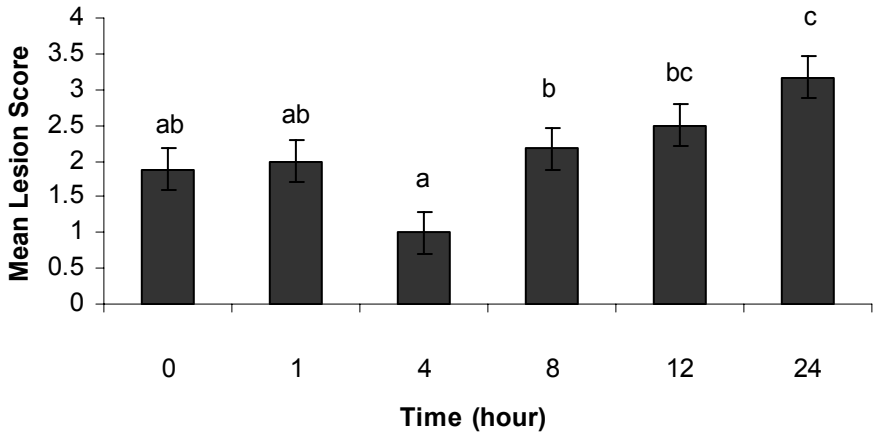

Fig. 42. Time analysis of mean vacuolation severity lesion scores for slices incubated with carprofen. Comparison of mean vacuolation severity lesion scores (±SE, n=12 except for 0 and 4 hour where n=8) across time for slices incubated in all concentrations of carprofen. Homogeneous groups for vacuolation severity two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Carprofen and Phenobarbital Incubation

Media and Supernatant Drug Concentrations

Hepatic slices were incubated for 24 hours in media containing phenobarbital (75 µg/ml) followed by incubation with carprofen. Media concentrations for 0 µg/ml carprofen media were below the lower limit of quantification. Supernatant concentrations measured for carprofen were below the lower limit of quantification for 0 and 10 µg/ml media.

The mean carprofen media concentrations (µg/ml), post-phenobarbital incubation were determined; for 0, 10, 50 and 100 µg/ml media, 0 and 24 hour concentrations were below the lower limit of quantification (Figure 43). For 10 µg/ml media, the AUC (hour*µg/ml) for peak B was higher than that of peak A (p-value=0.0057; without 0 hour p-value>0.05) (Table 40, Figure 44). No differences were noted between peaks for either 50 or 100 µg/ml media (Figure 44).

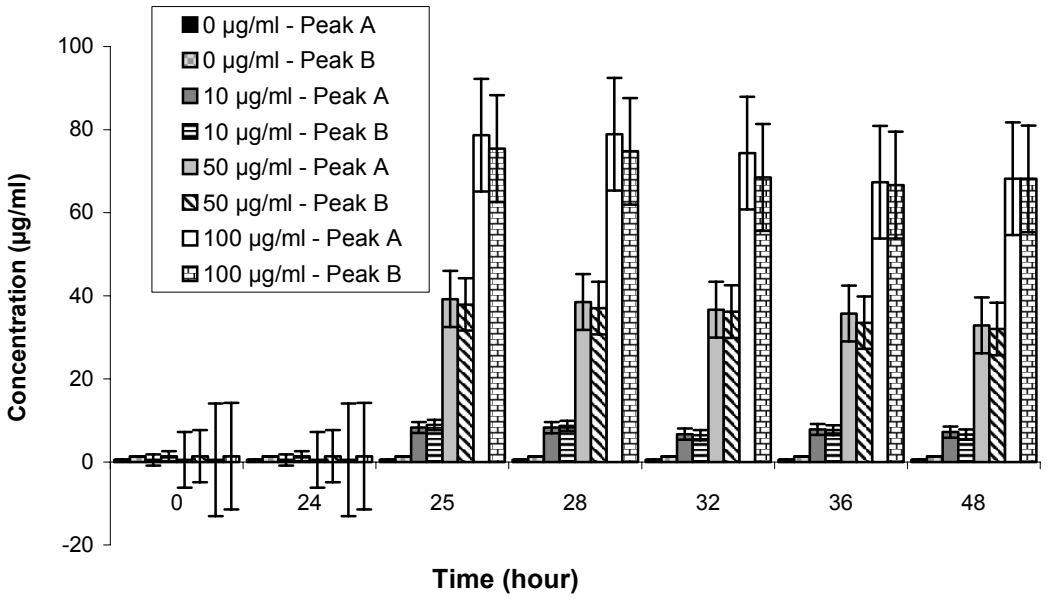

Fig. 43. Mean carprofen media concentrations following incubation with phenobarbital and carprofen. Mean concentration (µg/ml±SE) of carprofen enantiomers (A or B) in media after incubation for 24 hours with phenobarbital (75µg/ml) followed with an additional incubation in carprofen for 1, 4, 8, 12 and 24 hours. Values are means of nine samples per media concentration per time point. Carprofen concentrations of 0 µg/ml media were below the LOQ.

Table 40. AUC of carprofen in media following incubation with phenobarbital and carprofen. Mean AUC (hour*µg/ml, n=3) of carprofen in media (0, 10, 50 or 100 µg/ml) for slices incubated for 24 hours in phenobarbital (75µg/ml) followed by an additional 24 hour incubation in carprofen.

Carprofen and phenobarbital		Media
Concentration (µg/ml)	Peak	AUC (hour*µg/ml)
0	A	23.12
	B	66.48
10	A	189.77
	B	208.60
50	A	854.67
	B	844.60
100	A	1690.00
	B	1662.00

Carprofen and phenobarbital (without 0 hour)		Media
Concentration (µg/ml)	Peak	AUC (hour*µg/ml)
0	A	11.56
	B	33.24
10	A	178.20
	B	175.37
50	A	843.07
	B	811.37
100	A	1678.00
	B	1629.00

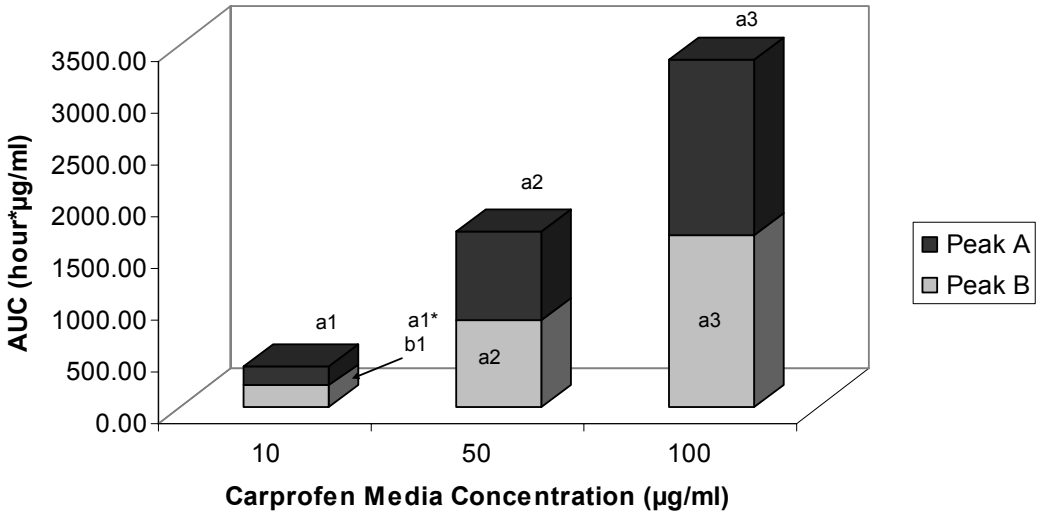

Fig. 44. AUC of carprofen in media following incubation with phenobarbital and carprofen. Mean AUC (hour*µg/ml, n=3) of carprofen in media (10, 50 or 100 µg/ml) for slices incubated for 24 hours in phenobarbital (75µg/ml) followed by an additional 24 hour incubation in carprofen. For each media concentration, peaks were compared for significant differences. Within each carprofen media concentration, peaks with the same letter are not significantly different. For 10 µg/ml media, no statistical difference was noted (a1*) when 0 hour carprofen concentrations were excluded.

The mean carprofen supernatant concentrations (ng/ml) following phenobarbital incubation were determined, and mean concentrations at 0 and 24 hours were below lower limit of quantification (Figure 45). The AUC (hour*ng/ml) of carprofen supernatant for peak B was higher than the AUC of peak A for both 50 µg/ml (p-value=0.0066; without 0 hour p-value=0.0069) and 100 µg/ml media (p-value=0.0078; without 0 hour p-value=0.0078) (Table 41, Figure 46).

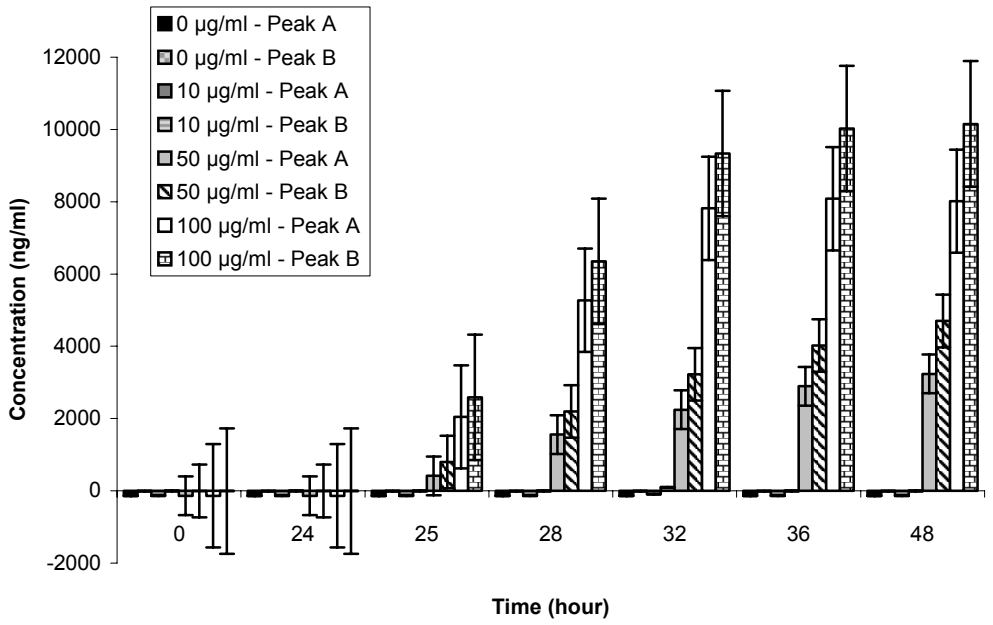

Fig. 45. Mean hepatic slice supernatant concentrations of carprofen following incubation with phenobarbital and carprofen. Mean concentrations (ng/ml±SE) of carprofen enantiomers (A or B) in hepatic slice supernatant after incubation for 24 hours with phenobarbital (75 µg/ml) followed with an additional incubation in carprofen for 1, 4, 8, 12 and 24 hours. The values are an average of six samples per media concentration per time point. Carprofen supernatant concentrations for slices incubated in 0 and 10 µg/ml media were below the LOQ.

Table 41. AUC of carprofen in hepatic slice supernatant following incubation with phenobarbital and carprofen. Mean AUC (hour*ng/ml, n=3) of carprofen in supernatant of hepatic slices incubated for 24 hours in phenobarbital (75µg/ml) followed by an additional 24 hour incubation in carprofen media (0, 10, 50 and 100 µg/ml).

Carprofen and phenobarbital		Supernatant
Concentration (µg/ml)	Peak	AUC (hour*ng/ml)
0	A	0.00
	B	0.00
10	A	0.00
	B	0.00
50	A	57822.00
	B	82576.33
100	A	166514.67
	B	205865.00

Carprofen and phenobarbital (without 0 hour)		Supernatant
Concentration (µg/ml)	Peak	AUC (hour*ng/ml)
0	A	0.00
	B	0.00
10	A	0.00
	B	0.00
50	A	57881.67
	B	82577.67
100	A	166518.67
	B	205865.00

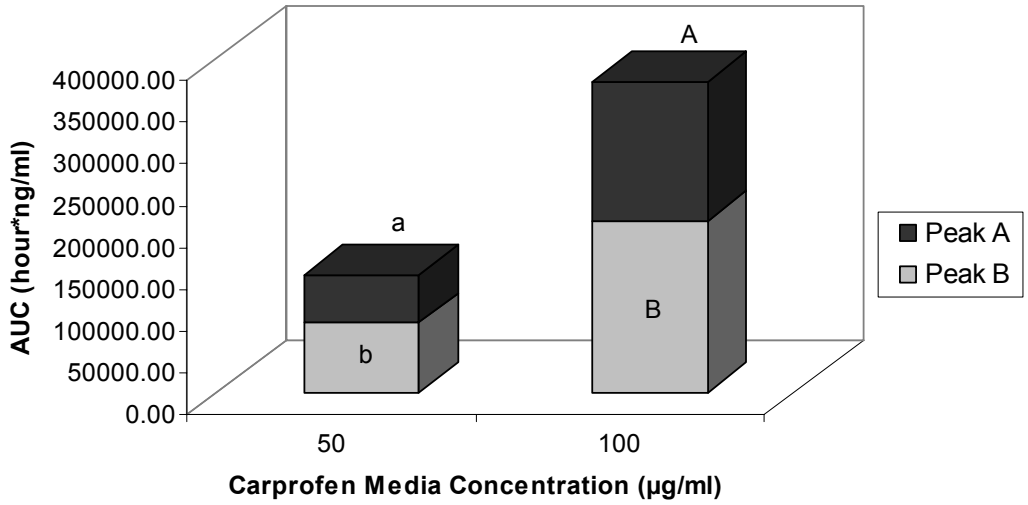

Fig. 46. AUC of carprofen in hepatic slice supernatant following incubation with phenobarbital and carprofen. Mean AUC (hour*ng/ml, n=3) of carprofen in supernatant of hepatic slices incubated for 24 hours in phenobarbital (75µg/ml) followed by an additional 24 hour incubation in carprofen media (50 and 100 µg/ml). For each media concentration, peaks were compared for significant differences. Within each carprofen media concentration, peaks with the same letter are not significantly different. For both media concentrations, Peak B AUC is greater than that of Peak A ($p<0.01$).

Potassium

The mean potassium concentrations (µmol/g-L) for slices incubated in phenobarbital followed by carprofen are displayed in Figure 47. Two-way ANOVA showed a significant difference across time (df=6, F-ratio=30.046, p-value=0.000). ANOVA groups were based on the means of all samples at each time point (n=24) or the means of all times for each concentration (n=42). Potassium concentrations at 0 hour were higher than those at all other time points, and 24 hour concentrations were higher than 48 hour concentrations (Table 42, Figure 48). The potassium content AUC (hour*µmol/g-L) was also determined for each concentration of carprofen (Table 43).

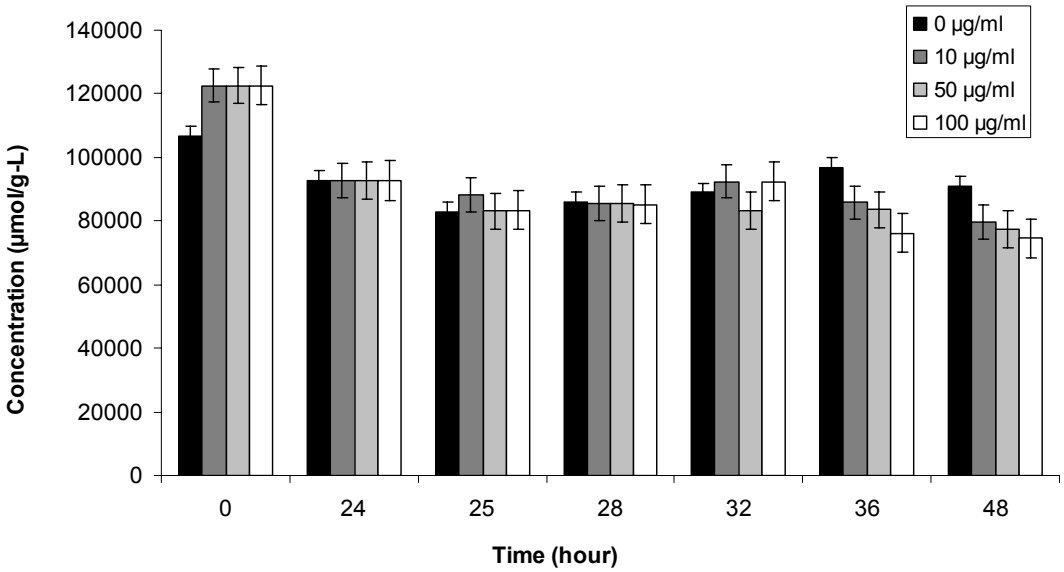

Fig. 47. Potassium concentrations of slices after incubation with carprofen and phenobarbital. Potassium concentrations (µmol/g-L±SE, mean) in hepatic slice supernatant during 24 hours of incubation phenobarbital (75µg/ml) followed by an additional 24 hour incubation in carprofen (0, 10, 50 and 100 µg/ml). Potassium concentrations are a mean of six samples per time point for each concentration.

Table 42. Time analysis of potassium concentrations for slices incubated with carprofen and phenobarbital. Comparison of mean potassium concentrations (µmol/g-L) of all potassium samples (n=24) at each time point for all carprofen concentrations. Slices were incubated for 24 hours in phenobarbital (75µg/ml) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for potassium two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups			Means (µmol/g-L)
0	a			118678.73
24		b		92757.17
25		b	c	84466.99
28		b	c	85598.34
32		b	c	89251.99
36		b	c	85623.27
48			c	80661.32

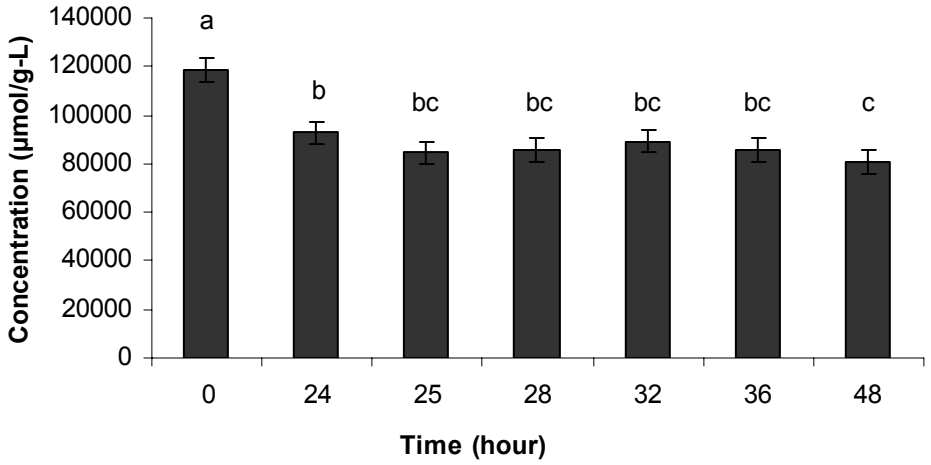

Fig. 48. Time analysis of potassium concentrations for slices incubated with carprofen and phenobarbital. Comparison of mean potassium concentrations (µmol/g-L±SE) of all potassium samples (n=24) at each time point for all carprofen concentrations. Slices were incubated for 24 hours in phenobarbital (75µg/ml) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for potassium two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Table 43. Potassium content AUC for slices incubated with carprofen and phenobarbital. Mean AUC (hour*µmol/g-L, n=3) of potassium content for slices incubated for 24 hours in phenobarbital (75 µg/ml) followed by an additional 24 hour incubation in media containing 0, 10, 50 and 100 µg/ml carprofen.

Carprofen and phenobarbital	Potassium Content
Concentration (µg/ml)	AUC (hour*µmol/g-L)
0	4.627E+06
10	4.494E+06
50	4.414E+06
100	4.375E+06

Carprofen and phenobarbital (without 0 hour)	Potassium Content
Concentration	AUC
0	2.192E+06
10	2.059E+06
50	1.980E+06
100	1.940E+06

ATP

Figure 49 illustrates the mean ATP concentrations (nmol/g) for slices incubated in phenobarbital followed by carprofen incubation. Two-way ANOVA indicated that a significant time-concentration (factor) interaction (df=18, F-ratio=1.761, p-value=0.039) and a significant time-related difference (df=6, F-ratio=3.243, p-value=0.006) in mean ATP concentrations.

ANOVA groups were based on the means of all samples at each time point (n=24) or the means of all times for each concentration (n=42). When compared across time, ATP concentrations at 25 hours were higher than those at 32 hours. With the disorderly factor interaction, the time effect is more difficult to interpret. The ATP content AUC (hour*nmol/g) was also determined for each concentration of carprofen (Table 44).

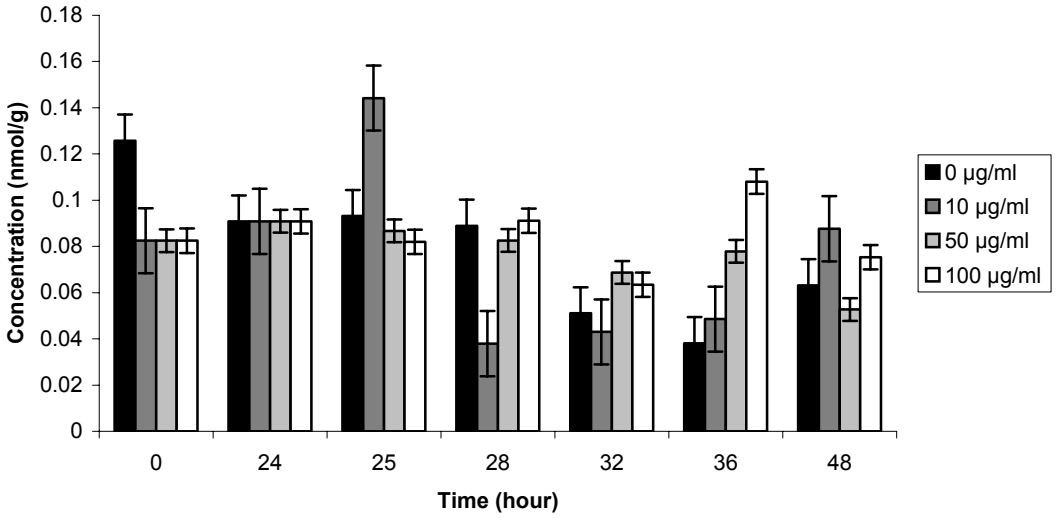

Fig. 49. ATP concentrations for slices incubated with carprofen and phenobarbital. ATP concentrations (nmol/g±SE, mean) in hepatic slice supernatant during 24 hours of incubation phenobarbital (75µg/ml) followed by an additional 24 hour incubation in carprofen (0, 10, 50 and 100 µg/ml). ATP concentrations are a mean of six samples per time point for each concentration.

Table 44. ATP content AUC for slices incubated with carprofen and phenobarbital. Mean AUC (hour*nmol/g, n=3) of ATP content for slices incubated for 24 hours in phenobarbital (75 µg/ml) followed by an additional 24 hour incubation in media containing 0, 10, 50 and 100 µg/ml carprofen.

Carprofen and phenobarbital	ATP Content
Concentration (µg/ml)	AUC (hour*nmol/g)
0	4.03
10	3.63
50	3.80
100	4.18

Carprofen and phenobarbital (without 0 hour)	ATP Content
Concentration (µg/ml)	AUC (hour*nmol/g)
0	1.43
10	1.55
50	1.72
100	2.10

Histology

Slices incubated in phenobarbital followed by incubation with carprofen were evaluated for the extent of necrosis (Table 45, Figure 50), extent of vacuolation (Table 45, Figure 51) and severity of vacuolation (Table 45, Figure 52). Two-way ANOVA was performed on mean lesion scores for necrosis, vacuolation extent and vacuolation severity for slices. Significant

Table 45. Hepatic slice lesion scores for slices incubated with carprofen and phenobarbital. Lesion scores of hepatic slices incubated in phenobarbital for 24 hours followed with incubation with carprofen for and additional 1, 4, 8, 12 and 24 hours.

		Lesion scores*								
Media Concentration	**Time (hour)**	**Necrosis : extent**			**Vacuolation : extent**			**Vacuolation : severity**		
0 µg/ml	0	0	0	0	0	2	0	0	1	0
0 µg/ml	24	2	1	2	1	4	1	2	4	2
phenobarbital	0	0	0	0	0	1	0	0	1	0
phenobarbital	24	1	2	1	2	4	2	3	4	3
0 µg/ml	25	2	2	2	4	4	4	4	4	4
0 µg/ml	28	1	2	1	4	4	4	3	3	3
0 µg/ml	32	2	2	2	3	4	3	3	4	3
0 µg/ml	36	2	1	2	4	4	4	3	3	3
0 µg/ml	48	1[b]	1	1	3[b]	4	3	3[b]	3	3
10 µg/ml	25	1	1	1	4	4	4	3	3	3
10 µg/ml	28	2[a]	2	n/a	4[a]	4	n/a	3[a]	4	n/a
10 µg/ml	32	2	2	1	4	4	4	3	3	4
10 µg/ml	36	2	2[a]	2[a]	4	4[a]	4[a]	4	3[a]	3[a]
10 µg/ml	48	3	3[a]	2	2	4[a]	4	2	4[a]	3
50 µg/ml	25	1	2	1	4	4	3	4	4	3
50 µg/ml	28	2	1	2	2	4	3	2	4	3
50 µg/ml	32	1	1	2	2	2	2	2	2	2
50 µg/ml	36	3	1	2	4	3	4	3	2	3
50 µg/ml	48	3[a]	2[a]	2[a]	2[a]	4[a]	4[a]	2[a]	3[a]	4[a]
100 µg/ml	25	2	2	1	4	2	4	4	2	4
100 µg/ml	28	2[a]	2	2	1[a]	2	3	1[a]	2	2
100 µg/ml	32	1	2[a]	3	1	3[a]	2	1	2[a]	2
100 µg/ml	36	2	3[a]	2	1	3[a]	2	1	2[a]	1
100 µg/ml	48	2	3	1	0	2	4	0	1	3

* Lesion scoring: (outermost zone ~10 hepatocytes wide was disregarded)

0	< 1% hepatocytes affected	3	21-40%
1	1-10%	4	41-90%
2	11-20%	5	>90%

a Slides showing marked difference in lesion extent and/or severity as move across the slide
b Surface contamination with yeasts
n/a No slide

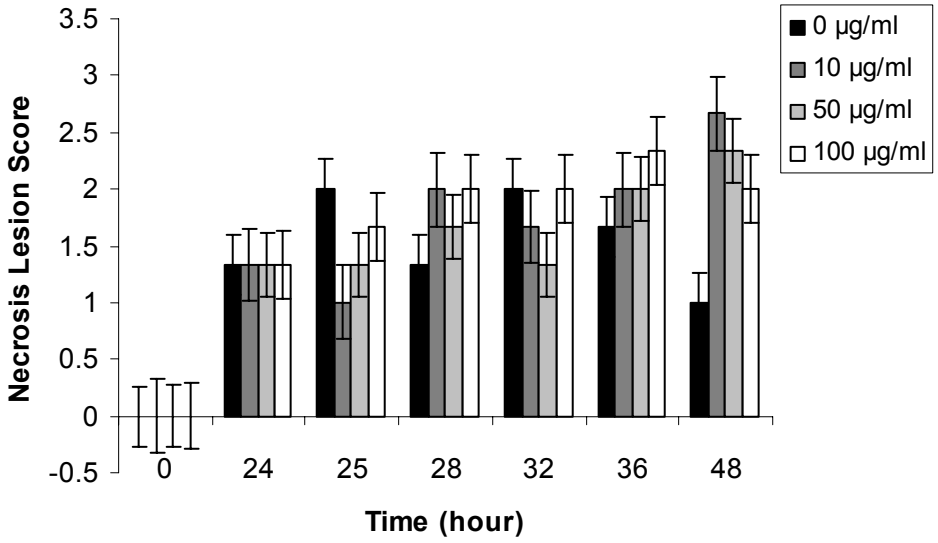

Fig. 50. Mean necrosis hepatic slice lesion scores for slices incubated with carprofen and phenobarbital. Mean necrosis lesion scores (±SE) for slices incubated for 24 hours in phenobarbital (75µg/ml) followed with an additional 24 hour incubation in carprofen (0, 10, 50 or 100 µg/ml). Scores are means of three samples per time point.

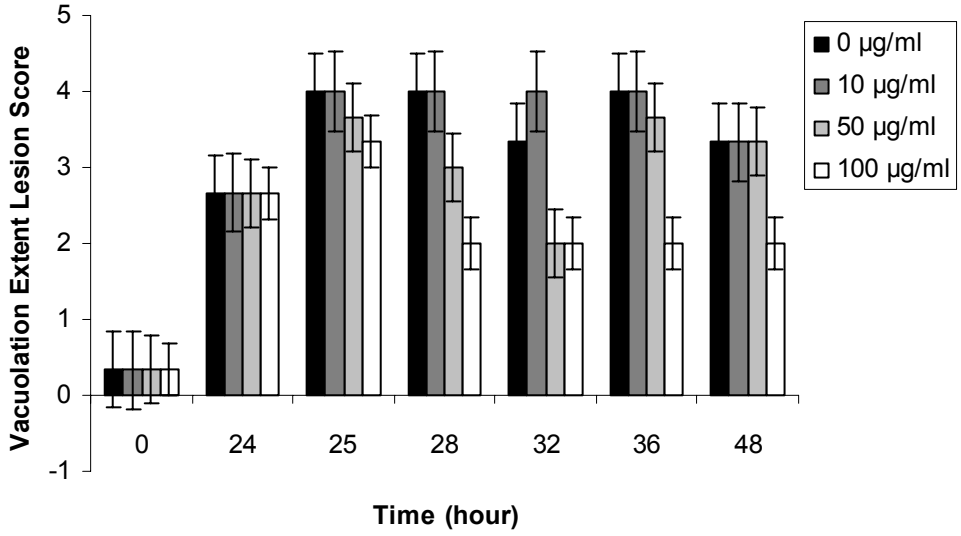

Fig. 51. Mean vacuolation extent hepatic slice lesion scores for slices incubated with carprofen and phenobarbital. Mean vacuolation extent lesion scores (±SE) for slices incubated for 24 hours in phenobarbital (75µg/ml) followed with an additional 24 hour incubation in carprofen (0, 10, 50 or 100 µg/ml). Scores are means of three samples per time point.

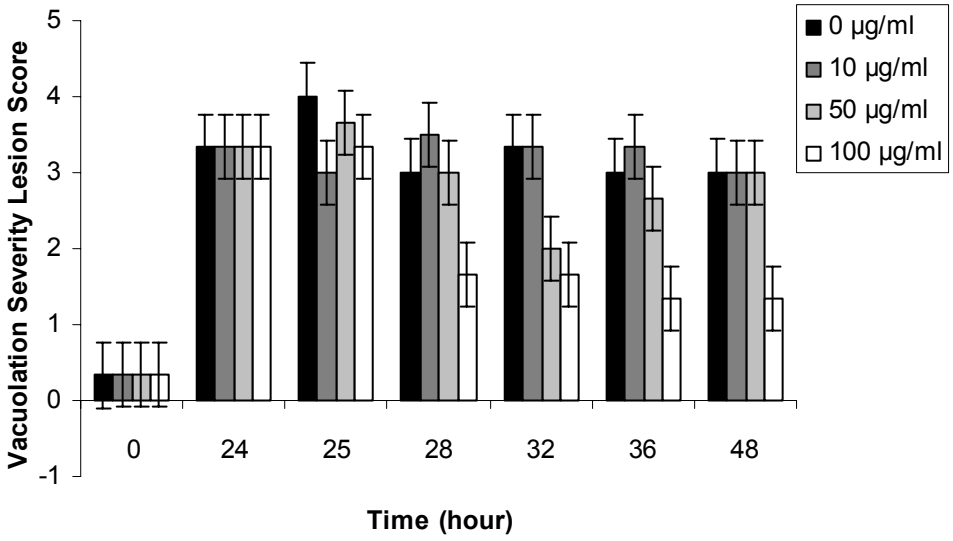

Fig. 52. Mean vacuolation severity hepatic slice lesion scores for slices incubated with carprofen and phenobarbital. Mean vacuolation severity lesion scores (±SE) for slices incubated for 24 hours in phenobarbital (75µg/ml) followed with an additional 24 hour incubation in carprofen (0, 10, 50 or 100 µg/ml). Scores are means of three samples per time point.

differences were seen across time for necrosis (df=6, F-ratio=20.735, p-value=0.000), across time (df=6, F-ratio=20.853, p-value=0.000) and among concentrations (df=3, F-ratio=7.703, p-value=0.000) for vacuolation extent and among concentrations (df=3, F-ratio=10.171, p-value=0.000) and across time (df=6, F-ratio=29.098, p-value=0.000) for vacuolation severity. For necrosis, mean lesion scores at time 0 were lower than at all other time points (Table 46, Figure 53). Twenty-four hour lesion scores were lower than those at 36 and 48 hours. With vacuolation extent, lesion scores were lower for slices incubated in 100 µg/ml media than for 0 and 10 µg/ml media (Table 47, Figure 54). Across time, lesion scores for vacuolation extent were lower at time 0 than for all other time points (Table 48, Figure 55). Twenty-four hour lesion scores were lower than 25 hour scores. For vacuolation severity, lesion scores at 0 hour were lower than at all other time points, and 25 hour lesion scores were higher than those at 32, 36 and 48 hours (Table 49, Figure 56). Additionally, for vacuolation severity, mean lesion scores for slices incubated in 100 µg/ml media were lower than all other concentrations (Table 50, Figure 57).

Table 46. Time analysis of mean necrosis lesion scores for slices incubated with carprofen and phenobarbital. Comparison of necrosis lesion scores (n=12) across time for slices incubated in all concentrations of carprofen. Slices were incubated for 24 hours in phenobarbital (75µg/ml) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for necrosis two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups			Mean lesion score
0	a			0.00
24		b		1.33
25		b	c	1.50
28		b	c	1.73
32		b	c	1.75
36			c	2.00
48			c	2.00

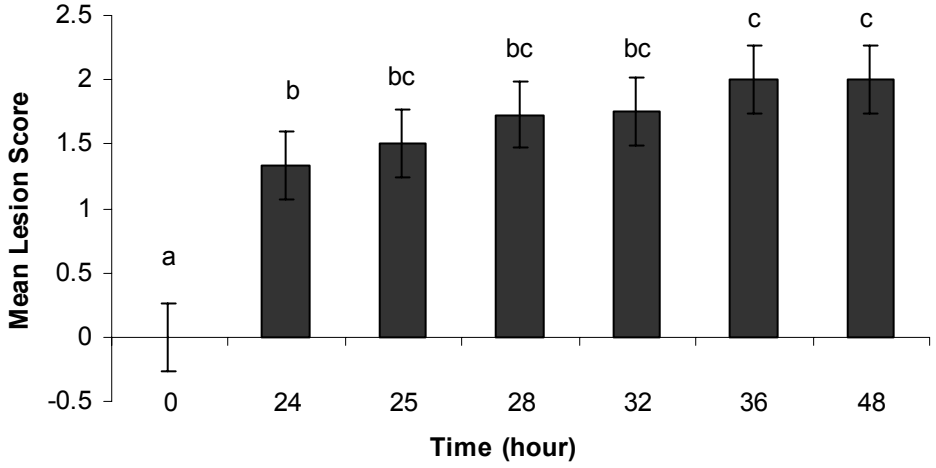

Fig. 53. Time analysis for mean necrosis lesion scores for slices incubated with carprofen and phenobarbital. Comparison of mean necrosis lesion scores (±SE, n=12) across time for slices incubated in all concentrations of carprofen. Slices were incubated for 24 hours in phenobarbital (75µg/ml) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for necrosis two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Table. 47. Concentration analysis for mean vacuolation extent lesion scores for slices incubated with carprofen and phenobarbital. Comparison of mean vacuolation extent lesion scores (n=18) among carprofen concentrations for all time points. Slices were incubated for 24 hours in phenobarbital (75µg/ml) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for vacuolation extent two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Concentration (µg/ml)	Homogeneous groups	Mean lesion score
0	a	3.10
10	a	3.15
50	a b	2.67
100	b	2.05

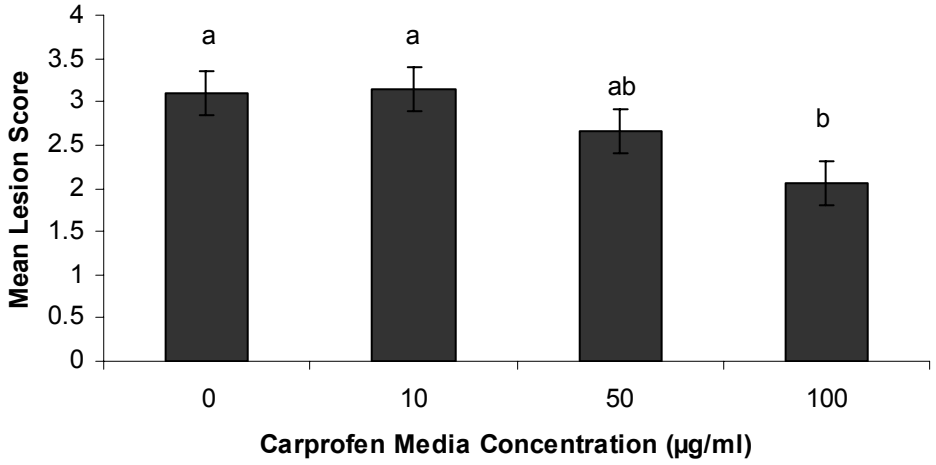

Fig. 54. Concentration analysis for mean vacuolation extent lesion scores for slices incubated with carprofen and phenobarbital. Comparison of mean vacuolation extent lesion scores (±SE, n=18) among carprofen concentrations for all time points. Slices were incubated for 24 hours in phenobarbital (75µg/ml) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for vacuolation extent two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Table 48. Time analysis of mean vacuolation extent lesion scores for slices incubated with carprofen and phenobarbital. Comparison of mean vacuolation extent lesion scores (n=12) across time for slices incubated in all concentrations of carprofen. Slices were incubated for 24 hours in phenobarbital (75µg/ml) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for vacuolation extent two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups			Mean lesion score
0	a			0.33
24		b		2.67
25			c	3.75
28		b	c	3.18
32		b	c	2.83
36		b	c	3.42
48		b	c	3.00

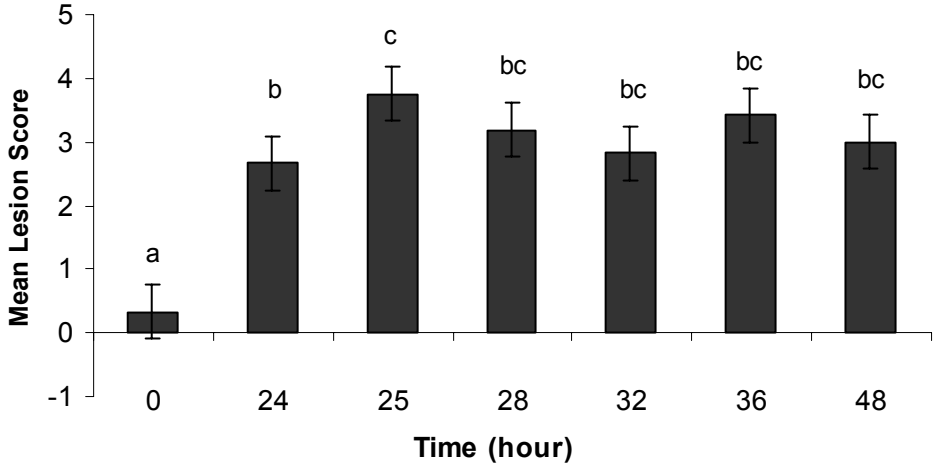

Fig. 55. Time analysis of mean vacuolation extent lesion scores for slices incubated with carprofen and phenobarbital. Comparison of mean vacuolation extent lesion scores (±SE, n=12) across time for slices incubated in all concentrations of carprofen. Slices were incubated for 24 hours in phenobarbital (75µg/ml) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for vacuolation extent two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Table 49. Concentration analysis for mean vacuolation severity lesion scores for slices incubated with carprofen and phenobarbital. Comparison of mean vacuolation severity lesion scores (n=18) among carprofen concentrations for all time points. Slices were incubated for 24 hours in phenobarbital (75µg/ml) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for vacuolation severity two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Concentration (µg/ml)	Homogeneous groups	Mean lesion score
0	a	2.86
10	a	2.80
50	a	2.57
100	b	1.86

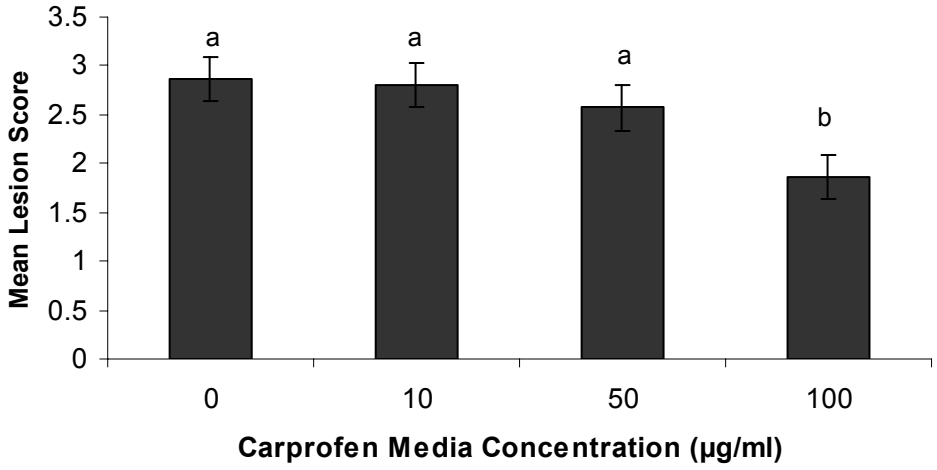

Fig. 56. Concentration analysis for mean vacuolation severity lesion scores for slices incubated with carprofen and phenobarbital. Comparison of mean vacuolation severity lesion scores (±SE, n=18) among carprofen concentrations for all time points. Slices were incubated for 24 hours in phenobarbital (75µg/ml) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for vacuolation severity two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Table 50. Time analysis of mean vacuolation severity lesion scores for slices incubated with carprofen and phenobarbital. Comparison of mean vacuolation severity lesion scores (n=12) across time for slices incubated in all concentrations of carprofen. Slices were incubated for 24 hours in phenobarbital (75µg/ml) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for vacuolation severity two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups			Mean lesion score
0	a			0.33
24		b	c	3.33
25		b		3.50
28		b	c	2.73
32			c	2.58
36			c	2.58
48			c	2.58

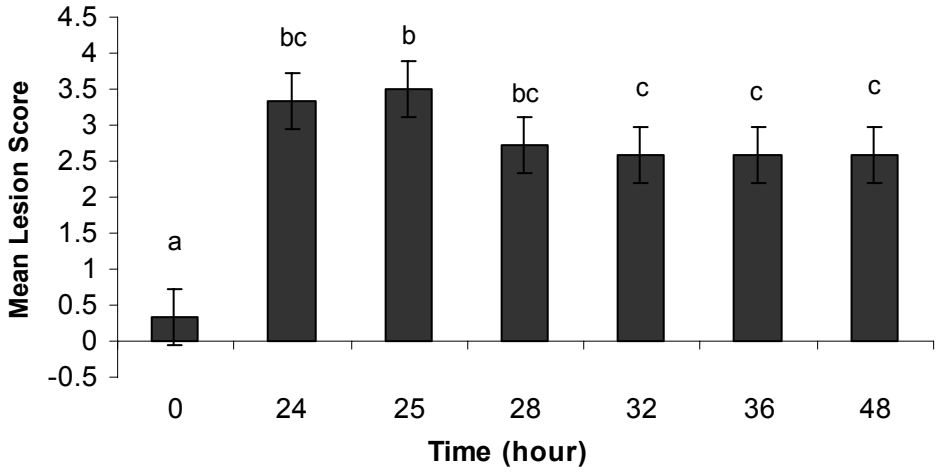

Fig. 57. Time analysis of mean vacuolation severity lesion scores for slices incubated with carprofen and phenobarbital. Comparison of mean vacuolation severity lesion scores (±SE, n=12) across time for slices incubated in all concentrations of carprofen. Slices were incubated for 24 hours in phenobarbital (75µg/ml) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for vacuolation severity two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Carprofen and Cimetidine Incubation

Media and Supernatant Drug Concentrations

Hepatic slices were incubated for 24 hours in media containing cimetidine followed by incubation with carprofen. Media concentrations for 0 µg/ml carprofen media were below the

lower limit of quantification. Supernatant concentrations (ng/ml) measured for carprofen were below the lower limit of quantification for 0 and 10 µg/ml media.

The carprofen concentrations in media (µg/ml) after slice incubation with cimetidine followed by incubation with carprofen (Figure 58) were evaluated for differences among media concentrations. The AUC (hour*µg/ml) of peak B was higher than the AUC of peak A at 10 µg/ml (p-value=0.0020; without 0 hour p-value=0.0321) and 100 µg/ml (p-value=0.0331; without 0 hour p-value>0.05) (Table 51, Figure 59). There were no differences in AUC of peaks for 50 µg/ml media.

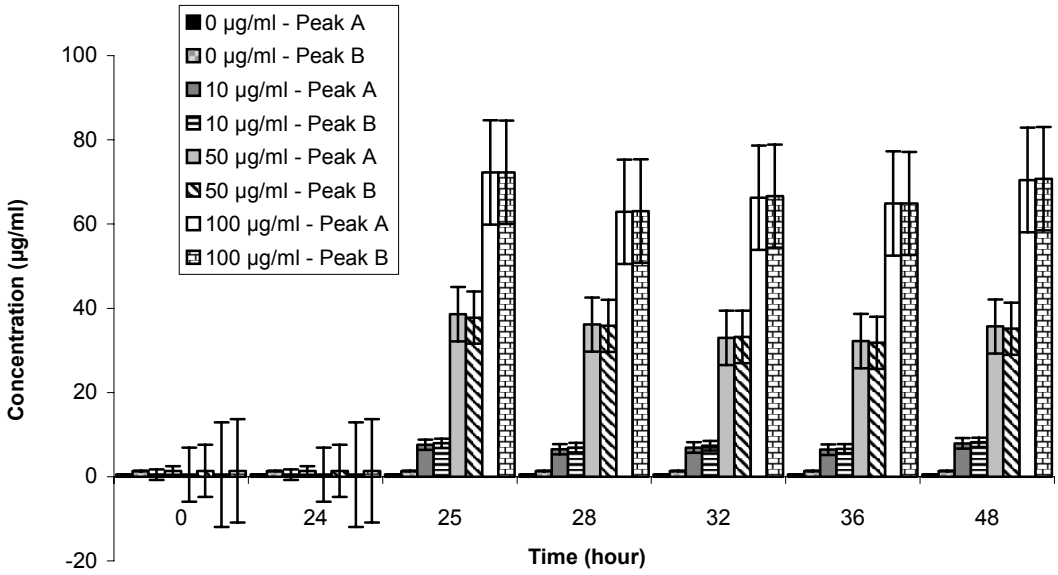

Fig. 58. Mean carprofen media concentrations following incubation with cimetidine and carprofen. Mean concentration (µg/ml±SE) of carprofen enantiomers (A or B) in media after incubation for 24 hours with cimetidine (1000µM) followed with an additional incubation in carprofen for 1, 4, 8, 12 and 24 hours. Values are means of nine samples per media concentration per time point. Carprofen concentrations of 0 µg/ml media were below the LOQ.

Table 51. AUC of carprofen in media following incubation with cimetidine and carprofen. Mean AUC (hour*µg/ml, n=3) of carprofen in media (0, 10, 50 or 100 µg/ml) for slices incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen.

Carprofen and cimetidine		Media
Concentration (µg/ml)	Peak	AUC (hour*µg/ml)
0	A	23.12
	B	66.48
10	A	176.47
	B	205.47
50	A	819.13
	B	833.10
100	A	1583.00
	B	1609.67

Carprofen and cimetidine (without 0 hour)		Media
Concentration (µg/ml)	Peak	AUC (hour*µg/ml)
0	A	11.56
	B	33.24
10	A	164.90
	B	172.17
50	A	807.53
	B	799.87
100	A	1571.67
	B	1576.33

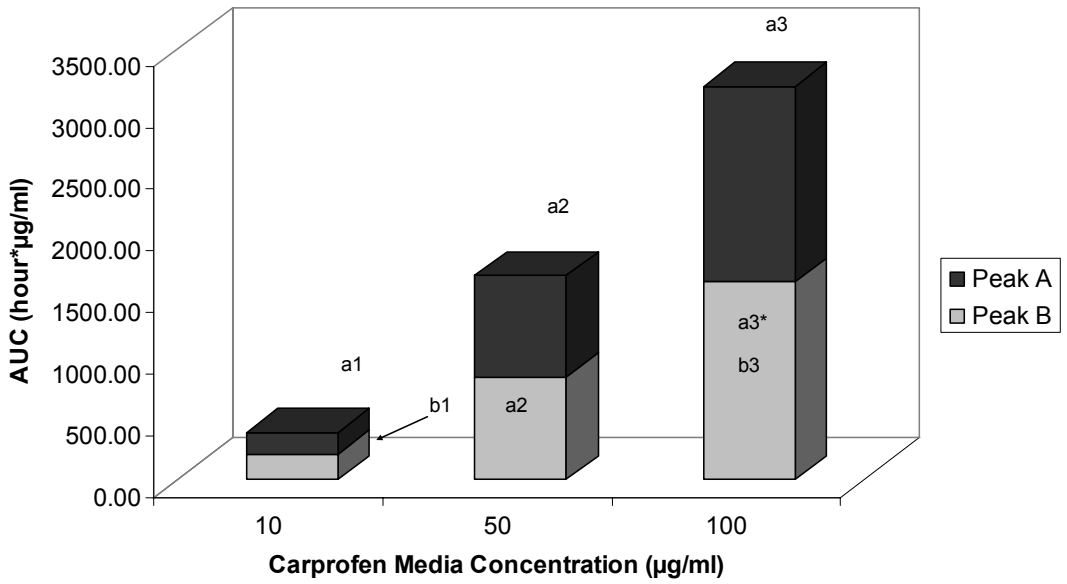

Fig. 59. AUC of carprofen in media following incubation with cimetidine and carprofen. Mean AUC (hour*µg/ml, n=3) of carprofen in media (10, 50 or 100 µg/ml) for slices incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen. For each media concentration, peaks were compared for significant differences. Within each carprofen media concentration, peaks with the same letter are not significantly different. For 100 µg/ml media, no statistical difference was noted (a3*) when 0 hour carprofen concentrations were excluded.

The mean supernatant concentrations for slices incubated in cimetidine (1000 µM) followed by carprofen (Figure 60) showed that 0 and 24 hour concentrations were below the lower limit of quantification. For 50 µg/ml media, the AUC (hour*ng/ml) of peak B was higher than peak A (p-value=0.0101) (Table 52, Figure 61). No differences in peak AUC values were noted for 100 µg/ml media.

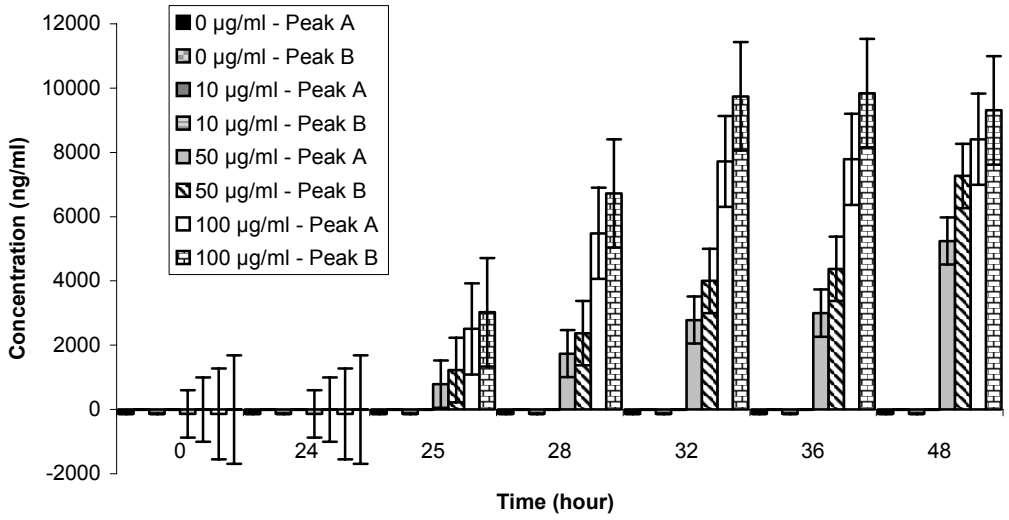

Fig. 60. Mean hepatic slice supernatant concentrations of carprofen following incubation with cimetidine and carprofen. Mean concentrations (ng/ml±SE) of carprofen enantiomers (A or B) in hepatic slice supernatant after incubation for 24 hours with cimetidine (1000 µM) followed with an additional incubation in carprofen for 1, 4, 8, 12 and 24 hours. The values are an average of six samples per media concentration per time point. Carprofen supernatant concentrations for slices incubated in 0 and 10 µg/ml media were below the LOQ.

Table 52. AUC of carprofen in hepatic slice supernatant following incubation with cimetidine and carprofen. Mean AUC (hour*ng/ml, n=3) of carprofen in supernatant of hepatic slices incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen media (0, 10, 50 and 100 µg/ml).

Carprofen and cimetidine		Supernatant
Concentration (µg/ml)	Peak	AUC (hour*ng/ml)
0	A	0.00
	B	0.00
10	A	0.00
	B	0.00
50	A	74169.67
	B	105371.00
100	A	167755.33
	B	203131.00

Carprofen and cimetidine (without 0 hour)		Supernatant
Concentration (µg/ml)	Peak	AUC (hour*ng/ml)
0	A	0.00
	B	0.00
10	A	0.00
	B	0.00
50	A	74179.67
	B	105371.00
100	A	167758.67
	B	203131.00

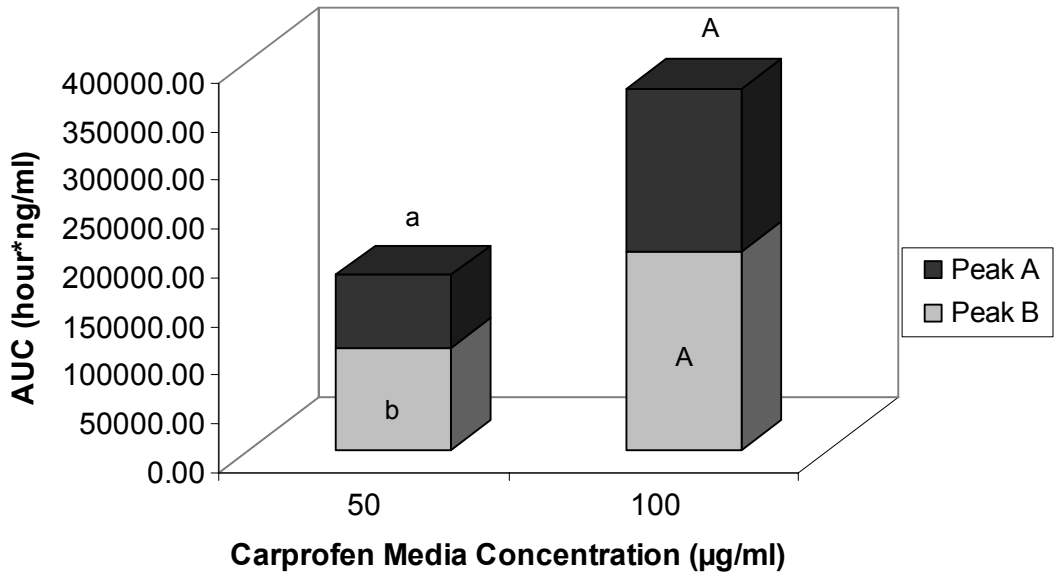

Fig. 61. AUC of carprofen in hepatic slice supernatant following incubation with cimetidine and carprofen. Mean AUC (hour*ng/ml, n=3) of carprofen in supernatant of hepatic slices incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen media (50 and 100 µg/ml). For each media concentration, peaks were compared for significant differences. Within each carprofen media concentration, peaks with the same letter are not significantly different. For 50 µg/ml media, Peak B AUC is greater than that of Peak A ($p<0.05$).

Potassium

Figure 62 shows the potassium concentrations for slices incubated in cimetidine followed by incubation with carprofen. Significant differences across time (df=6, F-ratio=64.516, p-value=0.000) and concentration (df=3, F-ratio=11.568, p-value=0.000) were noted by two-way ANOVA. ANOVA groups were based on the means of all samples at each time point (n=24) or the means of all times for each concentration (n=42). Zero hour mean potassium concentrations were higher than those at all other time points (Table 53, Figure 63). Forty-eight hour potassium concentrations were less than those at 25, 28, 32 and 36 hours. Among the media concentrations, mean potassium concentrations for slices incubated in 10 µg/ml media were higher than 0, 50 and 100 µg/ml potassium concentrations (Table 54, Figure 64). The potassium content AUC (hour*µmol/g-L) was also determined for each concentration of carprofen (Table 55).

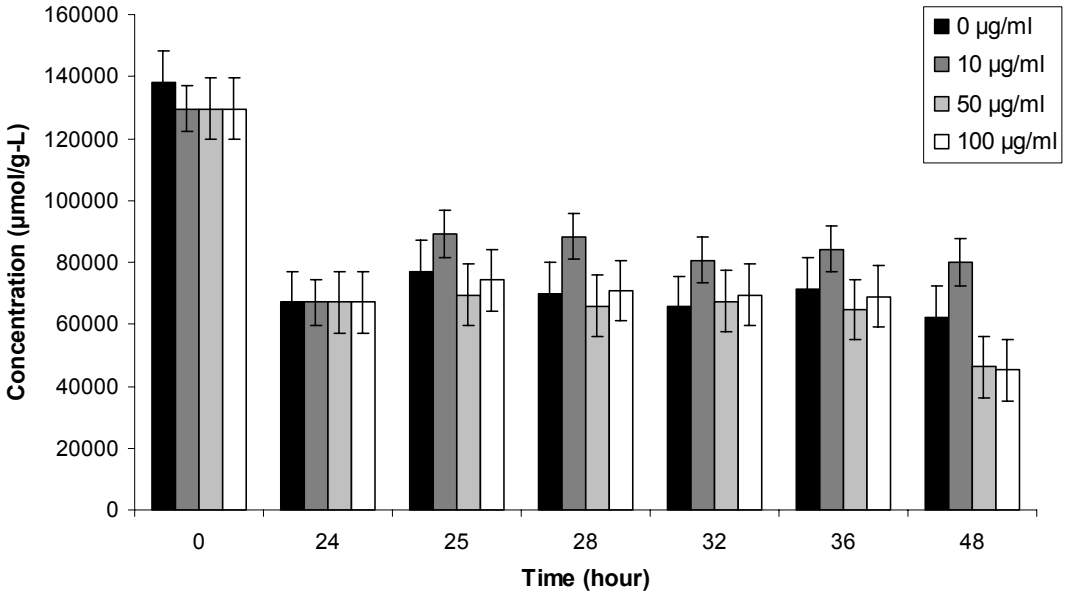

Fig. 62. Potassium concentrations for slices incubated with cimetidine and carprofen. Potassium concentrations (µmol/g-L±SE, mean) in hepatic slice supernatant during 24 hours of incubation cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen (0, 10, 50 and 100 µg/ml). Potassium concentrations are a mean of six samples per time point for each concentration.

Table 53. Time analysis of potassium concentrations for slices incubated with cimetidine and carprofen. Comparison of mean potassium concentrations (µmol/g-L) of all potassium samples (n=24) at each time point for all carprofen concentrations. Slices were incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for potassium two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups		Means (µmol/g-L)
0	a		131759.74
24	b	c	67108.92
25	b		77444.50
28	b		73777.79
32	b		70765.84
36	b		72323.46
48		c	58368.17

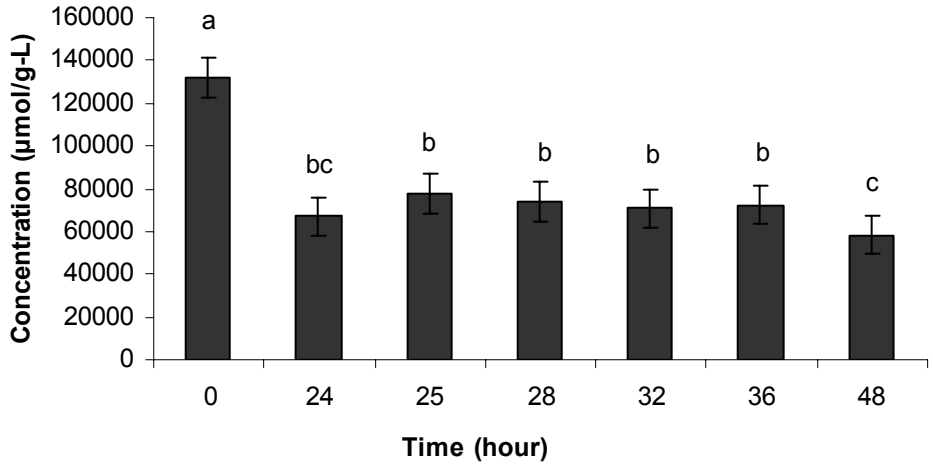

Fig. 63. Time analysis of potassium concentrations for slices incubated with carprofen and cimetidine. Comparison of mean potassium concentrations (µmol/g-L±SE) of all potassium samples (n=24) at each time point for all carprofen concentrations. Slices were incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for potassium two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Table 54. Concentration analysis of potassium concentrations for slices incubated with carprofen and cimetidine. Comparison of mean potassium concentrations (µmol/g-L, n=42) at all times for each carprofen media concentration. Slices were incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for potassium two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Concentration (µg/ml)	Homogeneous groups	Means (µmol/g-L)
0	a	78749.06
10	b	88475.23
50	a	72875.48
100	a	75070.76

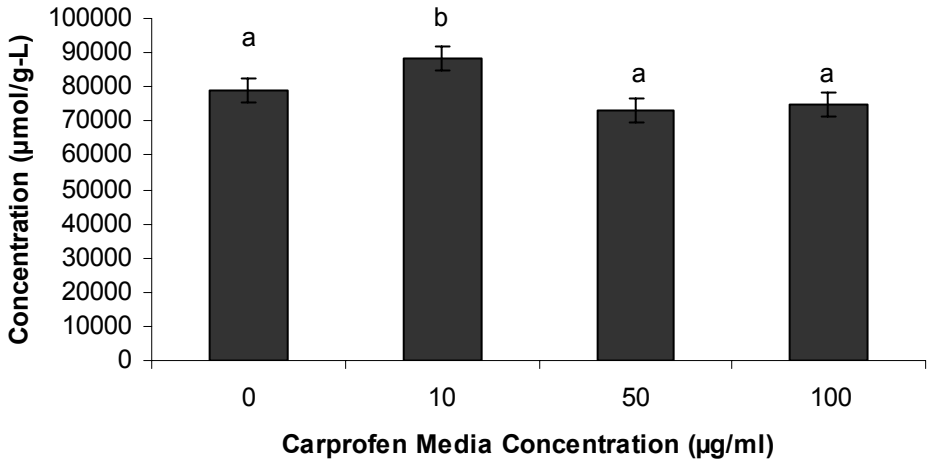

Fig. 64. Concentration analysis of potassium concentrations for slices incubated with carprofen and cimetidine. Comparison of mean potassium concentrations (µmol/g-L±SE, n=42) at all times for each carprofen media concentration. Slices were incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for potassium two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Table 55. Potassium content AUC for slices incubated with carprofen and cimetidine. Mean AUC (hour*µmol/g-L, n=3) of potassium content for slices incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in media containing 0, 10, 50 and 100 µg/ml carprofen.

Carprofen and cimetidine	Potassium Content
Concentration (µg/ml)	AUC (hour*µmol/g-L)
0	4.101E+06
10	4.461E+06
50	3.985E+06
100	4.047E+06

Carprofen and cimetidine (without 0 hour)	Potassium Content
Concentration (µg/ml)	AUC (hour*µmol/g-L)
0	1.638E+06
10	1.998E+06
50	1.522E+06
100	1.585E+06

ATP

Mean ATP concentrations (nmol/g) for slices incubated in cimetidine followed by incubation in carprofen (Figure 65) were evaluated for differences across time and among carprofen media concentrations. Two-way ANOVA demonstrated significant differences across time (df=6, F-ratio=7.833, p-value=0.000). ANOVA groups were based on the means of all samples at each time point (n=24) or the means of all times for each concentration (n=42). Mean ATP concentrations at 0 hour were less than those at 24, 25 and 32 hours (Table 56, Figure 66). Concentrations at 24 hours were higher than at 25, 28, 32, 36 and 48 hours. The ATP content AUC (hour*nmol/g) was also determined for each concentration of carprofen (Table 57).

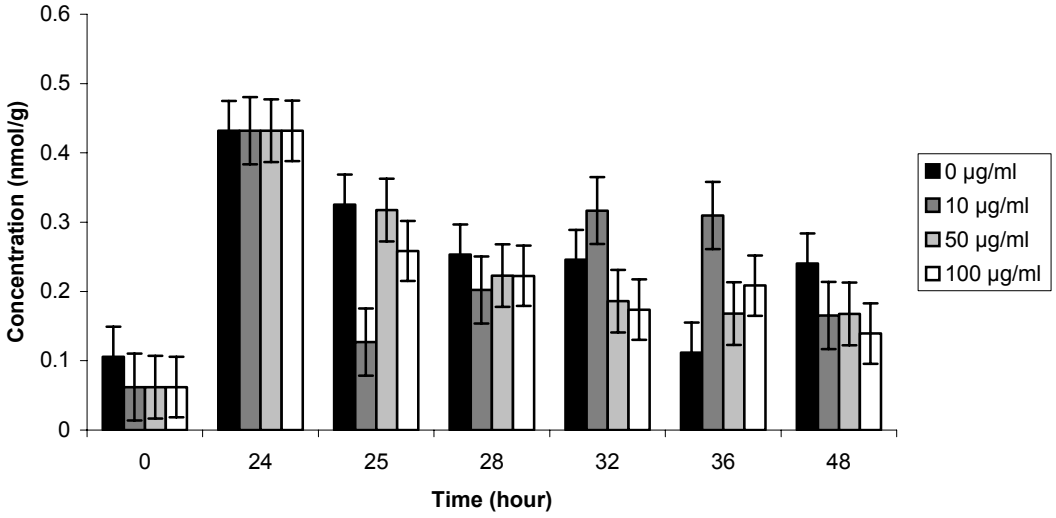

Fig. 65. ATP concentrations for slices incubated with carprofen and cimetidine. ATP concentrations (nmol/g±SE, mean) in hepatic slice supernatant during 24 hours of incubation cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen (0, 10, 50 and 100 µg/ml). ATP concentrations are a mean of six samples per time point for each concentration.

Table 56. Time analysis of ATP concentrations for slices incubated with carprofen and cimetidine. Comparison of mean ATP concentrations (nmol/g) of all ATP samples (n=24) at each time point for all carprofen concentrations. Slices were incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for ATP two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups		Means (nmol/g)
0	a		0.072966
24		b	0.431838
25		c	0.257108
28	a	c	0.225234
32		c	0.230605
36	a	c	0.199570
48	a	c	0.178108

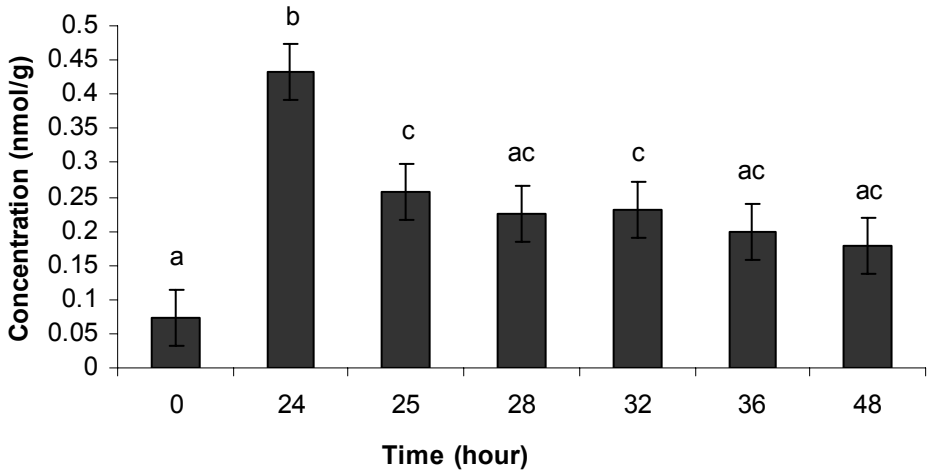

Fig. 66. Time analysis of ATP concentrations for slices incubated with carprofen and cimetidine. Comparison of mean ATP concentrations (nmol/g±SE) of all ATP samples (n=24) at each time point for all carprofen concentrations. Slices were incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for ATP two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Table 57. ATP content AUC for slices incubated with carprofen and cimetidine. Mean AUC (hour*nmol/g, n=3) of ATP content for slices incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in media containing 0, 10, 50 and 100 µg/ml carprofen.

Carprofen and cimetidine	ATP Content
Concentration (µg/ml)	AUC (hour*nmol/g)
0	11.52
10	11.84
50	10.65
100	10.64

Carprofen and cimetidine (without 0 hour)	ATP Content
Concentration (µg/ml)	AUC (hour*nmol/g)
0	5.07
10	5.91
50	4.73
100	4.71

Histology

The hepatic slice lesion scores for slices incubated in cimetidine and carprofen (Table 58, Figures 67 through 69) were examined by two-way ANOVA and showed significant differences for necrosis, vacuolation extent and vacuolation severity. For necrosis, mean lesion scores

Table 58. Hepatic slice lesion scores for slices incubated with carprofen and cimetidine. Lesion scores of hepatic slices incubated in cimetidine for 24 hours followed with incubation in carprofen for and additional 1, 4, 8, 12 and 24 hours.

		Lesion scores*								
Media Concentration	**Time (hour)**	**Necrosis : extent**			**Vacuolation : extent**			**Vacuolation : severity**		
0 µg/ml	0	0	0	0	0	0	1	0	0	1
0 µg/ml	24	3	3	1	4	4	4	4	4	3
cimetidine	0	0	0	0	0	0	3	0	0	2
cimetidine	24	1	1	2	1	1	4	2	2	4
0 µg/ml	25	2	2	1	3	3	4	4	4	4
0 µg/ml	28	1	1	2	4	4	4	4	4	4
0 µg/ml	32	2	2	3	4	4	4	4	4	4
0 µg/ml	36	2	2	4	4	4	4	3	3	3
0 µg/ml	48	2	2	4	3	3	3	3	3	2
10 µg/ml	25	3[a]	1	3	3[a]	4	4	4[a]	3	4
10 µg/ml	28	1	1	2	4	4	4	4	4	4
10 µg/ml	32	2	3[a]	1	4	4[a]	5	4	4[a]	4
10 µg/ml	36	2	3[a]	2	4	4[a]	4	4	4[a]	3
10 µg/ml	48	2	3[a]	3[a]	3	4[a]	4[a]	3	4[a]	4[a]
50 µg/ml	25	2	3[a]	2	3	4[a]	4	3	4[a]	4
50 µg/ml	28	3	3	3	4	4	4	4	3	3
50 µg/ml	32	2[a]	3	3	4[a]	3	2	4[a]	2	2
50 µg/ml	36	2	3	3	4	3	2	3	2	1
50 µg/ml	48	2	4	4	3	2	2	3	2	2
100 µg/ml	25	2	3	2	1	4	4	2	3	4
100 µg/ml	28	1	3	3	4	4	2	3	3	2
100 µg/ml	32	2	3	3	4	2	2	2	1	1
100 µg/ml	36	2	4	4	4	2	2	3	1	1
100 µg/ml	48	2	4	4	1	1	2	2	1	2

* Lesion scoring: (outermost zone ~10 hepatocytes wide was disregarded)

0	< 1% hepatocytes affected	3	21-40%
1	1-10%	4	41-90%
2	11-20%	5	>90%

a Slides showing marked difference in lesion extent and/or severity as move across the slide

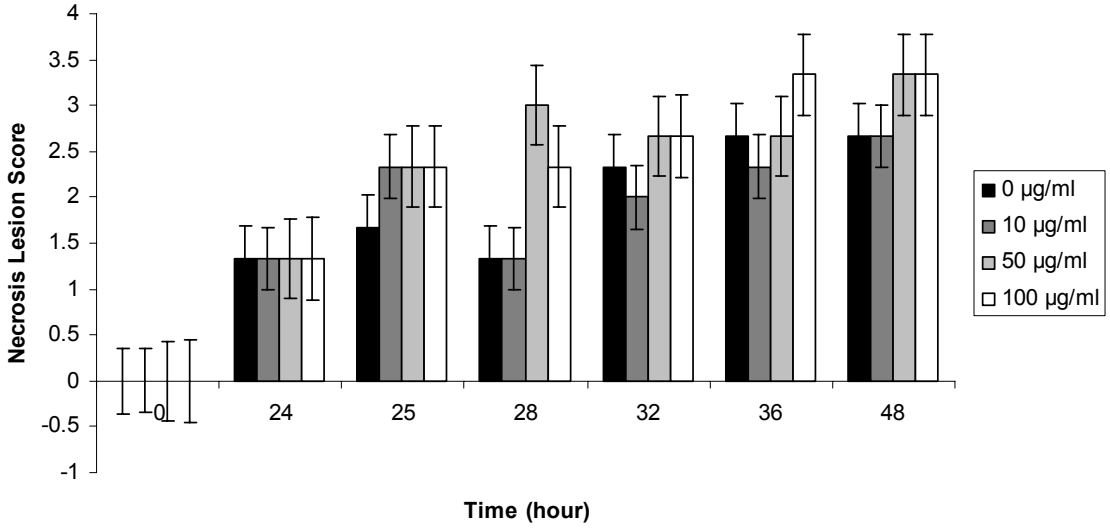

Fig. 67. Mean necrosis hepatic slice lesion scores for slices incubated with carprofen and cimetidine. Mean necrosis lesion scores (±SE) for slices incubated for 24 hours in cimetidine (1000 µM) followed with an additional 24 hour incubation in carprofen (0, 10, 50 or 100 µg/ml). Scores are means of three samples per time point.

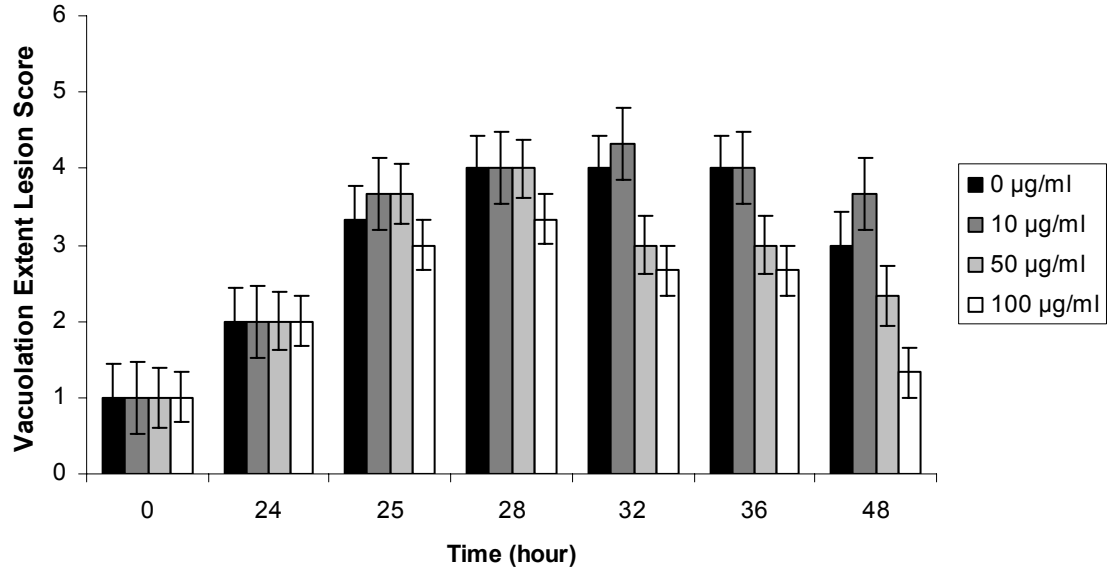

Fig. 68. Mean vacuolation extent hepatic slice lesion scores for slices incubated with carprofen and cimetidine. Mean vacuolation extent lesion scores (±SE) for slices incubated for 24 hours in cimetidine (1000 µM) followed with an additional 24 hour incubation in carprofen (0, 10, 50 or 100 µg/ml). Scores are means of three samples per time point.

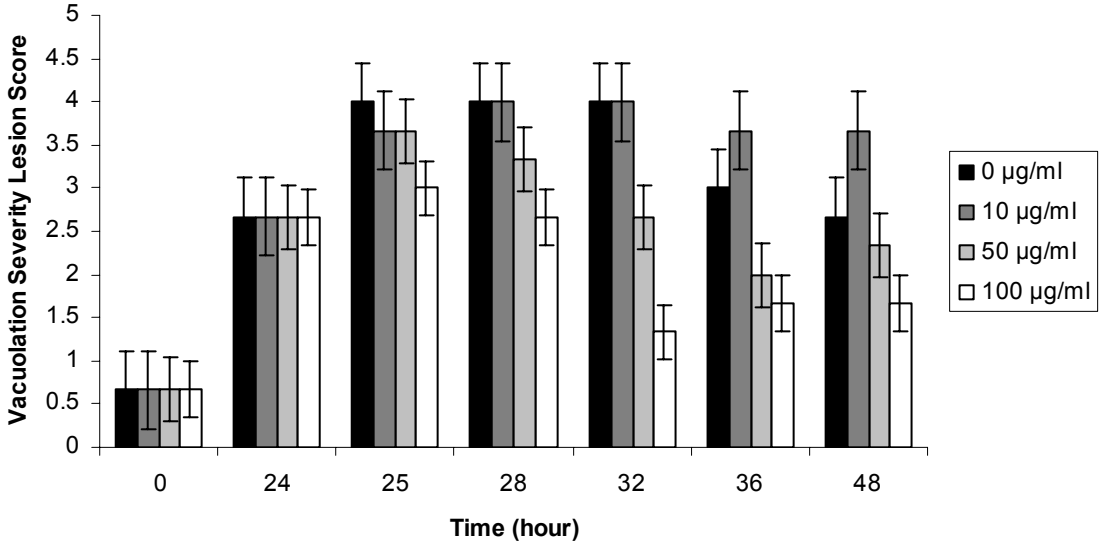

Fig. 69. Mean vacuolation severity hepatic slice lesion scores for slices incubated with carprofen and cimetidine. Mean vacuolation severity lesion scores (±SE) for slices incubated for 24 hours in cimetidine (1000 µM) followed with an additional 24 hour incubation in carprofen (0, 10, 50 or 100 µg/ml). Scores are means of three samples per time point.

across time (df=6, F-ratio=22.616, p-value=0.000) were lower at 0 hour than at all other times. Twenty-four hour scores were lower than those at 32, 36 and 48 hours; 48 hour scores were higher than 28 hour scores (Table 59, Figure 70). Mean vacuolation extent lesion scores for time (df=6, F-ratio=9.893, p-value=0.000) were lower at 0 hour than at 25, 28, 32, 36 and 48 hours (Table 60, Figure 71). Twenty-four hour scores were lower than 25, 32 and 36 hour scores. Among concentrations (df=3, F-ratio=2.903, p-value=0.043), mean vacuolation extent scores were lower for 100 µg/ml media than for 10 µg/ml media (Table 61, Figure 72). Mean lesion scores for vacuolation severity across time (df=6, F-ratio=16.976, p-value=0.000) were lower at 0 hour than at all other time points (Table 62, Figure 73). For vacuolation severity among concentrations (df=3, F-ratio=9.780, p-value=0.000), mean lesion scores for 100 µg/ml media were less than those for 0 and 10 µg/ml media, and lesion scores for 50 µg/ml media were less than for 10 µg/ml media (Table 63, Figure 74).

Table 59. Time analysis of mean necrosis lesion scores for slices incubated with carprofen and cimetidine. Comparison of necrosis lesion scores (n=12) across time for slices incubated in all concentrations of carprofen. Slices were incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for necrosis two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups			Mean lesion score
0	a			0.00
24		b		1.33
25		b		2.17
28		b	c	2.00
32			c	d	2.42
36			c	d	2.75
48				d	3.00

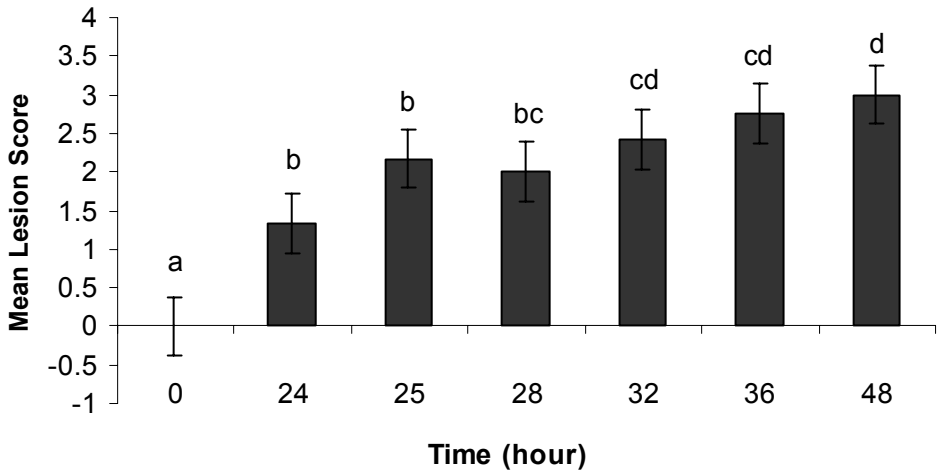

Fig. 70. Time analysis for mean necrosis lesion scores for slices incubated with carprofen and cimetidine. Comparison of mean necrosis lesion scores (±SE, n=12) across time for slices incubated in all concentrations of carprofen. Slices were incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for necrosis two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Table 60. Time analysis of mean vacuolation extent lesion scores for slices incubated with carprofen and cimetidine. Comparison of vacuolation extent lesion scores (n=12) across time for slices incubated in all concentrations of carprofen. Slices were incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for vacuolation extent two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups	Mean lesion score
0	a	1.00
24	a	2.00
25	b	3.58
28	b	3.83
32	b	3.50
36	b	3.25
48	b	2.83

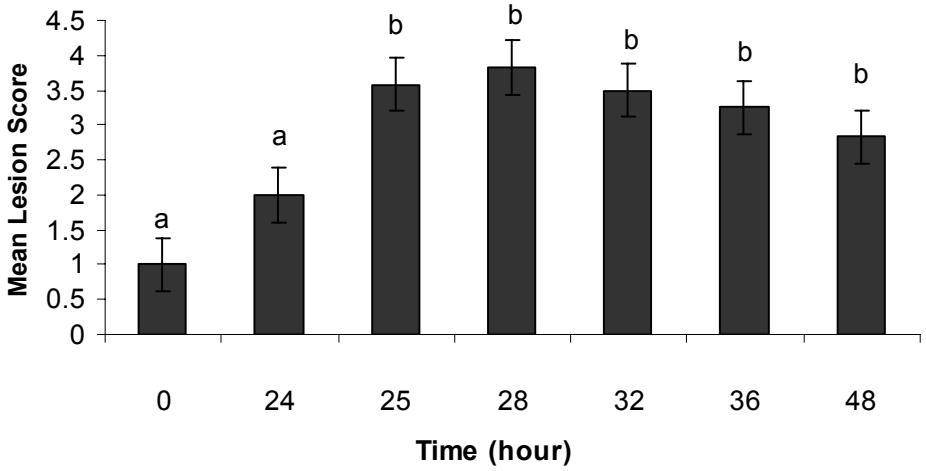

Fig. 71. Time analysis of mean vacuolation extent lesion scores for slices incubated with carprofen and cimetidine. Comparison of vacuolation extent lesion scores (±SE, n=12) across time for slices incubated in all concentrations of carprofen. Slices were incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for vacuolation extent two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Table 61. Concentration analysis for mean vacuolation extent lesion scores for slices incubated with carprofen and cimetidine. Comparison of mean vacuolation extent lesion scores (n=18) among carprofen concentrations for all time points. Slices were incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for vacuolation extent two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Concentration (µg/ml)	Homogeneous groups	Mean lesion score
0	a b	3.05
10	a	3.24
50	a b	2.71
100	b	2.43

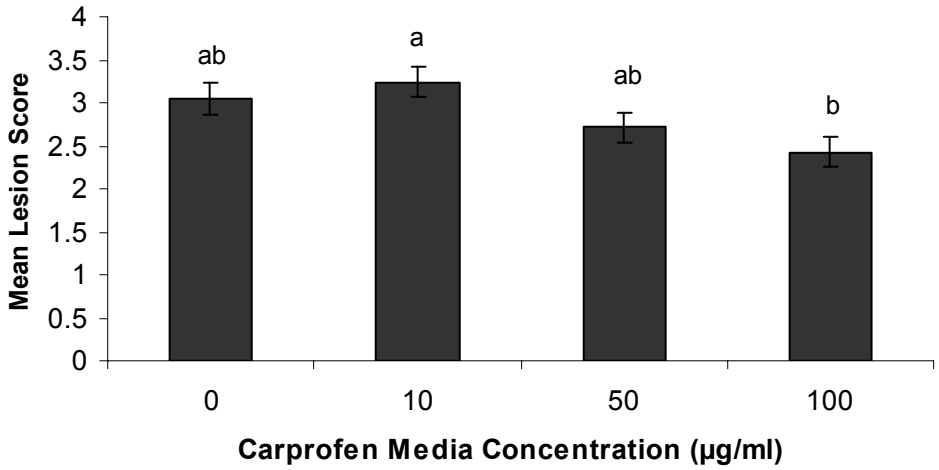

Fig. 72. Concentration analysis for mean vacuolation extent lesion scores for slices incubated with carprofen and cimetidine. Comparison of mean vacuolation extent lesion scores (±SE, n=18) among carprofen concentrations for all time points. Slices were incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for vacuolation extent two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Table 62. Time analysis of mean vacuolation severity lesion scores for slices incubated with carprofen and cimetidine. Comparison of vacuolation severity lesion scores (n=12) across time for slices incubated in all concentrations of carprofen. Slices were incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for vacuolation severity two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (h)	Homogeneous groups	Mean lesion score
0	a	0.67
24	b	2.67
25	b	3.42
28	b	3.50
32	b	3.00
36	b	2.75
48	b	2.33

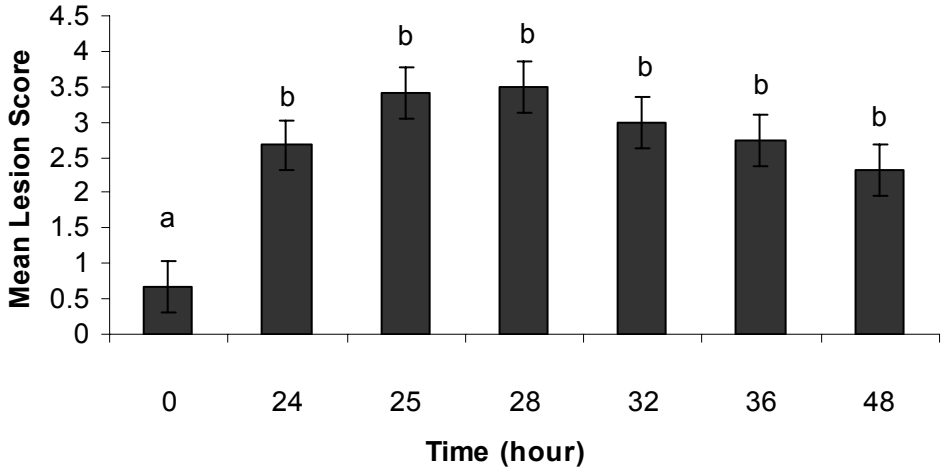

Fig. 73. Time analysis of mean vacuolation severity lesion scores for slices incubated with carprofen and cimetidine. Comparison of vacuolation severity lesion scores (±SE, n=12) across time for slices incubated in all concentrations of carprofen. Slices were incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for vacuolation severity two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Table 63. Concentration analysis for mean vacuolation severity lesion scores for slices incubated with carprofen and cimetidine. Comparison of mean vacuolation severity lesion scores (n=18) among carprofen concentrations for all time points. Slices were incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for vacuolation severity two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Concentration (µg/ml)	Homogeneous groups	Mean lesion score
0	a b	3.00
10	a	3.19
50	b c	2.33
100	c	1.95

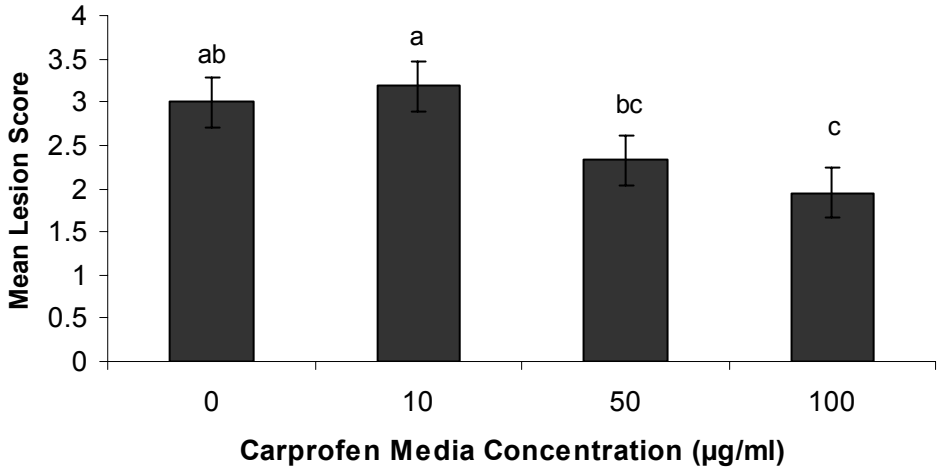

Fig. 74. Concentration analysis for mean vacuolation severity lesion scores for slices incubated with carprofen and cimetidine. Comparison of mean vacuolation severity lesion scores (±SE, n=18) among carprofen concentrations for all time points. Slices were incubated for 24 hours in cimetidine (1000 µM) followed by an additional 24 hour incubation in carprofen. Homogeneous groups for vacuolation severity two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Carprofen Treatment Comparisons

The relationship among the slice treatments—carprofen alone, carprofen with phenobarbital or carprofen with cimetidine—was investigated by examining carprofen concentrations in media and supernatant.

The mean media AUC (hour*µg/ml) for 10 µg/ml (Figure 75), 50 µg/ml (Figure 76) and 100 µg/ml (Figure 77) were examined for peak and treatment differences. When each peak was analyzed and treatments compared by ANOVA, the AUC of peak A media for 50 µg/ml (p-value=0.0260; without 0 hour p-value>0.05; Figure 76, Table 64) and 100 µg/ml (p-value=0.0142; without 0 hour p-value=0.0359; Figure 77, Table 65) were different among treatments. In both instances, treatment with carprofen yielded a higher AUC than carprofen with cimetidine. No significant differences were noted among treatments for peak A or peak B for 10 µg/ml media or for peak B of 50 and 100 µg/ml media. In addition, no significant differences were noted among treatments for either peak A or B for supernatant AUC values.

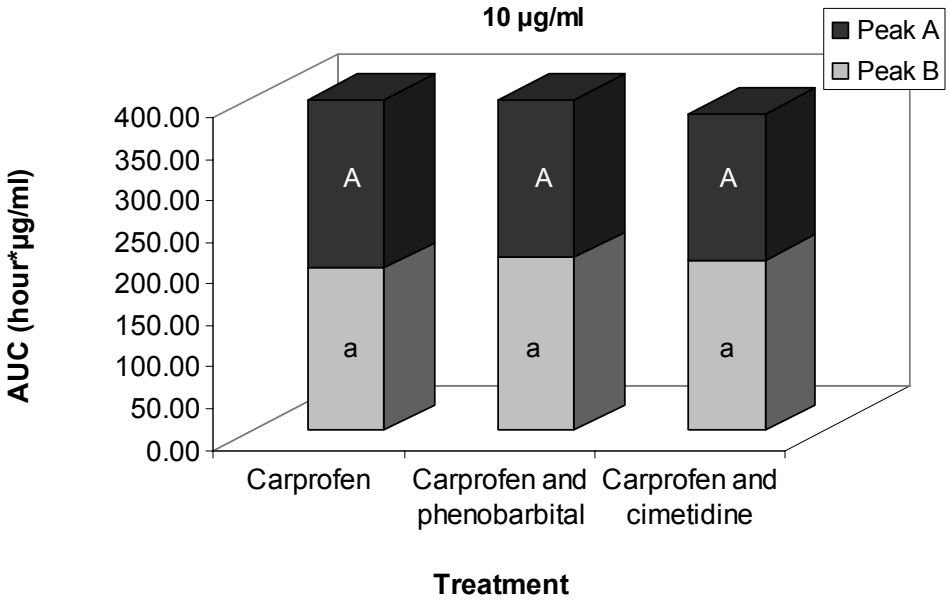

Fig. 75. Mean carprofen AUC for 10 µg/ml media. Mean peak A and B AUC (hour*µg/ml, n=3) for 10 µg/ml carprofen, carprofen following phenobarbital (75 µg/ml) incubation and carprofen following cimetidine (1000 µM) incubation. For all treatments, peak A or peak B was compared for significant differences. For each peak, treatments with the same letter are not significantly different.

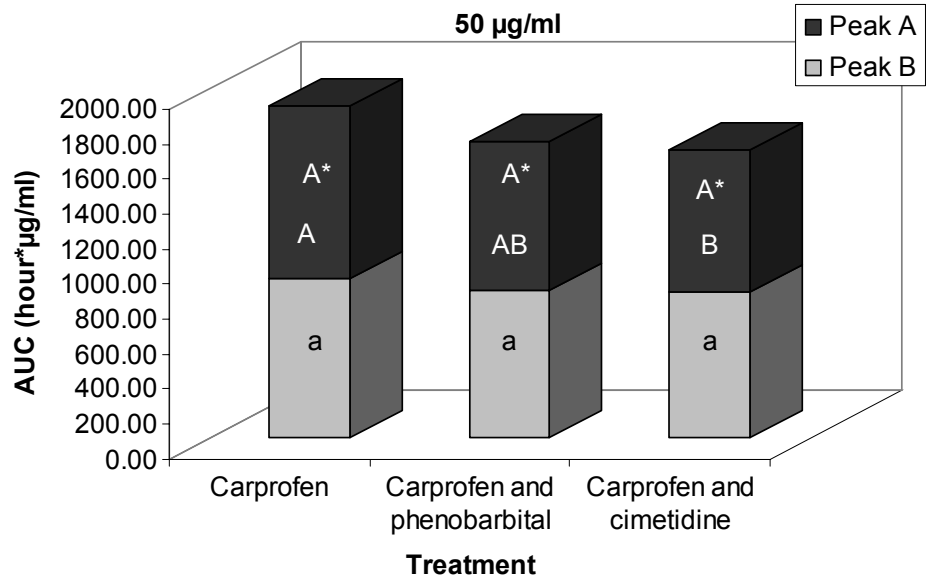

Fig. 76. Mean carprofen AUC for 50 µg/ml media. Mean peak A and B AUC (hour*µg/ml, n=3) for 50 µg/ml carprofen, carprofen following phenobarbital (75 µg/ml) incubation and carprofen following cimetidine (1000 µM) incubation. For all treatments, peak A or peak B was compared for significant differences. For each peak, treatments with the same letter are not significantly different. For peak A, no statistical differences were noted among treatments (A*) when 0 hour concentrations were excluded.

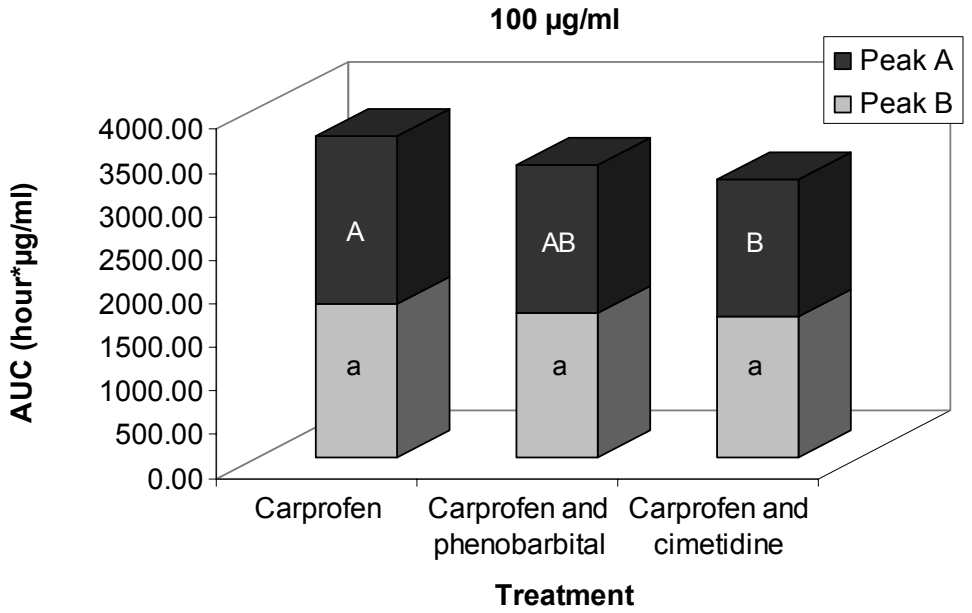

Fig. 77. Mean carprofen AUC for 100 µg/ml media. Mean peak A and B AUC (hour*µg/ml, n=3) for 100 µg/ml carprofen, carprofen following phenobarbital (75 µg/ml) incubation and carprofen following cimetidine (1000 µM)

incubation. For all treatments, peak A or peak B was compared for significant differences. For each peak, treatments with the same letter are not significantly different.

Table 64. Mean Peak A AUC for 50 µg/ml media. Mean peak A (hour*µg/ml, n=3) for 50 µg/ml carprofen, carprofen following phenobarbital (75 µg/ml) incubation and carprofen following cimetidine (1000 µM) incubation. Treatments with the same letter are not significantly different.

Treatment	Homogenous groups		Mean Peak A AUC (hour*µg/ml)
Carprofen	a		988.40
Carprofen with phenobarbital	a	b	854.67
Carprofen with cimetidine		b	819.13

Table 65. Mean Peak A AUC for 100 µg/ml media. Mean peak A (hour*µg/ml, n=3) for 100 µg/ml carprofen, carprofen following phenobarbital (75 µg/ml) incubation and carprofen following cimetidine (1000 µM) incubation. Treatments with the same letter are not significantly different.

Treatment	Homogenous groups		Mean Peak A AUC (hour*µg/ml)
Carprofen	a		1931.67
Carprofen with phenobarbital	a	b	1690.00
Carprofen with cimetidine		b	1583.00

Histology

Mean necrosis lesion scores were compared to identify differences among treatments and across time for slices incubated in 0 µg/ml, 10 µg/ml, 50 µg/ml and 100 µg/ml carprofen. For 0 µg/ml media (Figure 78), a significant time-treatment interaction was noted (df=8, F-ratio=2.411, p-value=0.039), and a significant treatment effect (df=2, F-ratio=8.644, p-value-0.001) was found. Mean necrosis lesion scores for carprofen alone were lower than for carprofen with phenobarbital or cimetidine (Table 66, Figure 79).

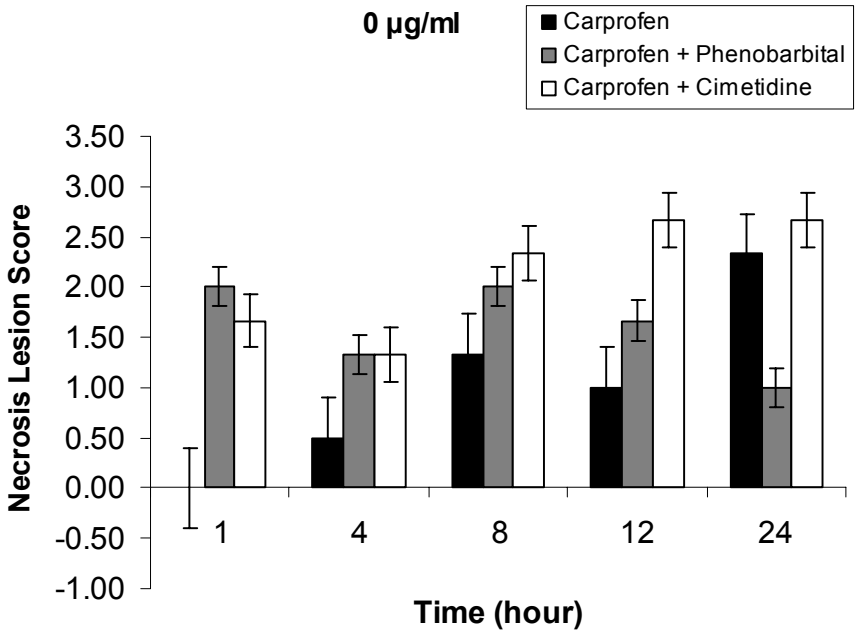

Fig. 78. Mean necrosis lesion scores among 0 µg/ml carprofen treatments. Mean necrosis lesion scores (±SE) for 0 µg/ml carprofen media for slices incubated in carprofen, carprofen with phenobarbital or carprofen with cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are means of three scores per treatment per time point.

Table 66. Mean necrosis lesion score 0 µg/ml treatment analysis. Comparison of mean necrosis lesion scores for slice treatments (n=15) for all time points. Homogeneous groups for necrosis two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Treatment	Homogeneous groups	Mean Lesion Score
carprofen alone	a	1.03
carprofen with phenobarbital	b	1.60
carprofen with cimetidine	b	2.13

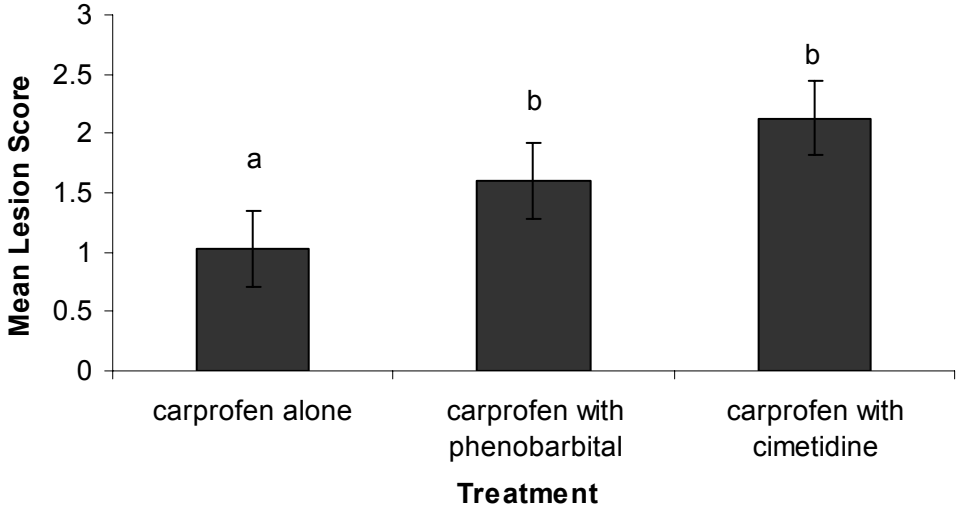

Fig. 79. Mean necrosis lesion score 0 µg/ml treatment analysis. Comparison of mean necrosis lesion scores for slice treatments (±SE, n=15) for all time points. Homogeneous groups for necrosis two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

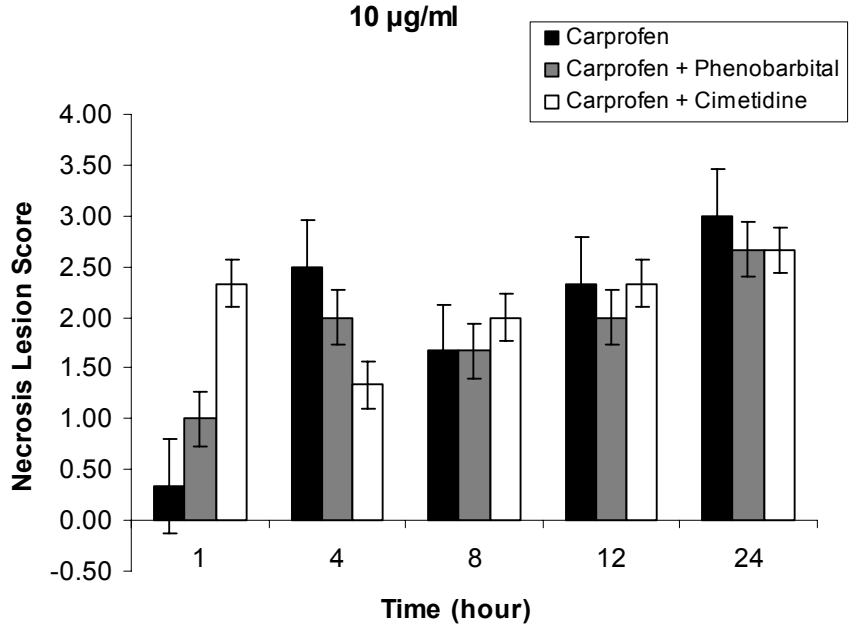

Fig. 80. Mean necrosis lesion scores among 10 µg/ml carprofen treatments. Mean necrosis lesion scores (±SE) for 10 µg/ml carprofen media for slices incubated in carprofen, carprofen with phenobarbital or carprofen with cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are means of three scores per treatment per time point.

For 10 µg/ml necrosis lesion scores (Figure 80), a significant factor interaction was noted (df=8, F-ratio=2.973, p-value=0.015). A significant time effect (df=4, F-ratio=11.250, p-value=0.000) was found with one hour mean lesion scores less than those at 12 and 24 hours (Table 67, Figure 81). Four and eight hour necrosis lesion scores were lower than 24 hour scores.

Table 67. Mean necrosis lesion score 10 µg/ml time analysis. Comparison of mean necrosis lesion scores (n=9) across time for all treatments. Homogeneous groups for necrosis two-way ANOVA for treatment are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups	Mean Lesion Score
1	a	1.22
4	a b	1.94
8	a b	1.78
12	b c	2.22
24	c	2.78

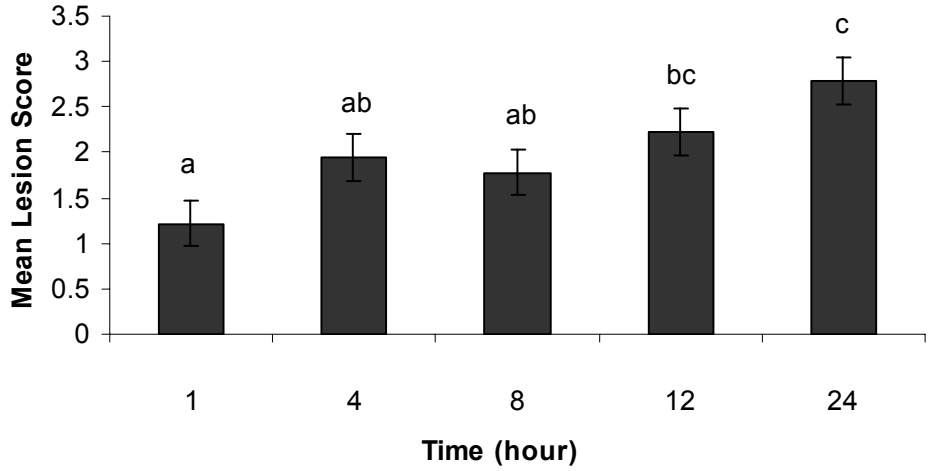

Fig. 81. Mean necrosis lesion score 10 µg/ml time analysis. Comparison of mean necrosis lesion scores (±SE, n=9) across time for all treatments. Homogeneous groups for necrosis two-way ANOVA for treatment are represented as letters. Times with the same letter are not significantly different.

The mean necrosis lesion scores for 50 µg/ml media (Figure 82) revealed significant time (df=4, F-ratio=4.938, p-value=0.004) and treatment (df=2, F-ratio=25.222, p-value=0.000)

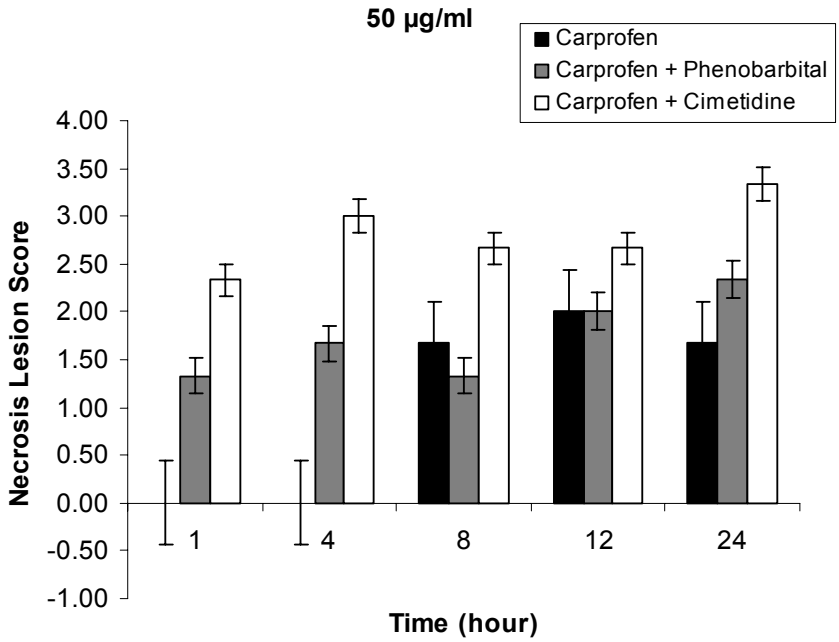

Fig. 82. Mean necrosis lesion scores among 50 µg/ml carprofen treatments. Mean necrosis lesion scores (±SE) for 50 µg/ml carprofen media for slices incubated in carprofen, carprofen with phenobarbital or carprofen with cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are means of three scores per treatment per time point.

effects. With time, one hour necrosis lesion scores were less than 12 and 24 hour scores (Table 68, Figure 83). Among treatments, mean necrosis lesion scores for carprofen alone were less than those for carprofen with phenobarbital or cimetidine; necrosis scores for carprofen with phenobarbital were less than those for carprofen with cimetidine (Table 69, Figure 84).

Table 68. Mean necrosis lesion score 50 µg/ml time analysis. Comparison of mean necrosis lesion scores (n=9) across time for all treatments. Homogeneous groups for necrosis two-way ANOVA for treatment are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups	Mean Lesion Score
1	a	1.22
4	a b	1.56
8	a b	1.89
12	b	2.22
24	b	2.44

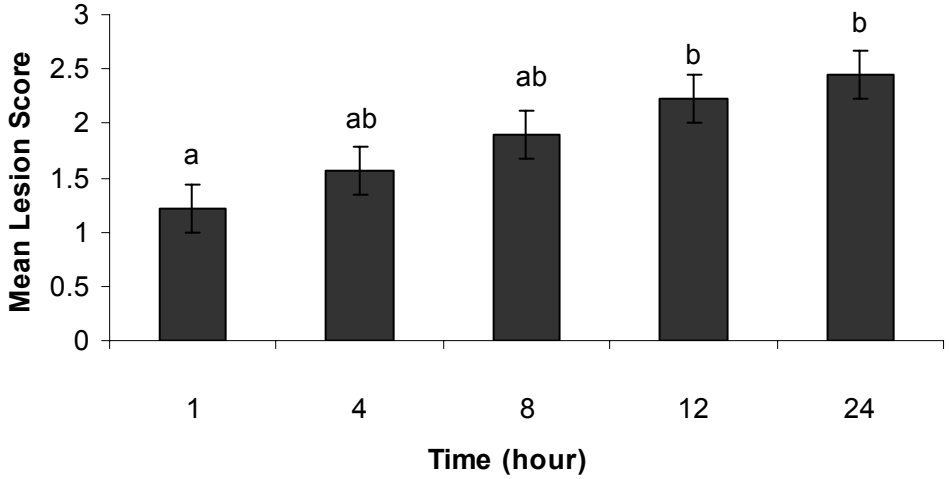

Fig. 83. Mean necrosis lesion score 50 µg/ml time analysis. Comparison of mean necrosis lesion scores (±SE, n=9) across time for all treatments. Homogeneous groups for necrosis two-way ANOVA for treatment are represented as letters. Times with the same letter are not significantly different.

Table 69. Mean necrosis lesion score 50 µg/ml treatment analysis. Comparison of mean necrosis lesion scores for slice treatments (n=15) for all time points. Homogeneous groups for necrosis two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Treatment	Homogeneous groups	Mean Lesion Score
carprofen alone	a	1.07
carprofen with phenobarbital	b	1.73
carprofen with cimetidine	c	2.80

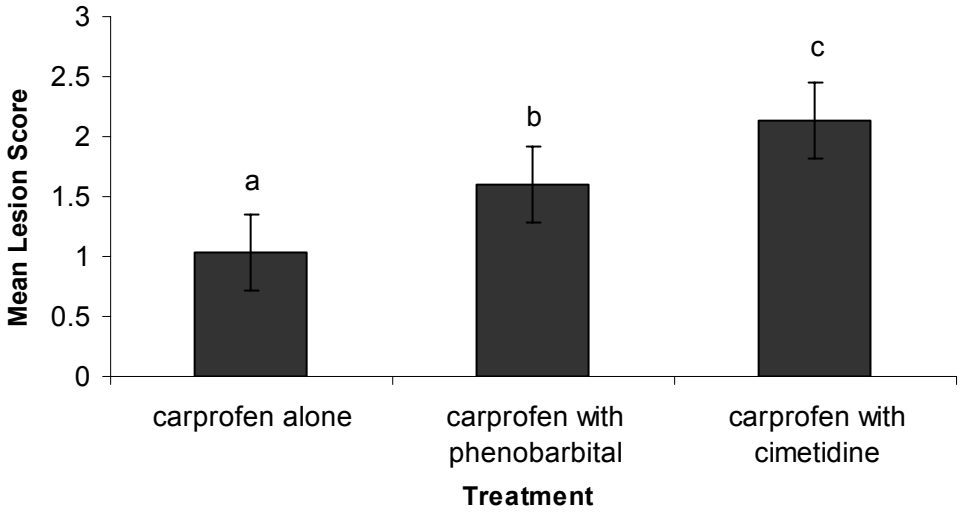

Fig. 84. Mean necrosis lesion score 50 µg/ml treatment analysis. Comparison of mean necrosis lesion scores for slice treatments (±SE, n=15) for all time points. Homogeneous groups for necrosis two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Mean necrosis scores for 100 µg/ml media (Figure 85) were analyzed by two-way ANOVA and significant time (df=4, F-ratio=4.856, p-value=0.004) and treatment (df=2, F-ratio=9.945, p-value=0.001) effects were noted. Across time, mean necrosis lesion scores at one hour were

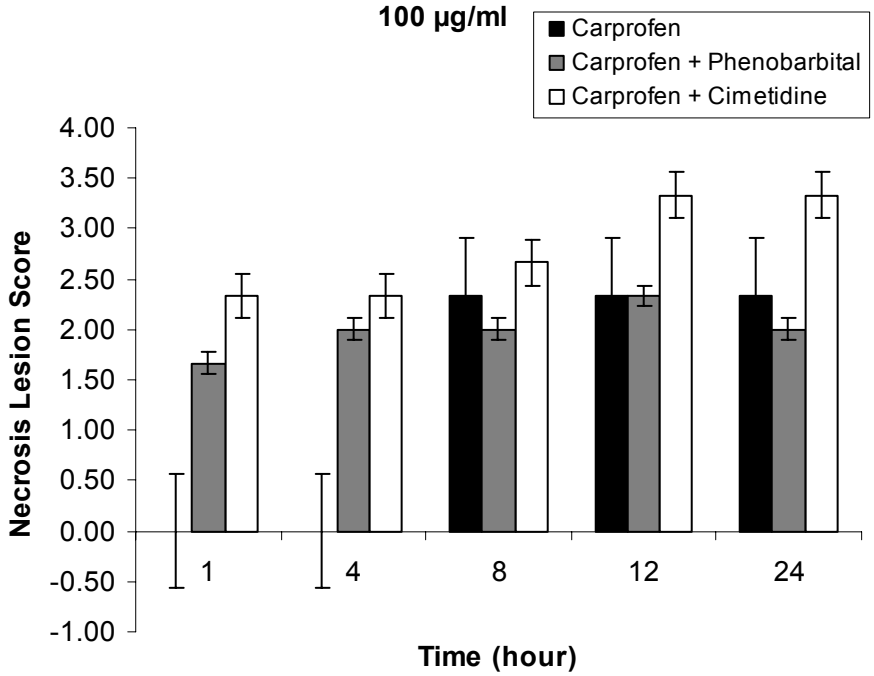

Fig. 85. Mean necrosis lesion scores among 100 µg/ml carprofen treatments. Mean necrosis lesion scores (±SE) for 100 µg/ml carprofen media for slices incubated in carprofen, carprofen with phenobarbital or carprofen with cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are means of three scores per treatment per time point.

less than those at 12 and 24 hours, and four hour lesion scores were less than those at 12 hours (Table 70, Figure 86). Among treatments, mean necrosis lesion scores for slices treated with carprofen and cimetidine were higher than those for slices treated with carprofen alone or with carprofen and phenobarbital (Table 71, Figure 87).

Table 70. Mean necrosis lesion score 100 µg/ml time analysis. Comparison of mean necrosis lesion scores (n=9) across time for all treatments. Homogeneous groups for necrosis two-way ANOVA for treatment are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups	Mean Lesion Score
1	a	1.33
4	a b	1.44
8	a b c	2.33
12	c	2.67
24	b c	2.56

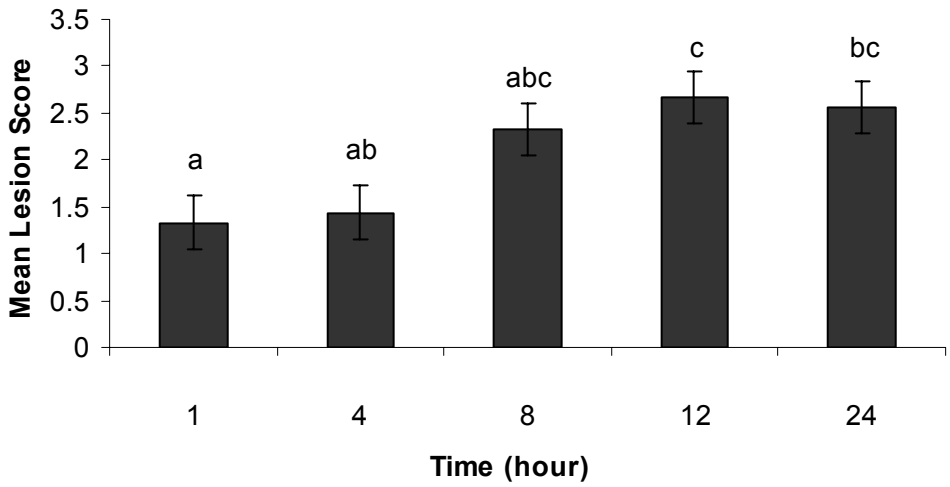

Fig. 86. Mean necrosis lesion score 100 µg/ml time analysis. Comparison of mean necrosis lesion scores (±SE, n=9) across time for all treatments. Homogeneous groups for necrosis two-way ANOVA for treatment are represented as letters. Times with the same letter are not significantly different.

Table 71. Mean necrosis lesion score 100 µg/ml treatment analysis. Comparison of mean necrosis lesion scores for slice treatments (n=15) for all time points. Homogeneous groups for necrosis two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Treatment	Homogeneous groups	Mean Lesion Score
carprofen alone	a	1.40
carprofen with phenobarbital	a	2.00
carprofen with cimetidine	b	2.80

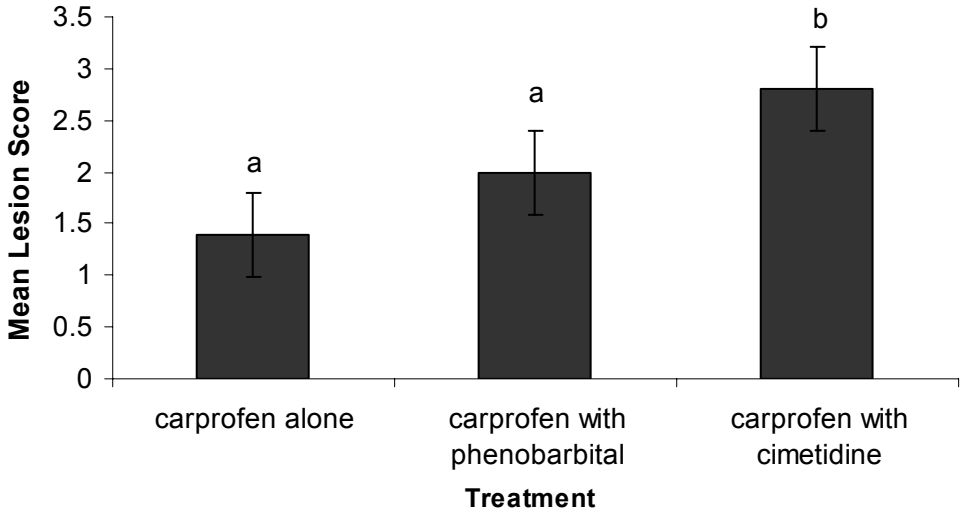

Fig. 87. Mean necrosis lesion score 100 µg/ml treatment analysis. Comparison of mean necrosis lesion scores for slice treatments (±SE, n=15) for all time points. Homogeneous groups for necrosis two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Mean vacuolation extent lesion scores were compared to identify differences among treatments and across time for slices incubated in 0 µg/ml (Figure 88), 10 µg/ml, 50 µg/ml and 100 µg/ml carprofen. No significant differences were found for mean vacuolation extent lesion scores for 0 µg/ml media. For 10 µg/ml media (Figure 89), a significant factor interaction (df=8, F-ratio=4.324, p-value=0.002) and significant treatment effect (df=2, F-ratio=27.165, p-value=0.000) were noted. Mean lesion scores for carprofen alone were lower than those for carprofen with phenobarbital or cimetidine (Table 72, Figure 90).

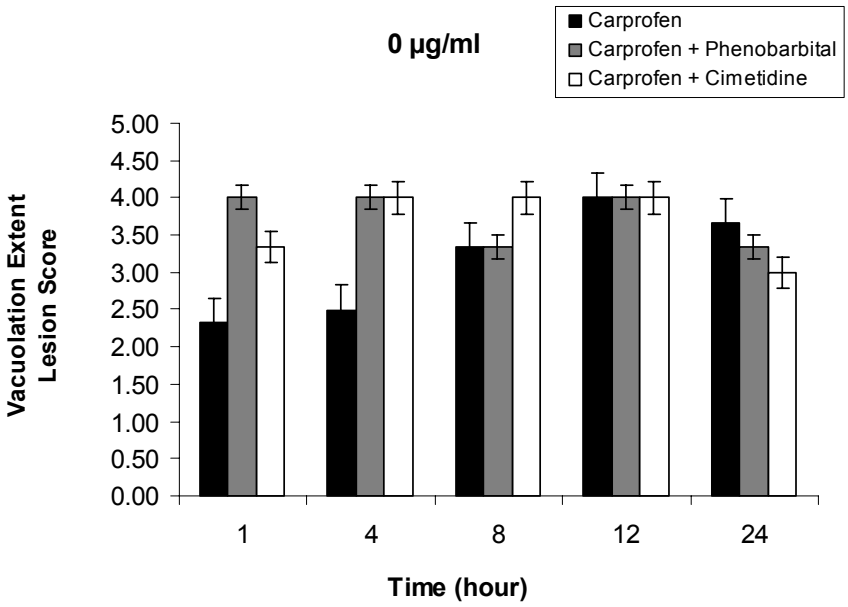

Fig. 88. Mean vacuolation extent lesion scores among 0 µg/ml carprofen treatments. Mean vacuolation extent lesion scores (±SE) for 0 µg/ml carprofen media for slices incubated in carprofen, carprofen with phenobarbital or carprofen with cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are means of three scores per treatment per time point.

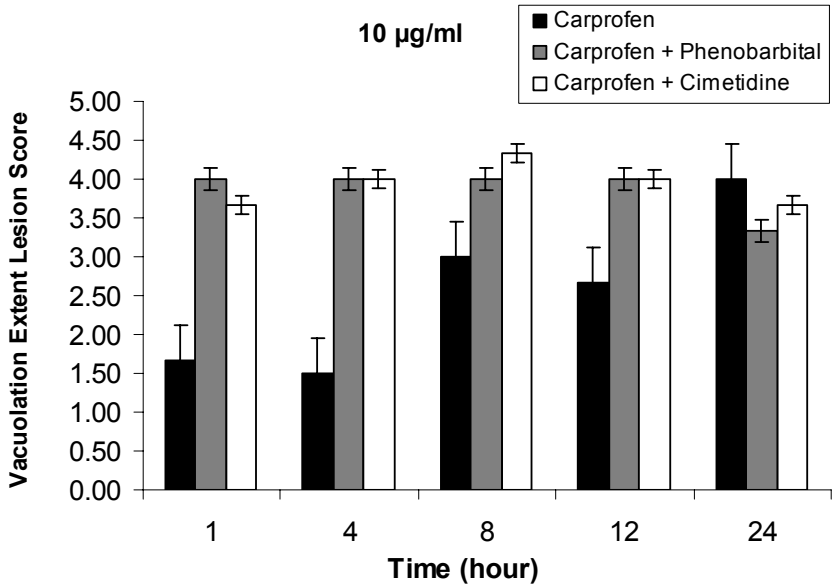

Fig. 89. Mean vacuolation extent lesion scores among 10 µg/ml carprofen treatments. Mean vacuolation extent lesion scores (±SE) for 10 µg/ml carprofen media for slices incubated in carprofen, carprofen with phenobarbital or carprofen with cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are means of three scores per treatment per time point.

Table 72. Mean vacuolation extent lesion score 10 µg/ml treatment analysis. Comparison of mean vacuolation extent lesion scores for slice treatments (n=15) for all time points. Homogeneous groups for vacuolation extent two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Treatment	Homogeneous groups	Mean Lesion Score
carprofen alone	a	2.57
carprofen with phenobarbital	b	3.87
carprofen with cimetidine	b	3.93

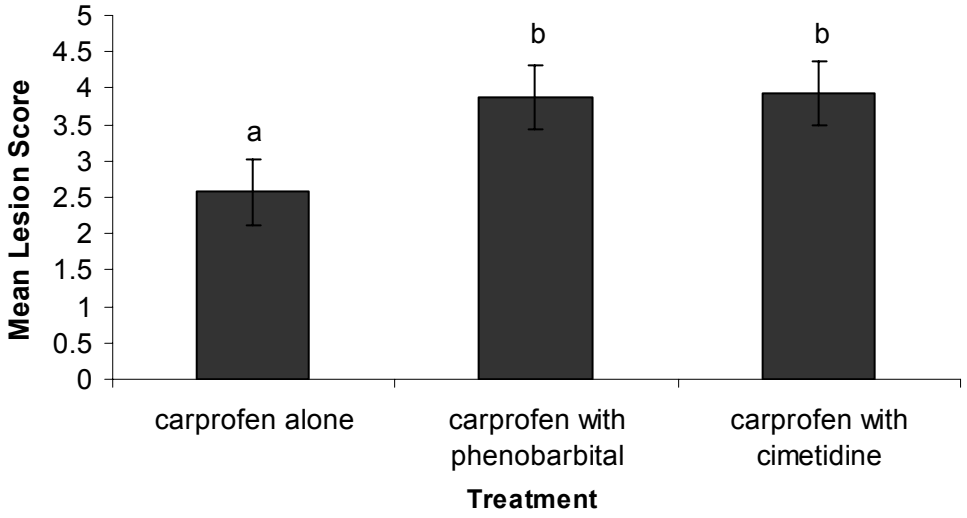

Fig. 90. Mean vacuolation extent lesion score 10 µg/ml treatment analysis. Comparison of mean vacuolation extent lesion scores for slice treatments (±SE, n=15) for all time points. Homogeneous groups for vacuolation extent two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Mean lesion scores for vacuolation extent with 50 µg/ml media (Figure 91) showed a significant time effect (df=4, F-ratio=2.815, p-value=0.043) by two-way ANOVA. Eight hour mean vacuolation extent lesion scores were lower than one hour lesion scores (Table 73, Figure 92).

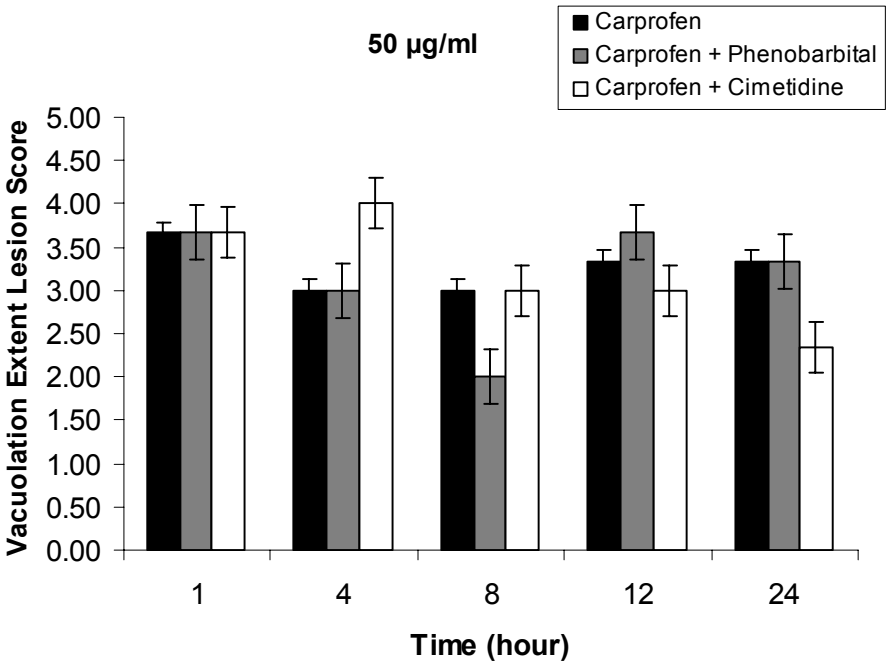

Fig. 91. Mean vacuolation extent lesion scores among 50 µg/ml carprofen treatments. Mean vacuolation extent lesion scores (±SE) for 50 µg/ml carprofen media for slices incubated in carprofen, carprofen with phenobarbital or carprofen with cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are means of three scores per treatment per time point.

Table 73. Mean vacuolation extent lesion score 50 µg/ml time analysis. Comparison of mean vacuolation extent lesion scores (n=9) across time for all treatments. Homogeneous groups for vacuolation extent two-way ANOVA for treatment are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups	Mean Lesion Score
1	a	3.67
4	a b	3.33
8	b	2.67
12	a b	3.33
24	a b	3.00

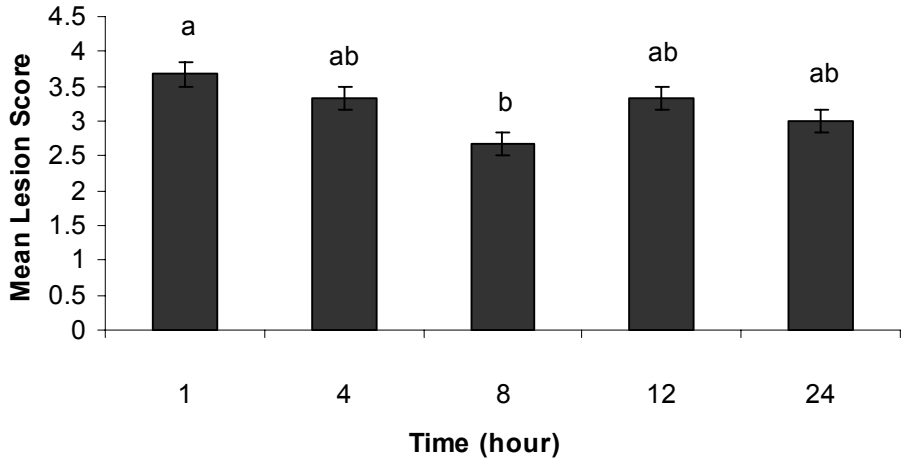

Fig. 92. Mean vacuolation extent lesion score 50 µg/ml time analysis. Comparison of mean vacuolation extent lesion scores (±SE, n=9) across time for all treatments. Homogeneous groups for vacuolation extent two-way ANOVA for treatment are represented as letters. Times with the same letter are not significantly different.

The mean vacuolation extent lesion scores for 100 µg/ml carprofen (Figure 93) did not show any significant differences across time or among treatments.

Mean vacuolation severity lesion scores were compared to identify differences among treatments and across time for slices incubated in 0 µg/ml, 10 µg/ml, 50 µg/ml and 100 µg/ml carprofen. For vacuolation severity of 0 µg/ml media (Figure 94), a significant factor interaction was noted (df=8, F-ratio=6.814, p-value=0.000); a signification treatment effect (df=2, F-ratio=12.615, p- value=0.000) was also found.

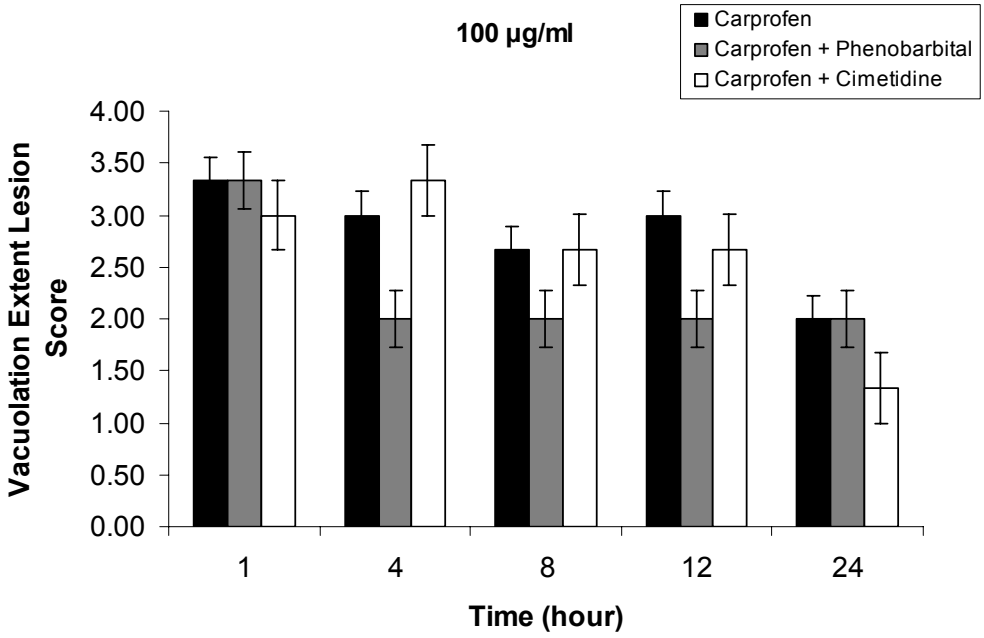

Fig. 93. Mean vacuolation extent lesion scores among 100 µg/ml carprofen treatments. Mean vacuolation extent lesion scores (±SE) for 100 µg/ml carprofen media for slices incubated in carprofen, carprofen with phenobarbital or carprofen with cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are means of three scores per treatment per time point.

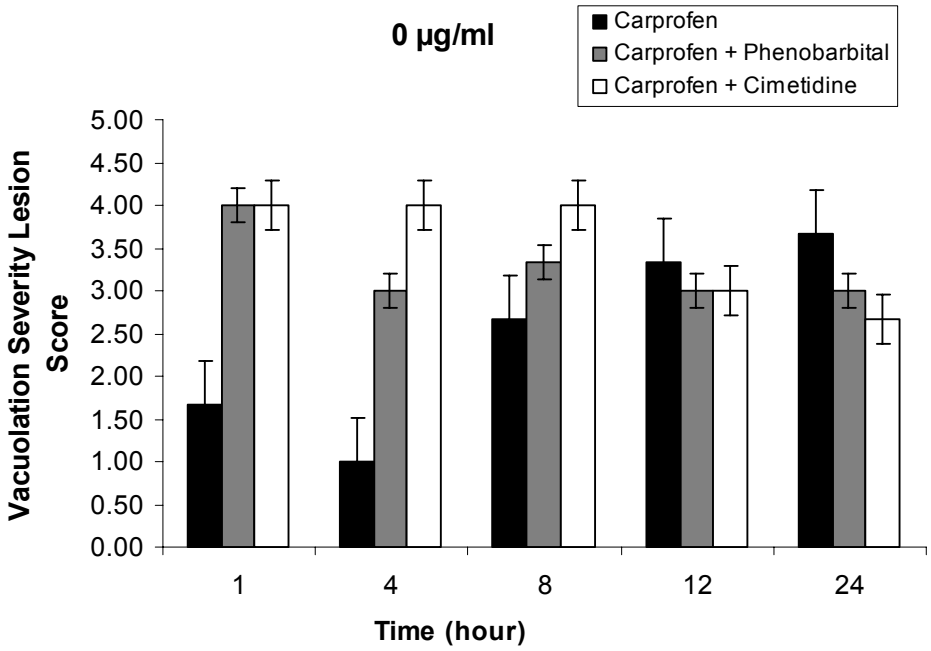

Fig. 94. Mean vacuolation severity lesion scores among 0 µg/ml carprofen treatments. Mean vacuolation severity lesion scores (±SE) for 0 µg/ml carprofen media for slices incubated in carprofen, carprofen with phenobarbital or carprofen with cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are means of three scores per treatment per time point.

The mean vacuolation severity lesion scores for carprofen alone were lower than those for carprofen with phenobarbital or cimetidine (Table 74, Figure 95).

Table 74. Mean vacuolation severity lesion score 0 µg/ml treatment analysis. Comparison of mean vacuolation severity lesion scores for slice treatments (n=15) for all time points. Homogeneous groups for vacuolation severity two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Treatment	Homogeneous groups	Mean Lesion Score
carprofen alone	a	2.47
carprofen with phenobarbital	b	3.27
carprofen with cimetidine	b	3.53

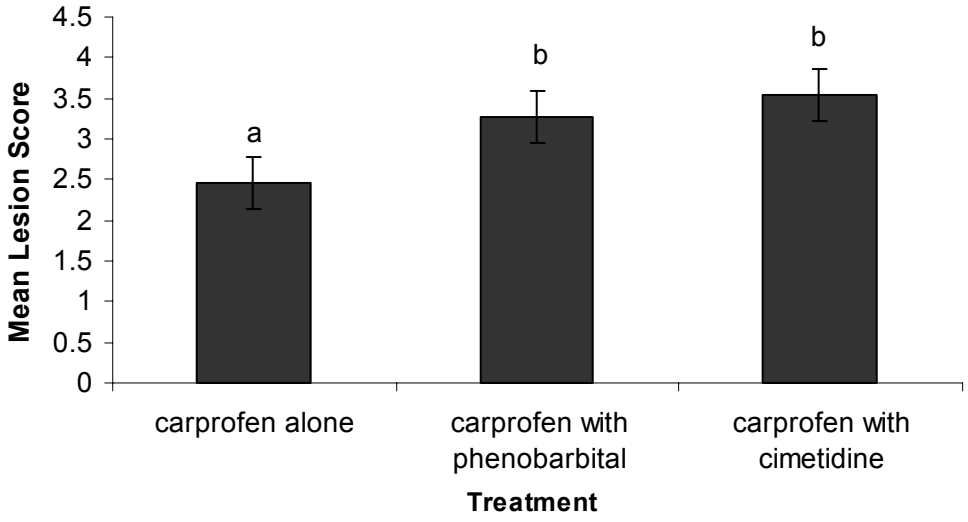

Fig. 95. Mean vacuolation severity lesion score 0 µg/ml treatment analysis. Comparison of mean vacuolation severity lesion scores for slice treatments (±SE, n=15) for all time points. Homogeneous groups for vacuolation severity two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

The mean vacuolation severity lesion scores for 10 µg/ml media (Figure 96) revealed a significant factor interaction (df=8, F-ratio=3.239, p-value=0.010), as well as, a significant

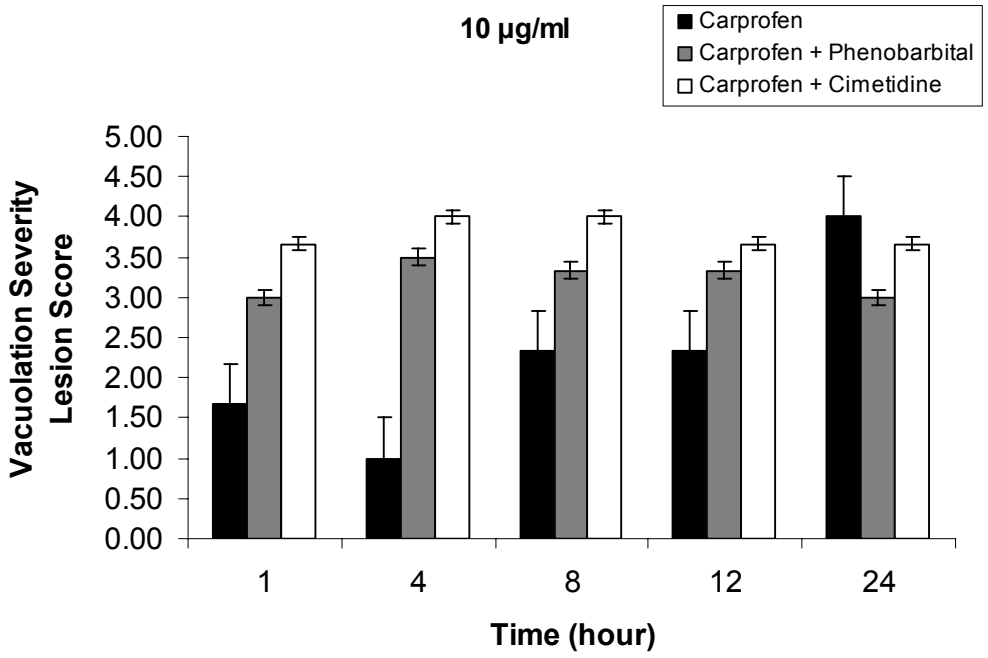

Fig. 96. Mean vacuolation severity lesion scores among 10 µg/ml carprofen treatments. Mean vacuolation severity lesion scores (±SE) for 10 µg/ml carprofen media for slices incubated in carprofen, carprofen with phenobarbital or carprofen with cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are means of three scores per treatment per time point.

treatment effect (df=2, F-ratio=20.161, p-value=0.000). Mean vacuolation severity lesion scores for carprofen alone were lower than those for carprofen with phenobarbital or cimetidine (Table 75, Figure 97).

Table 75. Mean vacuolation severity lesion score 10 µg/ml treatment analysis. Comparison of mean vacuolation severity lesion scores for slice treatments (n=15) for all time points. Homogeneous groups for vacuolation severity two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Treatment	Homogeneous groups	Mean Lesion Score
carprofen alone	a	2.27
carprofen with phenobarbital	b	3.23
carprofen with cimetidine	b	3.80

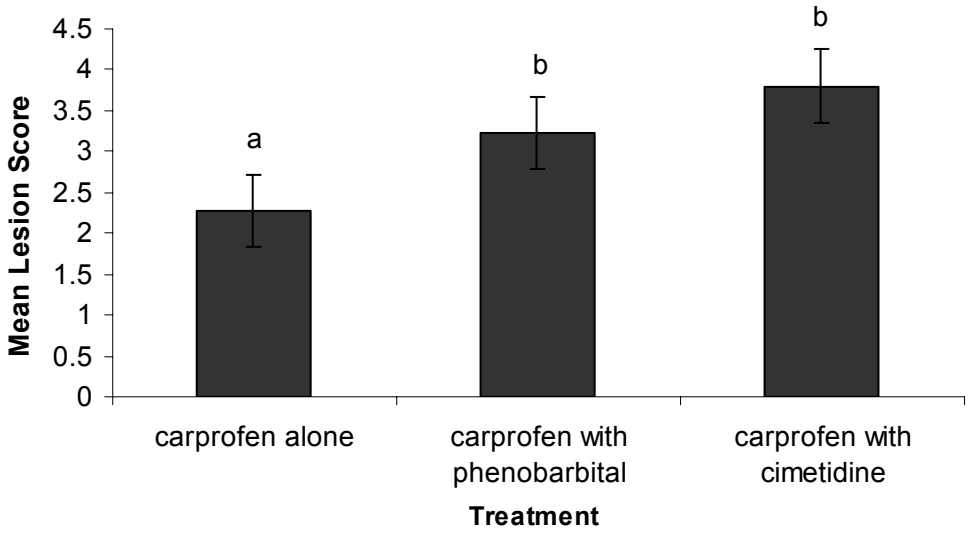

Fig. 97. Mean vacuolation severity lesion score 10 µg/ml treatment analysis. Comparison of mean vacuolation severity lesion scores for slice treatments (±SE, n=15) for all time points. Homogeneous groups for vacuolation severity two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Statistical analysis of the mean vacuolation severity lesion scores for 50 µg/ml media (Figure 98) demonstrated significant time (df=4, F-ratio=2.780, p-value=0.045) and treatment (df=2, F-ratio=4.376, p-value=0.022) effects. Across time, eight hour mean vacuolation severity lesion scores were lower than those at one hour (Table 76, Figure 99). Among treatments, mean lesion scores for carprofen alone were lower than those for carprofen with phenobarbital (Table 77, Figure 100).

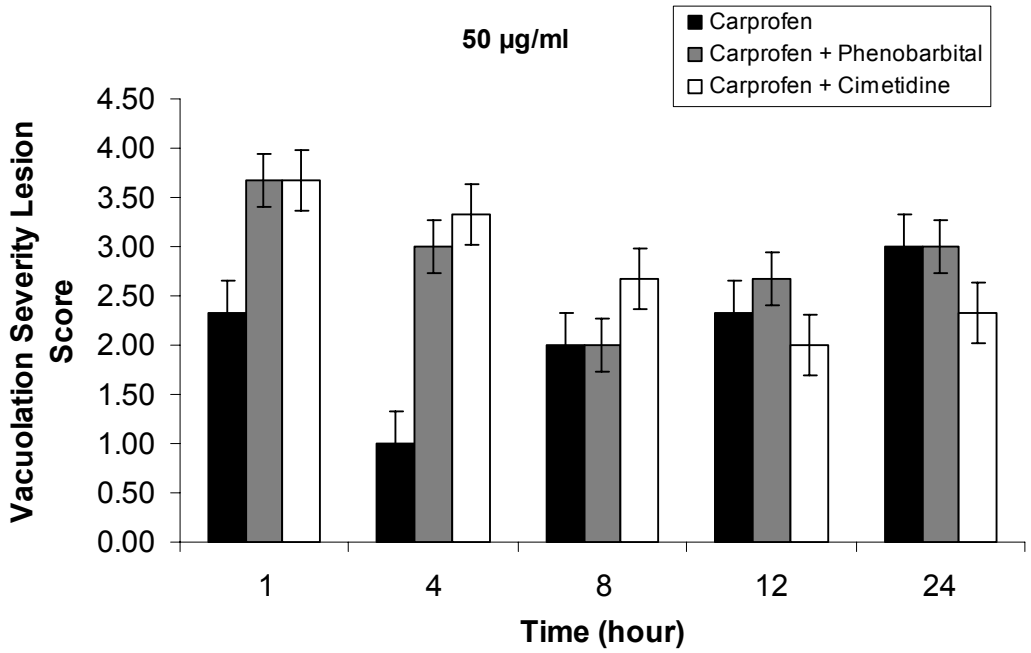

Fig. 98. Mean vacuolation severity lesion scores among 50 µg/ml carprofen treatments. Mean vacuolation severity lesion scores (±SE) for 50 µg/ml carprofen media for slices incubated in carprofen, carprofen with phenobarbital or carprofen with cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are means of three scores per treatment per time point.

Table 76. Mean vacuolation severity lesion score 50 µg/ml time analysis. Comparison of mean vacuolation severity lesion scores (n=9) across time for all treatments. Homogeneous groups for vacuolation severity two-way ANOVA for treatment are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups	Mean Lesion Score
1	a	3.22
4	a b	2.44
8	b	2.22
12	a b	2.33
24	a b	2.78

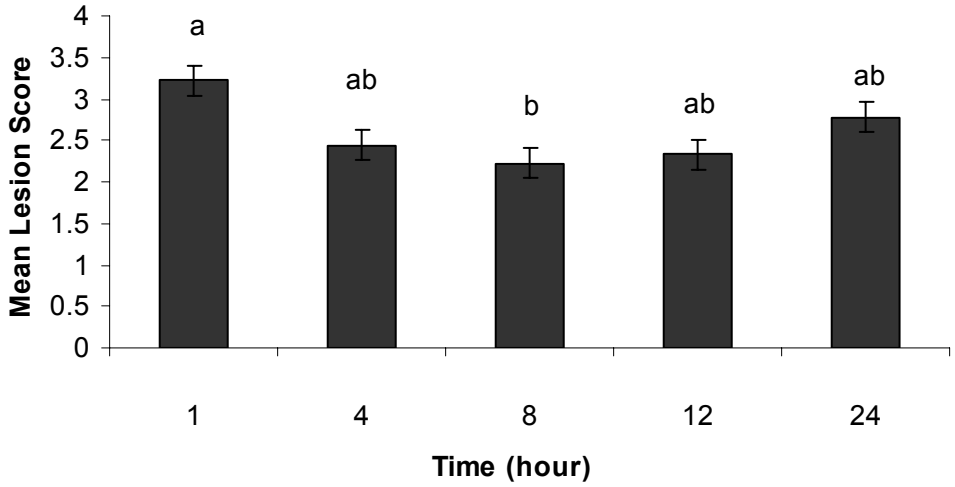

Fig. 99. Mean vacuolation severity lesion score 50 µg/ml time analysis. Comparison of mean vacuolation severity lesion scores (±SE, n=9) across time for all treatments. Homogeneous groups for vacuolation severity two-way ANOVA for treatment are represented as letters. Times with the same letter are not significantly different.

Table 77. Mean vacuolation severity lesion score 50 µg/ml treatment analysis. Comparison of mean vacuolation severity lesion scores for slice treatments (n=15) for all time points. Homogeneous groups for vacuolation severity two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Treatment	Homogeneous groups	Mean Lesion Score
carprofen alone	a	2.13
carprofen with phenobarbital	b	2.87
carprofen with cimetidine	a b	2.80

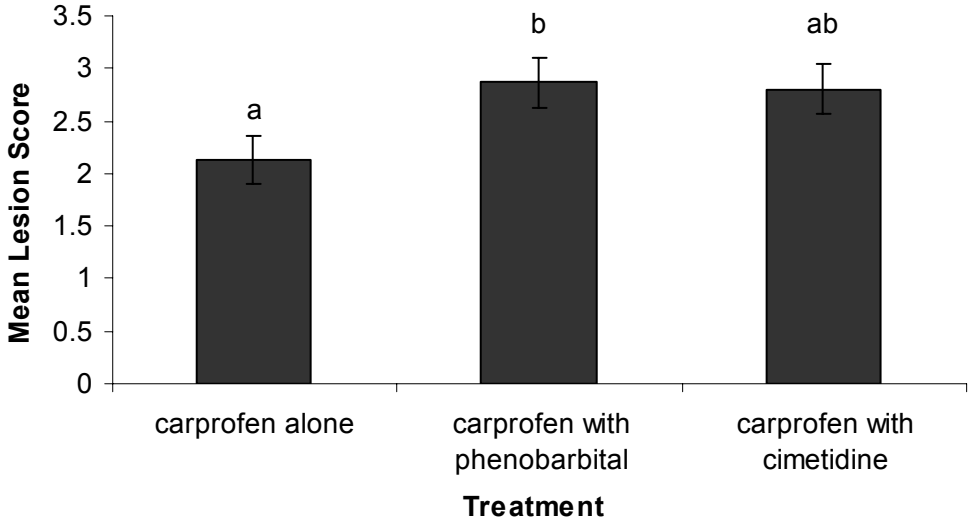

Fig. 100. Mean vacuolation severity lesion score 50 µg/ml treatment analysis. Comparison of mean vacuolation severity lesion scores for slice treatments (±SE, n=15) for all time points. Homogeneous groups for vacuolation severity two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

A significant time effect (df=4, F-ratio=4.108, p-value=0.009) was noted for 100 µg/ml vacuolation severity scores (Figure 101). One hour mean vacuolation severity lesion scores were higher than those at 12 and 24 hours (Table 78, Figure 102).

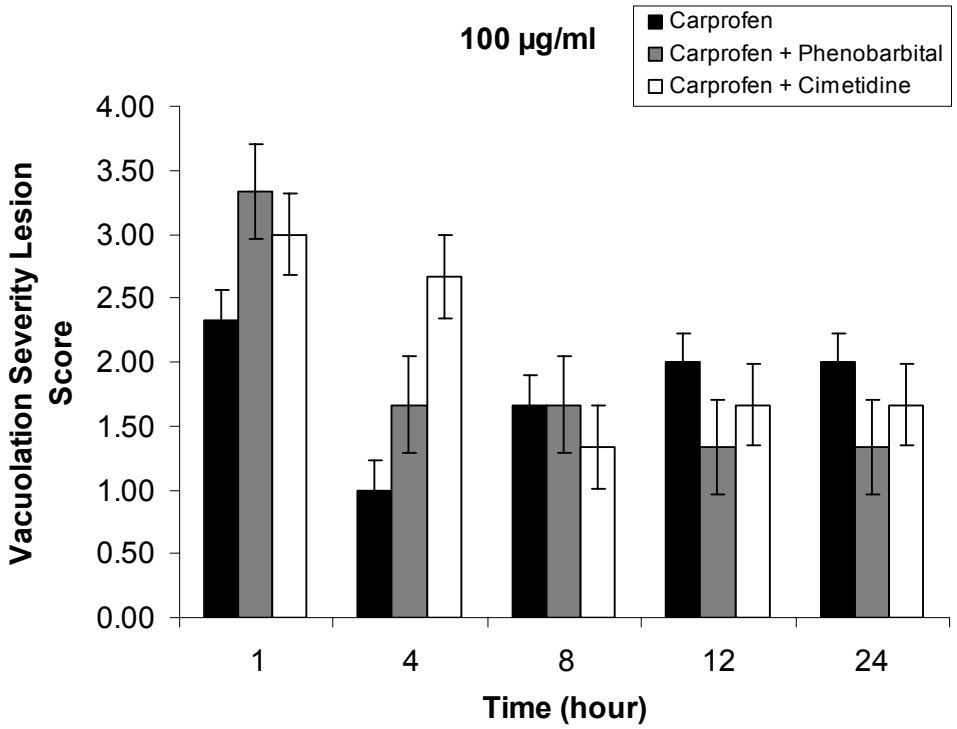

Fig. 101. Mean vacuolation severity lesion scores among 100 µg/ml carprofen treatments. Mean vacuolation severity lesion scores (±SE) for 100 µg/ml carprofen media for slices incubated in carprofen, carprofen with phenobarbital or carprofen with cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are means of three scores per treatment per time point.

Table 78. Mean vacuolation severity lesion score 100 µg/ml time analysis. Comparison of mean vacuolation severity lesion scores (n=9) across time for all treatments. Homogeneous groups for vacuolation severity two-way ANOVA for treatment are represented as letters. Times with the same letter are not significantly different.

Time (hour)	Homogeneous groups	Mean Lesion Score
1	a	2.89
4	a b	1.78
8	a b	1.56
12	b	1.67
24	b	1.67

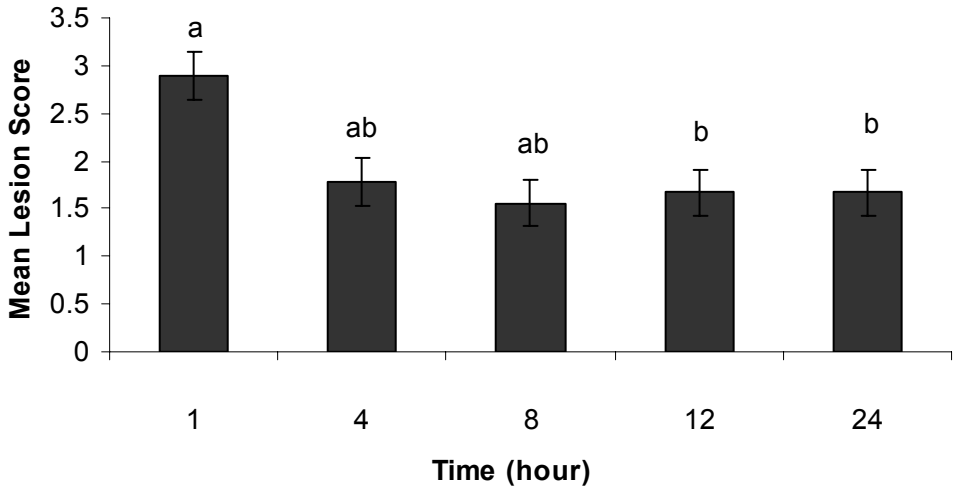

Fig. 102. Mean vacuolation severity lesion score 100 µg/ml time analysis. Comparison of mean vacuolation severity lesion scores (±SE, n=9) across time for all treatments. Homogeneous groups for vacuolation severity two-way ANOVA for treatment are represented as letters. Times with the same letter are not significantly different.

ELISA for Cytochrome P450 Enzymes

Slices were incubated in media containing no drug or phenobarbital to determine whether CYP2B11 enzymes were induced or in media containing no drug or cimetidine to determine whether CYP2C21 enzymes were inhibited. Mean enzyme concentrations were determined for CYP2B11 (Table 79) and CYP2C21 (Table 80). For CYP2B11, no differences were noted between 0 and 24 hour concentrations for slices incubated in media with no drug or in media with 75 µg/ml phenobarbital. Slices incubated in media containing 150 µg/ml phenobarbital showed a 1.2 increase in CYP2B11 concentrations (p-value for paired t-test=0.0115) after 24 hours of incubation. Slices incubated in media containing 0, 75 or 150 µg/ml phenobarbital show no differences in mean CYP2B11 concentrations between 0 and 48 hours. Twenty-four hour mean CYP2C21 concentrations were not different from 0 hour concentrations after incubation in cimetidine.

Table 79. Mean CYP2B11 concentrations. Mean CYP2B11 concentrations in the hepatic supernatant of slices incubated in media containing 0, 75 or 150 µg/ml phenobarbital. Means are an average of three (0 and 24 hour for 0 and 75 µg/ml media) or two (48 hour and 150 µg/ml media) samples.

Time	Concentration phenobarbital (µg/ml)	Mean CYP2B11 Concentration (pmol/mg protein)	standard deviation
0	0	0.1109	0.0115
24	0	0.3496	0.1976
48	0	0.1706	0.0023
0	75	0.1276	0.0046
24	75	0.2534	0.1365
48	75	0.1098	0.0044
0	150	0.1026	0.0090
24	150	0.1249	0.0084
48	150	0.1774	0.0354

Table 80. Mean CYP2C21 concentrations. Mean CYP2C21 concentrations in hepatic slice supernatant of slices incubated in media containing 0 or 1000 µM cimetidine. Means are an average of four samples per time point per concentration.

Time	Concentration cimetidine (µM)	Mean CYP2C21 Concentration (pmol/mg protein)	standard deviation
0	0	0.0581	0.0296
24	0	0.0542	0.0338
0	1000	0.0471	0.0158
24	1000	0.0715	0.0201

CHAPTER VI
DISCUSSION AND SUMMARY

Effects of Dynamic Organ Culture Incubation

Hepatic slices have been used to study phase I and II biotransformation of various drugs and xenobiotics, as well as, their cytotoxicity, genotoxicity and uptake into the slices (Bach *et al*., 1996; Gandolfi *et al*., 1996; Olinga *et al*., 2001). Freshly prepared rat hepatic slices remain viable for up to 48 hours, and human hepatic slices remain viable for up to 2 to 3 days (Lupp *et al*., 2001; Groneberg *et al*., 2002). Some studies have been performed using canine liver slices, but the use of canine tissue slices as a model for studying drug metabolism and hepatotoxicity in dogs has not been reported. Because rat and human hepatic slices have shown utility as an *in vitro* model, it was hypothesized that canine hepatic slices would also be a valuable tool for investigating drug-induced hepatotoxicity. Therefore, the major objective of this thesis research project was to test the utility of canine hepatic slices as a model for studying the toxicity and metabolism of selected drugs, including phenobarbital, primidone, lidocaine and carprofen.

Canine hepatic slices incubated in media without drug were evaluated for changes in supernatant potassium ion (K^+) levels and ATP concentrations and histologic lesions measured as necrosis, extent of vacuolation and severity of vacuolation. Potassium ion concentrations are used as the primary general index of hepatic slice viability; they are useful for determining the time course of toxicity and the concentration-response relationships of a toxic agent (Azri *et al*., 1990). Fisher *et al*. (1996a) used potassium ion levels to determine the viability of liver slices following cold and cryopreservation, and VandenBranden *et al*. (1998) used potassium ion retention as a measure of viability while studying the catalytic activity of eight cytochrome P450 isoforms. ATP concentrations reflect the energy status of hepatic slices following incubation with a xenobiotic (Azri *et al*., 1990). Obatomi *et al*. (1998) and Martignoni *et al*. (2004) used changes in ATP content to determine viability of liver slices following exposure to atractyloside or testosterone, respectively. Histologic evaluation can be used to detect injury to specific cell populations or slice regions (Azri *et al*., 1990). Lupp *et al*. (2001) and Behrsing *et al*. (2003) employed histopathology to assess liver slice viability. Martin *et al*. (2000) used ATP content and histology as measures of liver slice viability for fresh and cryopreserved liver slices. Fisher *et al*. (2001) used potassium ion concentrations, ATP levels and histology to evaluate the viability of liver slices of varying thickness under three different incubation systems.

Decreases in potassium ion and ATP concentrations were indicators of cellular injury or death. When potassium concentrations of slices incubated without drug were initially examined, 0 hour time point concentrations were higher than at all other time points, and no other significant declines were seen (Figure 2). Additionally, no significant changes in ATP concentrations were noted (Figure 3). Potassium and ATP levels suggest that canine hepatic slices are viable up to 24 hours.

Increases in hepatic lesion scores are another indication of hepatotoxic damage and viability. When hepatic slices were incubated without drug, 0 hour histologic lesion scores were lower than those observed after incubation for 8, 12 and 24 hours for each histologic lesion measured (Figures 4, 5 and 6). Unlike potassium and ATP levels, the histologic changes indicate that the viability of canine hepatic slices is much shorter than reported for rat and human hepatic slices (Lupp *et al*., 2001; Groneberg *et al*., 2002). Significant histologic changes were noted after incubating slices for 8 hours, and these changes may affect the interpretation of drug-induced studies. These results also suggest that histology is a more sensitive measure of toxicity than either potassium ion concentrations or ATP levels.

Hepatic Slice Potassium Content Response to Cooling and Incubation

For isolated liver cells, potassium ion levels decrease when cells are kept at low ($4^{\circ}C$) temperatures, and the levels subsequently increase to equal or higher levels when these cells are incubated at $37^{\circ}C$ (Barnabei *et al*., 1974). Potassium slice content remains relatively stable following an initial recovery period after slicing (Azri *et al*., 1990). A similar finding was noted in this study for canine hepatic slices.

Potassium ion concentrations from canine hepatic slices incubated for 1 and 3 hours in the absence of drug were lower than those observed at all other time points; the K^+ concentrations were increased by 4 hours and were significantly increased by 24 hours (Figure 2). When the mean potassium ion concentrations for all phenobarbital concentrations (0, 20, 45 and 75 µg/ml) were calculated, the concentrations at 1 hour were lower than those observed after incubation for 6 hours (Table 10, Figure 10). The potassium ion concentrations of slices incubated with lidocaine were lower at 1 hour than at all other time points with a significant increase in potassium ion concentration noted at 24 hours (Table 24, Figure 24). After incubating slices with 0, 10, 50 and 100 µg/ml carprofen, mean potassium levels at 1 hour were significantly lower than those at 4 hours (Table 31, Figure33). While not all of these early decreases were

statistically significant, a pattern was observed. In these experiments, prior to incubation at 37°C, the liver slices were kept at 4°C. This pattern of lowered potassium concentrations followed by recovery was seen for slices incubated with no drug, phenobarbital, lidocaine and carprofen. This finding is consistent with experiments using rat liver slices and isolated rat liver cells (McLaughlin, 1973; Barnabei *et al*., 1974). This cooling effect has not been reported for canine hepatic slices. Although significant differences in potassium ion concentrations were not noted for all experiments, the pattern was consistent for most treatments.

Hepatic Slice Incubation with Phenobarbital

The concentrations of phenobarbital in the media were relatively constant over time (Figure 7). The initial concentrations in the media were within 10% of expected concentrations indicating that the method used for adding drug to the media was successful. The appearance of phenobarbital in the hepatic slice supernatant indicates that phenobarbital penetrated into the slices and diffused into the hepatocytes (Figure 8). Additionally, as the concentration of phenobarbital in the media increased, the amount of phenobarbital found in the supernatant increased. The retention of phenobarbital in hepatic slices was also concentration-dependent; the concentration of phenobarbital in hepatic slice supernatant of slices incubated with 75 µg/ml phenobarbital was higher than that of slices treated with 45 µg/ml media (Figure 8). No phenobarbital was noted in the hepatic slice supernatant of slices treated with 0 or 20 µg/ml phenobarbital.

The potassium ion concentrations in hepatic slice supernatant for slices incubated with 75 µg/ml phenobarbital were lower than those observed in untreated slices (Table 11, Figure 11). This decrease in potassium ion concentrations indicated that the presence of high concentrations of phenobarbital caused damage to the liver cells causing them to release potassium ions into the media. A similar finding was noted for ATP concentrations. The ATP concentrations for slices incubated with 75 µg/ml phenobarbital were significantly lower than in slices that were untreated (Table 14, Figure 14). The ATP concentrations for slices incubated with 20 and 45 µg/ml were also lower than untreated slices, but the differences were not statistically significant (Table 14). For both potassium ions and ATP, the measured concentrations for slices incubated with 75 µg/ml phenobarbital were the lowest for all concentrations used in this study.

High serum concentrations of phenobarbital are thought to be associated with hepatotoxicity in dogs (Podell, 1998). The finding that both potassium and ATP concentrations were lower

following slice incubation with 75 µg/ml phenobarbital shows a correlation between results from the slices and *in vivo* data. These findings indicate that liver slices may be useful for examining the potential hepatotoxicity of drugs in dogs. Using the indices of hepatic slice supernatant content of potassium ions and ATP, 75 µg/ml phenobarbital was toxic to canine hepatic slices.

Phenobarbital is reported to cause hepatotoxicity in dogs (Dayrell-Hart *et al*., 1991; Müller *et al*., 2000). Chronic treatment with phenobarbital has been associated with pathologic hepatic changes—fibrosis, nodular hyperplasia and bile duct proliferation—as well as, serum biochemical changes—increased serum liver parameters (e.g., alkaline phosphatase, alanine aminotransferase) (Dayrell-Hart *et al*., 1991; Müller *et al*., 2000). Hepatic changes associated with phenobarbital are related not only to high serum concentrations but also to an individual animal's response to treatment (Müller *et al*., 2000). In these studies dose-dependent changes in canine hepatic slice viability measures were found which is similar to the *in vivo* hepatotoxicity associated with phenobarbital.

Hepatic Slice Incubation with Primidone

Canine hepatic slices were exposed to primidone to determine the drug metabolizing activity of the hepatic slices. Primidone is metabolized to phenobarbital and phenylethylmalonamide (Yeary, 1980). There was no evidence of primidone metabolism as indicated by the lack of quantifiable concentrations of phenobarbital in either the media or hepatic slice supernatant (Tables 16 and 17).

Potassium ion concentrations for slices incubated with all concentrations of primidone were lower than those for slices incubated without primidone indicating that treatment with primidone caused toxicity (Table 18, Figure 16). For ATP, mean concentrations of 75 µg/ml primidone were lower than those observed for untreated slices (Table 21, Figure 19). ATP levels for slices incubated with 20 and 45 µg/ml primidone were also lower than those for untreated slices, but the differences were not statistically significant (Table 21). The potassium ion levels and ATP data are consistent showing that treatment with 75 µg/ml primidone caused toxicity. The potassium ion concentrations for slices incubated with 20 and 45 µg/ml primidone were statistically lower than concentrations for slices incubated without primidone indicating the possibility of toxicity with the other concentrations of primidone.

Primidone has been reported to cause hepatic cirrhosis and increases in alanine aminotransferase and alkaline phosphatase in dogs (Bunch *et al*., 1985; Poffenbarger & Hardy,

1985). Liver toxicity due to primidone is associated with high doses of primidone administered chronically (Bunch *et al*., 1982; Schwartz-Porsche *et al*., 1985). The association of primidone with liver disease has caused it to fall out of favor for use in treatment of canine epilepsy. Results observed in this study show a dose-dependent *in vitro* hepatotoxicity associated with primidone which is similar to results observed *in vivo*.

Hepatic Slice Incubation with Lidocaine

Lidocaine was also used to determine the xenobiotics metabolizing ability of the liver slices. In dogs, lidocaine is metabolized to two active metabolites, MEGX and GX (Keenaghan & Boyes, 1972; Wilcke *et al*., 1983) (Figure 103). Production of MEGX from lidocaine is a rapid and sensitive indicator of hepatic drug metabolizing enzyme activity (Tanaka & Breimer, 1997). Lidocaine is metabolized to MEGX in humans by CYP3A4 (Tanaka & Breimer, 1997).

In the media the concentration of lidocaine decreased for each concentration of lidocaine used (3, 10 and 20 µg/ml) (Figures 20, 21 and 22). For 10 µg/ml lidocaine, the media concentrations of lidocaine were significantly lower after 6 hours compared to 1 hour (Figure 21). For 3 and 20 µg/ml lidocaine, the concentrations at 12 hours were significantly lower than those observed after 1 hour (Figures 20 and 22). The metabolite MEGX was produced, and quantifiable concentrations were noted for each concentration of lidocaine (3, 10 and 20 µg/ml). For 3 µg/ml lidocaine, MEGX was detected after 1 hour (Figure 20). For 10 and 20 µg/ml lidocaine, MEGX was present after 1 hour with concentrations increasing over time (Figures 21 and 22). MEGX concentrations were significantly higher by 6 hours for 10 µg/ml and by 8 hours for 20 µg/ml (Figures 20 and 21). No quantifiable concentrations of GX were found in the media, and no notable concentrations of lidocaine, MEGX or GX were observed in hepatic slice supernatants. Studies using human liver slices have also shown that only negligible amounts of MEGX are found in the slices (Olinga *et al*., 1998).

Lidocaine is rapidly metabolized in dogs with a half-life of 45 to 60 minutes (Keenaghan & Boyes, 1972). A rapid decrease in plasma concentrations due to rapid clearance has also been shown when the drug is given by the intraperitoneal route (Wilson *et al*., 2004). The rapid metabolism of lidocaine may account for the failure to detect its presence in the hepatic slice supernatant.

Human liver slices have been treated with lidocaine to determine the relationship among the metabolites produced (Parker *et al*., 1996). MEGX was shown to be one of the metabolites

produced by human liver slices, and GX concentrations were below the limit of quantification (Parker *et al*., 1996). Parker *et al*. (1996) also found an initial 10 to 20% decrease in media substrate concentrations indicating the distribution of the substrate into the liver tissue. The experiments of this study are in agreement with those found by Parker *et al*. (1996). The rapid elimination and clearance of lidocaine may be responsible for the non-detectable levels in the supernatant, and the decline of lidocaine in the media.

When all three concentrations of lidocaine are compared, the MEGX concentrations were higher for slices incubated with higher concentrations of lidocaine (Figure 104). The decreasing lidocaine and increasing MEGX concentrations indicate that canine liver slices take up and metabolize lidocaine. Potassium and ATP concentrations indicated lidocaine did not cause toxicity to the liver slices. Incubation of lidocaine with canine hepatic slices demonstrates the xenobiotic metabolizing activity of slices and the lack of toxic effects of lidocaine as measured by potassium ion and ATP concentrations in the hepatic slice supernatant.

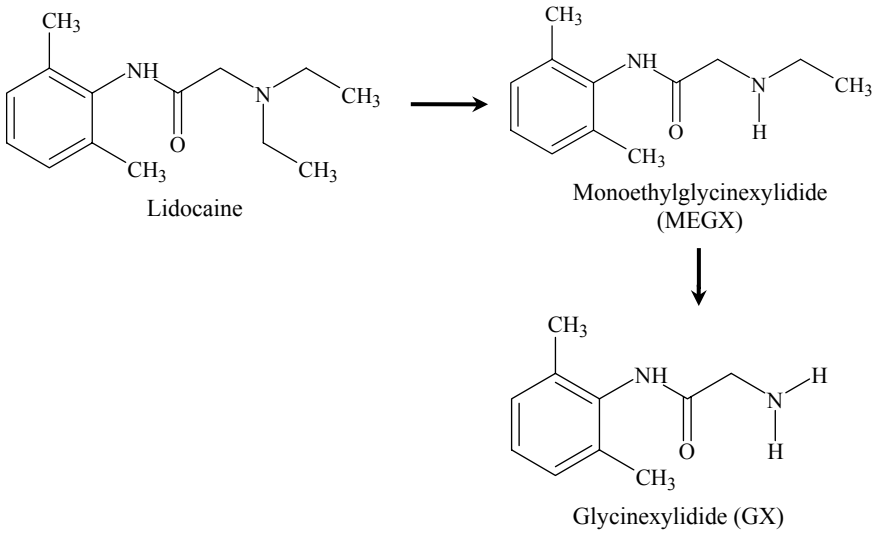

Figure 103. Metabolism of lidocaine. Lidocaine is metabolized to MEGX by N-deethylation. A second N-deethylation produces GX.

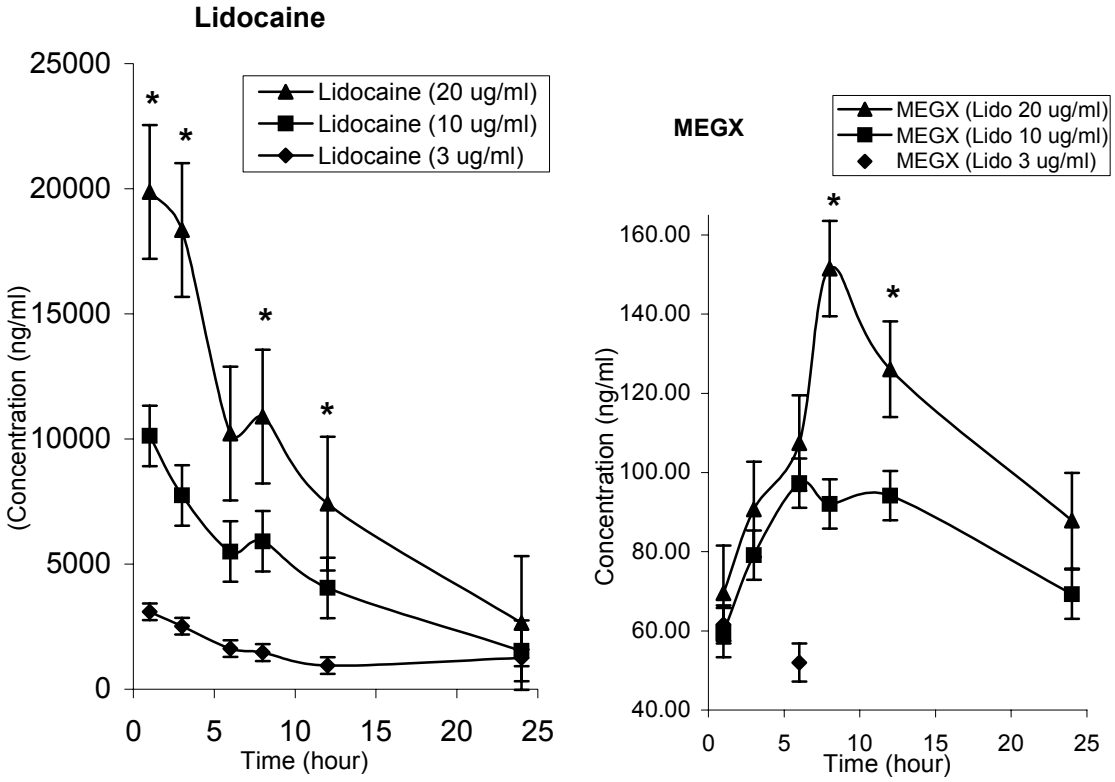

Fig. 104. Lidocaine and MEGX comparisons among media concentrations. Lidocaine (A) and MEGX (B) concentrations (ng/ml±SE) in media for slices incubated for 24 hours with 3, 10 and 20 µg/ml lidocaine. All concentrations shown are above the limit of quantification of 50 ng/ml for both lidocaine and MEGX. Media concentrations are an average of two samples per time point. Time points with an asterisk (*) indicate concentrations are significantly different.

Cytochrome P450 Induction and Inhibition

The ability of phenobarbital and cimetidine to induce or inhibit cytochrome P450 enzymes in canine hepatic slices was also investigated. No induction or inhibition was noted in the slices at the concentrations used in this study (Tables 79 and 80).

In vitro induction by phenobarbital has been performed in rat liver slices. The slices were incubated for 24 hours in carbogen saturated William's Medium E containing phenobarbital (100 µM); based on information from prior experiments, this concentration of phenobarbital induced CYP mRNA levels and enzyme activities (Lupp *et al*., 2001). Lupp *et al*. (2001) used a semiquantitative immunohistochemistry method for staining slices to determine the location and

induction of CYPs. With phenobarbital, induction of CYP2B1 and 3A2 was noted, as well as, hyperplasia of hepatocytes (Lupp *et al*., 2001).

Phenobarbital can induce liver CYPs by multiple mechanisms as shown by the discovery that higher doses of phenobarbital are required for induction of CYP3A1 mRNA than for CYP2B1 mRNA (Waxman, 1999). Phenobarbital (PB) and phenobarbital-like inducers induce CYPs by triggering the cytoplasmic-nuclear translocation of orphan nuclear receptor constitutive androstane receptor (CAR) (Waxman, 1999; Handschin & Meyer, 2003). CAR then binds with the PB-responsive enhancer (PBRE) and confers PB-inducible gene transcription (Waxman, 1999).

CAR, as a heterodimer with the retinoid X receptor RXR, binds to each of the nuclear receptor binding (NR) sites (Waxman, 1999; Handschin & Meyer, 2003). RXR serves as a common heterodimerization partner for several orphan nuclear receptors (Waxman, 1999; Handschin & Meyer, 2003). The CAR-RXR heterodimer binds to a hexameric DNA response element and activates CYP gene transcription (Waxman, 1999; Handschin & Meyer, 2003).

Cimetidine is an H_2 receptor antagonist, and this compound inhibits the metabolism of a number of xenobiotics *in vitro* and *in vivo* in rats and humans (Levine *et al*., 1998). Theophylline, warfarin and lidocaine are examples of drugs whose metabolism is inhibited by cimetidine (Furuta *et al*., 2001). In humans, cimetidine inhibits CYP2C9 (Furuta *et al*., 2001), whereas in rats, cimetidine inhibits CYP2C6 and CYP2C11 (Levine *et al*., 1998).

Cimetidine inhibition of CYPs *in vitro* displays competitive or mixed competitive/noncompetitive enzyme kinetics (Levine *et al*., 1998). Cimetidine acts by binding to the heme moiety of CYP which results in non-specific inhibition of CYP enzymatic activity (Furuta *et al*., 2001). The heme moiety is the oxygen binding site for oxidative metabolism (Furuta *et al*., 2001).

Incubation with Carprofen, Carprofen with Phenobarbital and Carprofen with Cimetidine

Carprofen

The media concentrations of carprofen were maintained over 24 hours, and no differences were observed between the enantiomers (Table 29, Figures 28 and 29). In the hepatic slice supernatant of slices incubated in 50 and 100 µg/ml media, the concentrations of carprofen increased over time for both enantiomers, and the AUC for peak B AUC was higher than that for peak A (Table 30, Figure 31). Other than an initial decline in potassium ion concentrations (1

hour), there were no other significant declines in potassium ion concentration (Table 31, Figure 33). For ATP concentrations, significant decreases were noted at 12 and 24 hours compared to concentrations at 1, 4 and 8 hours (Table 34, Figure 36). There were no significant differences in ATP concentrations among the media concentrations. For necrosis, vacuolation extent and vacuolation severity, no significant effects of carprofen were seen at any concentration used in this study. Significant time differences, however, were noted for necrosis and vacuolation severity (Tables 37 and 39, Figures 40 and 42). For necrosis, 0, 1 and 4 hour lesion scores were lower than those at 8, 12 and 24 hours (Table 37, Figure 40). With vacuolation severity, 0, 1 and 4 hour lesion scores were lower than those at 24 hours (Table 39, Figure 42).

There was no evidence of toxicity caused by carprofen using potassium ion or ATP concentrations or histologic changes as indicators. An effect of time of incubation was noted by the presence of histologic changes across time for slices incubated in all concentrations of carprofen (0, 10, 50 and 100 µg/ml). Treatment with carprofen does not appear to cause toxicity in the hepatic slices.

Carprofen with Phenobarbital

Incubation of hepatic slices with phenobarbital followed by incubation with carprofen did not result in differences in the concentration of carprofen enantiomers in the media (Table 40, Figure 44). Similar to incubation with carprofen alone, the hepatic slice supernatant AUC for peak B was higher than that for peak A for slices incubated in media containing 50 and 100 µg/ml carprofen (Table 41, Figure 46). After an initial decline in potassium ion concentrations at 24 hours, the only other statistically significant decline in potassium ion concentrations occurred at 48 hours where concentrations were lower than those observed at 24 hours (Table 42, Figure 48). No significant differences were noted for ATP concentrations. The examination of histologic lesions did not reveal consistent differences for the range of carprofen media concentrations. Zero hour lesion scores were lower than all other time points for necrosis, vacuolation extent and vacuolation severity (Tables 46, 48 and 50, Figures 53, 55 and 57). There is some indication that the length of incubation may be the cause of the histologic changes. There were no toxic effects associated with the various concentrations of carprofen in the media since the histologic lesion scores for untreated slices were not lower than those incubated with 10, 50 or 100 µg/ml carprofen (Tables 47 and 49, Figures 54 and 56). No toxicity appears to be related to hepatic

slice incubation with phenobarbital and carprofen based on potassium ion and ATP concentrations and histologic lesions.

Carprofen with Cimetidine

Differences for media and hepatic slice supernatant were noted for slices incubated with cimetidine followed by incubation with carprofen (Table 51 and 52, Figure 59 and 61). The media peak B AUC for 10 µg/ml carprofen was higher than that for peak A (Table 51, Figure 59), and the hepatic slice supernatant peak B AUC for 50 µg/ml carprofen was higher than that for peak A (Table 52, Figure 61). After an initial decline in potassium ion concentrations at 24 hours, there were no significant declines in potassium ion concentrations (Table 53, Figure 63). When ATP concentrations were compared, concentrations at 24 hours were higher than at all other time points (Table 56, Figure 66). After the decline in ATP levels at 25 hours, there were no significant differences in concentrations (Figure 66). For histologic lesions, significant increases in necrosis were noted across time (Table 59, Figure 70). No significant changes in vacuolation extent lesion scores were noted after 25 hours of incubation (Table 60, Figure 71), and for vacuolation severity, no significant lesion changes were noted after 24 hours (Table 62, Figure 73). No toxicity was noted for slices incubated with cimetidine and carprofen. Any toxic differences appear to be related to length of incubation and not the concentration of carprofen in the media.

Treatment Comparisons

The only AUC differences noted were for slices incubated with 100 µg/ml carprofen; peak A AUC for carprofen was higher than that for slices incubated with cimetidine and carprofen (Figure 77, Table 65). The histologic lesion scores for slices incubated with carprofen for up to 24 hours were compared with the lesion scores for slices incubated for up to 48 hours with phenobarbital and carprofen or cimetidine and carprofen. In most cases where differences were observed, incubation with carprofen alone produced lower lesion scores than incubation with cimetidine and carprofen (Table 81). The differences may be related to length of incubation rather than actual treatment differences. The hepatotoxicity associated with carprofen is thought to be idiosyncratic (MacPhail *et al*., 1998). These results support that conclusion since no dose-related hepatic damage was shown for canine slices incubated with carprofen.

Table 81. Summary of histologic results for treatment comparisons. Summary of histologic lesion findings for slices incubated with carprofen (C), carprofen and phenobarbital (PC) and carprofen and cimetidine (CC). Treatments with the same letter are not significantly different.

Treatment		Carprofen Concentration			
		0 µg/ml	10 µg/ml	50 µg/ml	100 µg/ml
Necrosis	C	L*	ND*	L	L
	PC	H*	ND*	M	L
	CC	H*	ND*	H	H
Vacuolation extent	C	ND	L*	ND	ND
	PC	ND	H*	ND	ND
	CC	ND	H*	ND	ND
Vacuolation severity	C	L*	L*	L	ND
	PC	H*	H*	H	ND
	CC	H*	H*	LH	ND

ND no difference * factor interaction
L low lesion score M middle lesion score H high lesion score

Table 82. Summary of experiment results. Summary of experiment results and findings for canine hepatic slices incubated with various drugs.

Drug Tested	Parent Compound Presence in Media	Parent Compound Presence in Supernatant	Metabolite Presence in Media	Metabolite Presence in Supernatant	Toxicity Indicated		
					K^+	ATP	Histo
Phenobarbital	√	√	NA	NA	√	√	NM
Primidone	NM	NM	**X**	**X**	√	√	NM
Lidocaine	√	**X**	√	**X**	**X**	**X**	NM
Carprofen	√	√	NM	NM	**X**	**X**	**X**
Carprofen with phenobarbital	√	√	NM	NM	**X**	**X**	**X**
Carprofen with cimetidine	√	√	NM	NM	**X**	**X**	**X**

NA not applicable NM not measured **X** not present/no √ present/yes
Histo histology

Summary

Tissue slices are a good alternative to *in vivo* studies since tissue slice experiments require fewer animals, are cheaper and the effects of multiple agents can be examined at the same time (Azri *et al*., 1990). Human and animal tissue slices have been used to gather information to

predict what might occur in humans (Fisher *et al*., 2001). The application of this technique to predict what might happen in dogs has not been reported.

The results of these studies have shown the utility of canine hepatic slices as a model for studying drug metabolism and toxicity in dogs. Drug uptake, drug metabolism and toxicity were demonstrated using this *in vitro* technique (Table 82). Potassium ion and ATP levels were found to be less sensitive indicators of hepatic slice viability compared with histologic evaluations. A similar finding was noted for canine liver slices used to test three different incubation systems (Fisher *et al*., 2001). Additionally, when compared with the potassium ion and ATP concentrations of the Fisher *et al*. (2001) study, the ATP levels of these studies were lower, and the potassium ion concentrations were comparable or slightly higher. In another experiment, canine liver slices were used to determine the viability of cold-preserved slices (Fisher *et al*., 1996a). Again, the potassium ion concentrations of these studies were comparable or slightly higher than those of the Fisher *et al*. (1996a) study.

Cooling canine liver slices caused a decrease in potassium ion concentrations while concentrations recovered during incubation (warming) of the slices. This cooling effect on potassium ion concentrations has not been reported for canine hepatic slices, but has been noted in rat liver slices and isolated cells (McLaughlin, 1973; Barnabei *et al*., 1974).

Phenobarbital was used to determine the response of canine hepatic cells to a known hepatotoxicant. Toxicity, as indicated by potassium ion and ATP concentrations, was demonstrated for slices incubated in 75 µg/ml media (Figures 11 and 14). When potassium ion and ATP concentrations in slices treated with phenobarbital (20, 45 and 75 µg/ml) were compared with control slices, a pattern of lower ATP and K^+ concentrations was associated with the treated slices (Tables 11 and 14).

Exposure of hepatic slices to primidone was performed to determine the drug metabolizing enzyme activity of the slices. Unfortunately, no phenobarbital was detected to provide evidence of metabolism (Tables 16 and 17). Toxicity was noted, however, as lowered potassium ion and ATP concentrations were observed for slices incubated with 75 µg/ml primidone (Figures 16 and 19).

For both phenobarbital and primidone, the potassium ion concentrations after incubation with 75 µg/ml were the lowest of all treatments (Tables 11 and 18). The same was true for ATP where treatment with 75 µg/ml phenobarbital or primidone gave the lowest ATP concentrations (Table 14 and 21). In these experiments, incubation of hepatic slices with equal concentrations

of primidone and phenobarbital yielded similar results. A trend of high drug concentrations being associated with toxicity was observed since both primidone and phenobarbital were toxic to canine hepatic slices at high concentrations. This is similar to what is seen *in vivo* (Bunch *et al*., 1985; Poffenbarger & Hardy, 1985; Podell, 1998).

Phase I and phase II biotransformation have been demonstrated in canine hepatic slices using testosterone as a marker drug for phase I biotransformation and glucuronidation and sulfation of 7-hydroxycoumarin as indicators of phase II biotransformation (Martignoni *et al*., 2004). In these studies, lidocaine was metabolized to MEGX in canine hepatic slices indicating that the CYP enzyme responsible for the biotransformation of lidocaine (CYP3A4 in humans) was functional. Since lidocaine metabolism has been used as a hepatic function test in humans, these studies indicate that canine hepatic slices are functional for up to 24 hours.

The results of the phenobarbital, primidone and lidocaine studies should be confirmed by exposing additional slices to these drugs, and the experiments should be repeated using more slices to reduce variability. The use of additional slices would also help to determine whether any of the lower concentrations of phenobarbital or primidone could cause hepatotoxicity. Since histologic evaluation was not performed for slices incubated in phenobarbital, primidone or lidocaine, the use of histology for these studies would help determine the presence of other toxic changes and confirm the toxicity noted in these studies. Finally, a more sensitive primidone assay would be useful to determine whether canine slices are capable of metabolizing primidone to phenobarbital. Increasing the amount of primidone in the media may also lead to formation of higher concentrations of phenobarbital; however, increasing the primidone concentrations may enhance the apparent toxic effects.

Since induction and inhibition did not occur in the experiments performed, these studies should be repeated. Higher concentrations of phenobarbital and cimetidine could be used to induce and inhibit cytochrome P450 enzymes, respectively. Alternatively, different CYP inducers (i.e., rifampin) and inhibitors (i.e., ketoconazole) could also be used.

No toxicity was noted for slices incubated with carprofen, with phenobarbital and carprofen or with cimetidine and carprofen. The toxicity associated with carprofen in dogs is unlike that associated with phenobarbital and primidone. Phenobarbital and primidone-induced hepatotoxicity is associated with exposure of liver to high serum levels of these drugs while carprofen is thought to cause an idiosyncratic hepatotoxicity (MacPhail *et al*., 1998; Podell, 1998).

Canine hepatic slices were highly responsive to drugs with established *in vivo* hepatotoxic profiles in dogs. For drugs with inconsistent or idiosyncratic causes of hepatotoxicity, the use of canine hepatic slices has not been predictive in these *in vitro* slice experiments. The use of slices from breeds that are more sensitive to the toxicity of a drug may be useful for determining the utility of canine hepatic slices as a more global *in vitro* model of hepatotoxicity. Slices incubated in media without drug showed no toxicity over 24 hours based on potassium ion and ATP supernatant concentrations whereas significant increases in histologic lesions were noted at 8, 12 and 24 hours. Based on the results of these experiments, histologic parameters were more sensitive than biochemical measurements for determining the viability of these slices. Canine hepatic slices were found to be a useful model for examining drug metabolism and toxicity for up to 24 hours, and current studies are designed to further validate the use of this *in vitro* model.

REFERENCES

Azri, S., Gandolfi, A.J. & Brendel K. (1990) Precision-cut liver slices: an *in vitro* system for profiling potential hepatotoxicants. *In Vitro Toxicology*, **3**, 309-320.

Bach, P.H., Vickers, A.E.M., Fisher, R., Baumann, A., Brittebo, E., Carlile, D.J., Koster, H.J., Lake, B.G., Salmon, F., Sawyer, T.W. & Skibinski, G. (1996) The use of tissue slices for pharmacotoxicological studies. *Alternatives to Laboratory Animals*, **24**, 893-923.

Barnabei, O., Leghissa, G. & Tomasi, V. (1974) Hormonal control of the potassium level in isolated rat liver cells. *Biochimica et Biophysica Acta*, **362**, 316-325.

Behrsing, H.P., Vickers, A.E.M. & Tyson, C.A. (2003) Extended rat liver slice survival and stability monitored using clinical biomarkers. *Biochemical and Biophysical Research Communications*, **312**, 209-213.

Bidlack, W.R., Brown, R.C. & Mohan, C. (1986) Nutritional parameters that alter hepatic drug metabolism, conjugation, and toxicity. *Federation Proceedings*, **45**, 142-148.

Boelsterli, U.A., Zimmerman, H.J. & Kretz-Rommel, A. (1995) Idiosyncratic liver toxicity of nonsteroidal antiinflammatory drugs: molecular mechanisms and pathology. *Critical Reviews in Toxicology*, **25**, 207-235.

Bunch, S.E. (1993) Hepatotoxicity associated with pharmacologic agents in dogs and cats. *Veterinary Clinics of North America: Small Animal Practice*, **23**, 659-670.

Bunch, S.E., Castleman, W.L., Baldwin, B.H., Hornbuckle, W.E. & Tennant, B.C. (1985) Effects of long-term primidone and phenytoin administration on canine hepatic function and morphology. *American Journal of Veterinary Research*, **46**, 105-115.

Bunch, S.E., Castleman, W.L., Hornbuckle, W.E. & Tennant, B.C. (1982) Hepatic cirrhosis associated with long-term anticonvulsant drug therapy in dogs. *Journal of American Veterinary Medical Association*, **181**, 357-362.

Caldwell, J. (1980) Comparative aspects of detoxication in mammals. In *Enzymatic Basis of Detoxication*. Ed. Jakoby, W.B. pp. 85-114. Academic Press, New York.

Cervenkova, K., Belejova, M., Vesely, J., Chmela, Z., Rypka, M., Ulrichova, J., Modriansky, M. & Maurel, P. (2001) Cell suspensions, cell cultures, and tissue slices—important metabolic *in vitro* systems. *Biomedical Papers*, **145**, 57-60.

Dayrell-Hart, B., Steinberg, S.A., VanWinkle, T.J. & Farnbach, G.C. (1991) Hepatotoxicity of phenobarbital in dogs: 18 cases. *Journal of American Veterinary Medical Association*, **199**, 1060-1066.

Eguchi, K., Nishibe, Y., Baba, T. & Ohno, K. (1996) Quantitation of cytochrome P450 enzymes (CYP1A1/2, 2B11, 2C21 and 3A12) in dog liver microsomes by enzyme-linked immunosorbent assay. *Xenobiotica*, **26**, 755-764.

Ekins, S., Ring, B.J., McRobie-Belle, D.J. & Wrighton, S.A. (2000) Present and future *in vitro* approaches for drug metabolism. *Journal of Pharmacological and Toxicological Methods*, **44**, 313-324.

Ekins, S., Williams, J.A., Murray, G.I., Burke, M.D., Marchant, N.C., Engeset, J. & Hawksworth, G.M. (1996) Xenobiotic metabolism in rat, dog, and human precision-cut liver slices, freshly isolated hepatocytes, and vitrified precision-cut liver slices. *Drug Metabolism and Disposition*, **24**, 990-995.

Farber, J.L. & Gerson, R.J. (1984) Mechanisms of cell injury with hepatotoxic chemicals. *Pharmacological Reviews*, **36**, 71S-75S.

Fisher, R.L., Hasal, S.J., Sanuik, J.T., Hasal, K.S., Gandolfi, A.J. & Brendel, K. (1996a) Cold- and cryopreservation of dog liver and kidney slices. *Cryobiology*, **33**, 163-171.

Fisher, R.L., Jenkins, P.M., Hasal, S.J., Sanuik, J.T., Gandolfi, A.J. & Brendel, K. (1996b) Rainbow trout liver slices: a tool for aquatic toxicology. *Toxic Substance Metabolism*, **15**, 13-26.

Fisher, R.L., Putnam, C.W., Koep, L.J., Sipes, I.G., Gandolfi, A.J. & Brendel, K. (1991) Cryopreservation of pig and human liver slices. *Cryobiology*, **28**, 131-142.

Fisher, R.L., Ulreich, J.B., Nakazato, P.Z. & Brendel, K. (2001) Histological and biochemical evaluation of precision-cut liver slices. *Toxicology Methods*, **11**, 59-79.

Furuta, S., Kamada, E., Suzuki, T., Sugimoto, T., Kawabata, Y., Shinozaki, Y. & Sano, H. (2001) Inhibition of drug metabolism in human liver microsomes by nizatidine, cimetidine and omeprazole. *Xenobiotica*, **31**, 1-10.

Gandolfi, A.J., Brendel, K., Fisher, R.L. & Michaud, J.-P. (1995) Use of tissue slices in chemical mixture toxicology and interspecies investigations. *Toxicology*, **105**, 285-290.

Gandolfi, A.J., Wijeweera, J. & Brendel, K. (1996) Use of precision-cut liver slices as an *in vitro* tool for evaluating liver function. *Toxicologic Pathology*, **24**, 58-61.

Glockner, R., Steinmetzer, P., Drobner, C. & Müller, D. (1999) Use of fresh and cryopreserved human liver slices in toxicology with special reference to *in vitro* induction of cytochrome P450. *Toxicology in Vitro*, **13**, 531-535.

Graham, R.A., Downey, A., Mudra, D., Krueger, L., Carroll, K., Chengelis, C., Madan, A. & Parkinson, A. (2002) *In vivo* and *in vitro* induction of cytochrome P450 enzymes in beagle dogs. *Drug Metabolism and Disposition*, **30**, 1206-1213.

Griffin, S.J. & Houston, J.B. (2005) Prediction of *in vitro* intrinsic clearance from hepatocytes: comparison of suspensions and monolayer cultures. *Drug Metabolism and Disposition*, **33**, 115-120.

Groneberg, D.A., Grosse-Siestrup, C. & Fischer, A. (2002) *In vitro* models to study hepatotoxicity. *Toxicologic Pathology*, **30**, 394-399.

Handschin, C. & Meyer, U.A. (2003) Induction of drug metabolism: the role of nuclear receptors. *Pharmacological Reviews*, **55**, 649-673.

Hay Kraus, B.L., Greenblatt, D.J., Venkatakrishnan, K. & Court, M.H. (2000) Evidence for propofol hydroxylation by cytochrome P4502B11 in canine liver microsomes: breed and gender differences. *Xenobiotica*, **30**, 575-588.

Herbert, R.A., Hailey, J.R., Seely, J.C., Shackelford, C.C., Jokinen, M.P., Wolf, J.C. & Travlos, G.S. (2002) Nomenclature. In *Handbook of Toxicologic Pathology*, 2nd edn. Eds Haschek, W.M., Rousseaux, C.G. & Wallig, M.A. pp. 157-167. Academic Press, San Diego.

Hojo, T., Ohno, R., Shimoda, M. & Kokue, E. (2002) Enzyme and plasma protein induction by multiple oral administrations of phenobarbital at a therapeutic dosage regimen in dogs. *Journal of Veterinary Pharmacology and Therapeutics*, **25**, 121-127.

Kaplowitz, N. (2004) Drug-induced hepatotoxicity. *Clinical Infectious Diseases*, **38**, S44-S48.

Kaplowitz, N., Aw, T.Y., Simon, F.R. & Stolz, A. (1986) Drug-induced hepatotoxicity. *Annals of Internal Medicine*, **104**, 826-839.

Keenaghan, J.B. & Boyes, R.N. (1972) The tissue distribution, metabolism and excretion of lidocaine in rats, guinea pigs, dogs and man. *Journal of Pharmacology and Experimental Therapeutics*, **180**, 454-463.

Kurihara, N., Paulson, G.D., Otto, S., Miyamoto, J. & Hollingworth, R.M. (1993) Use of isolated cells to study the metabolism of agrochemicals in animals. *Pure and Applied Chemistry*, **65**, 2299-2312.

Lee, W.M. (2003) Drug-induced hepatotoxicity. *New England Journal of Medicine*, **349**, 474-485.

Leemann, T.D., Transon, C., Bonnabry, P. & Dayer, P. (1993) A major role for cytochrome P450TB (CYP2C subfamily) in the actions of non-steroidal anti-inflammatory drugs. *Drugs under Experimental and Clinical Research*, **19**, 189-195.

Levine, M. & Bellward, G.D. (1995) Effect of cimetidine on hepatic cytochrome P450: evidence for formation of a metabolite-intermediate complex. *Drug Metabolism and Disposition*, **23**, 1407-1411.

Levine, M., Law, E.Y.W., Bandiera, S.M., Chang, T.K.H. & Bellward, G.D. (1998) *In vivo* cimetidine inhibits hepatic CYP2C6 and CYP2C11 but not CYP1A1 in adult male rats. *Journal of Pharmacology and Experimental Therapeutics*, **284**, 493-499.

Lupp, A., Danz, M. & Müller, D. (2001) Morphology and cytochrome P450 isoforms expression in precision-cut rat liver slices. *Toxicology*, **161**, 53-66.

Lupp, A., Glockner, R., Danz, M. & Müller, D. (2002) Cryopreserved precision-cut rat liver slices: morphology and cytochrome P450 isoforms expression after prolonged incubation. *Toxicology in Vitro*, **16**, 749-758.

MacPhail, C.M., Lappin, M.R., Meyer, D.J., Smith, S.G., Webster, C.R.L. & Armstrong, P.J. (1998) Hepatocellular toxicosis associated with administration of carprofen in 21 dogs. *Journal of the Veterinary Medical Association*, **212**, 1895-1901.

Martignoni, M., Monshouwer, M., de Kanter, R., Pezzetta, D., Moscone, A. & Grossi, P. (2004) Phase I and phase II metabolic activities are retained in liver slices from mouse, rat, dog, monkey and human after cryopreservation. *Toxicology in Vitro*, **18**, 121-128.

Martin, H., Bournique, B., Sarsat, J.-P., Albaladejo, V. & Lerche-Langrand, C. (2000) Cryopreserved rat liver slices: a critical evaluation of cell viability, histological integrity, and drug-metabolizing enzymes. *Cryobiology*, **41**, 135-144.

McLaughlin, C.W. (1973) Control of sodium, potassium and water content and utilization of oxygen in rat liver slices, studied by affecting cell membrane permeability with calcium and active transport with ouabain. *Biochimica et Biophysica Acta*, **323**, 285-296.

Meyer, U.A. (1996) Overview of enzymes of drug metabolism. *Journal of Pharmacokinetics and Biopharmaceutics*, **24**, 449-459.

Müller, P.B., Taboada, J., Hosgood, G., Partington, B.P., VanSteenhouse, J.L., Taylor, H.W. & Wolfsheimer, K.J. (2000) Effects of long-term phenobarbital treatment on the liver in dogs. *Journal of Veterinary Internal Medicine*, **14**, 165-171.

Nebert, D.W. & Dieter, M.Z. (2000) The evolution of drug metabolism. *Pharmacology*, **61**, 124-135.

Nishibe, Y. & Hirata, M. (1993) Effect of phenobarbital and other model inducers on cytochrome P450 isoenzymes in primary culture of dog hepatocytes. *Xenobiotica*, **23**, 681-692.

Obatomi, D.K., Thanh, N.T.K, Brant, S. & Bach, P.H. (1998) The toxic mechanism and metabolic effect of atractyloside in precision-cut pig kidney and liver slices. *Archives of Toxicology*, **72**, 524-530.

Oddy, E.A., Manchee, G.R. & Coughtrie, M.W.H. (1997) Assessment of rat liver slices as a suitable model system for studying the simultaneous sulphation and glucuronidation of phenolic xenobiotics. *Xenobiotica*, **27**, 369-377.

Olinga, P., Hof, I.H., Merema, M.T., Smit, M., de Jager, M.H., Swart, P.J., Slooff, M.J.H., Meijer, D.K.F. & Groothuis, G.M.M. (2001) The applicability of rat and human liver slices to the study of mechanisms of hepatic drug uptake. *Journal of Pharmacological and Toxicological Methods*, **45**, 55-63.

Olinga, P., Merema, M., Hof, I.H., de Jong, K.P., Slooff, M.J.H., Meijer, D.K.F & Groothuis, G.M.M. (1998) Effect of human liver source on the functionality of isolated hepatocytes and liver slices. *Drug Metabolism and Disposition*, **26**, 5-11.

Parker, R.J., Collins, J.M. & Strong, J.M. (1996) Identification of 2,6-xylidine as a major lidocaine metabolite in human liver slices. *Drug Metabolism and Disposition*, **21**, 1167-1173.

Parkinson, A. (2001) Biotransformation of xenobiotics. In *Casarett & Doull's Toxicology: The Basic Science of Poisons*, 6th edn. Ed. Klaassen, C.D. pp. 133-224. McGraw-Hill, New York.

Podell, M. (1998) Antiepileptic drug therapy. *Clinical Techniques in Small Animal Practice*, **13**, 185-192.

Poffenbarger, E.M. & Hardy, R.M. (1985) Hepatic cirrhosis associated with long-term primidone therapy in a dog. *Journal of American Veterinary Medical Association*, **186**, 978-980.

Powis, G., Melder, D.C. & Wilke, T.J. (1989) Human and dog, but not rat, isolated hepatocytes have decreased foreign compound-metabolizing activity compared to liver slices. *Drug Metabolism and Disposition*, **17**, 526-531.

Rozman, K.K. & Klaassen, C.D. (2001) Absorption, distribution, and excretion of toxicants. In *Casarett & Doull's Toxicology: The Basic Science of Poisons*, 6th edn. Ed. Klaassen, C.D. pp. 107-132. McGraw-Hill, New York.

Schwartz-Porsche, D., Loscher, W. & Frey, H.-H. (1982) Treatment of canine epilepsy with primidone. *Journal of American Veterinary Medical Association*, **181**, 592-595.

Schwartz-Porsche, D., Loscher, W. & Frey, H.-H. (1985) Therapeutic efficacy of phenobarbital and primidone in canine epilepsy: a comparison. *Journal of Veterinary Pharmacology and Therapeutics*, **8**, 113-119.

Smith, P.F., Krack, G., McKee, R.L., Johnson, D.G., Gandolfi, A.J., Hruby, V.J., Krumdieck, C.L. & Brendel, K. (1986) Maintenance of adult rat liver slices in dynamic organ culture. *In Vitro Cellular and Developmental Biology*, **22**, 706-712.

Spahn, H., Spahn, I., Pflugmann, G., Mutschler, E. & Benet, L.Z. (1988) Measurement of carprofen enantiomer concentrations in plasma and urine using L-leucinamide as the chiral coupling component. *Journal of Chromatography*, **433**, 331-338.

Tanaka, E. & Breimer, D.D. (1997) *In vivo* function tests of hepatic drug-oxidizing capacity in patients with liver disease. *Journal of Clinical Pharmacy and Therapeutics*, **22**, 237-249.

Tibbitts, J. (2003) Issues related to the use of canines in toxicologic pathology—issues with pharmacokinetics and metabolism. *Toxicologic Pathology*, **31**, 17-24.

Treinen-Moslen, M. (2001) Toxic responses of the liver. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 6th edn. Ed. Klaassen, C.D. pp. 471-489. McGraw-Hill, New York.

Van Steenbergen, W., Peeters, P., De Bondt, J., Staessen, D., Büscher, H., Laporta, T., Roskams, T. & Desmet, V. (1998) Nimesulide-induced acute hepatitis: evidence from six cases. *Journal of Hepatology*, **29**, 135-141.

VandenBranden, M., Wrighton, S.A., Ekins, S., Gillespie, J.S., Binkley, S.N., Ring, B.J., Gadberry, M.G., Mullins, D.C., Strom, S.C. & Jensen, C.B. (1998) Alterations of the catalytic activities of drug-metabolizing enzymes in cultures of human liver slices. *Drug Metabolism and Disposition*, **26**, 1063-1068.

Vanhulle, V.P., Martiat, G.A., Bontemps, F., Vincent, M.-F., Pycke, J.-M., Verbeeck, R.K., Horsmans, Y. & Delzenne, N. (2003) Cryopreservation of rat precision-cut liver slices is associated with major metabolic stress and ionic perturbations. *Cellular Physiology and Biochemistry*, **13**, 103-112.

Vickers, A.E.M. (1994) Use of human organ slices to evaluate the biotransformation and drug-induced side-effects of pharmaceuticals. *Cell Biology and Toxicology*, **10**, 407-414.

Vickers, A.E.M., Fischer, V., Connors, S., Fisher, R.L., Baldeck, J.-P., Maurer, G. & Brendel, K. (1992) Cyclosporin A metabolism in human liver, kidney, and intestine slices. Comparison to rat and dog slices and human cell lines. *Drug Metabolism and Disposition*, **20**, 802-809.

Warburg, O. (1923) Versuche an überlebendem Karzinomgewebe. *Biochemische Zeitung*, **142**, 317-333.

Watkins, J.B. & Klaassen, C.D. (1986) Xenobiotic biotransformation in livestock: comparison to other species commonly used in toxicity testing. *Journal of Animal Science*, **63**, 933-942.

Watkins, P.B. (1990) Role of cytochromes P450 in drug metabolism and hepatotoxicity. *Seminars in Liver Disease*, **10**, 235-250.

Waxman, D.J. (1999) P450 gene induction by structurally diverse xenochemicals: central role of nuclear receptors CAR, PXR, and PPAR. *Archives of Biochemistry and Biophysics*, **369**, 11-23.

Wilcke, J.R., Davis, L.E., Neff-Davis, C.A. & Koritz, G.D. (1983) Pharmacokinetics of lidocaine and its active metabolites in dogs. *Journal of Veterinary Pharmacology and Therapeutics*, **6**, 49-57.

Williams, R.T. (1974) Inter-species variations in the metabolism of xenobiotics. *Biochemical Society Transaction*s, **2**, 13-377.

Wilson, D.V., Barnes, K.S. & Hauptman, J.G. (2004) Pharmacokinetics of combined intraperitoneal and incisional lidocaine in the dog following ovariohysterectomy. *Journal of Veterinary Pharmacology and Therapeutics*, **27**, 105-109.

Yeary, R.A. (1980) Serum concentrations of primidone and its metabolites, phenylethylmalonamide and phenobarbital, in the dog. *American Journal of Veterinary Research*, **41**, 1643-1645.

Supplemental References

Batt, A.M., Magdalou, J., Vincent-Viry, M., Ouzzine, M., Fournel-Gigleux, S., Galteau, M.M. & Siest, G. (1994) Drug metabolizing enzymes related to laboratory medicine: cytochromes P-450 and UDP-glucuronosyltransferases. *Clinica Chimica Acta*, **226**, 171-190.

Baur, H., Kasperek, S. & Pfaff, E. (1975) Criteria of viability of isolated liver cells. *Hoppe-Seyler's Zeitschrift fur physiologische Chemie*, **356**, 827-839.

Bayne, K.A.L. (2003) Environmental enrichment of nonhuman primates, dogs and rabbits used in toxicology studies. *Toxicologic Pathology*, **31**, 132-137.

Bolon, B., Campagnuolo, G., Zhu, L., Duryea, D., Zack, D. & Feige, U. (2004) Interleukin-1ß and tumor necrosis factor-α produce distinct, time-dependent patterns of acute arthritis in the rat knee. *Veterinary Pathology*, **41**, 235-243.

Bunch, S.E., Baldwin, B.H., Hornbuckle, W.E. & Tennant, B.C. (1984) Compromised hepatic function in dogs treated with anticonvulsant drugs. *Journal of American Veterinary Medical Association*, **184**, 444-448.

Bunch, S.E., Conway, M.B., Center, S.A., Castleman, W.L., Baldwin, B.H., Hornbuckle, W.E. & Tennant, B.C. (1987) Toxic hepatopathy and intrahepatic cholestasis associated with phenytoin administration in combination with other anticonvulsant drugs in three dogs. *Journal of American Veterinary Medical Association*, **190**, 194-198.

Cunningham, J.G., Ford, R.B., Gifford, J.A., Hulce, V.D., Chandler, M.L. & LeVier, R.R. (1981) Clinical evaluation of the new compound diphenylsilanediol for anti-epileptic efficacy and toxicity. *American Journal of Veterinary Research*, **42**, 2178-2181.

Cupp, M.J. & Tracy, T.S. (1998) Cytochrome P450: new nomenclature and clinical implications. *American Family Physician*, **57**, 107-116.

Fox, S.M. & Johnston, S.A. (1997) Use of carprofen for the treatment of pain and inflammation in dogs. *Journal of the American Veterinary Medical Association*, **210**, 1493-1498.

Hickford, F.H., Barr, S.C. & Erb, H.N. (2001) Effect of carprofen on hemostatic variables in dogs. *American Journal of Veterinary Research*, **62**, 1642-1646.

Iwakawa, S., Suganuma, T., Lee, S-F., Spahn, H., Benet, L.Z. & Lin, E.T. (1989) Direct determination of diastereomeric carprofen glucuronides in human plasma and urine and preliminary measurements of stereoselective metabolic and renal elimination after oral administration of carprofen in man. *Drug Metabolism and Disposit*ion, **17**, 414-419.

Kay-Mugford, P., Benn, S.J., La Marre, J. & Conlon, P. (2000) *In vitro* effects of nonsteroidal anti-inflammatory drugs on cyclooxygenase activity in dogs. *American Journal of Veterinary Research*, **61**, 802-810.

Larrey, D. & Pageaux, G.P. (1997) Genetic predisposition to drug-induced hepatotoxicity. *Journal of Hepatology*, **26**, 12-21.

Martin, F.L. & McLean, A.E.M. (1996) Cell protection by fructose is independent of adenosine triphosphate (ATP) levels in paracetamol injury to rat liver slices. *Toxicology*, **107**, 177-187.

McKellar, Q.A., Delatour, P. & Lees, P. (1994) Stereospecific pharmacodynamics and pharmacokinetics of carprofen in the dog. *Journal of Veterinary Pharmacology and Therapeutics*, **17**, 447-454.

Paulson, S.K., Engel, L., Reitz, B., Bolten, S., Burton, E.G., Maziasz, T.J., Yan, B. & Schoenhard, G.L. (1999) Evidence for polymorphism in the canine metabolism of the cyclooxygenase 2 inhibitor, celecoxib. *Drug Metabolism and Disposition*, **27**, 1133-1142.

Priymenko, N., Garnier, F., Ferre, J.P., Delatour, P. & Toutain, P.L. (1998) Enantioselectivity of the enterohepatic recycling of carprofen in the dog. *Drug Metabolism and Disposition*, **26**, 170-176.

Reinhardt, V. (2003) Compassion for animals in the laboratory: impairment or refinement of research methodology? *Journal of Applied Animal Welfare Science*, **6**, 123-130.

Rollin, B.E. (2003) Toxicology and new social ethics of animals. *Toxicologic Pathology*, **31**, 128-131.

Rubio, F., Seawall, S., Pocelinko, R., DeBarbieri, B., Benz, W., Berger, L., Morgan, L., Pao, J., Williams, T.H. & Koechlin, B. (1980) Metabolism of carprofen, a nonsteroidal anti-inflammatory agent, in rats, dogs, and humans. *Journal of Pharmaceutical Sciences*, **69**, 1245-1253.

Schmitt, M. & Guentert, T.W. (1990) Biopharmaceutical evaluation of carprofen following single intravenous, oral, and rectal doses in dogs. *Biopharmaceutics and Drug Disposition*, **11**, 585-594.

Sivapathasundaram, S., Howells, L.C., Sauer, M.J. & Ioannides, C. (2004) Functional integrity of precision-cut liver slices from deer and cattle. *Journal of Veterinary Pharmacology and Therapeutics*, **27**, 79-84.

Sohlenius-Sternbeck, A.-K., Floby, E., Svedling, M. & Orzechowski, A. (2000) High conservation of both phase I and phase II drug-metabolizing activities in cryopreserved rat liver slices. *Xenobiotica*, **30**, 891-903.

Taylor, P.M., Delatour, P., Landoni, F.M., Deal, C., Pickett, C., Aliabadi, F.S., Foot, R. & Lees, P. (1996) Pharmacodynamics and enantioselective pharmacokinetics of carprofen in the cat. *Research in Veterinary Science*, **60**, 144-151.

Worboys, P.D., Bradbury, A. & Houston, J.B. (1997) Kinetics of drug metabolism in rat liver slices III. Relationship between metabolic clearance and slice uptake rate. *Drug Metabolism and Disposition*, **25**, 460-467.

Yoshida, T., Arisaka, Y., Nakagawa, S. & Takahashi, H. (2004) Rotation culture with a newly developed holder enables long-term liver slice culture for study of liver fibrosis. *Hepatology Research*, **28**, 198-206.

APPENDIX I
STOCK SOLUTIONS

Phenobarbital

Measure 0.1 ml phenobarbital sodium injectable and q.s. to 20.2 ml with prepared media to make a phenobarbital solution of concentration 295.074 µg/ml

Primidone

Measure 0.0502 g powder and q.s. to 20 ml with ethanol to make a 2.51 mg/ml solution

Lidocaine

Measure 0.5 ml of 2% lidocaine HCl injectable and q.s. with prepared media to 20 ml to make a 405µg/ml lidocaine solution

Cimetidine

Measure 0.513 ml of 150 mg/ml cimetidine injectable and q.s. with prepared media to 305 ml to make a solution of 0.2523 mg/ml cimetidine

Diazepam

Measure 0.1 ml of 5 mg/ml diazepam injectable and q.s to 10 ml with prepared media to make a 50,000 ng/ml solution

Carprofen

Racemic – 10,000 µg/ml

Measure 0.1 g powder and q.s. to 10 ml with ethanol

R or S enantiomer – 20,000 µg/ml

Measure 0.2 g powder and q.s. to 10 ml with ethanol

APPENDIX II
CHEMICAL STRUCTURES

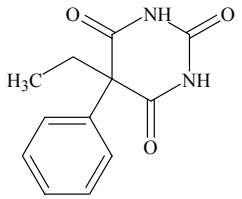

Fig. A-1. Phenobarbital

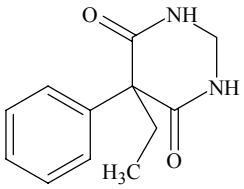

Fig. A-2. Primidone

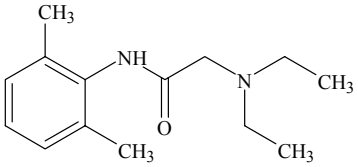

Fig. A-3. Lidocaine

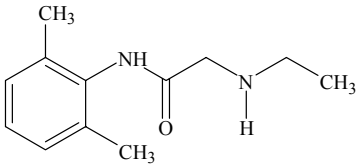

Fig. A-4. Monoethylglycinexylidide

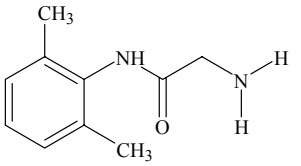

Fig. A-5. Glycinexylidide

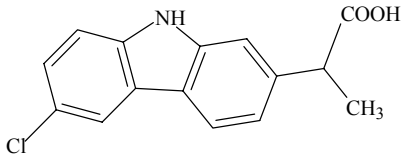

Fig. A-6. Carprofen

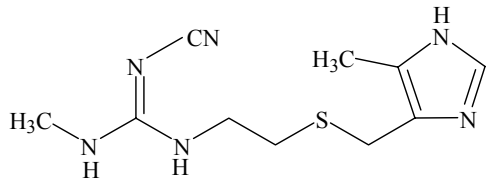

Fig. A-7. Cimetidine

APPENDIX III

HEPATIC SLICE WEIGHT TABLE

Table A-1. Hepatic slice weights. Hepatic slice weights with means and standard deviations (std dev) represent variability across time, among day and between day.

	2/25/2003				5/1/2003							7/3/2003			
Time (hour)	0 µg/ml	Carp 10 µg/ml	Carp 50 µg/ml	Carp 100 µg/ml	0 µg/ml	Pheno 20 µg/ml	Pheno 45 µg/ml	Pheno 75 µg/ml	Lido 3 µg/ml	Lido 10 µg/ml	Lido 20 µg/ml	0 µg/ml	Prim 20 µg/ml	Prim 45 µg/ml	Prim 75 µg/ml
1	0.0276	0.0265	0.0364	0.0304	0.0312	0.0278	0.0294	0.0264	0.0283	0.0244	0.0311	0.0249	0.0238	0.0284	0.0228
	0.0299	0.0334	0.0292	0.0366	0.0231	0.0313	0.0320	0.0269	0.0316	0.0222	0.0294	0.0273	0.0258	0.0267	0.0272
3					0.0264	0.0232	0.0228	0.0313	0.0301	0.0230	0.0259	0.0277	0.0213	0.0206	0.0226
					0.0275	0.0300	0.0280	0.0241	0.0263	0.0272	0.0232	0.0195	0.0254	0.0189	0.0273
6	0.0315	0.0264	0.0272	0.0221	0.0279	0.0261	0.0267	0.0297	0.0209	0.0220	0.0255	0.0186	0.0231	0.0207	0.0278
	0.0258	0.0290	0.0225	0.0248	0.0235	0.0249	0.0227	0.0290	0.0249	0.0263	0.0267	0.0228	0.0215	0.0237	0.0265
8	0.0305	0.0236	0.0241	0.0266	0.0260	0.0284	0.0248	0.0295	0.0256	0.0273	0.0226	0.0187	0.0195	0.0175	0.0270
	0.0276	0.0272	0.0283	0.0215	0.0224	0.0225	0.0271	0.0257	0.0227	0.0242	0.0218	0.0184	0.0191	0.0210	0.0200
12	0.0272	0.0252	0.0232	0.0243	0.0257	0.0286	0.0265	0.0223	0.0280	0.0238	0.0249	0.0174	0.0171	0.0171	0.0212
	0.0220	0.0259	0.0325	0.0265	0.0235	0.0237	0.0207	0.0229	0.0237	0.0238	0.0278	0.0171	0.0177	0.0219	0.0186
24	0.0215	0.0267	0.0280	0.0260	0.0251	0.0271	0.0269	0.0281	0.0222	0.0279	0.0235	0.0183	0.0183	0.0182	0.0180
	0.0249	0.0280	0.0256	0.0318	0.0229	0.0227	0.0260	0.0261	0.0251	0.0246	0.0225	0.0200	0.0210	0.0162	0.0200
mean	0.0269	0.0272	0.0277	0.0271	0.0254	0.0264	0.0261	0.0268	0.0258	0.0247	0.0254	0.0209	0.0211	0.0209	0.0233
std dev	0.0034	0.0026	0.0043	0.0046	0.0026	0.0030	0.0031	0.0028	0.0032	0.0020	0.0029	0.0038	0.0029	0.0038	0.0037
mean	0.0272				0.0258							0.0215			
std dev	0.0037				0.0028							0.0036			

Means across time for all treatments

Time (hour)	mean (g)	std dev
0	0.0252	0.0038
1	0.0260	0.0044
3	0.0251	0.0035
4	0.0229	0.0036
6	0.0250	0.0030
8	0.0236	0.0035
12	0.0227	0.0033
24	0.0234	0.0034

Mean for all times and treatments = 0.0241

Std Dev for all times and treatments = 0.0038

	10/15/2003								10/14/2003										
Time (hour)	0 ng/ml	0 ng/ml	0 ng/ml	Diaz 100 ng/ml	Diaz 250 ng/ml	Diaz 500 ng/ml	Pheno 75 µg/ml	Cimet 1000 µM	0 ng/ml	Diaz 100 ng/ml	Diaz 250 ng/ml	Diaz 500 ng/ml	0 µg/ml	Carp 10 µg/ml	Carp 50 µg/ml	Carp 100 µg/ml	Carp 10 µg/ml	Carp 50 µg/ml	Carp 100 µg/ml
0	0.0269	0.0261	0.0261	0.0250	0.0226	0.0185	0.0302	0.0227	0.0287	0.0272	0.0226	0.0239	0.0247	0.0329	0.0241	0.0225	0.0283	0.0235	0.0187
	0.0231	0.0247	0.0275	0.0272	0.0284	0.0243	0.0231	0.0314	0.0267	0.0236	0.0235	0.0305	0.0219	0.0270	0.0209	0.0239	0.0351	0.0175	0.0234
1	0.0194			0.0273	0.0172	0.0233			0.0257	0.0238	0.0349	0.0198	0.0226	0.0234	0.0230	0.0266	0.0318	0.0256	0.0220
	0.0266			0.0201	0.0208	0.0192			0.0251	0.0250	0.0217	0.0192	0.0221	0.0271	0.0266	0.0240	0.0241	0.0199	0.0225
4	0.0216			0.0238	0.0201	0.0235			0.0266	0.0242	0.0255	0.0199	0.0222	0.0302	0.0224	0.0222	0.0231	0.0238	0.0218
	0.0160			0.0216	0.0284	0.0191			0.0234	0.0178	0.0199	0.0271	0.0208	0.0276	0.0191	0.0246	0.0317	0.0192	0.0210
8	0.0196			0.0205	0.0240	0.0200			0.0249	0.0260	0.0268	0.0235	0.0235	0.0273	0.0205	0.0187	0.0338	0.0221	0.0196
	0.0269			0.0217	0.0222	0.0215			0.0257	0.0217	0.0260	0.0261	0.0222	0.0220	0.0230	0.0184	0.0269	0.0230	0.0173
12	0.0218			0.0218	0.0198	0.0243			0.0271	0.0193	0.0181	0.0250	0.0210	0.0263	0.0222	0.0217	0.0228	0.0233	0.0224
	0.0197			0.0192	0.0198	0.0229			0.0280	0.0214	0.0235	0.0217	0.0194	0.0195	0.0234	0.0212	0.0250	0.0200	0.0216
24	0.0253	0.0220	0.0229	0.0227	0.0205	0.0266	0.0267	0.0260	0.0242	0.0212	0.0281	0.0281	0.0224	0.0263	0.0177	0.0222	0.0215	0.0246	0.0220
	0.0243	0.0171	0.0197	0.0192	0.0193	0.0203	0.0223	0.0216	0.0220	0.0281	0.0260	0.0246	0.0232	0.0208	0.0231	0.0231	0.0287	0.0213	0.0196
mean	0.0226	0.0225	0.0241	0.0225	0.0219	0.0220	0.0256	0.0254	0.0257	0.0233	0.0247	0.0241	0.0222	0.0259	0.0222	0.0224	0.0277	0.0220	0.0210
std dev	0.0035	0.0040	0.0035	0.0028	0.0035	0.0026	0.0036	0.0044	0.0019	0.0031	0.0043	0.0035	0.0014	0.0039	0.0024	0.0023	0.0046	0.0024	0.0018
mean	0.0228								0.0237										
std dev	0.0033								0.0035										

APPENDIX IV

DIAZEPAM RESULTS

Abbreviation: Time (h) = Time (hour)

Diazepam was used as a drug to determine the drug-metabolizing ability of the hepatic slices. The results showed the potential formation of metabolites, but because of assay limitations, the metabolite concentrations could not be quantified. Additionally, there appeared to be a cross-reactivity of phenobarbital and cimetidine with diazepam in detection. Another predicament was the loss of supernatant samples during preparation for drug analysis. Because of these issues, the diazepam results are presented here. The author suggests that the diazepam experiment be tried again using the concentrations presented here, as well as, higher concentrations. Also, extension of the limits of quantification should be attempted.

Chemical Structures

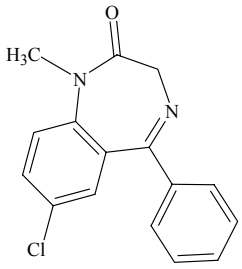

Fig. A-8. Diazepam

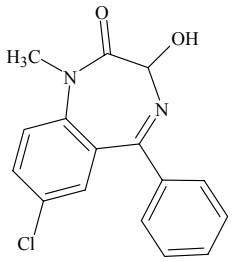

Fig. A-9. Temazepam

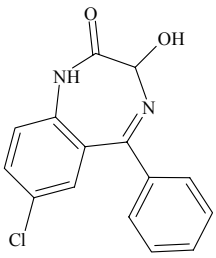

Fig. A-10. Oxazepam

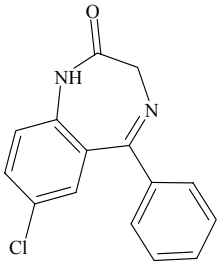

Fig. A-11. Nordiazepam

Media and supernatant drug concentrations

Figures A-12 through A-15 show the concentrations of diazepam and its metabolites, oxazepam, temazepam and nordiazepam, in media and supernatant without phenobarbital or cimetidine incubation. Media concentrations are a mean of three samples per time point per concentration. Concentrations of diazepam in 0 ng/ml media were below the lower limit of quantification (LOQ).

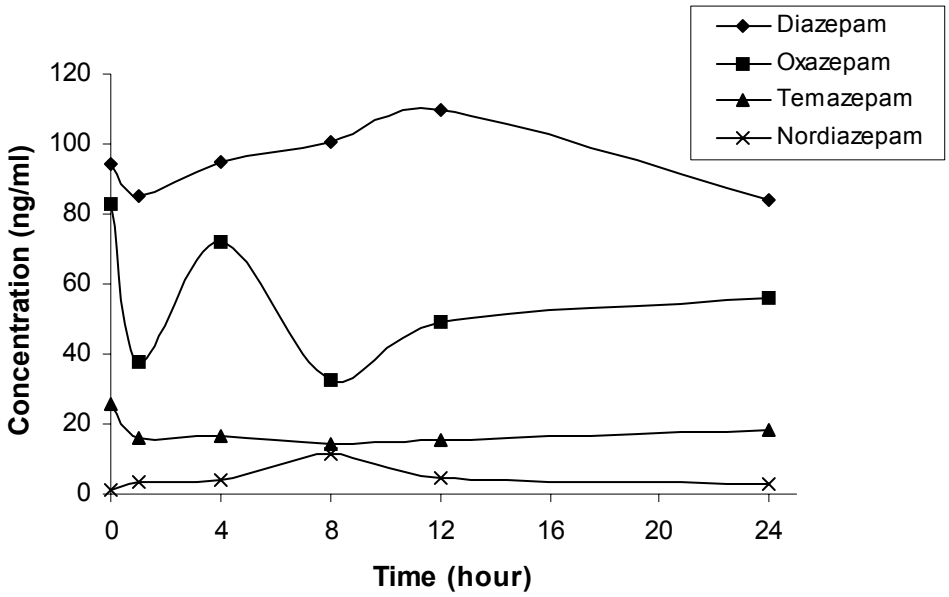

Fig. A-12. Concentration of diazepam and metabolites in media containing 100 ng/ml diazepam. Limit of quantification for diazepam is 40 ng/ml and for all metabolites is 80 ng/ml.

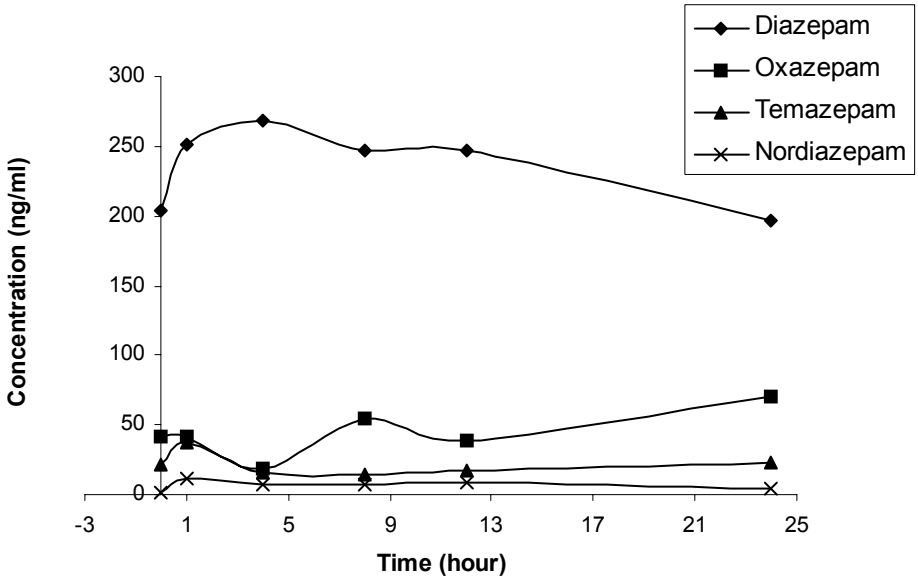

Fig. A-13. Concentration of diazepam and metabolites in media containing 250 ng/ml diazepam. Limit of quantification for diazepam is 40 ng/ml and for all metabolites is 80 ng/ml.

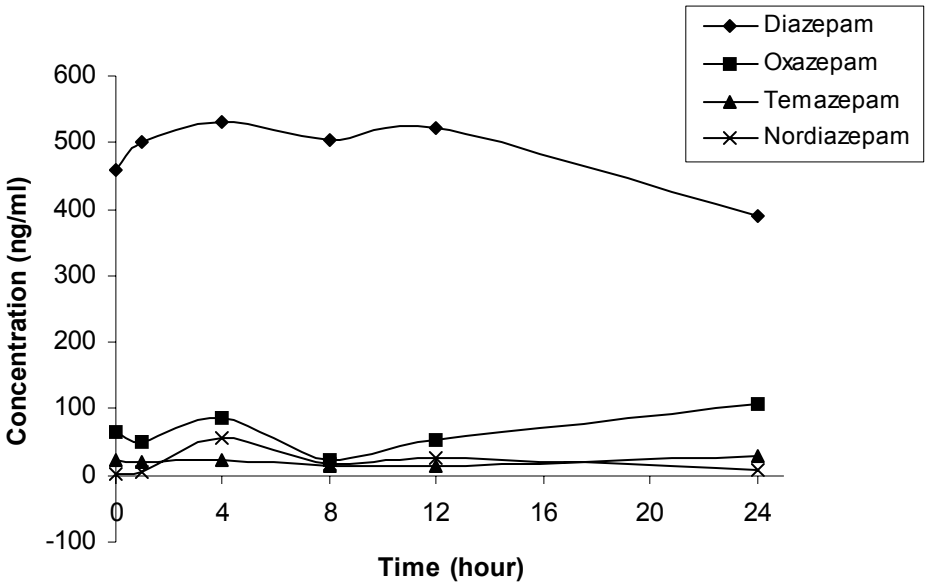

Fig A-14. Concentration of diazepam and metabolites in media containing 500 ng/ml diazepam. Limit of quantification for diazepam and nordiazepam is 40 ng/ml; for all other metabolites, it is 80 ng/ml.

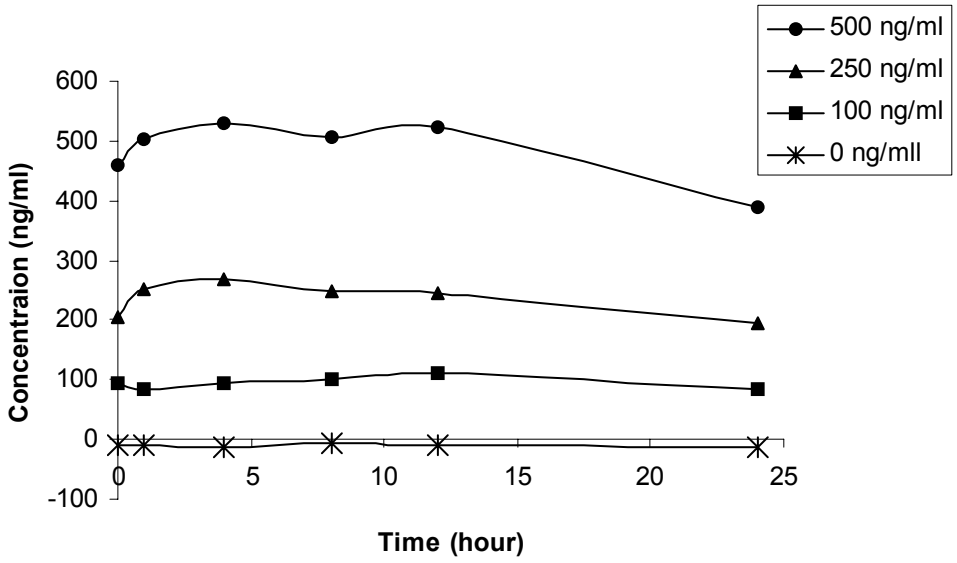

Fig. A-15. Concentrations of diazepam in media containing 0, 100, 250 and 500 ng/ml diazepam. Limit of quantification for diazepam is 40 ng/ml.

The concentrations of the diazepam metabolites in the media were below the lower limit of quantification (LOQ). A few samples did have concentrations above the lower LOQ for oxazepam and nordiazepam (Figures A-16 and A-17).

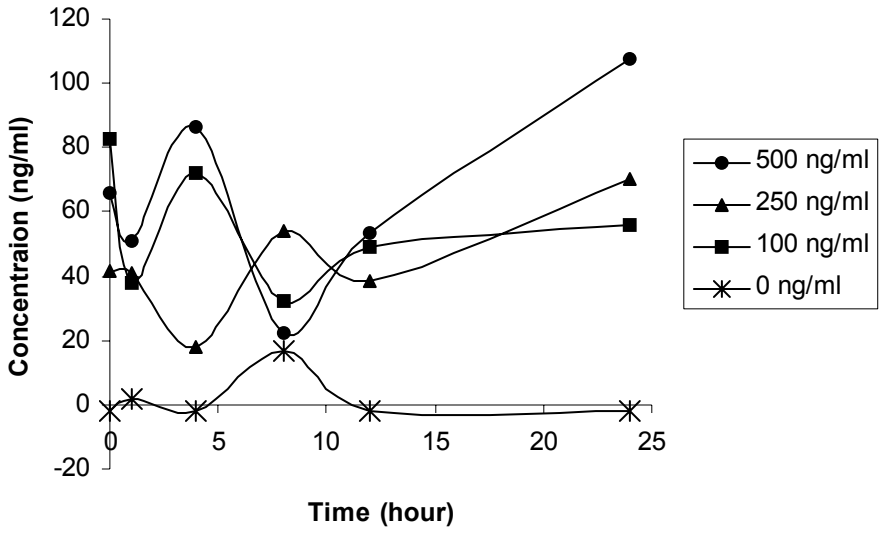

Fig. A-16. Concentrations of oxazepam in media containing 0, 100, 250 and 500 ng/ml diazepam. Limit of quantification for oxazepam is 80 ng/ml.

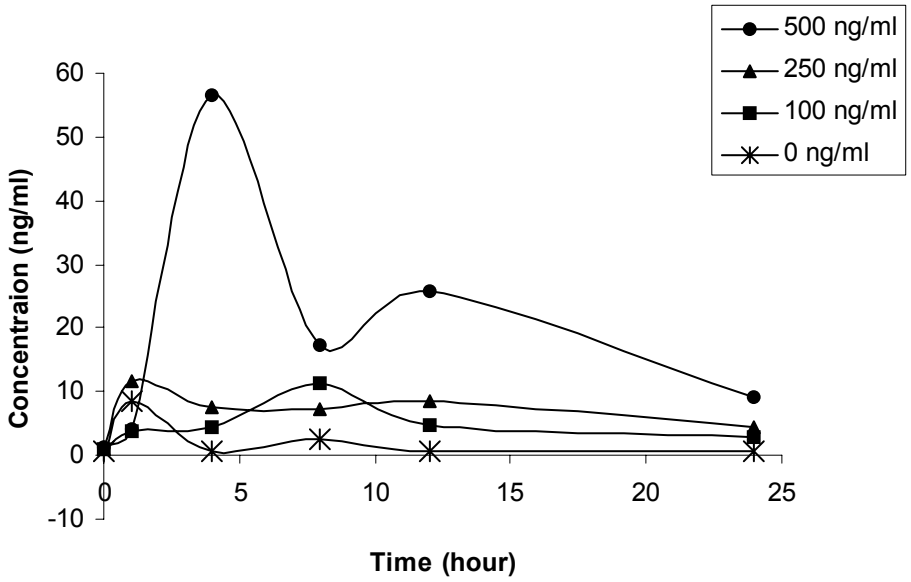

Fig. A-17. Concentrations of nordiazepam in media containing 0, 100, 250 and 500 ng/ml diazepam. Limit of quantification for nordiazepam is 40 ng/ml.

Figures A-18 through A-25 show the concentrations of diazepam and its metabolites in media with no treatment or following treatment with phenobarbital or cimetidine. Concentrations are a mean of three samples per time point per treatment. Diazepam concentrations in media containing 0 ng/ml diazepam were below the lower LOQ.

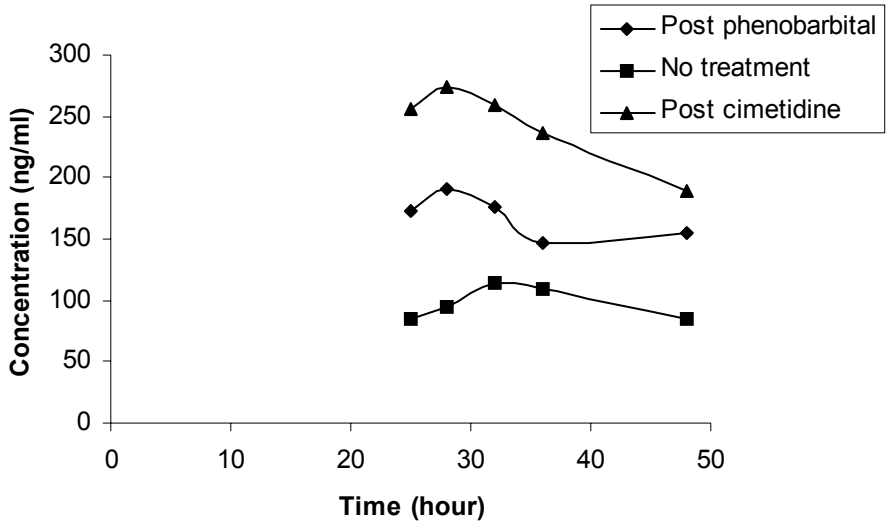

Fig. A-18. Concentration of diazepam in media containing 100 ng/ml diazepam following no treatment or treatment with phenobarbital or cimetidine. The limit of quantification is 40 ng/ml for diazepam.

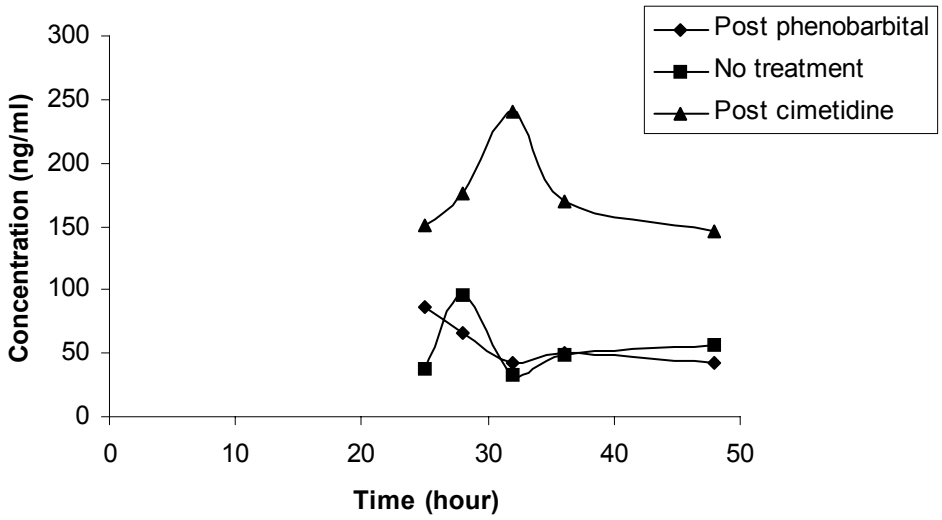

Fig. A-19. Concentration of oxazepam in media containing 100 ng/ml diazepam following no treatment or treatment with phenobarbital or cimetidine. The limit of quantification is 80 ng/ml.

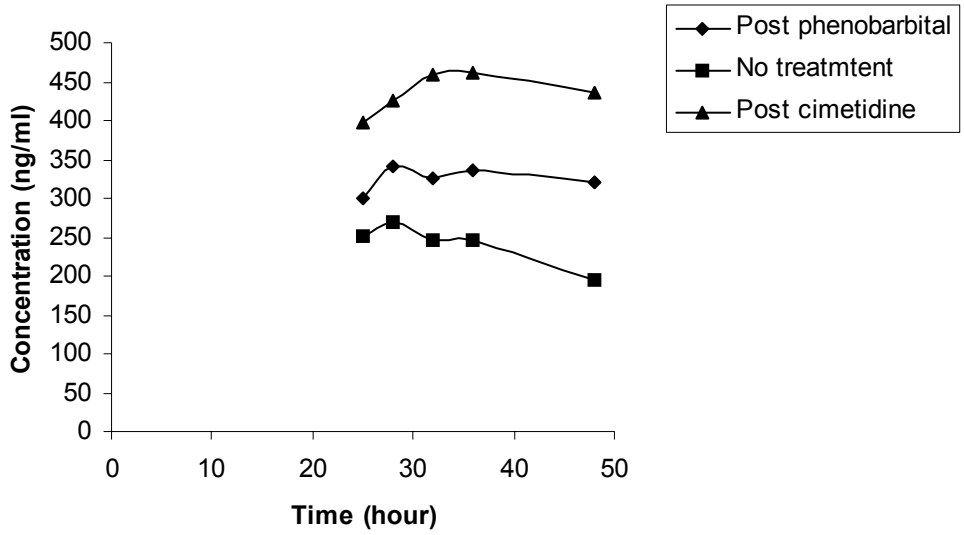

Fig. A-20. Concenation of diazepam in media containing 250 ng/ml diazepam following no treatment or treatment with phenobarbital or cimetidine. The limit of quantification is 40 ng/ml for diazepam.

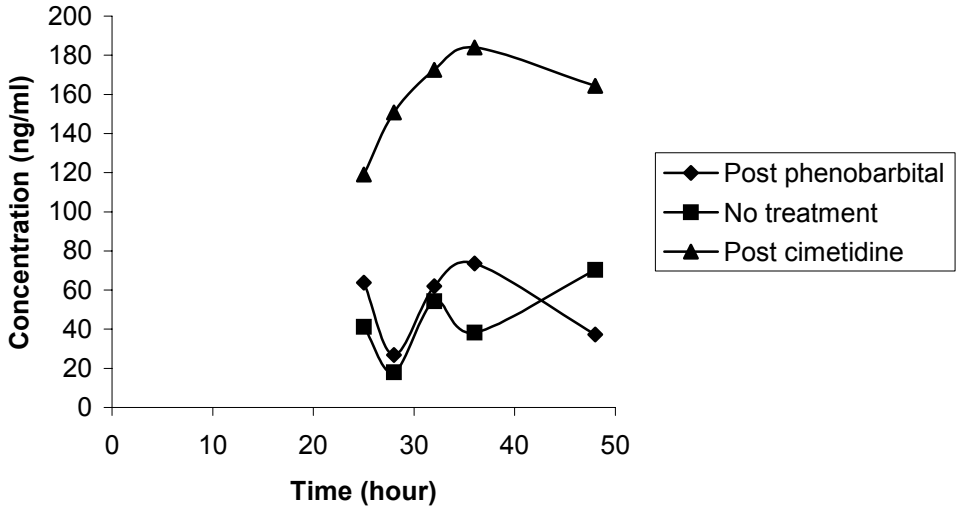

Fig. A-21. Concentration of oxazepam in media containing 250 ng/ml diazepam following no treatment or treatment with phenobarbital or cimetidine. The limit of quantification is 80 ng/ml.

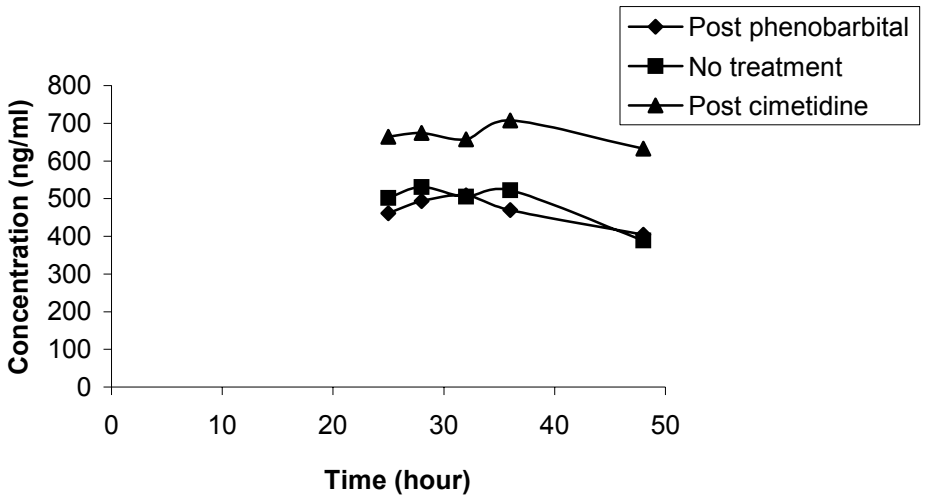

Fig. A-22. Concentration of diazepam in media containing 500 ng/ml diazepam following no treatment or treatment with phenobarbital or cimetidine. The limit of quantification is 40 ng/ml for diazepam.

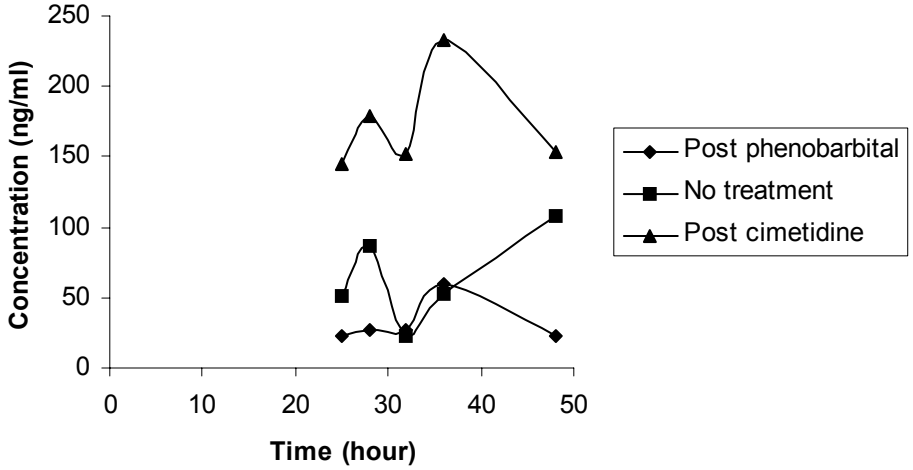

Fig. A-23. Concentration of oxazepam in media containing 500 ng/ml diazepam following no treatment or treatment with phenobarbital or cimetidine. The limit of quantification is 80 ng/ml.

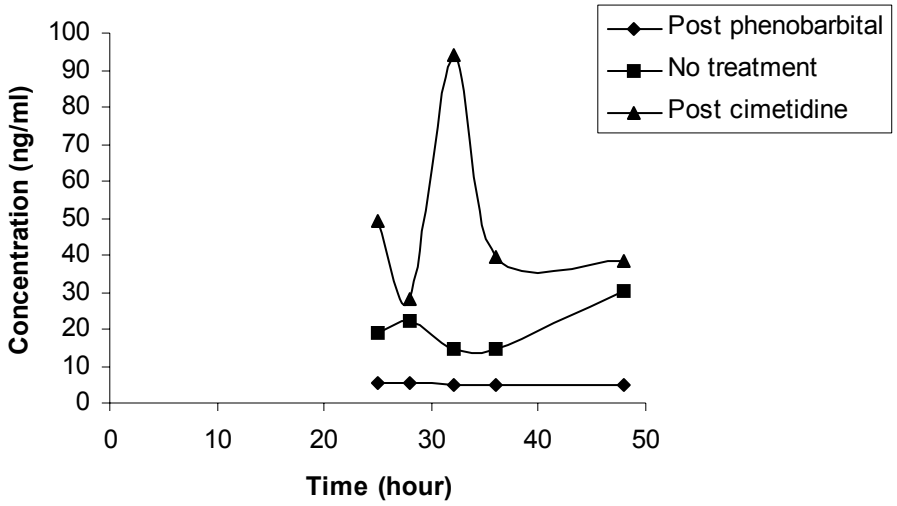

Fig. A-24. Concentration of temazepam in media containing 500 ng/ml diazepam following no treatment or treatment with phenobarbital or cimetidine. The limit of quantification is 80 ng/ml.

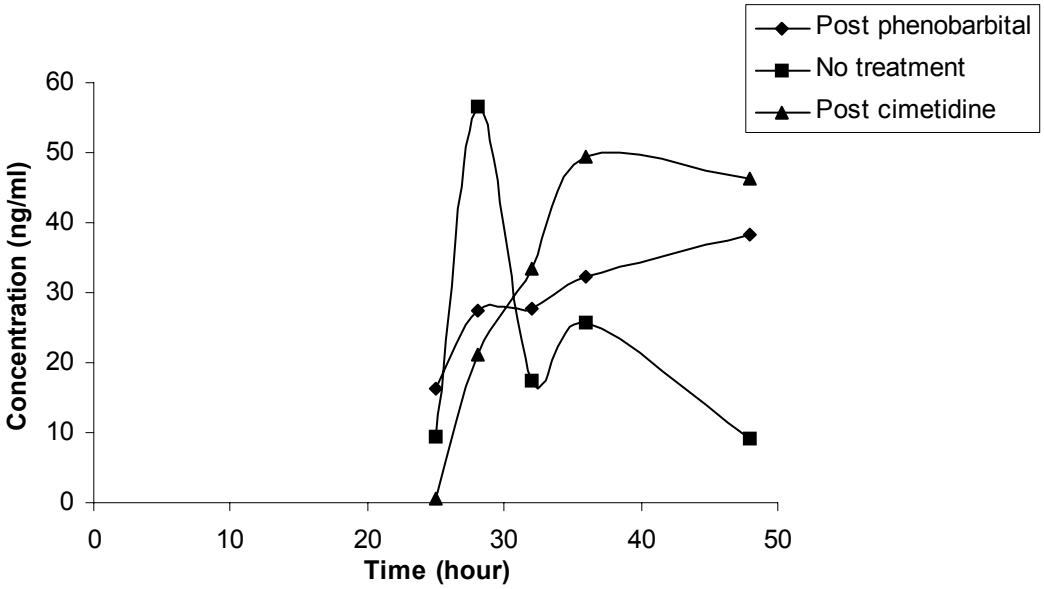

Fig. A-25. Concentration of nordiazepam in media containing 500 ng/ml diazepam following no treatment or treatment with phenobarbital or cimetidine. The limit of quantification is 40 ng/ml.

The hepatic supernatant drug levels were below the limit of quantification for the slices exposed to diazepam alone. Figures A-26 and A-27 show the changes that occurred following exposure to phenobarbital and cimetidine for samples that were above the limit of quantification.

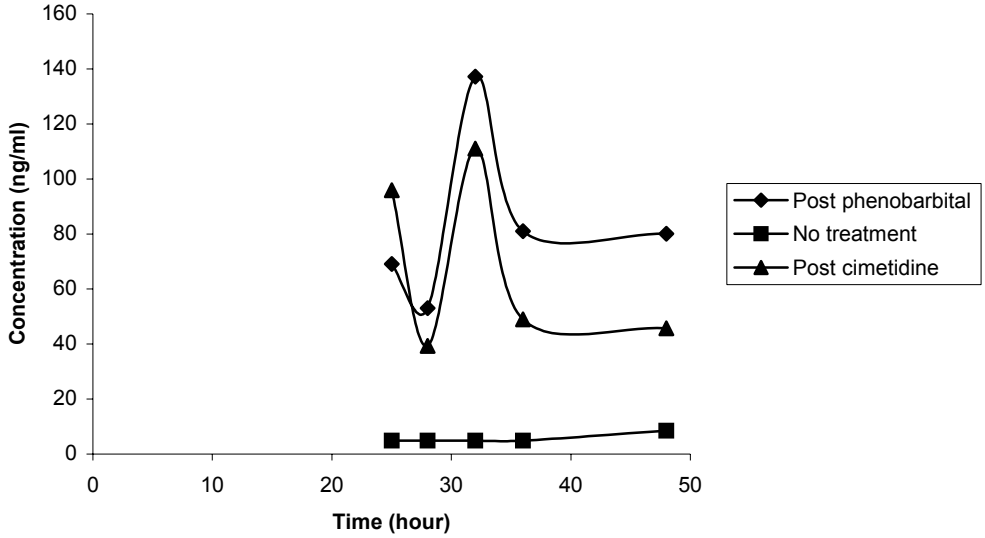

Fig. A-26. Hepatic supernatant concentration of temazepam. Slices were incubated in media containing 100 ng/ml diazepam following no treatment or treatment with phenobarbital or cimetidine. The limit of quantification is 80 ng/ml. Values are an average of two samples per time point per treatment.

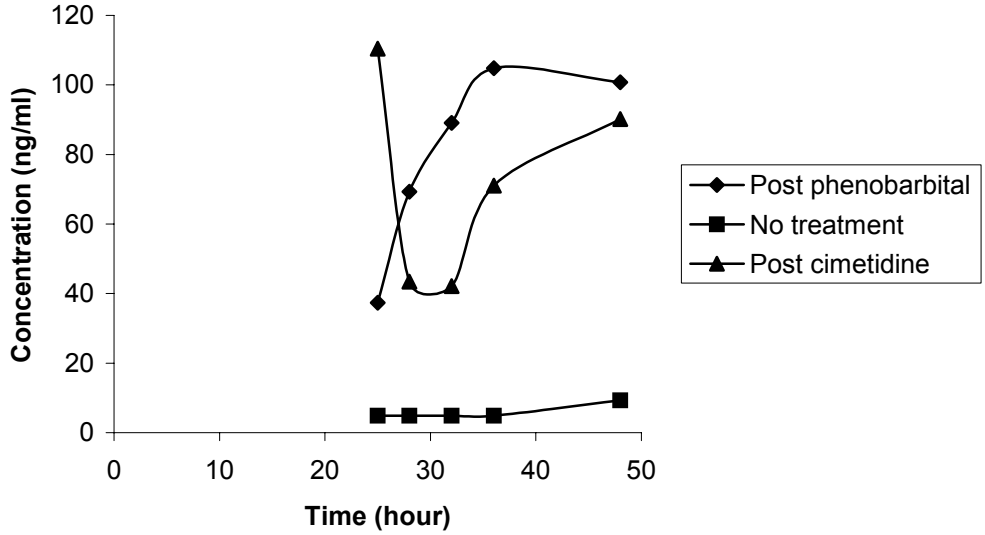

Fig. A-27. Hepatic supernatant concentration of temazepam. Slices were incubated in media containing 500 ng/ml diazepam following no treatment or treatment with phenobarbital or cimetidine. The limit of quantitation is 80 ng/ml. Values are an average of two samples per time point per treatment.

Potassium

Mean potassium values across time and by concentration are shown in Figure A-28. The two-way ANOVA results showed that mean potassium values for time were significantly different (df=5, F-ratio=28.442, p-value=0.000). Concentrations at time 0 were higher than

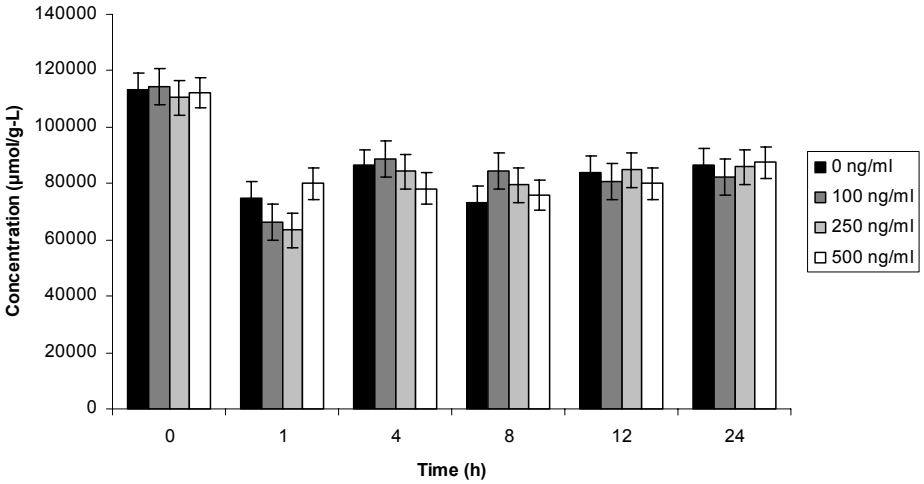

Fig. A-28. Potassium concentration in hepatic supernatant following incubation in media containing diazepam. Potassium concentrations are means of four samples per time point per media concentration.

those for all other time points, and concentrations at 1 hour were lower than concentrations at 4, 12 and 24 hours. Table A-2 displays the homogeneous groups for time.

Table A-2. Homogeneous groups for mean potassium values across time.

Time (h)	Homogeneous groups		
0	a		
1		b	
4			c
8		b	c
12			c
24			c

No significant differences were found for concentration (df=3, F-ratio=0.116, p-value=0.950) or the time-concentration interaction (df=15, F-ratio=0.808, p-value=0.665).

Figure A-29 shows the mean potassium values following incubation with phenobarbital and diazepam. Mean potassium values for time 0 were significantly higher than for all other concentrations (df=6, F-ratio=19.690, p-value=0.000) (Table A-3). For concentration (df=3, F-ratio=2.0317, p-value=0.132) and time-concentration interaction (df=18, F-ratio=0.496, p-value=0.938), no significant differences were found.

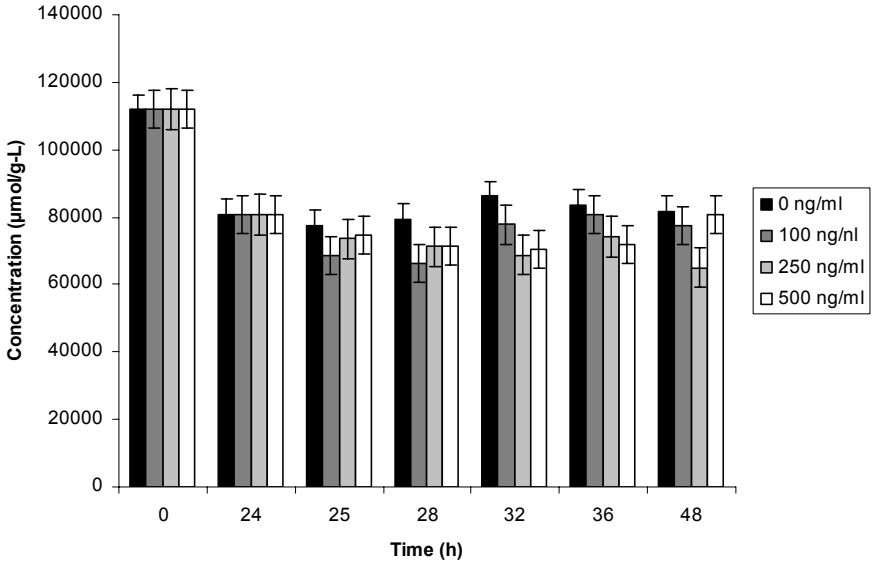

Fig. A-29. Potassium concentration in hepatic supernatant following exposure to phenobarbital and diazepam. Values are an average of two samples per time point per concentration.

Table A-3. Homogeneous groups for mean potassium value

Time (h)	Homogeneous groups
0	a
24	b
25	b
28	b
32	b
36	b
48	b

The potassium values following exposure to cimetidine and diazepam are shown in Figure A-30. Theean values for potassium are significantly higher at time 0 compared to times 25 through 48 (df=6, F-ratio=9.841, p-value=0.000) (Table A-4). Additionally, potassium values at time 48 are significantly lower than time 24. No significant differences were found for concentration (df=3, F-ratio=0.147, p-valu%=0.931) or the time*concentration interaction (df=18, F-ratio=1.085, p-value=0.413).

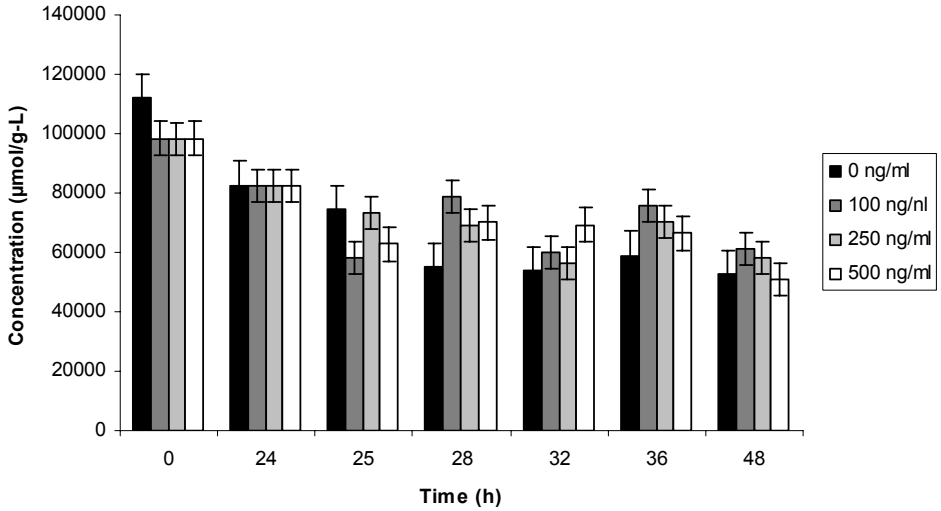

Fig. A-30. Potassium concentration in hepatic supernatant following exposure to cimetidine and diazepam. Concentrations are an average of two samples per time and media concentration.

Table A-4. Homogeneous groups for mean potassium values by time.

Time (h)	Homogeneous groups		
0	a		
24	a	b	
25		b	c
28		b	c
32		b	c
36		b	c
48			c

ATP

Figure A-31 shows the mean ATP values across time and concentration for hepatic slices incubated with diazepam. Table A-5 shows the groups with significantly different means. For time (df=5, F-ratio=2.904, p-value=0.019), concentration (df=3, F-ratio=0.180, p-value=0.910) and the time*concentration interaction (df=15, F-ratio=0.407, p-value=0.973) only mean ATP values across time were found to be significantly different. Mean ATP values at 8 hours were significantly higher than those for 24 hours.

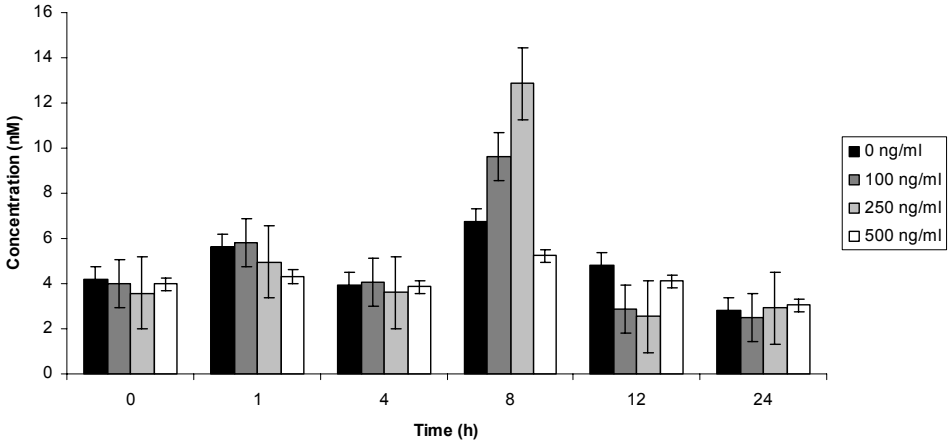

Fig. A-31. ATP values for slices exposed to diazepam over 24 hours. Concentrations are a mean of four supernatant samples per time point and concentration.

Table A-5. Homogeneous groups for ATP across time following exposure to diazepam.

Time (h)	Homogeneous groups	
0	a	b
1	a	b
4	a	b
8	a	
12	a	b
24		b

Figure A-32 and Table A-6 show the ATP values and significantly different groups for slices incubated in the presence of phenobarbital and diazepam. The two-way ANOVA analysis showed a significant difference in time (df=6, F-ratio=81.994, p-value=0.000) but not in concentration (df=3, F-ratio=0.835, p-value=0.486) or interaction (df=18, F-ratio=1.080, p-value=0.417). Mean ATP values for 0 hour were significantly higher than all other times. At times 24, 32 and 36 values were higher than for times 25 and 28. Concentrations at 48 hours were higher than at hours 24, 25, 28, 32 and 36.

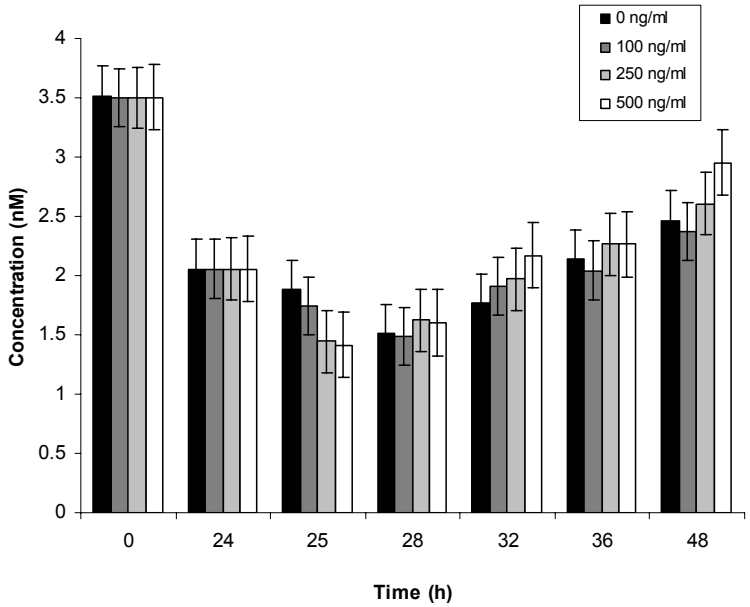

Fig. A-32. ATP values for slices exposed to phenobarbital and diazepam.

Table A-6. Homogeneous groups for ATP by time.

Time (h)	Homogeneous groups
0	a
24	b
25	c
28	c
32	b
36	b
48	d

Figure A-33 and Tables A-7 and A-8 show the significant differences across time and concentration for ATP values of slices exposed to cimetidine and diazepam. Mean ATP values for time 32, 36 and 48 were significantly higher than time 0 (df=6, F-ratio=24.247, p-value=0.000). Values at 24 and 28 hours were less than at 36 hours. Hour 25 values were less than those at times 32 and 36. ATP values at 48 hours were higher than at times 24, 25, 28 and 32. For concentration, mean ATP values for 500 were significantly higher than those for concentrations 0 and 100 (df=3, F-ratio=4.076, p-value=0.016). The concentration*time interaction was not significantly different (df=18, F-ratio=1.288, p-value=0.267).

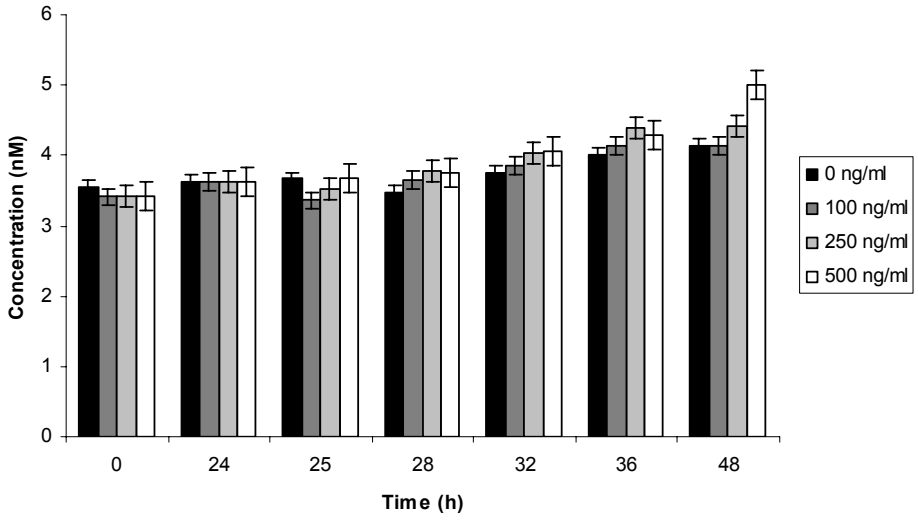

Fig. A-33. ATP values for slices exposed to cimetidine and diazepam.

Table A-7. Homogeneous groups for ATP by time

Time (h)	Homogeneous groups		
0	a		
24	a	b	
25	a		
28	a	b	
32		b	
36			c
48			c

Table A-8. Homogeneous groups for ATP by concentration

Media concentration (ng/ml)	Homogeneous groups	
0	a	
100	a	
250	a	b
500		b

Histology

Table A-9. Diazepam Hepatic Slice Lesion Scores. Lesion scores of hepatic slices incubated in diazepam for up to twenty-four hours.

		Lesion scores*			
Media Concentration	**Time (hour)**	**Necrosis : extent**	**Vacuolation : extent**	**Vacuolation : severity**	**Comments**
0 ng/ml	0	0	0	0	
0 ng/ml	1	0	0	0	
0 ng/ml	4	1	2	1	
0 ng/ml	8	1	3	1	a
0 ng/ml	12	1	1	1	
0 ng/ml	24	2	2	2	
100 ng/ml	0	0	0	0	
100 ng/ml	1	0	0	0	
100 ng/ml	4	1	2	1	
100 ng/ml	8	1	3	1	
100 ng/ml	12	2	1	1	
100 ng/ml	24	1	1	1	
250 ng/ml	0	0	0	0	
250 ng/ml	1	0	2	1	
250 ng/ml	4	0	2	1	
250 ng/ml	8	1	2	1	
250 ng/ml	12	1	2	2	a
250 ng/ml	24	2	1	1	
500 ng/ml	0	0	0	0	
500 ng/ml	1	0	2	1	
500 ng/ml	4	1	2	1	
500 ng/ml	8	1	3	2	
500 ng/ml	12	1	1	1	a
500 ng/ml	24	1	2	2	

* Lesion scoring: (outermost zone ~10 hepatocytes wide was disregarded)

0	< 1% hepatoc9tes affected	3	21-40%
1	1-10%	4	41-90%
2	11-20%	5	>90%

a Slides showing marked difference in lesion extent and/or severity as move across the slide

Table A-10. Diazepam 2 Hepatic Slice Lesion Scores. Lesion scores of hepatic slices incubated in diazepam for up to twenty-four hours.

		Lesion scores*			
Media Concentration	**Time (hour)**	**Necrosis : extent**	**Vacuolation : extent**	**Vacuolation : severity**	**Comments**
0 ng/ml	0	0	1	1	
0 ng/ml	1	0	0	0	
0 ng/ml	4	1	2	1	
0 ng/ml	8	2	3	2	
0 ng/ml	12	1	4	4	
0 ng/ml	24	2	4	4	
100 ng/ml	0	0	1	1	
100 ng/ml	1	0	1	1	
100 ng/ml	4	1	2	1	
100 ng/ml	8	2	3	2	
100 ng/ml	12	1	2	1	
100 ng/ml	24	2	4	4	a
250 ng/ml	0	0	1	1	
250 ng/ml	1	0	1	1	
250 ng/ml	4	0	3	2	
250 ng/ml	8	1	4	3	
250 ng/ml	12	1	3	1	
250 ng/ml	24	2	4	3	
500 ng/ml	0	0	1	1	
500 ng/ml	1	0	2	1	
500 ng/ml	4	1	3	2	
500 ng/ml	8	1	4	3	
500 ng/ml	12	1	3	2	
500 ng/ml	24	1	4	4	

* Lesion scoring: (outermost zone ~10 hepatocytes wide was disregarded)

0	< 1% hepatocytes affected	3	21-40%
1	1-10%	4	41-90%
2	11-20%	5	>90%

a Slides showing marked difference in lesion extent and/or severity as move across the slide

Table A-11. Diazepam and phenobarbital Hepatic Slice Lesion Scores. Lesion scores of hepatic slices incubated in phenobarbital for 24 hours followed by incubation in diazepam for 1, 4, 8, 12 and 24 hours.

		Lesion scores*			
Media Concentration	**Time (hour)**	**Necrosis : extent**	**Vacuolation : extent**	**Vacuolation : severity**	**Comments**
0 ng/ml	0	0	0	0	
0 ng/ml	24	2	0	0	
phenobarbital	0	0	0	0	
phenobarbital	24	1	3	3	
0 ng/ml	25	1	2	3	
0 ng/ml	28	2	4	4	a
0 ng/ml	32	2	4	4	
0 ng/ml	36	2	3	3	
0 ng/ml	48	2	3	3	a
100 ng/ml	25	1	3	3	
100 ng/ml	28	3	3	2	
100 ng/ml	32	2	3	2	
100 ng/ml	36	2	4	4	
100 ng/ml	48	2	2	2	
250 ng/ml	25	2	2	2	
250 ng/ml	28	3	3	2	a
250 ng/ml	32	3	4	4	
250 ng/ml	36	3	4	4	
250 ng/ml	48	2	4	4	
500 ng/ml	25	2	3	3	a
500 ng/ml	28	2	4	4	a
500 ng/ml	32	3	3	2	
500 ng/ml	36	3	4	4	
500 ng/ml	48	2	3	3	

* Lesion scoring: (outermost zone ~10 hepatocytes wide was disregarded)

0	< 1% hepatocytes affected	3	21-40%
1	1-10%	4	41-90%
2	11-20%	5	>90%

a Slides showing marked difference in lesion extent and/or severity as move across the slide

Table A-12. Diazepam and cimetidine Hepatic Slice Lesion Scores. Lesion scores of hepatic slices incubated in cimetidine for 24 hours followed by incubation in diazepam for 1, 4, 8, 12 and 24 hours.

		Lesion scores*			
Media Concentration	**Time (hour)**	**Necrosis : extent**	**Vacuolation : extent**	**Vacuolation : severity**	**Comments**
0 ng/ml	0	0	0	0	
0 ng/ml	24	3	1	2	
cimetidine	0	0	0	0	
cimetidine	24	2	0	0	
0 ng/ml	25	2	2	2	
0 ng/ml	28	2	3	3	
0 ng/ml	32	3	3	2	a
0 ng/ml	36	3	3	2	a
0 ng/ml	48	2	2	2	
100 ng/ml	25	2	1	2	a
100 ng/ml	28	3	2	2	
100 ng/ml	32	3	2	2	
100 ng/ml	36	2	4	3	
100 ng/ml	48	4	2	2	
250 ng/ml	25	2	2	2	
250 ng/ml	28	3	2	2	
250 ng/ml	32	3	2	1	
250 ng/ml	36	4	3	2	
250 ng/ml	48	4	2	2	
500 ng/ml	25	3	0	0	
500 ng/ml	28	3	2	2	a
500 ng/ml	32	3	2	1	a
500 ng/ml	36	4	3	2	a
500 ng/ml	48	3	3	3	a

* Lesion scoring: (outermost zone ~10 hepatocytes wide was disregarded)

0	< 1% hepatocytes affected	3	21-40%
1	1-10%	4	41-90%
2	11-20%	5	>90%

a Slides showing marked difference in lesion extent and/or severity as move across the slide

APPENDIX V

CARPROFEN SINGLE STUDY DATA

Abbreviation: Time (h) = Time (hour)

Carprofen Single Study Data

Media

Carprofen A, B and C were used to designate the different racemic studies. Figure A-34 show3 the media concentrations of carprofen A following 1, 3, 6, 8, 12 and 24 hours incubation. The carprofen concentrations of media containing 0 µg/ml carprofen were below the lower limit of quantification.

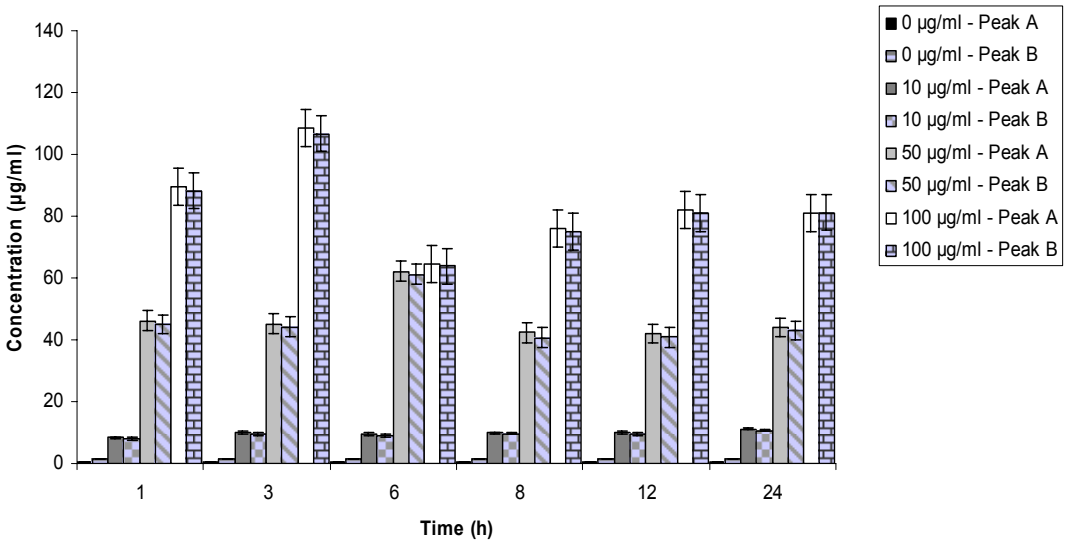

Fig. A-34. Carprofen A media concentrations. Concentrations of carprofen enantiomers (peak A and B) in media during twenty-four hours of incubation. Carprofen concentrations are an average of two samples per media concentration per time point.

Two-way ANOVA of carprofen A media concentration3 for time and peak for 10 and 50 revealed no significant differences across time or between peaks. For 100 µg/ml, concentrations at 3 hours were greater than those at 6 hours (df=5, F-ratio=3.288, p-value=0.042). Table A-13 shows the homogeneous groups for 100 µg/ml carprofen.

Table A-13. Carprofen A – 100 µg/ml analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups	
1	a	b
3	a	
6		b
8	a	b
12	a	b
24	a	b

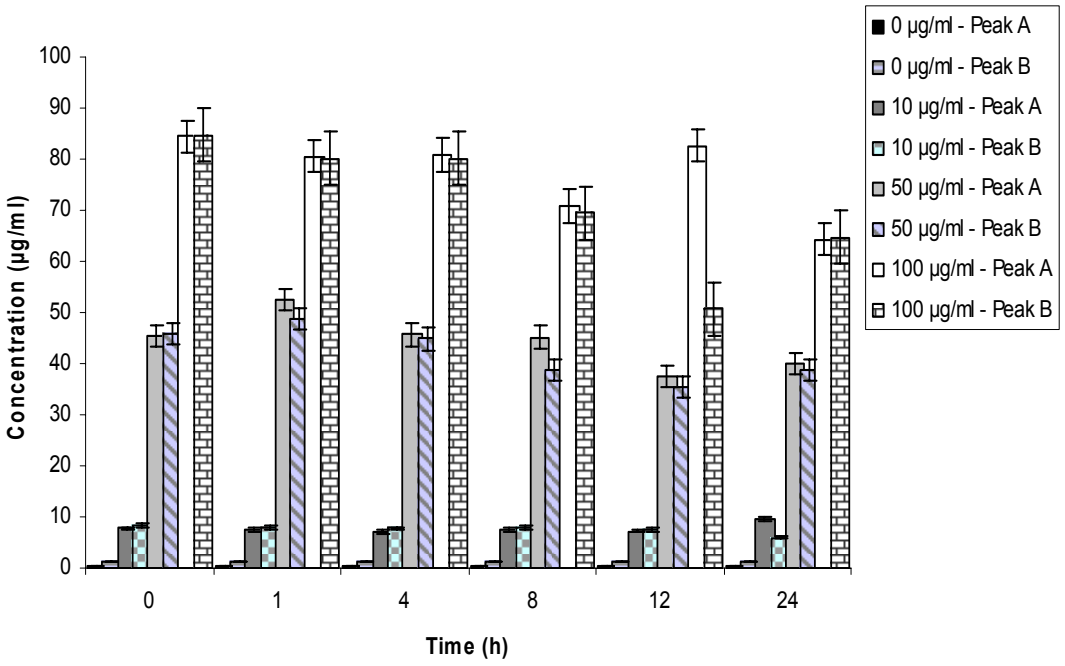

Fig. A-35. Carprofen B media concentrations. Concentrations of carprofen enantiomers in media following incubation for 0, 1, 4, 8, 12 and 24 hours. The values are means of three samples per media concentration per time point.

Figure A-35 illustrates the concentrations of carprofen in carprofen B media following 0, 1, 4, 8, 12 and 24 hours incubation. The two-way ANOVA of carprofen B media concentrations for time and peak showed differences across time for media containing 50 (df=5, F-ratio=6.273, p-value=0.01) and 100 µg/ml (df=5, F-ratio=4.145, p-value=0.007) carprofen. For 50 µg/ml, media concentrations at 12 hours were less than those at 0 and 1 hour, and concentrations at 24 hours were less than those at 1 hour. Table A-14 shows the homogeneous groups for 50 µg/ml carprofen B. The concentrations for 100 µg/ml carprofen B were higher at 0 hour than at 24 hours. Table A-15 shows the homogeneous groups for time for 100 µg/ml carprofen B.

Table A-14. Carprofen B – 50 µg/ml analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		
0	a		c
1	a		
4	a	b	c
8	a	b	c
12		b	
24		b	c

Table A-15. Carprofen B – 100 µg/ml analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups	
0	a	
1	a	b
4	a	b
8	a	b
12	a	b
24		b

Figure A-36 shows the concentration of carprofen enantiomers in media following hepatic slice incubation in carprofen C media. The two-way ANOVA for media concentrations with time and peak as factors indicated that for 10 µg/ml carprofen C, peak B was higher than peak A (df=1, F-ratio=7.610, p-value=0.011). For 50 µg/ml carprofen C, differences were found across time (df=5, F-ratio=4.984, p-value=0.003) and between the peaks (df=1, F-ratio=5.636, p-value=0.026). At 1 hour, carprofen enantiomer concentrations were higher than those at 8, 12 and 24 hours. The enantiomer concentrations at 4 hours were higher than those at 12 hours. Peak A concentrations were higher than peak B concentrations. Table A-16 shows the homogeneous groups for time for 50 µg/ml carprofen C. For 100 µg/ml carprofen C, enantiomer concentrations at 24 hours were less than those at 1 hour. Table A-17 shows the homogeneous groups for time during incubation with 100 µg/ml carprofen C.

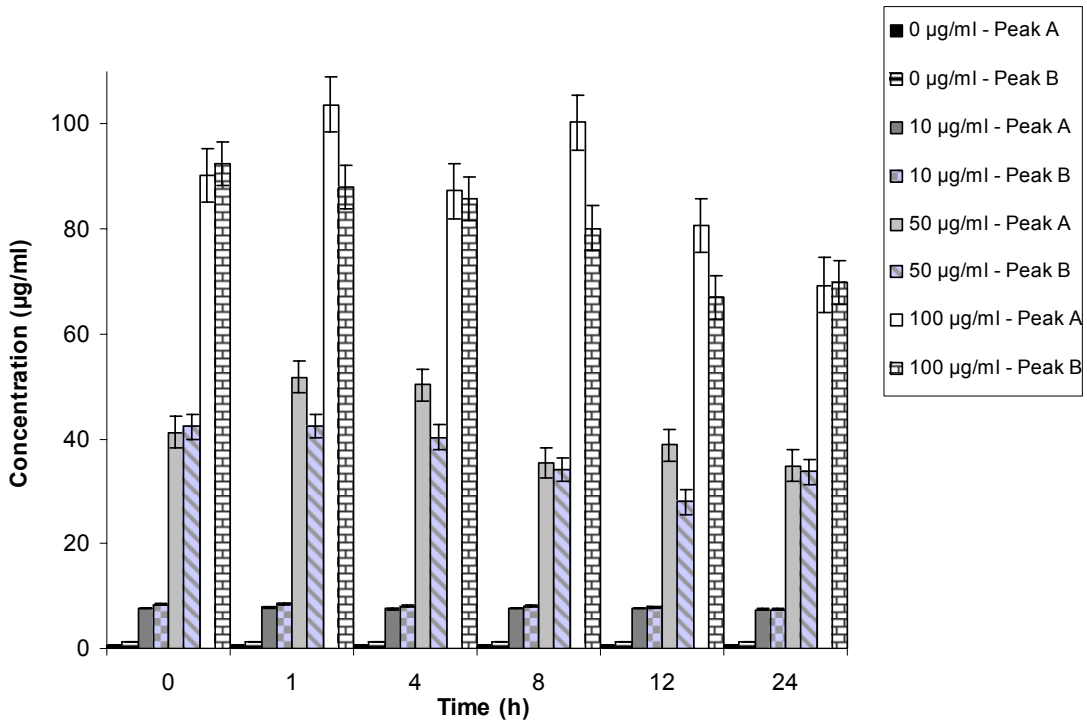

Fig. A-36. Carprofen C media concentrations. Concentrations of carprofen enantiomers in media following incubation for 0, 1, 4, 8, 12 and 24 hours. The values are means of three samples per media concentration per time point.

Table A-16. Carprofen C – 50 µg/ml analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		
0	a	b	c
1	a		
4	a	b	
8		b	c
12			c
24		b	c

Table A-17. Carprofen C – 100 µg/ml analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups	
0	a	b
1	a	
4	a	b
8	a	b
12	a	b
24		b

Supernatant

The concentrations of carprofen enantiomers in hepatic slice supernatant for slices incubated in carprofen A media are shown in Figure A-37. Significant differences were noted across time for 50 (df=5, F-ratio=5.550, p-value=0.007) and 100 µg/ml (df=5, F-ratio=6.295, p-value=0.004) media using two-way ANOVA. At 6 hours, slices incubated in media containing 50 µg/ml carprofen A had supernatant concentrations higher than at 1 and 3 hours. Table A-18 shows the 50 µg/ml carprofen A homogenous groups for time. At 12 hours, slices incubated in 100 µg/ml media had supernatant concentrations higher than at 1 and 6 hours. Concentrations were also higher at 24 hours than at 1 hour. Table A-19 shows the homogeneous groups across time for 100 µg/ml carprofen A media.

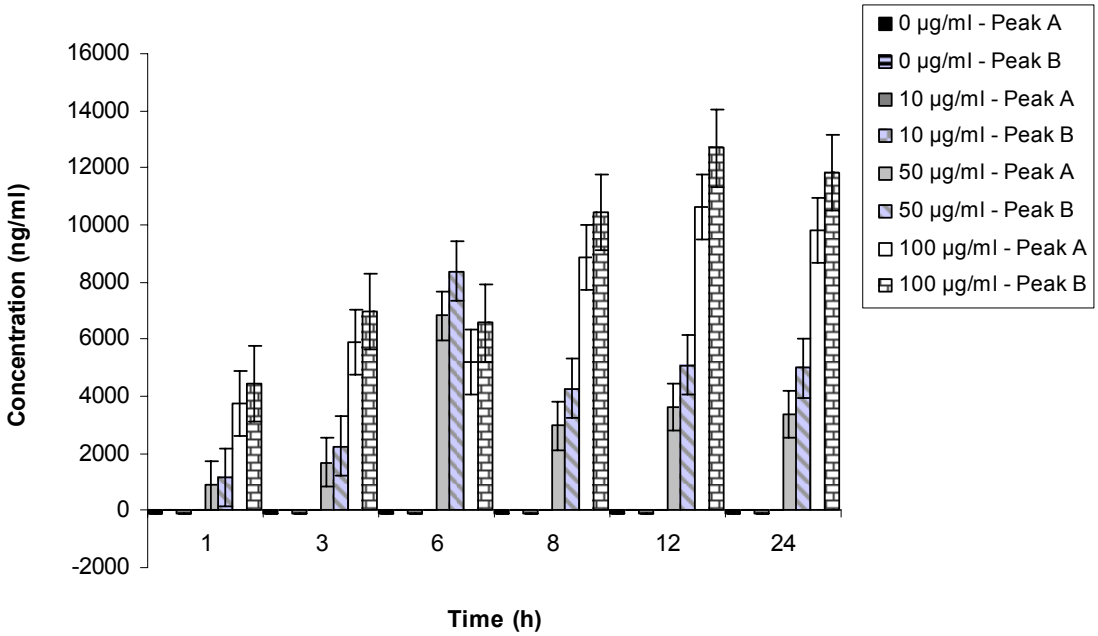

Fig. A-37. Carprofen A supernatant concentrations. Concentrations of carprofen enantiomers in hepatic slice supernatant following incubation for 1, 3, 6, 8, 12 and 24 hours. The values are an average of two samples per media concentration per time point.

Table A-18. Supernatant carprofen A – 50 µg/ml analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		
1	a		
3	a		
6		b	
8	a	b	
12	a	b	
24	a	b	

Table A-19. Supernatant carprofen A – 100 µg/ml analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		
1	a		
3	a	b	c
6	a	b	
8	a	b	c
12			c
24		b	c

Figure A-38 shows the supernatant concentrations of carprofen following incubation in carprofen B. Significant differences in carprofen hepatic slice concentrations were noted with two-way ANOVA across time for slices incubated in 50 (df=5, F-ratio=34.086, p-value=0.000) and 100 (df=5, F-ratio=190.018, p-

value=0.000) µg/ml media. For the 50 µg/ml media, supernatant concentrations at 0 hours were less than those at 4, 8, 12 and 24 hours. At 1 hour the concentrations were also lower than at 4, 8, 12 and 24 hours. At 24 hours, the supernatant concentrations were higher than at 4 hours. Table A-20 depicts the

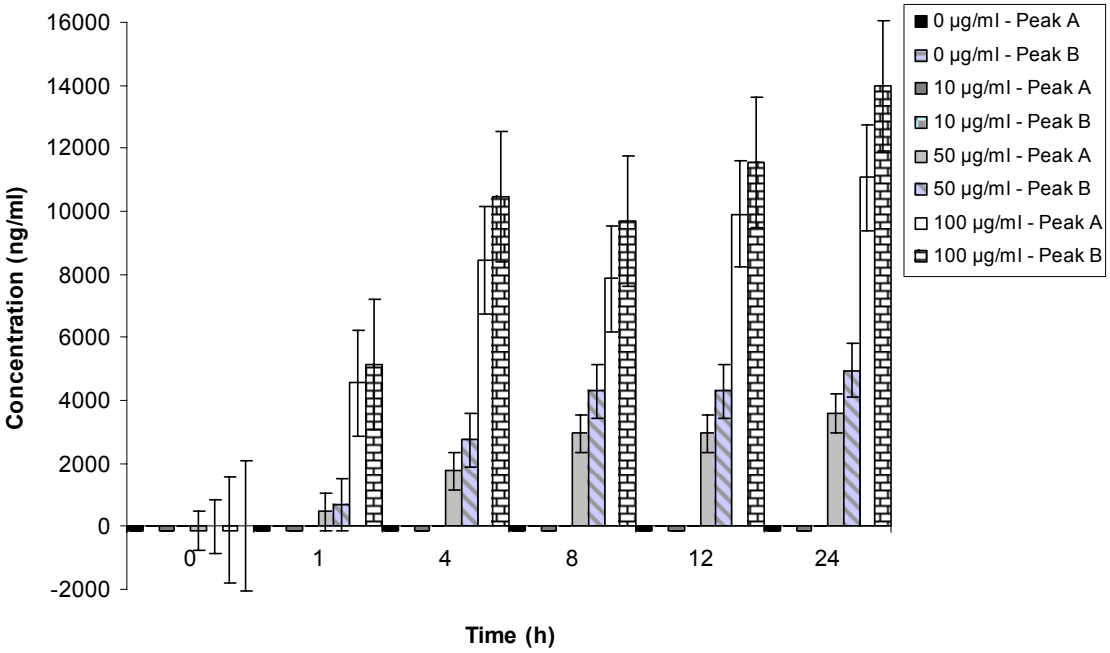

Fig. A-38. Carprofen B supernatant concentrations. Concentrations of carprofen enantiomers in hepatic slice supernatant following incubation for 0, 1, 4, 8, 12 and 24 hours. The values are an average of two samples per media concentration per time point.

homogeneous groups for time for 50 µg/ml media. Hepatic slice supernatant for slices incubated in media containing 100 µg/ml carprofen B were higher at 1, 4, 8, 12 and 24 hours than at 0 or 1 hour. Supernatant concentrations at 24 hours were higher than for 4 or 12 hours, and at 8 hours the concentrations were less than at 12 and 24 hours. Table A-21 shows the homogeneous groups for time for slices incubated in 100 µg/ml carprofen B. Peak B had higher hepatic supernatant concentrations than peak A for both 50 (df=1, F-ratio=13.163, p-value=0.003) and 100 (df=1, F-ratio=31.234, p-value=0.000) µg/ml media.

Table A-20. Supernatant carprofen B – 50 µg/ml analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		
0	a		
1	a		
4		b	
8		b	c
12		b	c
24			c

Table A-21. Supernatant carprofen B – 100 µg/ml analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups				
0	a				
1		b			
4			c	d	
8			c		
12				d	
24					e

Figure A-39 illtrates the concentrations of carprofen in hepatic slice supernatant following incubation with carprofen C. Significant differences in supernatant concentrations were found across time and between peaks for slices incubated in 50 (time: df=5, F-ratio=222.991, p-value=0.000; peak: df=1, F-ratio=100.711, p-v!lue=0.000) and 100 (time: df=5, F-ratio=173.733, p-value=0.000; peak: df=1, F-ratio=18.636, p-value=0.001) µg/ml carprofen C media. With 50 µg/ml media, supernatant concentrations were higher for 4, 8, 12 and 24 hours than at 0 or 1 hour. Concentrations at 4 hours were less than those at 8, 12 and 24 hours, and concentrations at 8 hours were less than those at 24 hours. With 100 µg/ml media, supernatant concentrations were higher at 4, 8, 12 and 24 hours than at 0 or 1 hour. At 4 hours, supernatant concentrations were less than at 8, 12 and 24 hours; at 24 hours, the concentrations were higher than at 8 and 12 hours. Tables A-22 and A-23 show the homogeneous groups time for 50 and 100 µg/ml carprofen C, respectively. For 50 and 100 µg/ml media, the concentrations of peak B in the supernatant were higher than those of peak A.

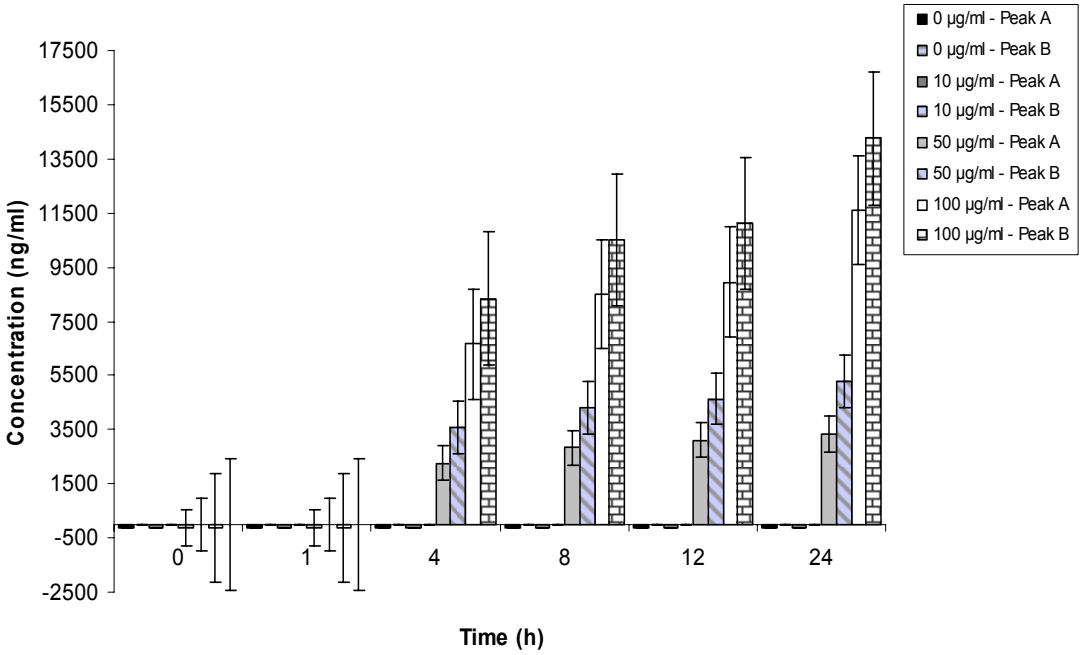

Fig. A-39. Carprofen C supernatant concentrations. Concentrations of carprofen. enantiomers in hepatic slice supernatant following incubation for 0, 1, 4, 8, 12 and 24 hours. The values are an average of two samples per media concentration per time point.

Table A-22. Supernatant carprofen C – 50 µg/ml analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups			
0	a			
1	a			
4		b		
8			c	
12			c	d
24				d

Table A-23. Supernatant carprofen C – 100 µg/ml analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups			
0	a			
1	a			
4		b		
8			c	
12			c	
24				d

*Potassiu*m

Figure A-40 shows the supernatant potassium concentrations during 24 hours of incubation with carprofen A. The two-way ANOVA for time and concentration revealed a significant difference across time (df=4, F-ratio=3.313, p-value=0.034); however, no significant pairwise differences were found. The pairs that were closest to significance which may have been responsible for the statistical significance were the higher 6 hour concentrations compared to 1 hour (p-value=0.064) and 24 hours (p-value=0.088).

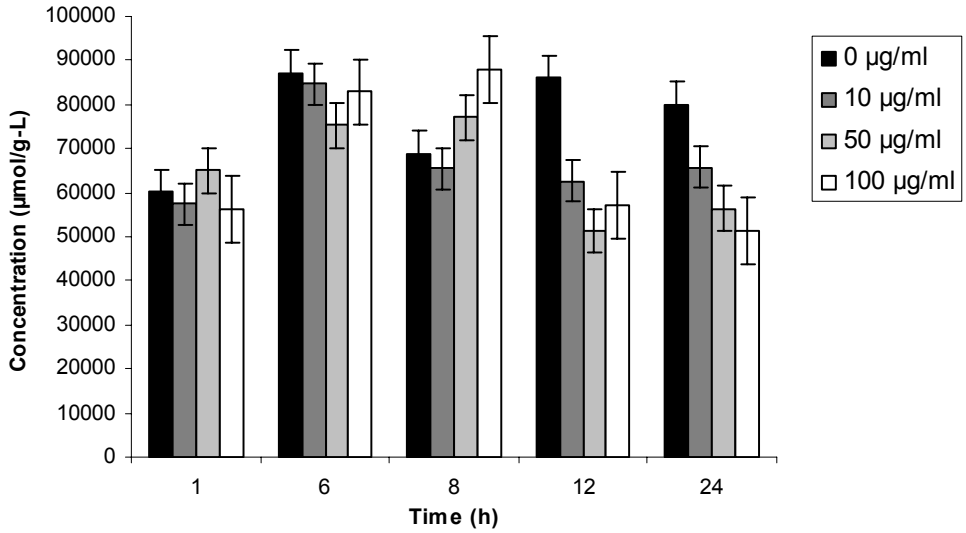

Fig. A-40. Carprofen A potassium concentrations. Potassium concentration in hepatic slice supernatant during 24 hours of incubation in media containing 0, 10, 50 and 100 µg/ml carprofen. Potassium concentrations are an average of two samples per time point for each concentration.

Figure A-41 depicts the potassium concentrations for slices incubated in carprofen B. A significant difference was detected across time (df=5, F-ratio=16.685, p-value=0.000). The potassium values at 0 hour were higher than those at 1, 4, 8, 12 and 24 hours. At 24 hours, the concentrations were higher than at 1 hour. Table A-24 illustrates the homogeneous groups for time.

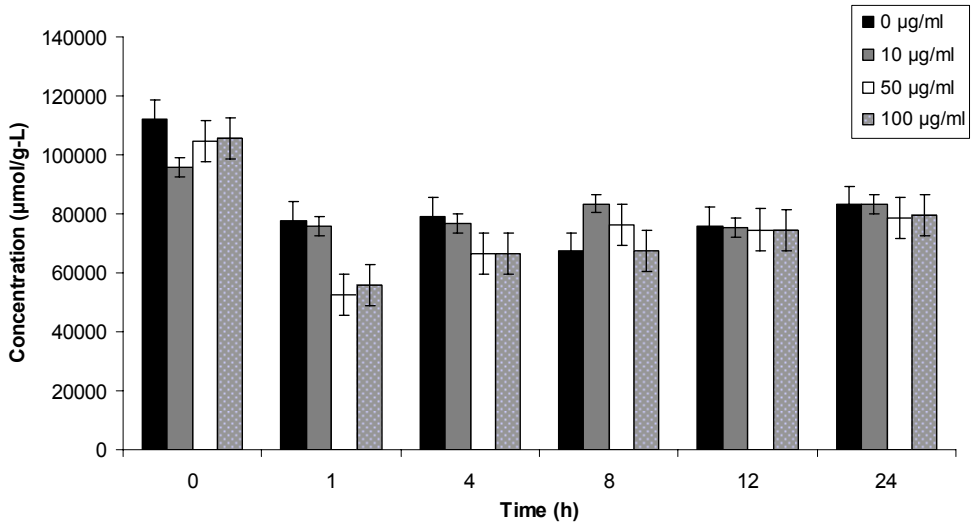

Fig. A-41. Carprofen B potassium concentrations. Potassium concentration in hepatic slice supernatant during 24 hours of incubation in media containing 0, 10, 50 and 100 µg/ml carprofen. Potassium concentrations are an average of two samples per time point for each concentration.

Table A-24. Carprofen B potassium analysis for time. Homogeneous groups for potassium two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (h)	Homogeneous groups		
0	a		
1		b	
4		b	c
8		b	c
12		b	c
24			c

Figure A-42 shows the potassium concentrations for slices incubated in carprofen C. The two-way ANOVA for time and concentration revealed a significant did th4hence across time (df=5, F-ratio=29.459, p-value=0.000). The concentrations at 0 hour were higher than those at 1, 4, 8, 12 and 24 hours. The concentrations at 1 hour were less 4han those at 4 and 24 hours. Table A-25 shows the homogenous groups for time.

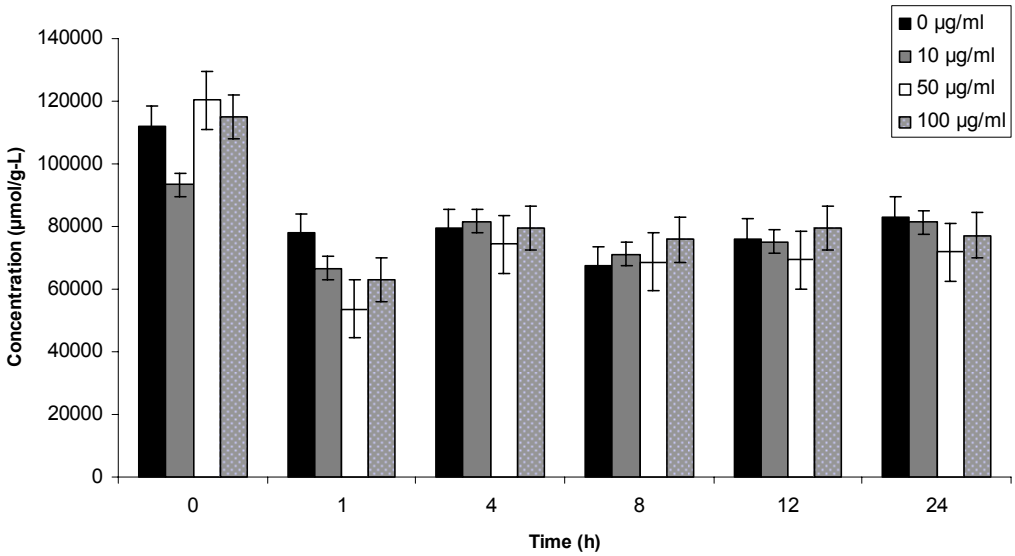

Fig. A-42. Carprofen C potassium concentrations. Potassium concentration in hepatic slice supernatant during 24 hourof incubation in media containing 0, 10, 50 and 100 µg/ml carprofen. Potassium concentrations are an average of two samples per time point for each concentration.

Table A-25. Carprofen C potassium analysis for time. Homogeneous groups for potassium two-way ANOVA for time are represented as letters. Tim%s with the same letter are not significantly different.

Time (h)	Homogeneous groups		
0	a		
1		b	
4			c
8		b	c
12		b	c
24			c

ATP

Figure A-43 shows the hepatic slice supernatant concentrations of ATP following incubation in carprofen A media for up to 24 hours. Two-way ANOVA of the concentrations revealed a significant factor interaction for time and concentration (df=12, F-ratio=5.840, p-value=0.000). Because of the significant factor interaction, it is difficult to assess the main effects. There were significant main effects for time (df=4, F-ratio=152.493, p-value=0.000) and concentration (df=3, F-ratio=11.420, p-value=0.000), and the information is stated in Tables A-26 and A-27.

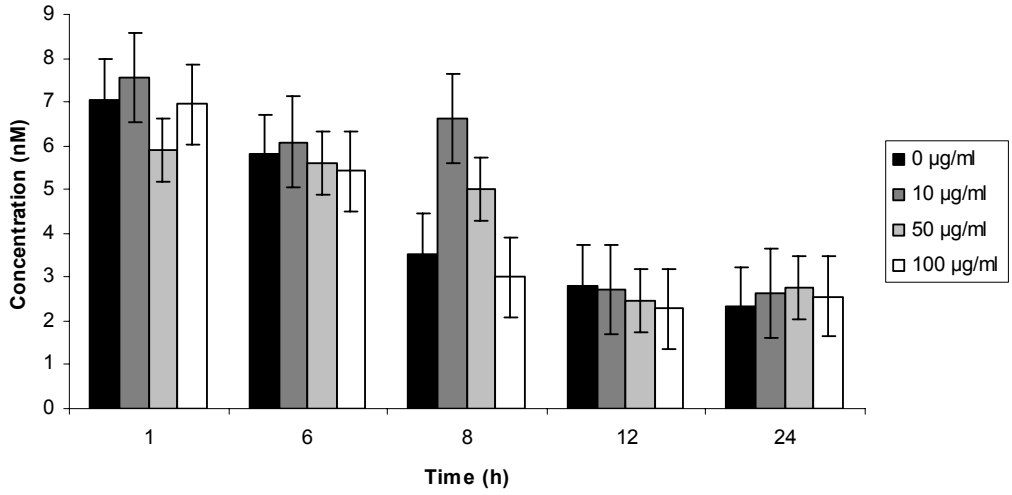

Fig. A-43. Carprofen A ATP concentrations. ATP concentration in hepatic slice supernatant during 24 hours of incubation in media containing 0, 10, 50 and 100 µg/ml carprofen. ATP concentrations are an average of two samples per time point for each concentration.

Table A-26. Carprofen A ATP analysis for time. Homogeneous groups for ATP two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different. ATP concentrations at 1 hour were higher than those at 6, 8, 12 and 24 hours. Concentrations at 6 hours were higher than at 8, 12 and 24 hours. Eight hour concentrations were higher than those at 12 and 24 hours. Note: significant factor interaction present.

Time (h)	Homogeneous groups
1	a
6	b
8	c
12	d
24	d

Table A-27. Carprofen A ATP analysis for concentration. Homogeneous groups for ATP two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different. ATP Concentrations for 10 µg/ml were higher than those for 0, 50 and 100 µg/ml. Note: significant factor interaction present.

Concentration (µg/ml)	Homogeneous groups
0	a
10	b
50	a
100	a

Figure A-44 represents the concentration of ATP in hepatic slice supernatant following incubation in carprofen B media. The two-way ANOVA for time and concentration demonstrated significant differences in ATP concentrations across time (df=5, F-ratio=20.392, p-value= 0.000) and concentration (df=3, F-ratio=9.378, p-value=0.000). For time, concentrations at 0 hour were less than those at 1, 4 and 8 hours. At 12 hours the concentrations were less than at 1, 4 and 8 hours. The same was true for 24 hour concentrations as they were lower than those at 1, 4 and 8 hours. For both 0 and 10 µg/ml, ATP

concentrations were higher than those at 50 and 100 µg/ml. Tables A-28 and A-29 display the homogeneous groups for time and concentration, respectively.

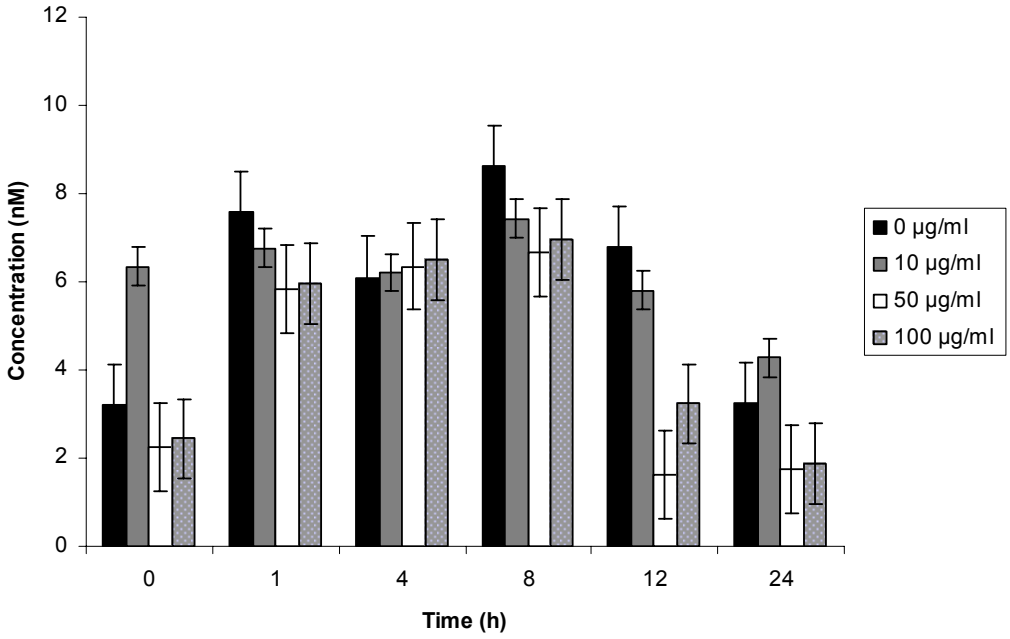

Fig. A-44. Carprofen B ATP concentrations. ATP concentration in hepatic slice supernatant during 24 hours of incubation in media containing 0, 10, 50 and 100 µg/ml carprofen. ATP concentrations are an average of two samples per time point for each concentration.

Table A-28. Carprofen B ATP analysis for time. Homogeneous groups for ATP two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (h)	Homogeneous groups	
0	a	
1		b
4		b
8		b
12	a	
24	a	

Table A-29. Carprofen B ATP analysis for concentration. Homogeneous groups for ATP two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Concentration (µg/ml)	Homogeneous groups	
0	a	
10	a	
50		b
100		b

Figure A-45 demonstrates the hepatic slice supernatant ATP concentrations following incubation in carprofen C media. Two-way ANOVA for time and concentration revealed significant diffences across time (df=5, F-ratio=7.552, p-value=0.000). ATP concentrations at 0 and 24 hours were lower than those at 1, 4 and 8 hours. Homogeneous groups for time are represented in Table A-30.

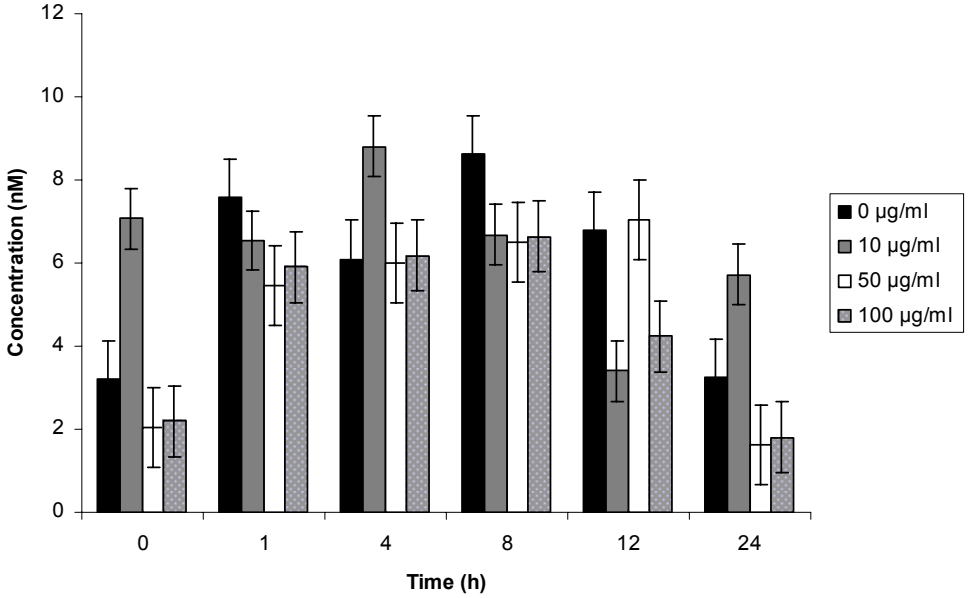

Fig. A-45. Carprofen C ATP concentrations. ATP concentration in hepatic slice supernatant during 24 hours /f incubation in media containing 0, 10, 50 and 100 µg/ml carprofen. ATP concentrations are an average of two samples per time point for each concentration.

Table A-30. Carprofen C ATP analysis for time. Homogeneous groups for ATP two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (h)	Homogeneous groups	
0	a	
1		b
4		b
8		b
12	a	b
24	a	

Histology

Hepatic slices were examined for extent of necrosis and extent and severity of vacuolation as indicators of toxicity. Slices were evaluated on a scale from 0 to 5. When one-way ANOVAs were run for carprofen A, B and C comparing lesion and concentration, only vacuolation extent for carprofen C revealed a significant difference across the media concentrations (df=3, F-ratio=3.736, p-value=0.028). The vacuolation extent lesion scores for 10 µg/ml carprofen C media were lower than those for 0 µg/ml carprofen C media (see Table A-31 for homogeneous groups).

Table A-31. Carprofen C vacuolation extent analysis for concentration. Homogeneous groups for vacuolation extent one-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Concentration (µg/ml)	Homogeneous groups
0	a
10	b
50	a b
100	a b

Carprofen with Phenobarbital Single Study Data

Media

Figure A-46 depicts the media concentrations of carprofen A following 24 hours incubation with phenobarbital. The two-way ANOVA for time and peak revealed significant differences across time for 10 (df=6, F-ratio=27.735, p-value=0.000), 50 (df=6, F-ratio=353.152, p-value=0.000) and 100 (df=6, F-ratio=195.505, p-value=0.000) µg/ml carprofen A media. Media concentrations for 10, 50 and 100 µg/ml carprofen were lower at 0 and 24 hours than at 25, 28, 32, 36 and 48 hours. Additionally for 50 µg/ml media, carprofen concentrations were lower at 48 hours than at 25, 32 and 36 hours. Tables A-32 through A-34 illustrate the homogeneous groups for time for 10, 50 and 100 µg/ml media.

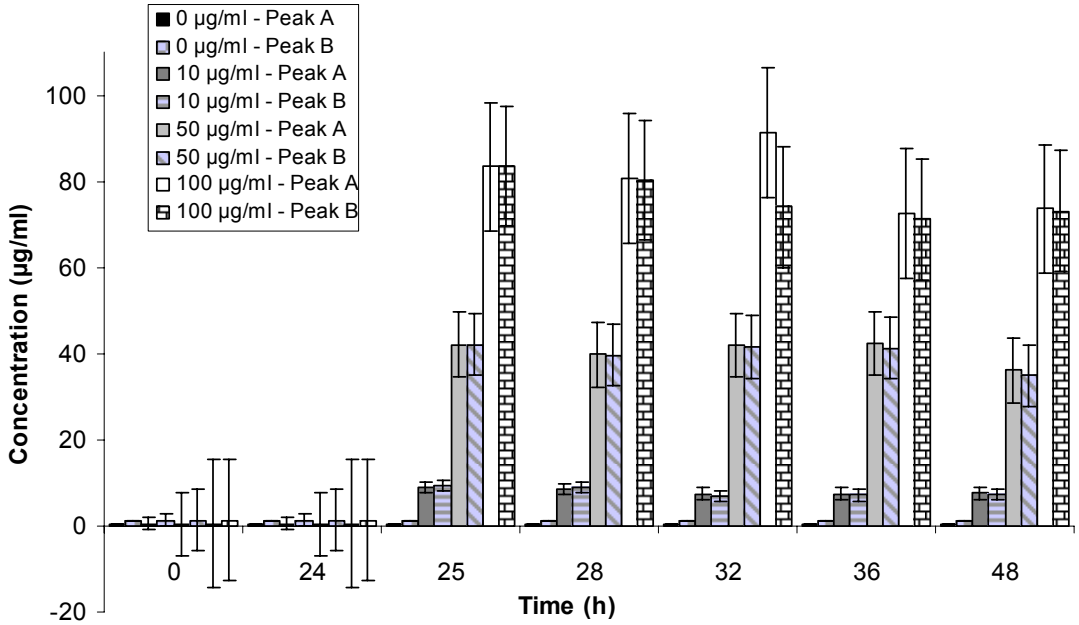

Fig. A-46. Carprofen A media concentrations following phenobarbital incubation. Concentrations of carprofen enantiomers in media after incubation for 24 hours with phenobarbital and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen A. The values are means of three samples per media concentration per time point.

Table A-32. 10 µg/ml carprofen A with phenobarbital incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		
0	a		
24	a		
25		b	
28		b	
32		b	
36		b	
48		b	

Table A-33. 50 µg/ml carprofen A with phenobarbital incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		
0	a		
24	a		
25		b	
28		b	c
32		b	
36		b	
48			c

Table A-34. 100 µg/ml carprofen A with phenobarbital incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups	
0	a	
24	a	
25		b
28		b
32		b
36		b
48		b

The media concentrations for carprofen B following phenobarbital incubation are shown in Figure A-47. Similarly to carprofen A, significant differences were detected among the concentrations for all media concentrations. Zero and 24 hour concentrations for 10, 50 and 100 µg/ml carprofen B media were lower than those at 25, 28, 32, 36 and 48 hours. For 50 µg/ml media, concentrations at 36 hours were lower than those at 25 and 28 hours. Tables A-35 through A-37 delineate the homogeneous groups for time for each media concentration.

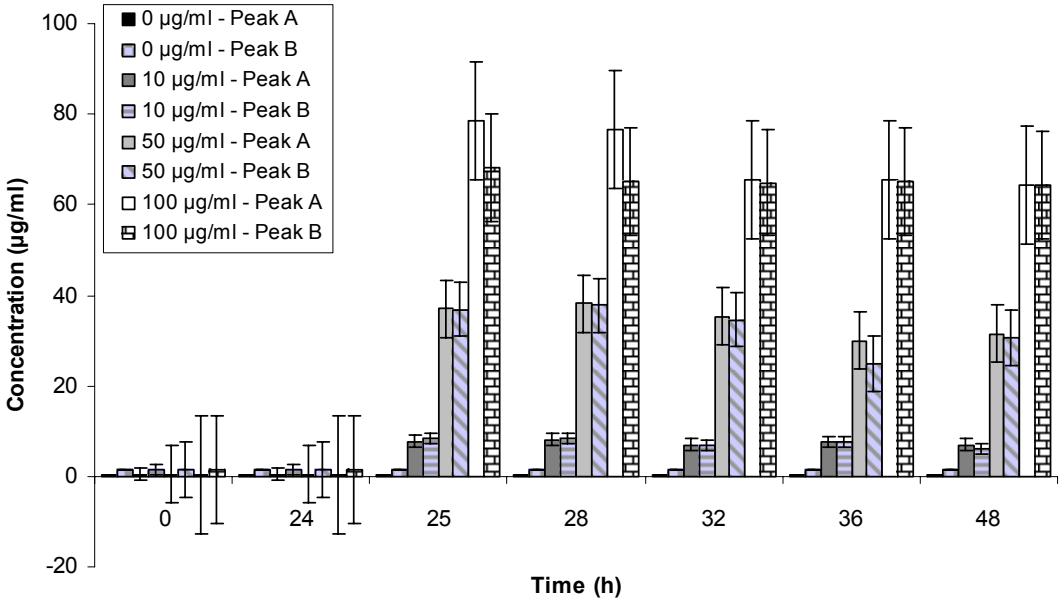

Fig. A-47. Carprofen B media concentrations following phenobarbital incubation. Concentrations of carprofen enantiomers in media after incubation for 24 hours with phenobarbital and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen B. The values are means of three samples per media concentration per time point.

Table A-35. 10 µg/ml carprofen B with phenobarbital incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		
0	a		
24	a		
25		b	
28		b	
32		b	
36		b	
48		b	

Table A-36. 50 µg/ml carprofen B with phenobarbital incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		
0	a		
24	a		
25		b	
28		b	
32		b	c
36			c
48		b	c

Table A-37. 100 µg/ml carprofen B with phenobarbital incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups	
0	a	
24	a	
25		b
28		b
32		b
36		b
48		b

Carprofen C media concentrations following phenobarbital incubation are displayed in Figure A-48. The two-way ANOVA for time and peak revealed significant differences for time for 10 (df=6, F-ratio=55.096, p-value=0.000), 50 (df=6, F-ratio=117.041, p-value=0.000) and 100 (df=6, F-ratio=333.296, p-value=0.000) µg/ml media. For these media, carprofen concentrations at 0 and 24 hours were less than those at 25, 28, 32, 36 and 48 hours. Additional differences were noted for 10 and 100 µg/ml media. Thirty-two hour carprofen concentrations for 10 µg/ml were lower than those at 25 and 28 hours but higher than those at 36 hours. Twenty-five hour carprofen concentrations for 100 µg/ml media were higher than 36 hour concentrations, and 28 hour concentrations were higher than those at 32, 36 and 48 hours. Tables A-38 through A-40 show the homogeneous groups for time for carprofen C media.

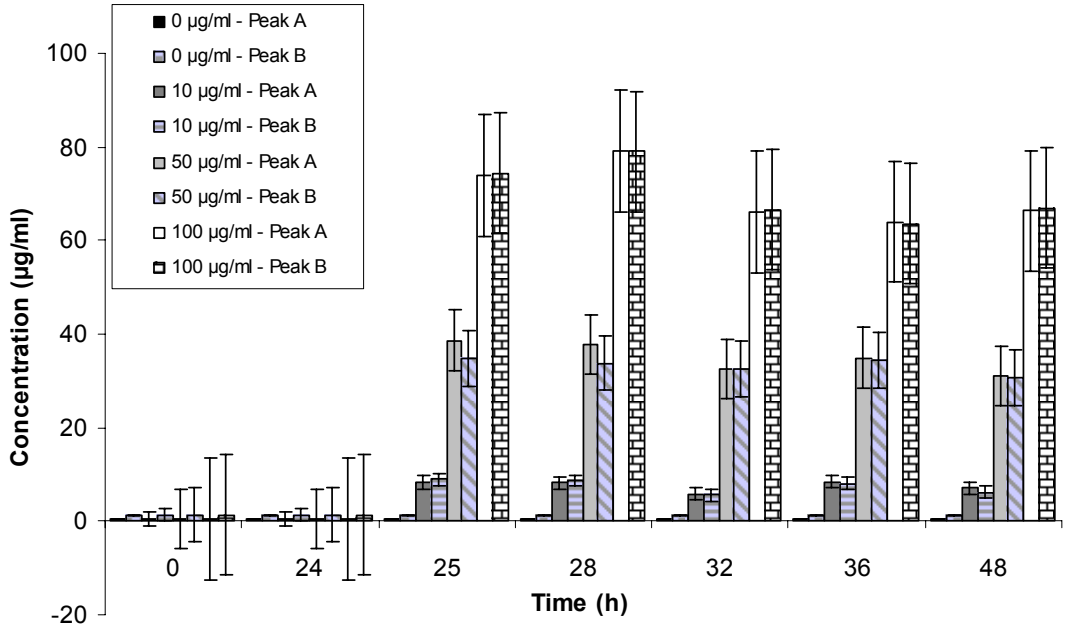

Fig. A-48. Carprofen C media concentrations following phenobarbital incubation. Concentrations of carprofen enantiomers in media after incubation for 24 hours with phenobarbital and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen C. The values are means of three samples per media concentration per time point.

Table A-38. 10 µg/ml carprofen C with phenobarbital incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		
0	a		
24	a		
25		b	
28		b	
32			c
36		b	
48		b	c

Table A-39. 50 µg/ml carprofen C with phenobarbital incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups	
0	a	
24	a	
25		b
28		b
32		b
36		b
48		b

Table A-40. 100 µg/ml carprofen C with phenobarbital incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		
0	a		
24	a		
25		b	c
28			c
32		b	d
36			d
48		b	d

*Supernatan*t

Carprofen A concentrations in hepatic slice supernatant after incubation with phenobarbital are sho7n in Figure A-49. The two-way ANOVA for time and peak revealed significant differences for time and peak for slices incubated in 50 (time: df=6, F-ratio=247.024, p-value=0.000; peak: df=1, F=45.692, p-value=0.000) and 100 (time: df=6, F-ratio=113.798, p-value=0.000; peak: df=1, F=11.493, p-value=0.004) µg/ml media. There was a significant time-peak interaction for 50 µg/ml media; the interaction appears to be orderly so examination of main effects is reasonable. At 0, 24 and 25 hours the supernatant concentrations of 50 µg/ml media were lower than those at 28, 32, 36 and 48 hours. At 28 hours, supernatant concentrations were lower than at 32, 36 and 48 hours. At 32 hours the concentrations were lower than at 36 and 48 hours, and at 36 hours supernatant concentrations were less than at 48 hours. For 100 µg/ml media, supernatant concentrations at 0 and 24 hours were lower than at 25, 28, 32, 36 and 48 hours. At 25 hours, the supernatant concentrations were lower than at 28, 32, 36 and 48 hours; at 28 hours supernatant concentrations of carprofen were lower than at 36 hours. For both 50 and 100 µg/ml, peak B

concentrations were higher than for peak A. Tables A-41 and A-42 show the homogeneous groups for time for 50 and 100 µg/ml media.

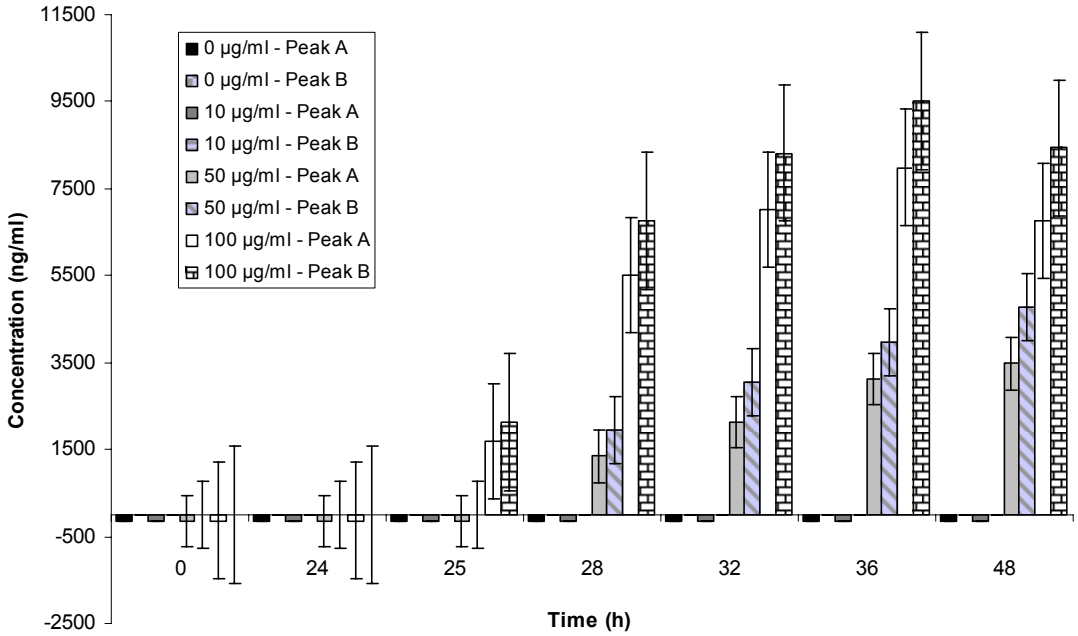

Fig. A-49. Carprofen A concentrations in hepatic slice supernatant following phenobarbital incubation. Concentrations of carprofen enantiomers in hepatic slice supernatant after incubation for 24 hours with phenobarbital and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen A. The values are an average of two samples per media concentration per time point.

Table A-41. 50 µg/ml carprofen A hepatic slice supernatant with phenobarbital incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups
0	a
24	a
25	a
28	b
32	c
36	d
48	e

Table A-42. 100 µg/ml carprofen A hepatic slice supernatant with phenobarbital incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups			
0	a			
24	a			
25		b		
28			c	
32			c	d
36				d
48			c	d

Figure A-50 displays the concentrations of carprofen B in the slice supernatant following incubation in phenobarbital with additional incubation in carprofen B. Similar to the results of analysis for supernatant concentrations for carprofen A (fig. 34), the supernatant concentrations for carprofen B showed significant differences across time and between peaks for 50 (time: df=6, F-ratio=407.840, p-value=0.000; peak: df=1, F-ratio=133.464), p-value=0.000) and 100 (time: df=6, F-ratio=229.723, p-value=0.000; peak: df=1, F-ratio=31.634, p-value=0.000) µg/ml. As seen with 50 µg/ml carprofen A, there was a significant and orderly significant interaction (df=6, F-ratio=10.613, p-value=0.000) between the factors time and peak. The concentrations for peak B were higher than those for peak A for 50 and 100 µg/ml media. For 50 µg/ml media, carprofen supernatant concentrations at 0 and 24 hours were less than those at 25, 28, 32, 36 and 48 hours. At 25 hours, the supernatant concentrations were less than at 28, 32, 36 and 48 hours. Twenty-eight hour concentrations were lower than 32, 36 and 48 hour concentrations. Thirty-two hour concentrations were lower than at 36 and 48 hours, and concentrations at 36 hours were less than those at 48 hours.

For 100 µg/ml media, supernatant concentrations of carprofen were lower at 0 and 24 hours than at 25, 28, 32, 36 and 48 hours. Twenty-five hour concentrations were lower than 28, 32, 36 and 48 hour concentrations; concentrations at 28 hours were lower than at 32, 36 and 48 hours. Tables A-43 and A-44 show the homogeneous groups for time for 50 and 100 µg/ml media.

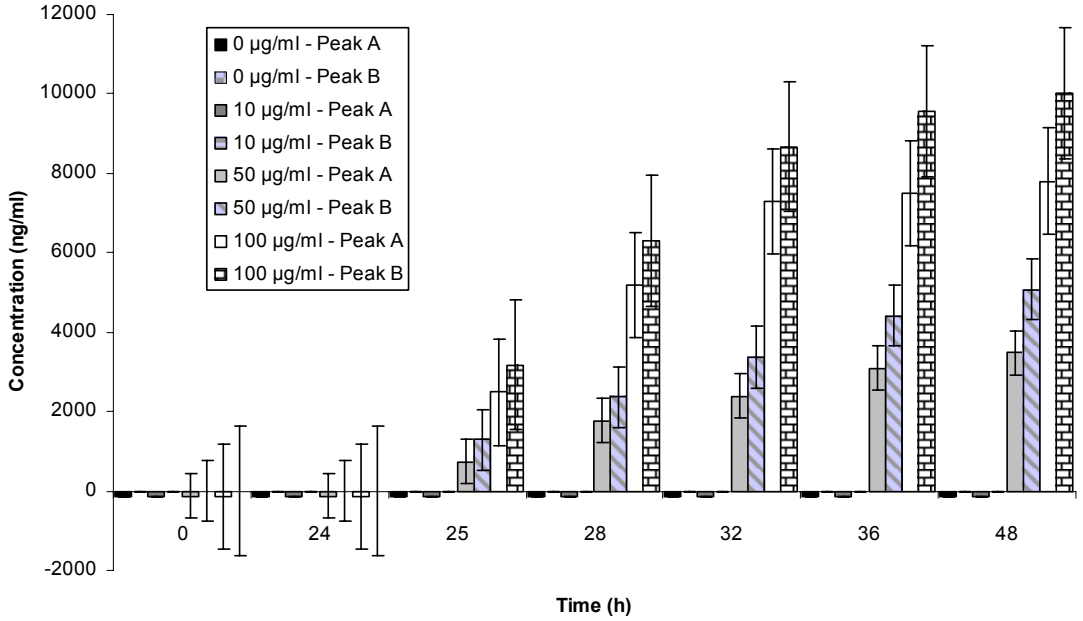

Fig. A-50. Carprofen B concentrations in hepatic slice supernatant following phenobarbital incubation. Concentrations of carprofen enantiomers in hepatic slice supernatant after incubation for 24 hours with phenobarbital and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen B. The values are an average of two samples per media concentration per time point.

Table A-43. 50 µg/ml carprofen B hepatic slice supernatant with phenobarbital incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups
0	a
24	a
25	b
28	c
32	d
36	e
48	f

Table A-44. 100 µg/ml carprofen B hepatic slice supernatant with phenobarbital incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups
0	a
24	a
25	b
28	c
32	d
36	d
48	d

The supernatant concentrations of carprofen C following phenobarbital incubation are illustrated in Figure A-51. Significant differences were noted in the two-way ANOVA of time and peak for 50 (time: df=6, F-ratio=115.672, p-value=0.000; peak: df=1, F-ratio=284.134, p-value=0.000) and 100 (time: df=6, F-ratio=656.537, p-value=0.000; peak: df=1, F=11.988, p-value=0.004) µg/ml media. For 50 and 100 µg/ml media, the significant differences for time and peak were the same as for carprofen B. Concentrations of peak B were higher than those of peak and the homogeneous groups were the same as seen in tables 60 and 61. There was also a significant factor interaction (df=6, F-ratio=20.479, p-value=0.000) for 50 µg/ml carprofen C as there was for 50 µg/ml carprofen B.

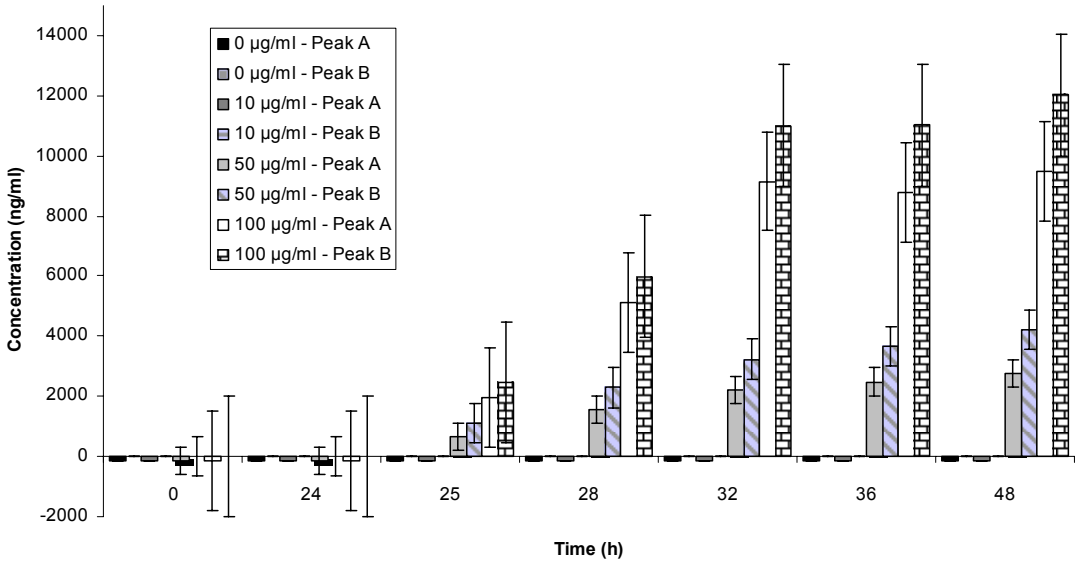

Fig. A-51. Carprofen C concentrations in hepatic slice supernatant following phenobarbital incubation. Concentrations of carprofen enantiomers in hepatic slice supernatant after incubation for 24 hours with phenobarbital and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen C. The values are an average of two samples per media concentration per time point.

Potassium

Potassium concentrations for slices incubated in phenobarbital followed by carprofen A are shown in Figure A-52. The two-way ANOVA for time and concentration revealed a significant difference across time (df=6, F-ratio=21.184, p-value=0.000) and a significant factor interaction (df=18, F-ratio=3.058, p-value=0.004). Because the interaction is disorderly, the main effects can be obscured.

Figure A-53 depicts the potassium concentrations of slices incubated in phenobarbital followed by carprofen B. Two-way ANOVA of time and concentrations expressed a significant across time (df=6, F-ratio=23.406, p-value=0.000). Concentrations at 0 hour were higher than those at 24, 25, 28, 32, 36 and 48 hours; concentrations at 24 hours were higher than those at 25 hours. Table A-45 depicts the homogeneous groups for time.

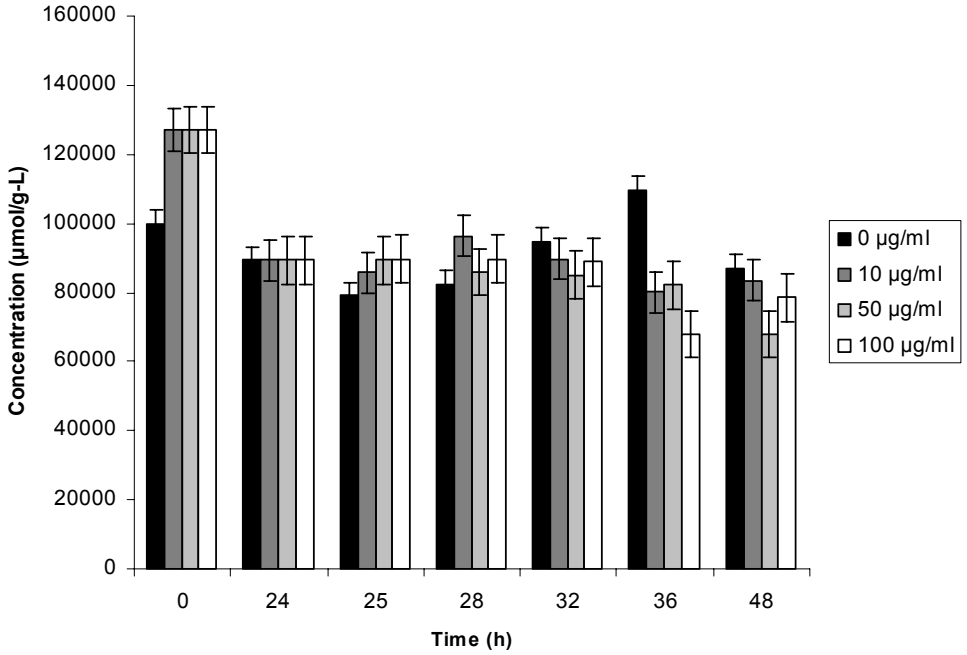

Fig. A-52. Carprofen A potassium concentrations following phenobarbital incubation. Potassium concentration in hepatic slice supernatant after incubation for 24 hours with phenobarbital and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen A. The values are an average of two samples per media concentration per time point.

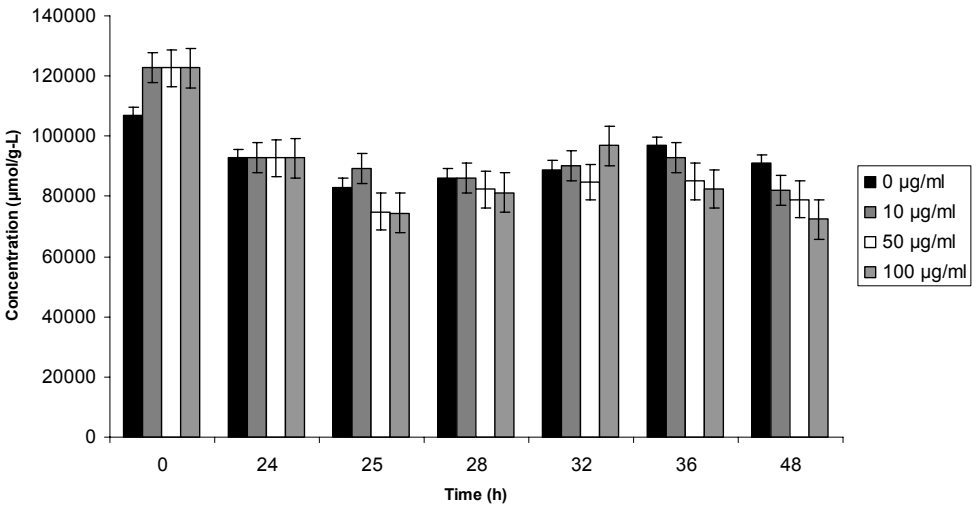

Fig. A-53. Carprofen B potassium concentrations following phenobarbital incubation. Potassium concentration in hepatic slice supernatant after incubation for 24 hours with phenobarbital and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen B. The values are an average of two samples per media concentration per time point.

Table A-45. Carprofen B with phenobarbital potassium analysis for time. Homogeneous groups for potassium two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (h)	Homogeneous groups		
0	a		
24		b	
25			c
28		b	c
32		b	c
36		b	c
48		b	c

Potassium concentrations for slices incubated in phenobarbital followed by carprofen C are shown in Figure A-54. Again, two-way ANOVA revealed a significant difference for time (df=6, F-rati13.635, p-value=0.000). Concentrations at 0 hour were higher than those for all other hours. Homogeneous groups are shown in Table A-46.

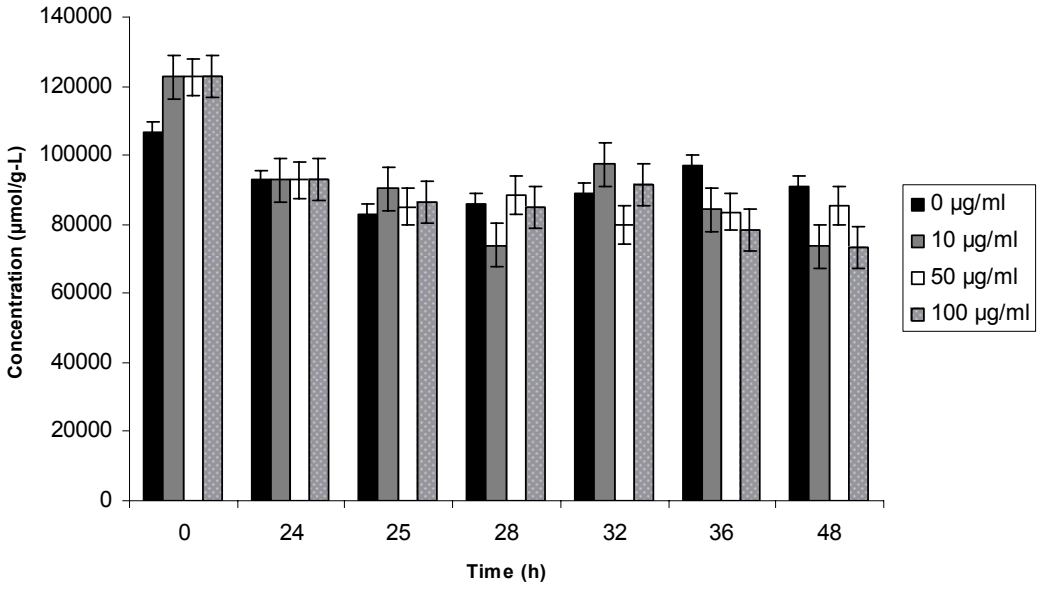

Fig. A-54. Carprofen C potassium concentrations following phenobarbital incubation. Potassium concentration in hepatic slice supernatant after incubation for 24 hou2s with phenobarbital and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen C. The values are an average of two samples per media concentration per time point.

Table A-46. Carprofen C with phenobarbital potassium analysis for time. Homogeneous groups for potassium two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (h)	Homogeneous groups
0	a
24	b
25	b
28	b
32	b
36	b
48	b

ATP

The ATP concentrations for slices incubated in phenobarbital followed by carprofen A are depicted in Figure A-55. The two-way ANOVA revealed significant differences across time (df=6, F-ratio=6.435, p-value=0.000) but not across media concentrations. The ATP concentrations at 0 and 24 hours were higher than those at 32 and 36 hours. At 25 hours, the ATP concentrations were higher than those at 32, 36 and 48 hours. Table A-47 shows the homogeneous groups for time.

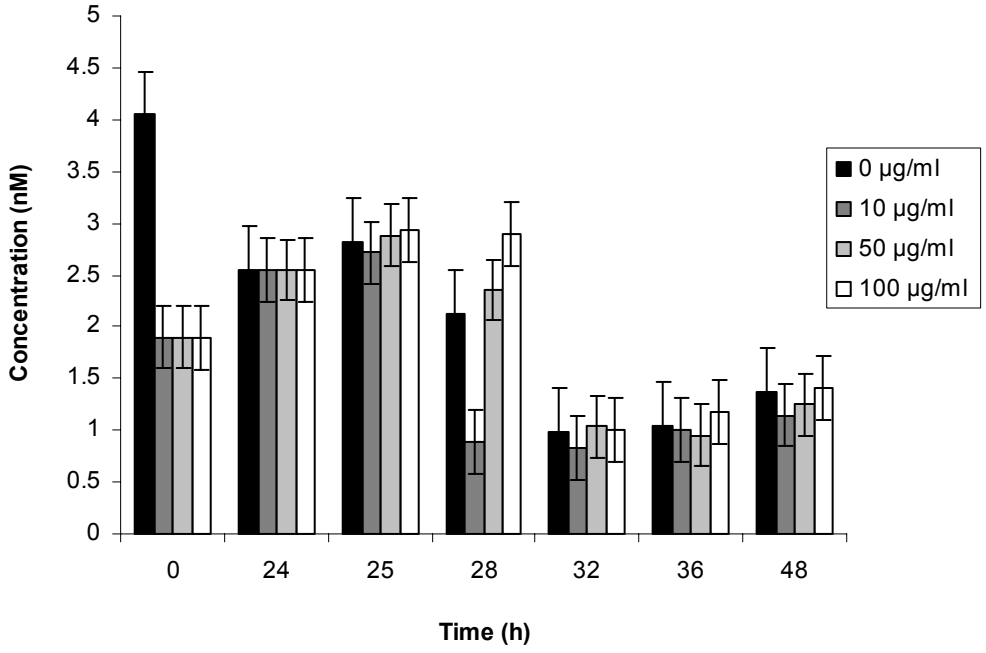

Fig. A-55. Carprofen A ATP concentrations following phenobarbital incubation. ATP concentration in hepatic slice supernatant after incubation for 24 hours with phenobarbital and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen A. The values are an average of two samples per media concentration per time point.

Table A-47. ATP analysis for time of carprofen A with phenobarbital. Homogeneous groups for ATP two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (h)	Homogeneous groups		
0	a	b	
24	a	b	
25	a		
28	a	b	c
32			c
36			c
48		b	c

The ATP concentrations for slices incubated in phenobarbital with subsequent carprofen B incubation are represented in Figure A-56. A significant factor interaction was revealed by two-way ANOVA. The interaction (df=18, F-ratio=3.456, p-value=0.002) was disorderly, and a main effect for time (df=6, F-ratio=6.407, p-value=0.000) was noted.

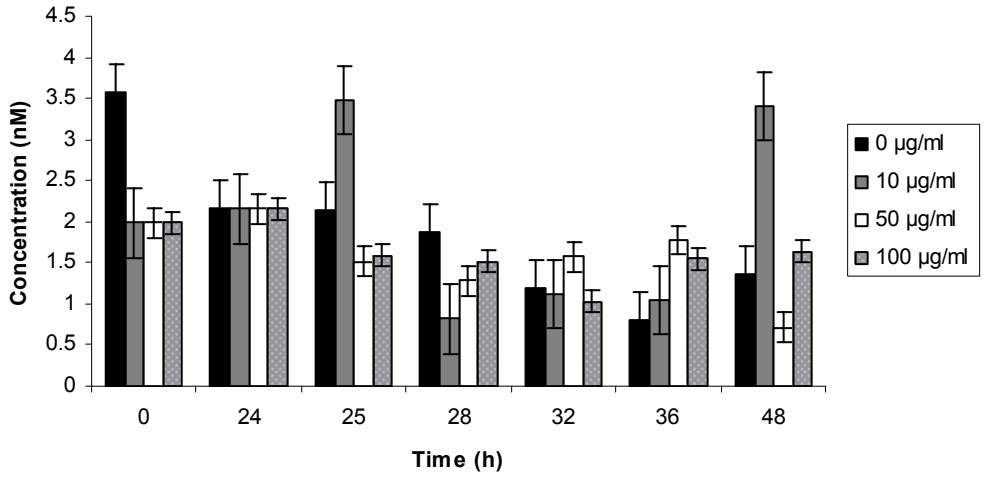

Fig. A-56. Carprofen B ATP concentrations following phenobarbital incubation. ATP concentration in hepatic slice supernatant after incubation for 24 hours with phenobarbital and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen B. The values are an average of two samples per media concentration per time point.

Figure A-57 displays the ATP concentrations for slices incubated in phenobarbital followed with carprofen C incubation. Two-way ANOVA revealed no significant differences for time or concentration.

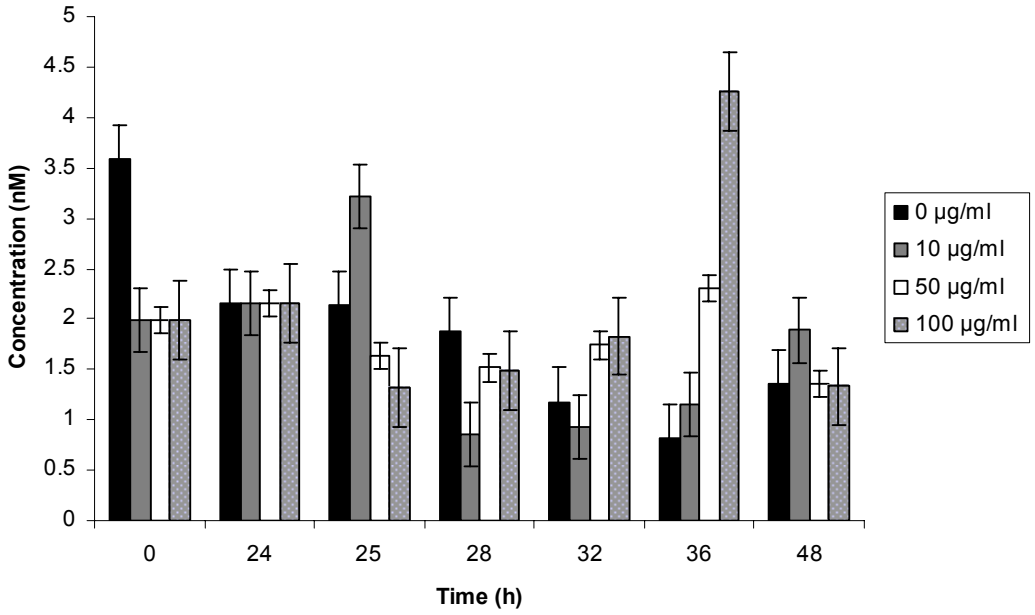

Fig. A-57. Carprofen C ATP concentrations following phenobarbital incubation. ATP concentration in hepatic slice supernatant after incubation for 24 hours with phenobarbital and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen C. The values are an average of two samples per media concentration per time point.

Histology

For the phenobarbital-carprofen A slices, one-way ANOVA of vacuolation extent revealed a significant difference among media concentrations (df=3, F-ratio=4.681. p=0.016). Slices incubated in media containing 100 µg/ml carprofen A had lower lesion scores than those incubated in 0 and 10 µg/ml media. A significant difference was also noted for one-way ANOVA of 6acuolation severity (df=3, F-ratio=3.421, p-value=0.043). Lesion scores for slices incubated in 100 µg/ml media were lower than those of 0 µg/ml media. Tables A-48 and A-49 demonstrate the homogeneous groups for vacuolation extent and severity.

Table A-48. Vacuolation extent analysis for concentration of carprofen A with phenobarbital. Homogeneous groups for vacuolation extent one-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Concentration (µg/ml)	Homogeneous groups
0	a
10	a
50	a b
100	b

Table A-49. Vacuolation severity analysis for concentration of carprofen A with phenobarbital. Homogeneous groups for vacuolation severity one-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Concentration (µg/ml)	Homogeneous groups
0	a
10	a b
50	a b
100	b

Similar findings were noted for slices incubated in phenobarbital followed by carprofen B. Significant differences were seen for one-way ANOVA of vacuolation extent (df=3, F-ratio=10.364, p-value=0.000) and vacuolation severity (df=3, F-ratio=6.370, p-value=0.005). For vacuolation extent, lesion scores associated with 100 µg/ml media were lower than those at 0, 10 and 50 µg/ml. The lesion scores for vacuolation severity were lower at 100 µg/ml than at 0 or 10 µg/ml. Tables A-50 and A-51 show the homogeneous groups for one-way ANOVA of vacuolation extent and vacuolation severity.

Table A-50. Vacuolation extent analysis for concentration of carprofen B with phenobarbital. Homogeneous groups for vacuolation extent one-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Concentration (µg/ml)	Homogeneous groups
0	a
10	a
50	a
100	b

Table A-51. Vacuolation severity analysis for concentration of carprofen B with phenobarbital. Homogeneous groups for vacuolation severity one-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Concentration (µg/ml)	Homogeneous groups
0	a
10	a
50	a b
100	b

Carprofen with Cimetidine Single Study Data

Media

Media concentrations of carprofen A after incubation with cimetidine followed by carprofen A are shown in Figure A-58. Two-way ANOVA for time and peak revealed significant differences across time for 10 (df=6, F-ratio=307.422, p=0.000), 50 (df=6, F=2282.393, p-value=0.000) and 100 (df=6, F-ratio=337.555, p-value=0.000) µg/ml media and between peaks (df=1, F=11.859, p-value=0.002) for 10 µg/ml media.

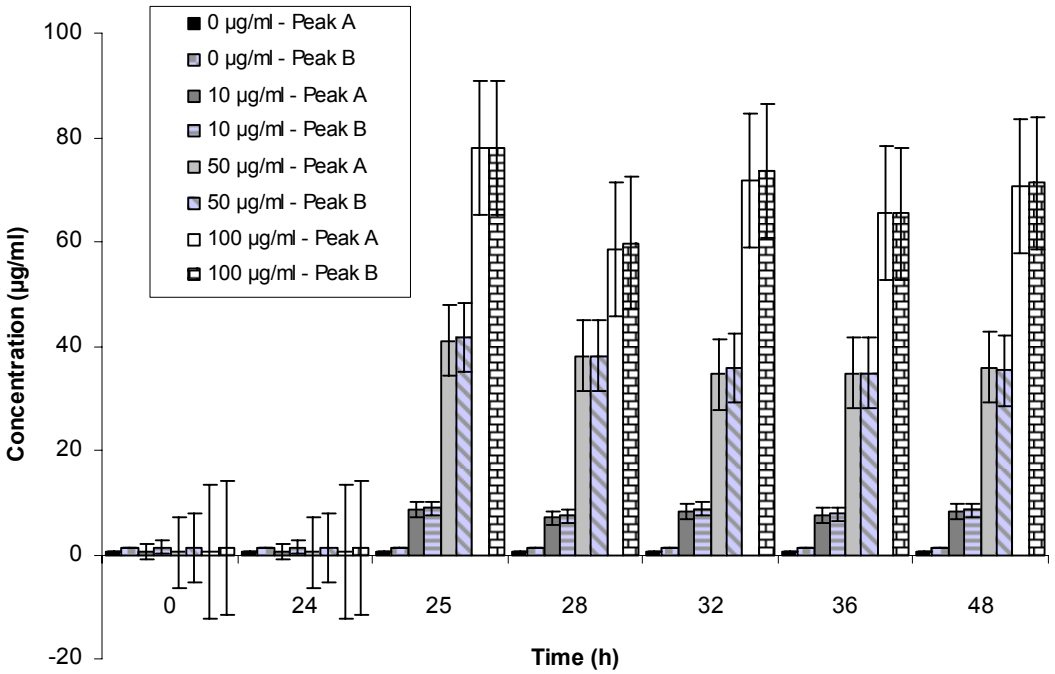

Fig. A-58. Carprofen A media concentrations following cimetidine incubation. Concentrations of carprofen enantiomers in media after incubation for 24 hours with cimetidine and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen A. The values are means of three samples per media concentration per time point.

For 10 µg/ml media, 0 and 24 hour concentrations were lower than those at 25, 28, 32, 36 and 48 hours. At 25 hours, carprofen concentrations were higher than at 28 and 36 hours, and 28 hour concentrations were less than those at 32 and 48 hours. Peak B concentrations were higher than those for peak A. Zero and 24 hour carprofen concentrations for 50 µg/ml media were lower than those at 25, 28, 32, 36 and 48 hours. Concentrations at 25 hours were higher than at 28, 32, 36 and 48 hours, and 28 hour concentrations were higher than those at 32, 36 and 48 hours. The pairwise differences across time for 100 µg/ml media were the same as for 10 µg/ml media. Tables A-52 through A-54 depict the homogeneous groups for 10, 50 and 100 µg/ml media.

Table A-52. 10 µg/ml carprofen A with cimetidine incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		
0	a		
24	a		
25		b	
28		b	c
32		b	c
36			c
48		b	

Table A-53. 50 µg/ml carprofen A with cimetidine incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups			
0	a			
24	a			
25		b		
28			c	
32				d
36				d
48				d

Table A-54. 100 µg/ml carprofen A with cimetidine incubation analysis for time. Homogeneous groups for 4wo-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups			
0	a			
24	a			
25		b		
28			c	
32		b		d
36			c	d
48		b		d

Figure A-59 displays the media concentrations of carprofen after incubation with cimetidine followed by carprofen B. The two-way ANOVA for time and peak revealed significant differences for time for 10 (df=6, F-ratio=60.934, p-value=0.000), 50 (df=6, F-ratio=198.658, p-value=0.000) and 100 (df=6, F-ratio=139.613, p-value=0.000) µg/ml media. Ten µg/ml carprofen concentrations at 0 and 24 hours were lower than those at 25, 28, 32, 36 and 48 hours. Concentrations at 28 and 32 hours were less than those at 48 hours. For 50 and 100 µg/ml media, 0 and 24 hour concentrations were less than 25, 28, 32, 36 and 48 hour concentrations. Tables A-55 through A-57 denote the homogeneous groups for each media concentration.

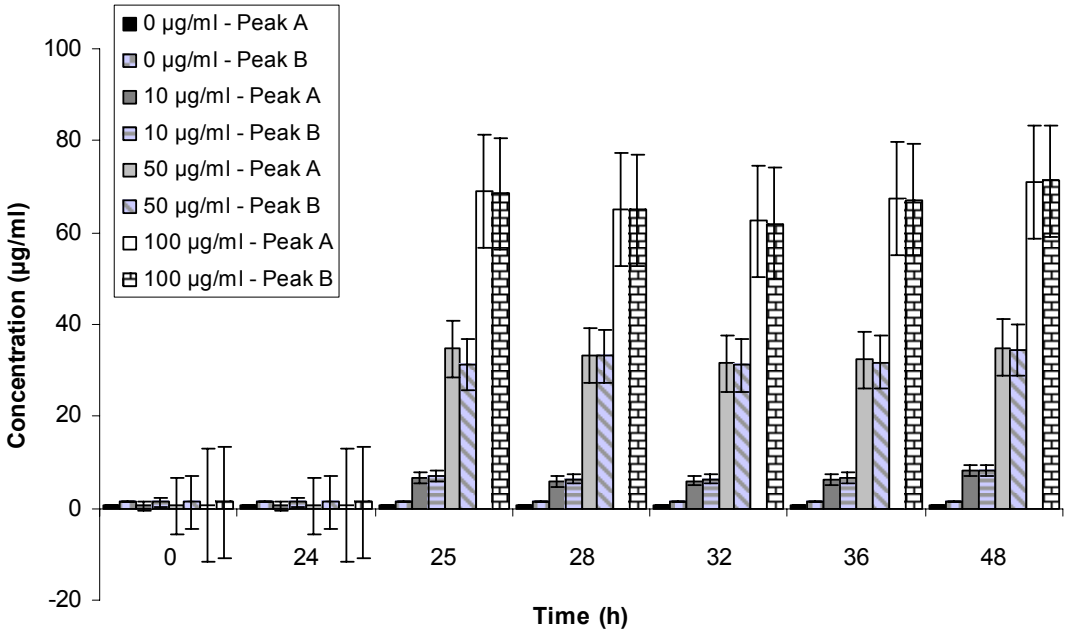

Fig. A-59. Carprofen B media concentrations following cimetidine incubation. Concentrations of carprofen enantiomers in media after incubation for 24 hours with cimetidine and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen B. The values are means of three samples per media concentration per time point.

Table A-55. 10 µg/ml carprofen B with cimetidine incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		
0	a		
24	a		
25		b	c
28		b	
32		b	
36		b	c
48			c

Table A-56. 50 µg/ml carprofen B with cimetidine incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups
0	a
24	a
25	b
28	b
32	b
36	b
48	b

Table A-57. 100 µg/ml carprofen B with cimetidine incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups	
0	a	
24	a	
25		b
28		b
32		b
36		b
48		b

The carprofen C media concentrations for slices incubated in cimetidine followed by carprofen C are shown in Figure A-60. Again, two-way ANOVA showed significant differences across time for 10 (df=6, F-ratio=48.922, p-value=0.000), 50 (df=6, F-ratio=241.616, p-value=0.000) and 100 (df=6, F-ratio=504.113, p-value=0.000) µg/ml media. Pairwise differences were the same for 10 and 100 µg/ml media. For those media, 0 and 24 hour carprofen concentrations were lower than at 25, 28, 32, 36 and 48 hours, and 36 hour concentrations were lower than 25 and 48 hour concentrations. For 50 µg/ml media, 0 and 24 hour concentrations were lower than 25, 28, 32, 36 and 48 hour concentrations. Additionally, 25 hour concentrations were higher than at 32 and 36 hours, and 36 hour concentrations were lower than at 28 and 48 hours. Tables A-58 through A-60 illustrate the homogeneous groups for time for each media concentration.

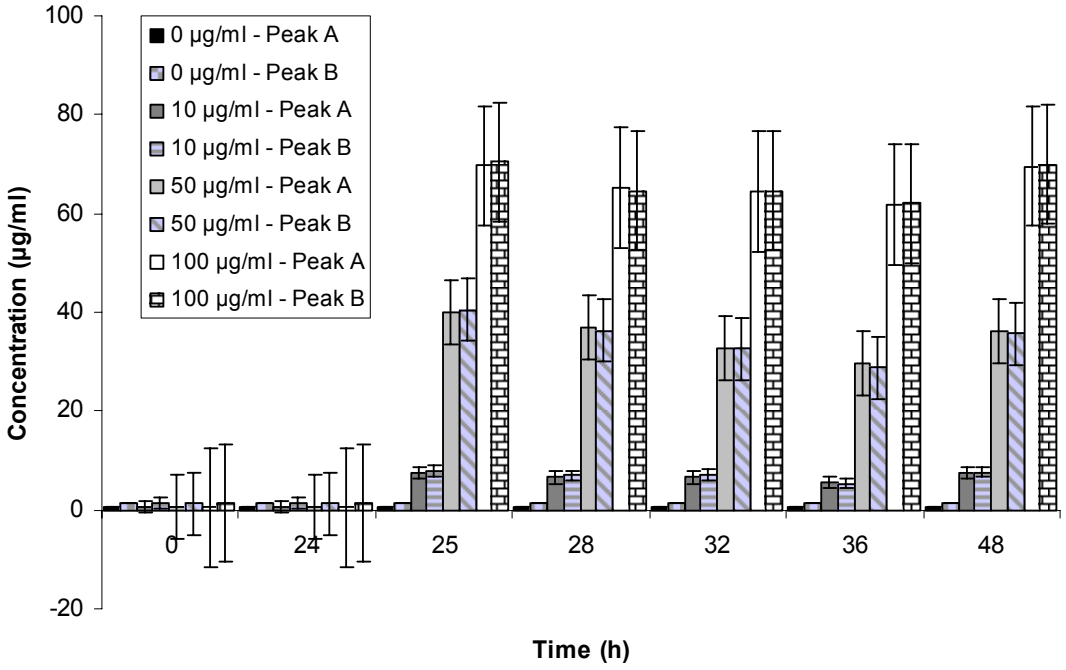

Fig. A-60. Carprofen C media concentrations following cimetidine incubation. Concentrations of carprofen enantiomers in media after incubation for 24 hours with cimetidine and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen C. The values are means of three samples per media concentration per time point.

Table A-58. 10 µg/ml carprofen C with cimetidine incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		
0	a		
24	a		
25		b	
28		b	c
32		b	c
36			c
48		b	

Table A-59. 50 µg/ml carprofen C with cimetidine incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups			
0	a			
24	a			
25		b		
28		b	c	
32			c	d
36				d
48		b	c	

Table A-60. 100 µg/ml carprofen C with cimetidine incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		
0	a		
24	a		
25		b	
28		b	c
32		b	c
36			c
48		b	

Supernatant

Carprofen A concentrations in hepatic slice supernatant after cimetidine incubation followed by incubation in carprofen A are shown in Figure A-61. The two-way ANOVA for time and peak revealed a significant difference across time for 50 (df=4, F-ratio=49.914, p=0.000) and 100 (df=4, F-ratio=4.807, p-value=0.020) µg/ml media and between peaks (df=1, F-ratio=40.202, p-value=0.000) for 50 µg/ml media. For 50 µg/ml media, carprofen supernatant concentrations at 25 and 28 hours were less than those at 32, 36 and 48 hours. The supernatant concentrations at 36 hours were less than those at 48 hours. Peak B concentrations were higher than peak A concentrations. Supernatant concentrations at 25 hours for 100 µg/ml media were lower tan 32 and 36 hour concentrations. Tables A-61 and A-62 show the homogeneous groups for time for 50 and 100 µg/ml media.

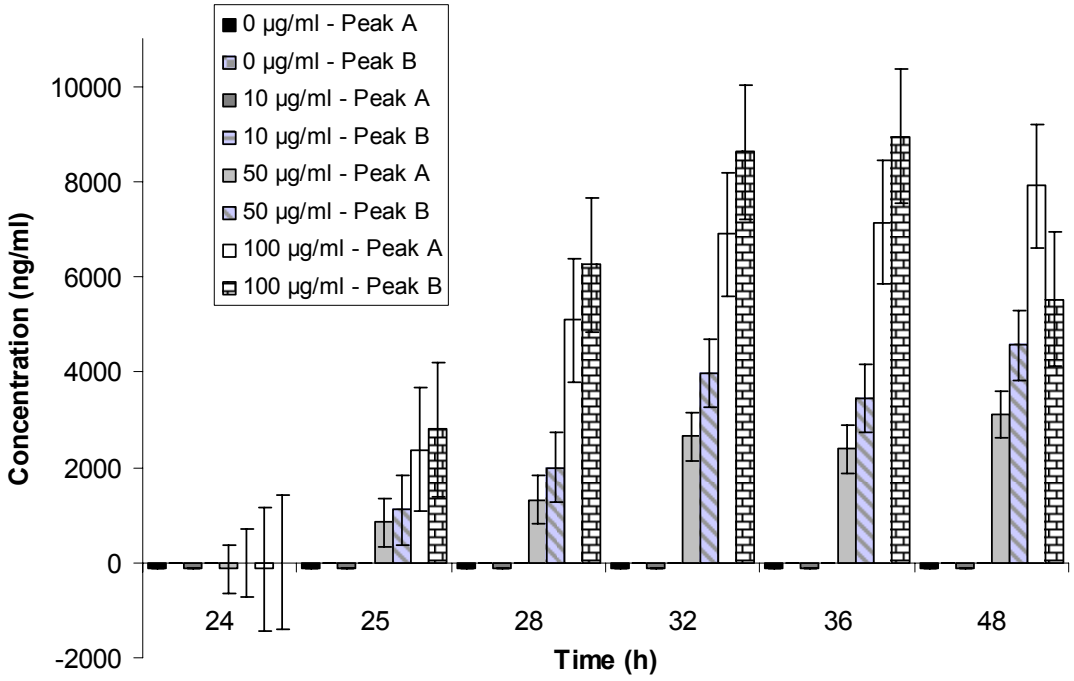

Fig. A-61. Carprofen A concentrations in hepatic slice supernatant following cimetidine incubation. Concentrations of carprofen enantiomers in hepatic slice supernatant after incubation for 24 hours with cimetidine and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen A. The values are an average of two samples per media concentration per time point.

Table A-61. 50 µg/ml carprofen A hepatic slice supernatant with cimetidine incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		
25	a		
28	a		
32		b	c
36		b	
48			c

Table A-62. 100 µg/ml carprofen A hepatic slice supernatant with cimetidine incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups	
25	a	
28	a	b
32		b
36		b
48	a	b

Figure A-62 displays the supernatant concentrations following cimetidine and carprofen B incubation. Significant differences by two-way ANOVA were seen for time (df=4, F-ratio=11.814, p-value=0.001) and peak (df=1, F-ratio=6.094, p-value=0.033) for 50 µg/ml media. Peak B concentrations were higher than peak A concentrations. Twenty-five hour supernatant concentrations were lower than those at 36 and 48 hours. Twenty-eight and 32 hour concentrations were lower than 48 hour concentrations. Table A-63 shows the homogeneous groups for time for 50 µg/ml media.

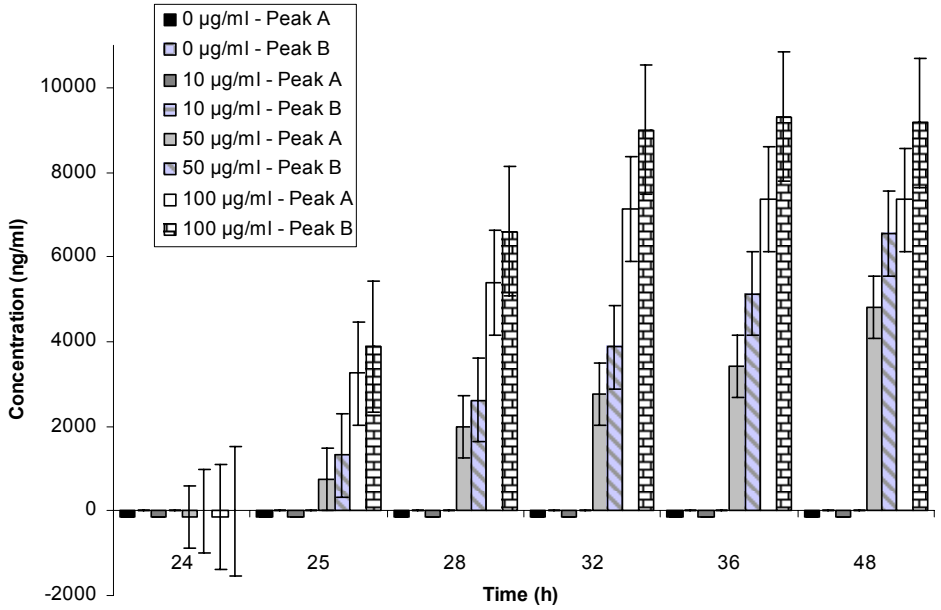

Fig. A-62. Carprofen B concentrations in hepatic slice supernatant following cimetidine incubation. Concentrations of carprofen enantiomers in hepatic slice supernatant after incubation for 24 hours with cimetidine and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen B. The values are an average of two samples per media concentration per time point.

Table A-63. 50 µg/ml carprofen B hepatic slice supernatant with cimetidine incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		
25	a		
28	a	b	
32	a	b	
36		b	c
48			c

Carprofen C supernatant concentrations following cimetidine and carprofen C incubation are displayed in Figure A-63. Two-way ANOVA for 50 µg/ml media revealed a difference across time (df=4, F-ratio=4.238, p-value=0.029). Twenty-five hour supernatant concentrations were less than those at 48 hours. For 100 µg/ml media, significant differences were noted for time (df=4, F-ratio=101.804, p-value=0.000) and peak (df=1, F-ratio=34.549, p-value=0.000). Supernatant concentrations for peak B were higher than those for peak A. For time, 25 hour supernatant concentrations were less than 28, 32, 36 and 48 hour concentrations. Twenty-eight hour concentrations were less than 32, 36 and 48 hour

supernatant concentrations. Tables A-64 and A-65 show the homogeneous groups for 50 and 100 µg/ml media.

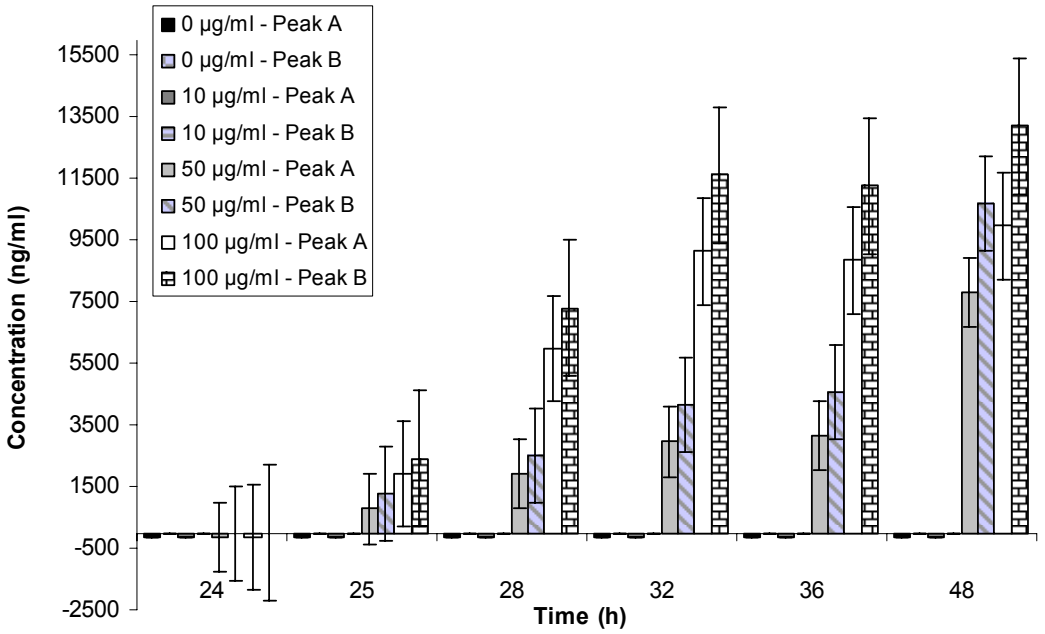

Fig. A-63. Carprofen C concentrations in hepatic slice supernatant following cimetidine incubation. Concentrations of carprofen enantiomers in hepatic slice supernatant after incubation for 24 hours with cimetidine and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen C. The values are an average of two samples per media concentration per time point.

Table A-64. 50 µg/ml carprofen C hepatic slice supernatant with cimetidine incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups	
25	a	
28	a	b
32	a	b
36	a	b
48		b

Table A-65. 100 µg/ml carprofen C hepatic slice supernatant with cimetidine incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		
25	a		
28		b	
32			c
36			c
48			c

Potassium

Figure A-64 shows the potassium concentrations for slices incubated in cimetidine followed by carprofen A. Two-way ANOVA revealed a significant difference across time (df=6, F-ratio=62.851, p-value=0.000) and among concentrations (df=3, F-ratio=3.522, p-value=0.027). For time, 0 hour potassium concentrations were higher than 24, 25, 28, 32, 36 and 48 hour concentrations. Potassium concentrations for 10 µg/ml media were higher than those for 50 µg/ml media. Tables A-66 and A-67 show the homogeneous groups for time and concentration.

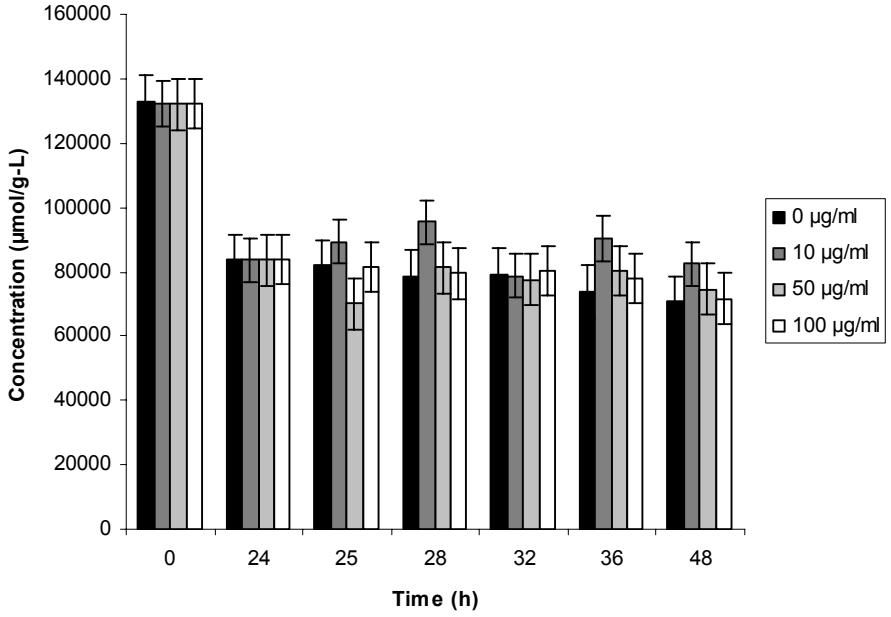

Fig. A-64. Carprofen A potassium concentrations following cimetidine incubation. Potassium concentration in hepatic slice supernatant after incubation for 24 hours with cimetidine and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen A. The values are an average of two samples per media concentration per time point.

Table A-66. Carprofen A with cimetidine potassium analysis for time. Homogeneous groups for potassium two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (h)	Homogeneous groups
0	a
24	b
25	b
28	b
32	b
36	b
48	b

Table A-67. Carprofen A with cimetidine potassium analysis for concentration. Homogeneous groups for potassium two-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Concentration (µg/ml)	Homogeneous groups
0	a b
10	a
50	b
100	a b

Figure A-65 show the potassium concentrations for slices incubated in cimetidine followed by carprofen B. Two-way ANOVA revealed a significant factor interaction for time and concentration (df=18, F-ratio=2.649, p-value=0.010). The disorderly interaction made interpretation of the main effects difficult. There were significant differences for time (df=6, F-ratio=62.602, p-value=0.000) and concentration (df=3, F-ratio=12.480, p-value=0.000).

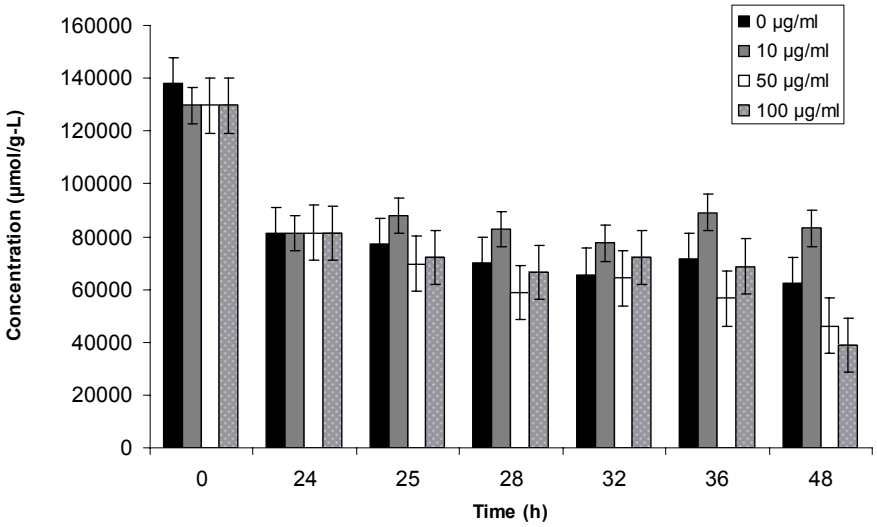

Fig. A-65. Carprofen B potassium concentrations following cimetidine incubation. Potassium concentration in hepatic slice supernatant after incubation for 24 hours with cimetidine and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen B. The values are an average of two samples per media concentration per time point.

Figure A-66 shows the potassium concentrations for slices incubated in cimetidine followed by carprofen C. Two-way ANOVA revealed a significant factor interaction for time and concentration (df=18, F-ratio=2.154, p-value=0.033). The disorderly interaction made interpretation of the main effects difficult. There were significant differences for the main effects of time (df=6, F-ratio=70.416, p-value=0.000) and concentration (df=3, F-ratio=16.191, p-value=0.000).

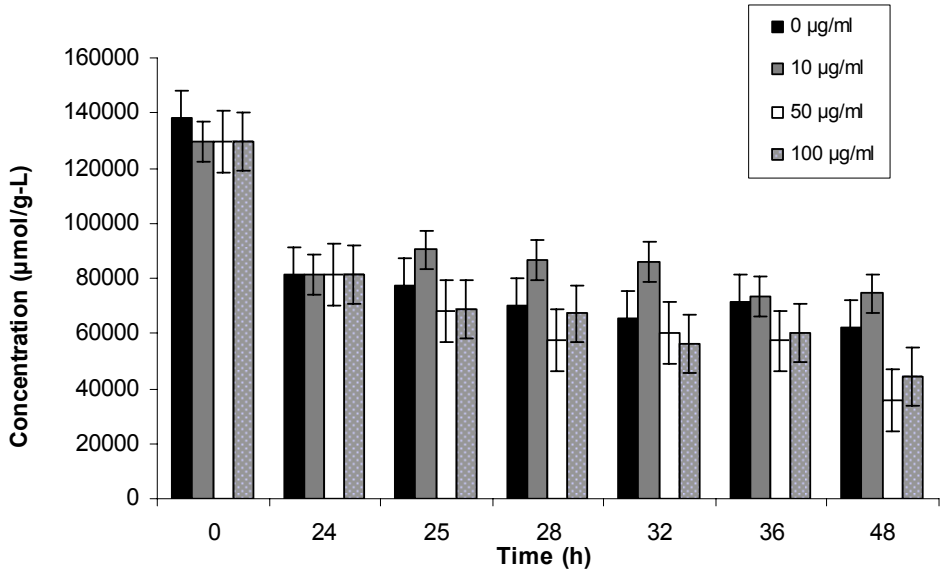

Fig. A-66. Carprofen C potassium concentrations following cimetidine incubation. Potassium concentration in hepatic slice supernatant after incubation for 24 hours with cimetidine and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen C. The values are an average of two samples per media concentration per time point.

ATP

ATP concentrations for slices incubated in cimetidine followed by carprofen A are illustrated in Figure A-67. Significant differences across time (df=6, F-ratio=39.658, p-value=0.000) were noted by two-way ANOVA. ATP concentrations at 0 hour were less than those at 24, 28, 32, 36 and 48 hours. Twenty-four

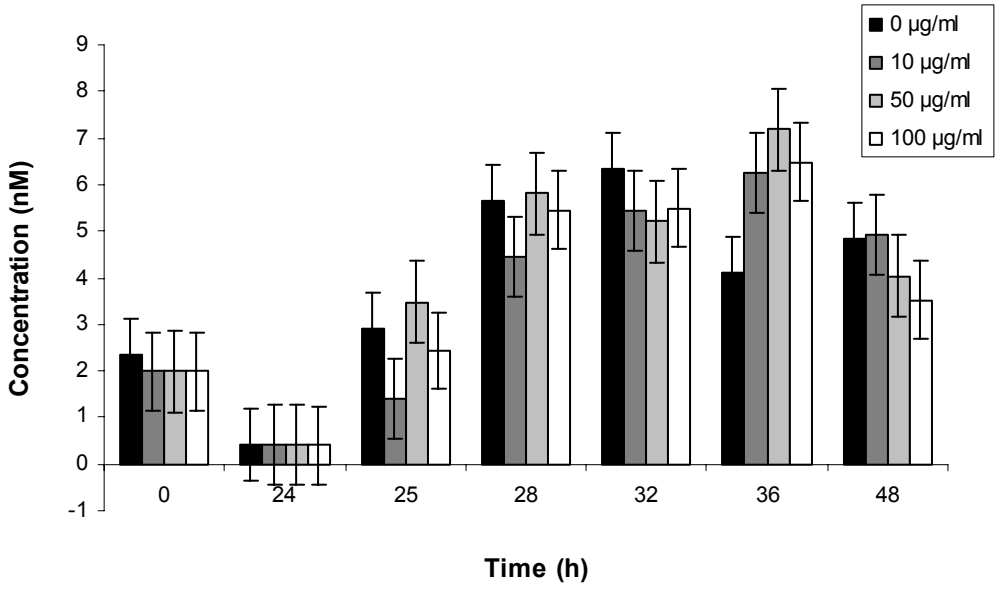

Fig. A-67. Carprofen A ATP concentrations following cimetidine incubation. ATP concentration in hepatic slice supernatant after incubation for 24 hours with cimetidine and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen A. The values are an average of two samples per media concentration per time point.

hour concentrations were less than those at 25, 28, 32, 36 and 48 hours. ATP concentrations at 25 hours were less than those at 28, 32, 36 and 48 hours; 48 hour concentrations were higher than 36 hour concentrations. Table A-68 delineated the homogeneous groups for time.

Table A-68. Carprofen A with cimetidine ATP analysis for time. Homogeneous groups for ATP two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (h)	Homogeneous groups			
0	a			
24		b		
25	a			
28			c	d
32			c	d
36			c	
48				d

Figures A-68 and A-69 display the ATP concentrations for slices incubated in cimetidine followed by carprofen B and carprofen C. No significant differences were noted by two-way ANOVA for either media.

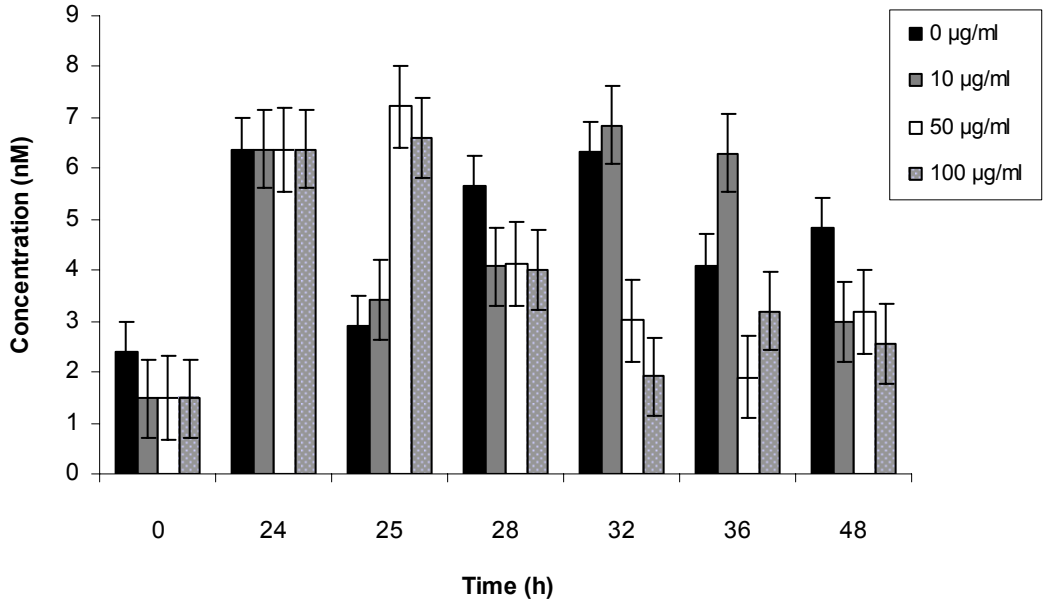

Fig. A-68. Carprofen B ATP concentrations following cimetidine incubation. ATP concentration in hepatic slice supernatant after incubation for 24 hours with cimetidine and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen B. The values are an average of two samples per media concentration per time point.

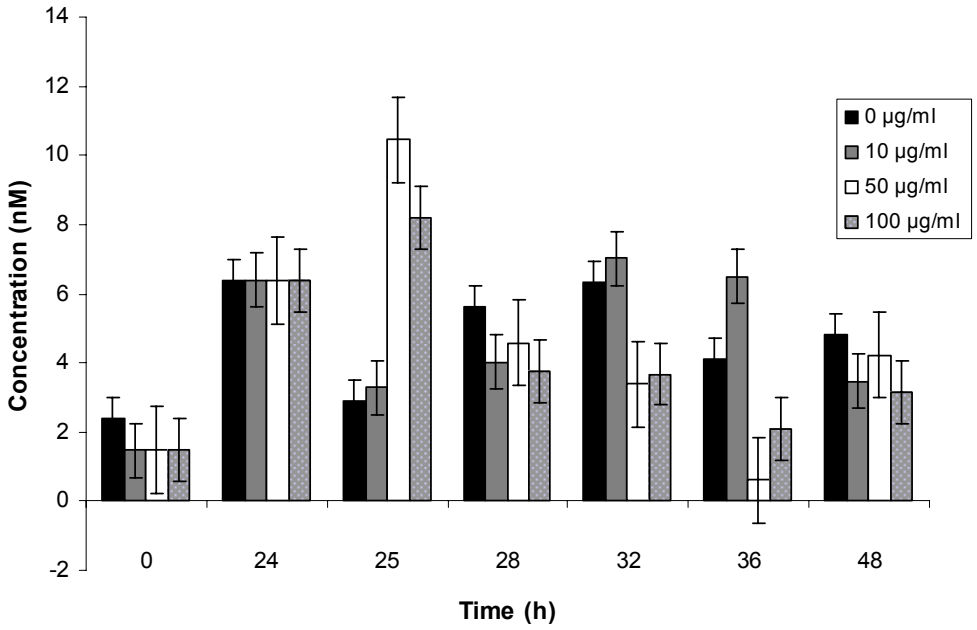

Fig. A-69. Carprofen C ATP concentrations following cimetidine incubation. ATP concentration in hepatic slice supernatant after incubation for 24 hours with cimetidine and incubated for an additional 1, 4, 8, 12 and 24 hours with carprofen C. The values are an average of two samples per media concentration per time point.

Histology

One-way ANOVA for vacuolation severity from cimetidine-carprofen A incubation expressed significant differences among the concentrations (df=3, F-ratio=7.030, p-value=0.003). Lesion scores for 100 µg/ml media were less than those for 0, 10 and 50 µg/ml media. Table A-69 shows the homogeneous groups for vacuolation severity associated with cimetidine-carprofen A incubation.

Table A-69. Carprofen A with cimetidine vacuolation severity analysis for concentration. Homogeneous groups for vacuolation severity one-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Concentration (µg/ml)	Homogeneous groups
0	a
10	a
50	a
100	b

One-way ANOVA of hepatic slice lesion scores for necrosis and vacuolation severity from cimetidine-carprofen B incubation revealed significant differences among concentrations. For necrosis (df=3, F-ratio=6.281, p-value=0.005), lesion scores for 0 µg/ml media were less than those for 50 and 100 µg/ml media. The lesion scores for vacuolation severity (df=3, F-ratio=6.907, p-value=0.003) for 100 µg/ml media were less than those for 0 and 10 µg/ml media. Tables A-70 and A-71 depict the homogeneous groups for necrosis and vacuolation severity.

Table A-70. Carprofen B with cimetidine necrosis analysis for concentration. Homogeneous groups for necrosis one-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Concentration (µg/ml)	Homogeneous groups
0	a
10	a b
50	b
100	b

Table A-71. Carprofen B with cimetidine vacuolation severity analysis for concentration. Homogeneous groups for vacuolation severity one-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Concentration (µg/ml)	Homogeneous groups
0	a
10	a
50	a b
100	b

Cimetidine-carprofen C hepatic lesion scores showed significant differences for vacuolation extent (df=3, F-ratio=5.889, p-value=0.007) and vacuolation severity (df=3, F-ratio=3.719, p-value=0.033) by one-way ANOVA. Lesion scores for vacuolation extent and vacuolation severity were lower for 100 µg/ml media than for 10 µg/ml media. Table A-72 shows the homogeneous groups for both vacuolation extent and vacuolation severity for cimetidine-carprofen B incubation.

Table A-72. Carprofen C with cimetidine vacuolation severity and extent analysis for concentration. Homogeneous groups for vacuolation severity and vacuolation extent one-way ANOVA for concentration are represented as letters. Concentrations with the same letter are not significantly different.

Concentration (µg/ml)	Homogeneous groups
0	a b
10	a
50	a b
100	b

Statistics for Mean Carprofen Data—replaced by AUC in Chapter VI

Carprofen Media

The mean values of carprofen enantiomers in media were measured over twenty-four hours of incubation. No significant differences were found across time or between peaks for 10 µg/ml media. For 50 µg/ml media, differences were seen across time (df=5, F-ratio=8.673, p-value=0.000) and between the peaks (df=1, F-ratio=6.239, p-value=0.015). The enantiomer concentrations at 0 hour were more than those at 12 hours. The concentrations at 1 hour were higher than at 8, 12 and 24 hours. At 4 hours, the concentrations were greater than at 12 and 24 hours. Mean concentrations for peak A (43.269 µg/ml) were higher than mean peak B concentrations (39.992 µg/ml). Four homogeneous groups for time (Table A-73) were noted for 50 µg/ml media. For the averaged 100 µg/ml carprofen media, concentrations were significantly different across time (df=5, F-ratio=5.161, p-value=0.000) but not between peaks. Concentrations at 0 hour were higher than those at 12 and 24 hours. At 1 hour the concentrations were

also higher than at 12 and 24 hours. There were two homogeneous groups for time for the mean 100 µg/ml carprofen media (Table A-74).

Table A-73. Comparison of mean 50 µg/ml carprofen media concentrations (A and B enantiomers) across time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups				Means (µg/ml)
0	a	b	c		43.69316
1	a				48.04358
4	a	b			45.24405
8		b	c	d	39.13747
12				d	36.58165
24			c	d	38.50213

Table A-74. Comparison of mean 100 µg/ml carprofen media concentrations (A and B enantiomers) across time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups			Means (µg/ml)
0	a			87.94843
1	a			88.29685
4	a	b		83.55151
8	a	b		78.96168
12		b		73.04642
24		b		70.55279

Carprofen Supernatant

For mean carprofen concentrations in hepatic slice supernatant, carprofen concentrations for slices incubated in 0 and 10 µg/ml carprofen were below the lower limit of quantification. The two-way ANOVA revealed significant differences across time and between peaks for the supernatant of slices incubated in the 50 (time: df=5, F-ratio=150.851, p-value=0.000; peak: df=1, F-ratio=65.558, p-value=0.000) and 100 µg/ml carprofen media (time: df=5, F-ratio=85.978, p-value=0.000; peak: df=1, F-ratio=12.384, p-value=0.001). There was a significant factor interaction for the 50 µg/ml media (df=5, F-ratio=4.862, p-value=0.001). The interaction appeared to be orderly indicating that the test on the main effects for time and peak could be meaningful.

At 4, 8, 12 and 24 hours the supernatant of slices incubated in 50 µg/ml carprofen contained higher mean concentrations than at 0 or 1 hour. The supernatant concentrations at 4 hours were less than those at 8, 12 and 24 hours, and at 8 hours the supernatant concentrations were less than at 24 hours. There were four homogeneous groups for time (Table A-75), and mean carprofen concentrations for peak B (3142.0423 ng/ml) were higher than those for peak A (2101.7894 ng/ml).

For slices incubated in 100 µg/ml carprofen media, mean supernatant concentrations at 1, 4, 8, 12 and 24 hours were higher than those at 0 hour. At 1 hour, the supernatant concentrations were less than those at 4, 8, 12 and 24 hours. For 4 hours, the supernatant concentrations were lower than at 12 and 24 hours, and at 8 hours, the concentrations were lower than at 24 hours. There were five homogeneous groups time for 100 µg/ml carprofen (Table A-76), and the mean concentrations of peak B (8402.3843 ng/ml) in the supernatant were higher than those of peak A (6888.1497 ng/ml).

Table A-75. Comparison of mean supernatant carprofen concentrations (A and B enantiomers) across time for 50 µg/ml media. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups			Means (ng/ml)
0	a			-70.4521
1	a			510.0783
4		b		2581.3911
8			c	3598.2027
12			c	d	3949.3921
24				d	4251.9186

Table A-76. Comparison of mean supernatant carprofen concentrations (A and B enantiomers) across time for 100 µg/ml media. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups				Means (ng/ml)
0	a				-70.4521
1		b			2961.8377
4			c		8477.9331
8			c	d	9308.6271
12				d	10803.9183
24					e	12095.3870

Carprofen with Phenobarbital Media

The two-way ANOVA revealed significant differences across time but not between peaks for 10 µg/ml (df=4, F-ratio=6.659, p-value=0.000), 50 µg/ml (df=4, F-ratio=3.668, p-value=0.009) and 100 µg/ml carprofen media (df=4, F-ratio=5.753, p-value=0.000). Twenty-five and 28 hour concentrations for 10 µg/ml media were higher than 32 and 48 hour concentrations (Table A-77).

Two-way ANOVA for time and peak revealed significant differences for 50 µg/ml (time: df=4, F-ratio=146.985, p-value=0.000; peak: df=1, F-ratio=87.602, p-value=0.000) and 100 µg/ml media (time: df=4, F-ratio=80.364, p-value=0.000; peak: df=1, F-ratio=24.365, p-value=0.000). A significant factor interaction (df=4, F-ratio=3.653, p=0.011) was noted for 50 µg/ml media. For 50 µg/ml media, 25 and 28 hour concentrations were higher than those at 48 hours (Table A-78). Media containing 100 µg/ml carprofen had higher concentrations at 25 and 28 hours than at 36 and 48 hours (Table A-79).

Table A-77. Comparison across time of mean media concentrations for 10 µg/ml carprofen (A and B enantiomers) with phenobarbital incubation. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		Means (µg/ml)
25	a		8.594
28	a		8.471
32		b	6.566
36	a	b	7.736
48		b	6.887

Table A-78. Comparison across time of mean media concentrations for 50 µg/ml carprofen (A and B enantiomers) with phenobarbital incubation. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		Means (µg/ml)
25	a		38.569
28	a		37.789
32	a	b	36.427
36	a	b	34.618
48		b	32.451

Table A-79. Comparison across time of mean media concentrations for 100 µg/ml carprofen (A and B enantiomers) with phenobarbital incubation. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups		Means (µg/ml)
25	a		77.020
28	a		76.808
32	a	b	71.402
36		b	66.961
48		b	68.149

Carprofen with phenobarbital supernatant

Mean supernatant concentrations for 50 µg/ml were lower at 25 hours than at 28, 32, 36, and 48 hours (Table A-80). At 28 hours, concentrations were lower than at 32, 36 and 48 hours. Thirty-two hour concentrations were lower than those at 36 and 48 hours; thirty-six hour concentrations were less than 48 hour concentrations. Mean peak B concentrations (2989.12 ng/ml) were higher than those for peak A (2069.56 ng/ml). Supernatant concentrations for 100 µg/ml media at 25 hours were less than those at 28, 32, 36 and 48 hours; concentrations at 28 hours were less than at 32, 36 and 48 hours (Table A-81). For 100 µg/ml media, mean peak B concentrations (7690.740137 ng/ml) were higher than peak A concentrations (6247.13528 ng/ml). Zero and 24 hour concentrations were below the lower limit of quantification for all media concentrations.

Table A-80. Mean 50 µg/ml carprofen A, B and C hepatic slice supernatant with phenobarbital incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups				Means (ng/ml)
25	a				607.412
28		b			1878.299
32			c		2733.933
36				d	3458.640
48				e	3968.394

Table A-81. Mean 100 µg/ml carprofen A, B and C hepatic slice supernatant with phenobarbital incubation analysis for time. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups	Means (ng/ml)
25	a	2318.528
28	b	5812.125
32	c	8575.214
36	c	9054.678
48	c	9084.143

Carprofen with Cimetidine Media

Two-way ANOVA for time and peak revealed significant differences across time for 10 µg/ml (df=4, F-ratio=5.581, p-value=0.001), 50 µg/ml (df=4, F-ratio=9.368, p-value=0.000) and 100 µg/ml media (df=4, F-ratio=7.121, p-value=0.000). Ten µg/ml media showed media concentrations at 25 hours were higher than those at 36 hours (Table A-82). Forty-eight hour concentrations were higher than at 28 and 36 hours. For 50 µg/ml media, 25 hour concentrations were higher than at 32 and 36 hours (Table A-83). Thirty-six hour concentrations were lower than at 28 and 48 hours. The 25 hour concentrations for 100 µg/ml media were higher than those at 28, 32 and 36 hours (Table A-84). The 28 hour concentrations were lower than the 48 hour concentrations.

Table A-82. Comparison of mean carprofen concentrations across time for 10 µg/ml media following cimetidine incubation. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups	Means (µg/ml)
25	a b	7.758
28	a c	6.709
32	a b c	7.148
36	c	6.529
48	b	8.043

Table A-83. Comparison of mean carprofen concentrations across time for 50 µg/ml media following cimetidine incubation. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups	Means (µg/ml)
25	a	38.172
28	a b	35.992
32	b c	33.090
36	c	32.012
48	a b	35.413

Table A-84. Comparison of mean carprofen concentrations across time for 100 µg/ml media following cimetidine incubation. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups			Means (µg/ml)
25	a			72.260
28		b		62.988
32		b	c	66.438
36		b	c	64.892
48	a		c	70.626

Carprofen with Cimetidine Supernatant

For 50 and 100 µg/ml, peak B concentrations were higher than peak A concentrations (50: df=1, F-ratio=5.630, p-value=0.022; 100: df=1, F-ratio=6.236, p-value=0.016). Supernatant concentrations across time were significantly different for 50 µg/ml media (df=4, F-ratio=13.628, p-value=0.000) and 100 µg/ml media (df=4, F-ratio=19.533, p-value=0.000). For 50 µg/ml media, mean 25 hour supernatant concentrations were lower than for 32, 36 and 48 hours (Table A-85). Forty-eight hour concentrations were higher than 28, 32 and 36 hour concentrations. Mean peak B concentrations were 3848.882 ng/ml while for peak A concentrations were 2710.508 ng/ml. For 100 µg/ml media, mean supernatant concentrations at 25 hours were lower than those at all other time points (Table A-86). Twenty-eight hour supernatant concentrations were lower than 32, 36 and 48 hour concentrations. Mean peak B concentrations (7728.308 ng/ml) were higher than peak A concentrations (6380.817 ng/ml).

Table A-85. Comparison across time of mean carprofen concentrations (A and B enantiomers) in hepatic slice supernatant for 50 µg/ml media with cimetidine. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups			Means (ng/ml)
25	a			1009.333
28	a	b		2056.396
32		b		3391.628
36		b		3685.621
48			c	6255.495

Table A-86. Comparison across time of mean carprofen concentrations (A and B enantiomers) in hepatic slice supernatant for 100 µg/ml media with cimetidine incubation. Homogeneous groups for two-way ANOVA of time are represented as letters. Time points with the same letter are not significantly different.

Time (h)	Homogeneous groups			Means (ng/ml)
25	a			2767.895
28		b		6102.338
32			c	8730.452
36			c	8813.921
48			c	8858.208

APPENDIX VI

CARPROFEN, CARPROFEN WITH PEHNOBARBITAL AND CARPROFEN WITH CIMETIDINE

Abbreviation: Time (h) = Time (hour)

This information was replaced by AUC in Chapter VI.

Single Study Media Charts

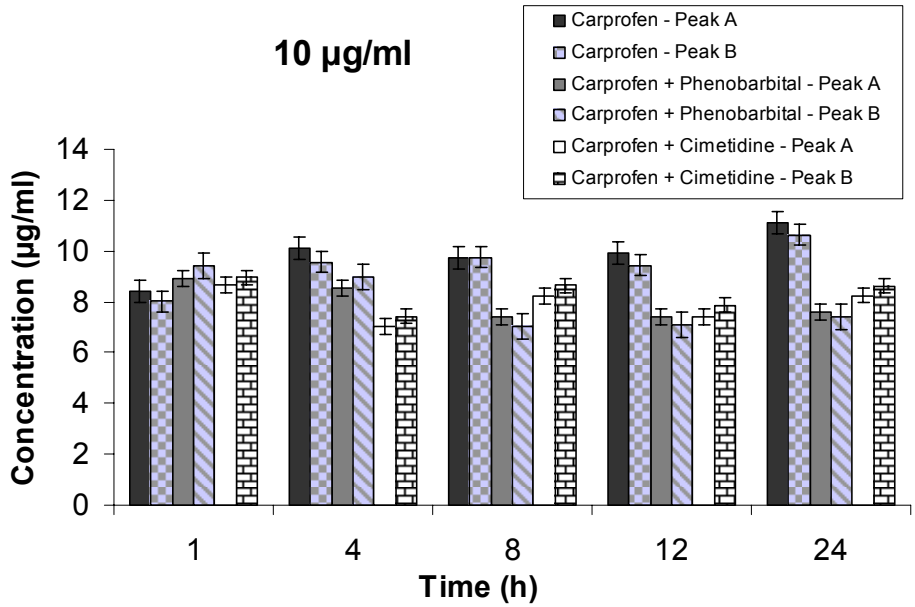

Fig. A-70. 10 µg/ml carprofen A media concentrations alone and after phenobarbital or cimetidine incubation. Concentrations of carprofen enantiomers in media alone and after incubation for 24 hours with phenobarbital or cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are a mean of three samples per treatment per time point.

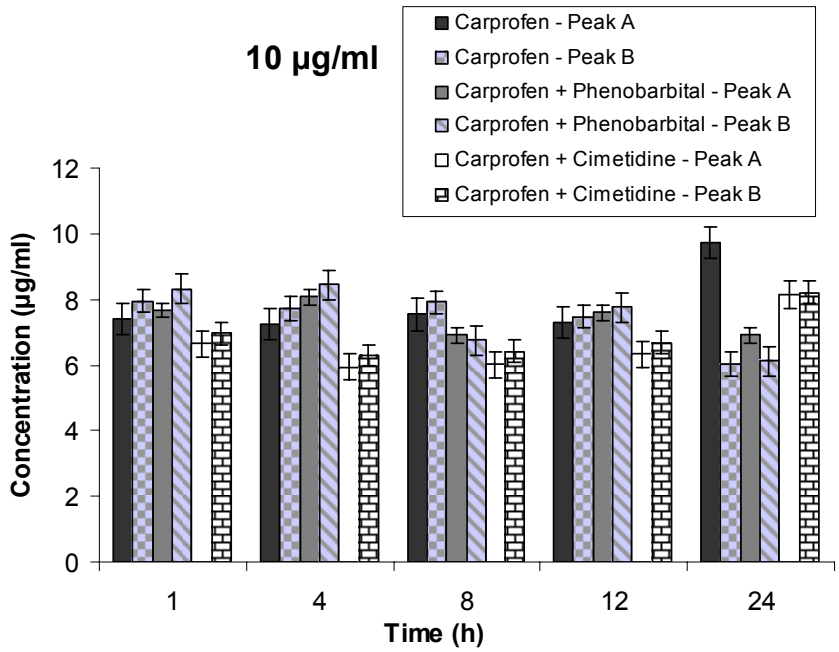

Fig. A-71. 10 µg/ml carprofen B media concentrations alone and after phenobarbital or cimetidine incubation. Concentrations of carprofen enantiomers in media alone and after incubation for 24 hours with phenobarbital or cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are a mean of three samples per treatment per time point.

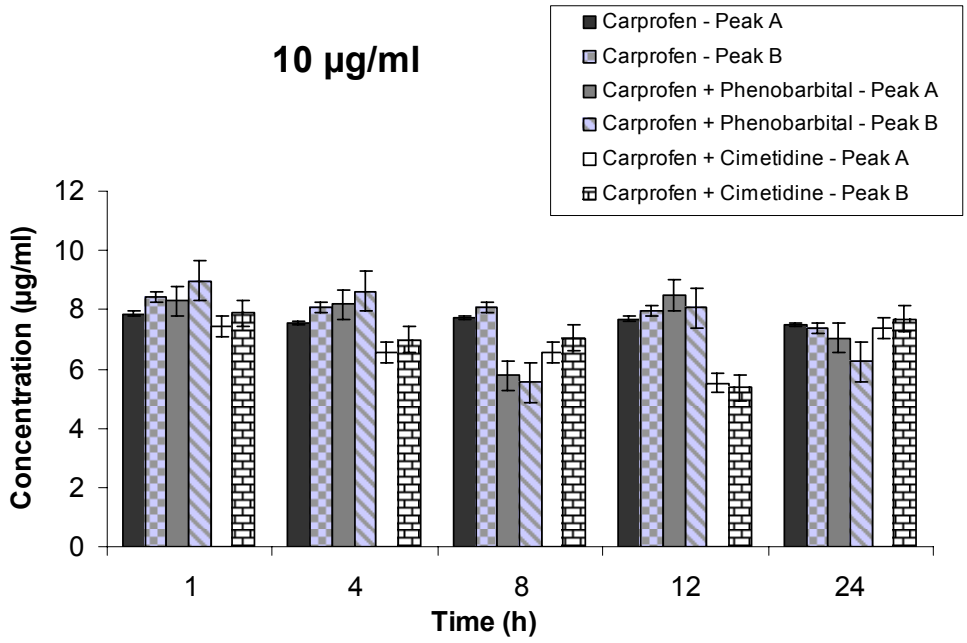

Fig. A-72. 10 µg/ml carprofen C media concentrations alone and after phenobarbital or cimetidine incubation. Concentrations of carprofen enantiomers in media alone and after incubation for 24 hours with phenobarbital or cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are a mean of three samples per treatment per time point.

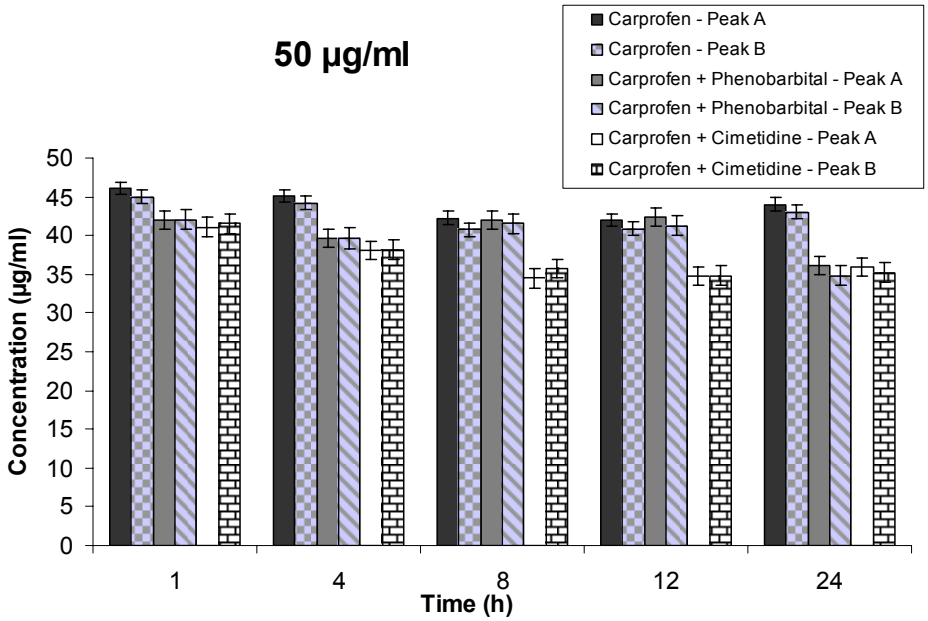

Fig. A-73. 50 µg/ml carprofen A media concentrations alone and after phenobarbital or cimetidine incubation. Concentrations of carprofen enantiomers in media alone and after incubation for 24 hours with phenobarbital or cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are a mean of three samples per treatment per time point.

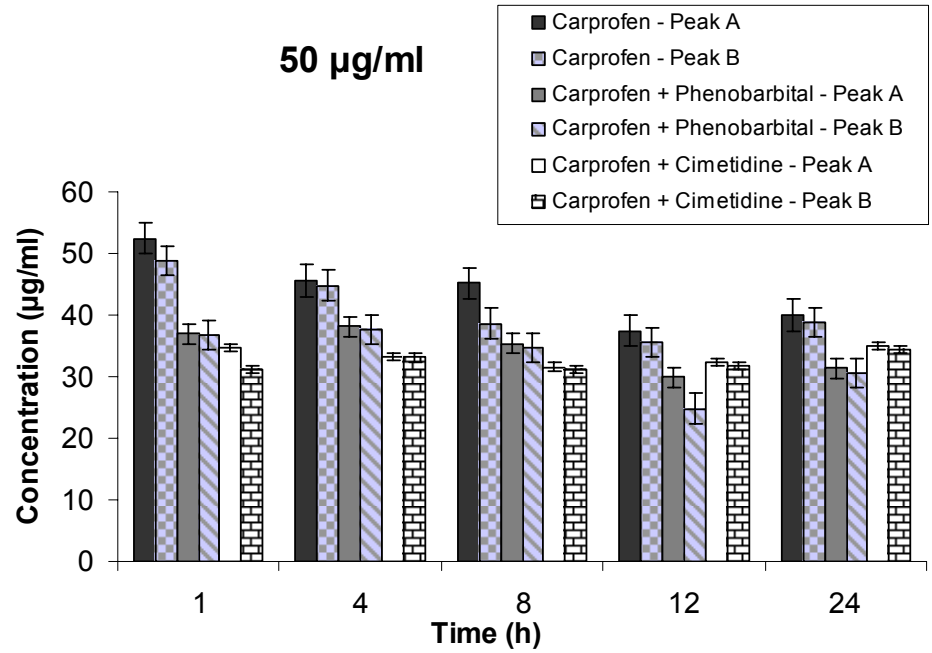

Fig. A-74. 50 µg/ml carprofen B media concentrations alone and after phenobarbital or cimetidine incubation. Concentrations of carprofen enantiomers in media alone and after incubation for 24 hours with phenobarbital or cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are a mean of three samples per treatment per time point.

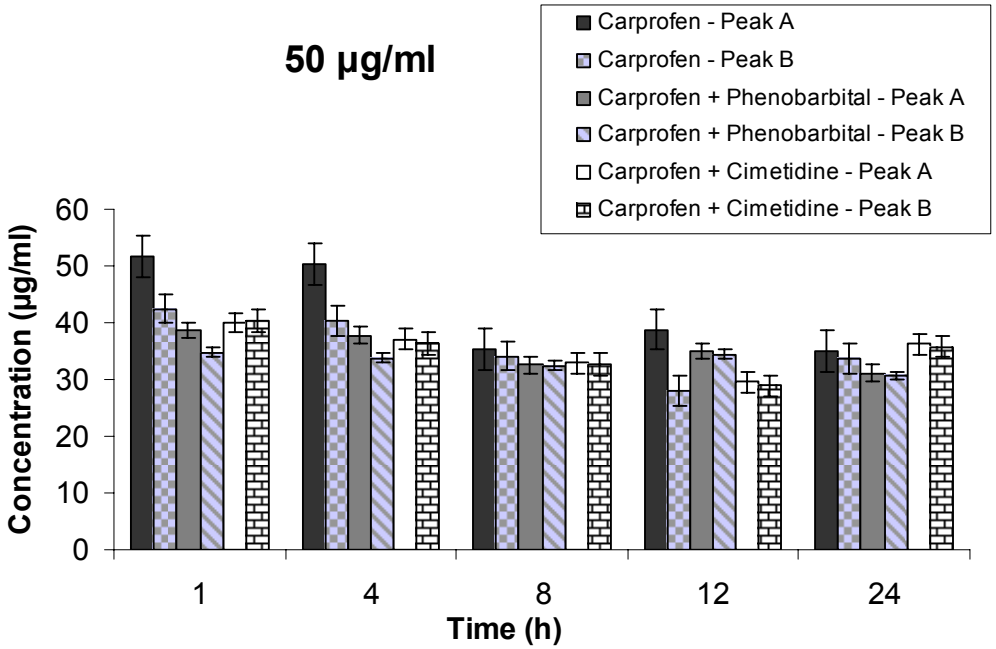

Fig. A-75. 50 µg/ml carprofen C media concentrations alone and after phenobarbital or cimetidine incubation. Concentrations of carprofen enantiomers in media alone and after incubation for 24 hours with phenobarbital or cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are a mean of three samples per treatment per time point.

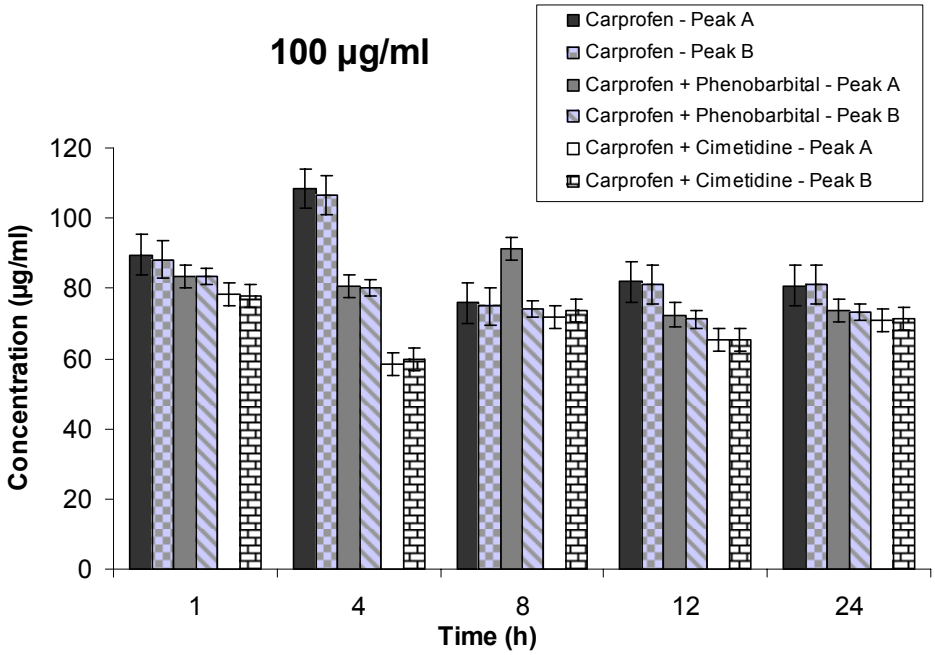

Fig. A-76. 100 µg/ml carprofen A media concentrations alone and after phenobarbital or cimetidine incubation. Concentrations of carprofen enantiomers in media alone and after incubation for 24 hours with phenobarbital or cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are a mean of three samples per treatment per time point.

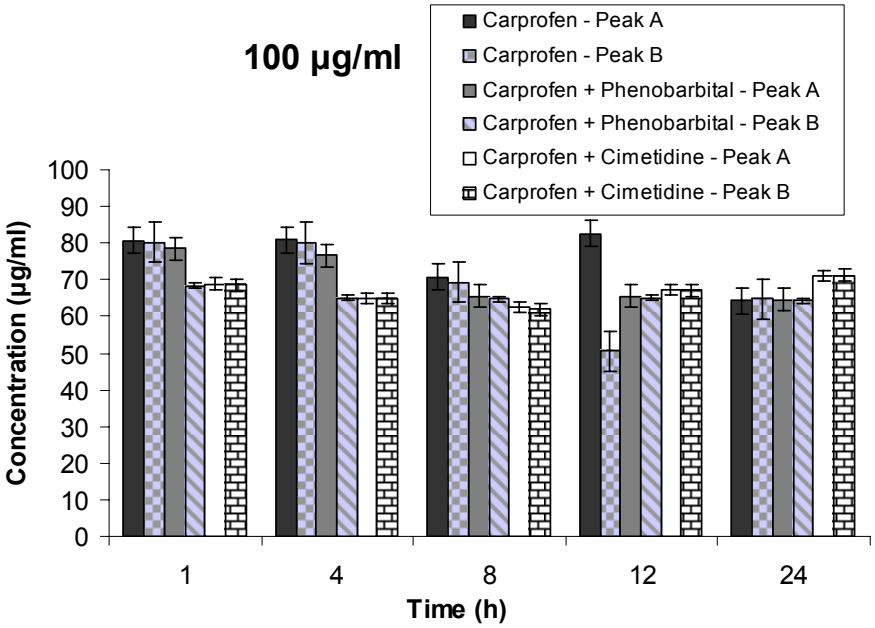

Fig. A-77. 100 µg/ml carprofen B media concentrations alone and after phenobarbital or cimetidine incubation. Concentrations of carprofen enantiomers in media alone and after incubation for 24 hours with phenobarbital or cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are a mean of three samples per treatment per time point.

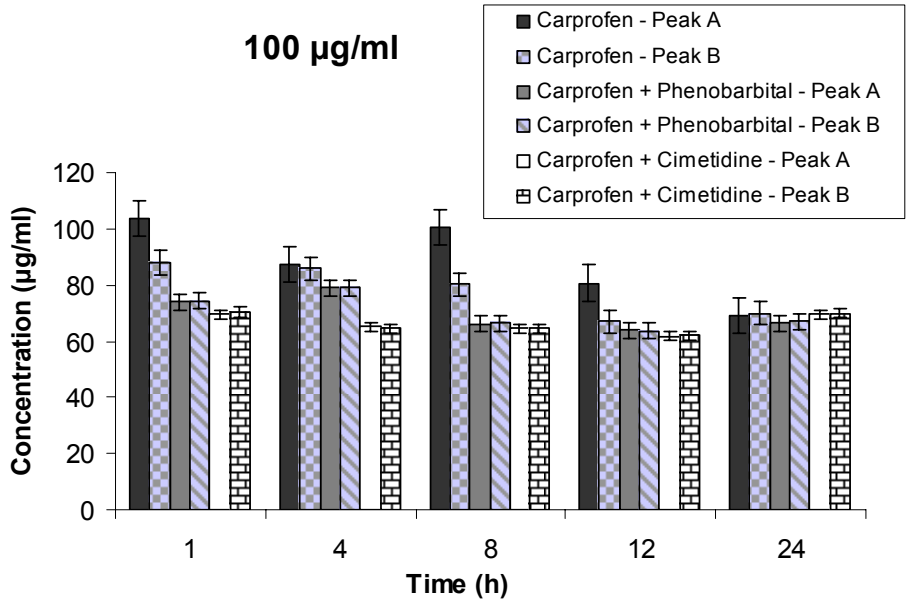

Fig. A-78. 100 µg/ml carprofen C media concentrations alone and after phenobarbital or cimetidine incubation. Concentrations of carprofen enantiomers in media alone and after incubation for 24 hours with phenobarbital or cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are a mean of three samples per treatment per time point.

Single Study Supernatant Charts

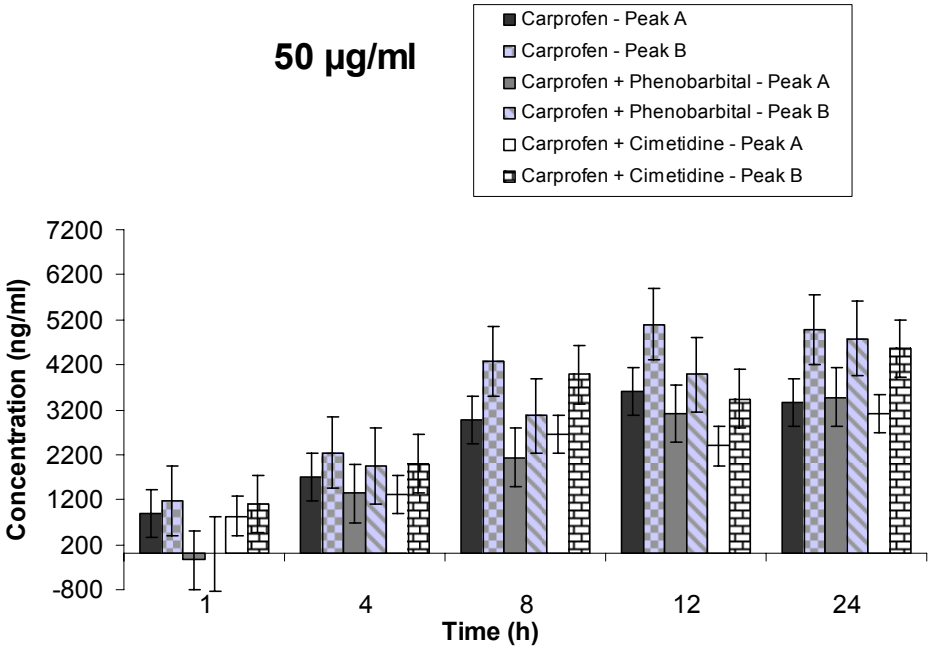

Fig. A-79. 50 µg/ml carprofen A hepatic slice supernatant concentrations alone and after phenobarbital or cimetidine incubation. Concentrations of carprofen enantiomers in supernatant alone and after incubation for 24 hours with phenobarbital or cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are an average of two samples per treatment per time point.

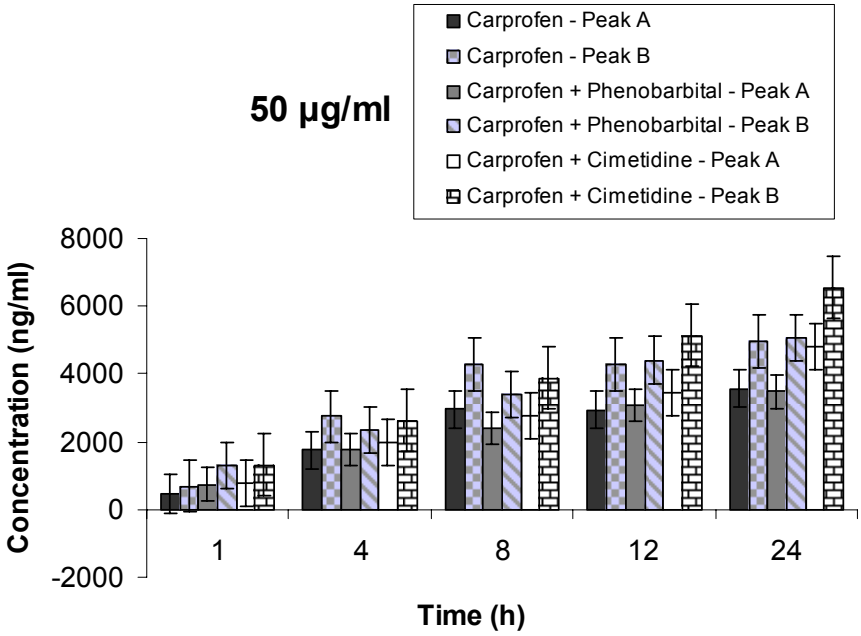

Fig. A-80. 50 µg/ml carprofen B hepatic slice supernatant concentrations alone and after phenobarbital or cimetidine incubation. Concentrations of carprofen enantiomers in supernatant alone and after incubation for 24 hours with phenobarbital or cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are an average of two samples per treatment per time point.

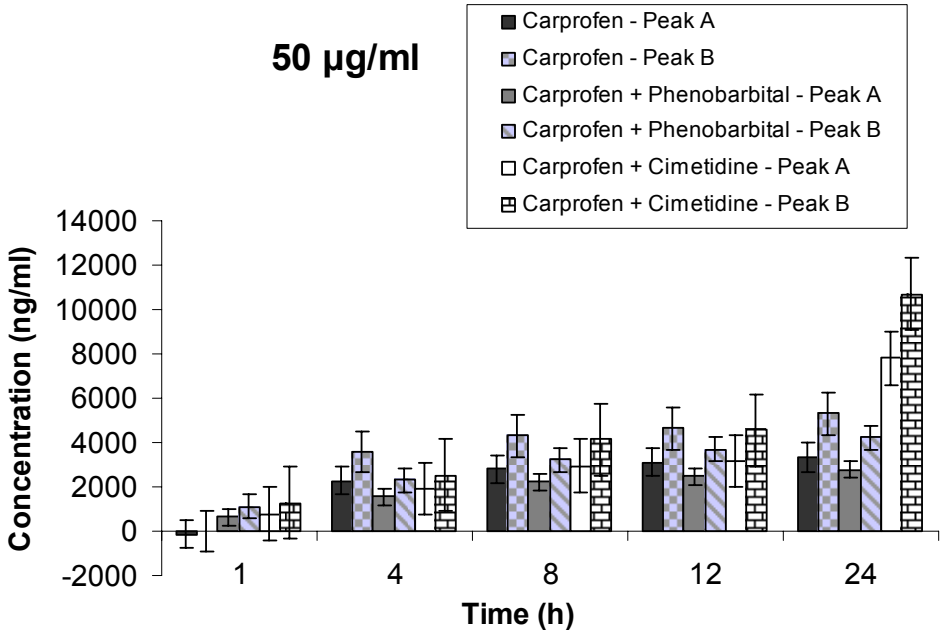

Fig. A-81. 50 µg/ml carprofen C hepatic slice supernatant concentrations alone and after phenobarbital or cimetidine incubation. Concentrations of carprofen enantiomers in supernatant alone and after incubation for 24 hours with phenobarbital or cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are an average of two samples per treatment per time point.

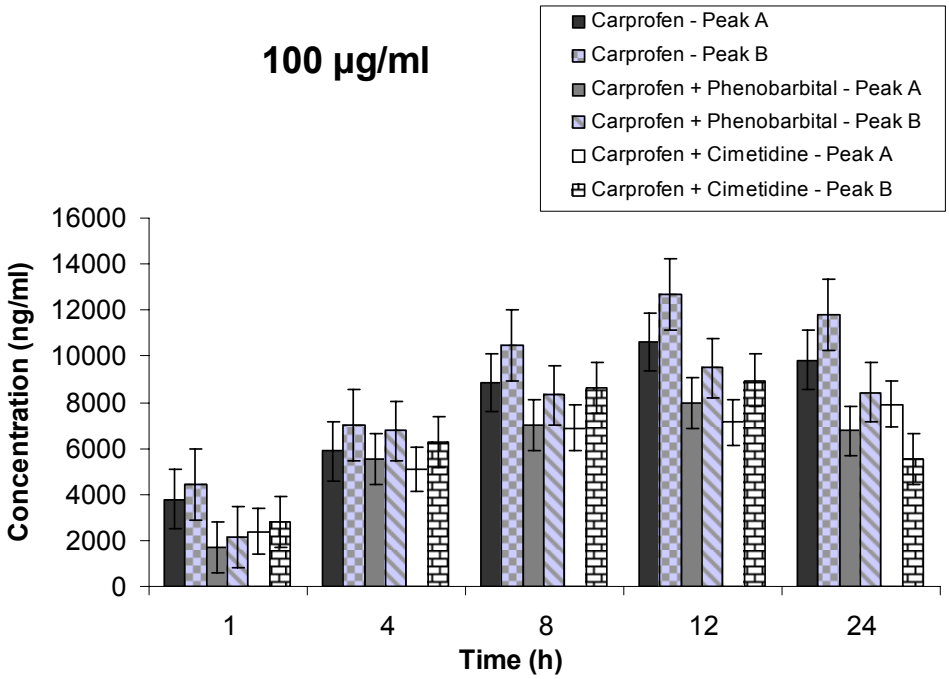

Fig. A-82. 100 µg/ml carprofen A hepatic slice supernatant concentrations alone and after phenobarbital or cimetidine incubation. Concentrations of carprofen enantiomers in supernatant alone and after incubation for 24 hours with phenobarbital or cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are an average of two samples per treatment per time point.

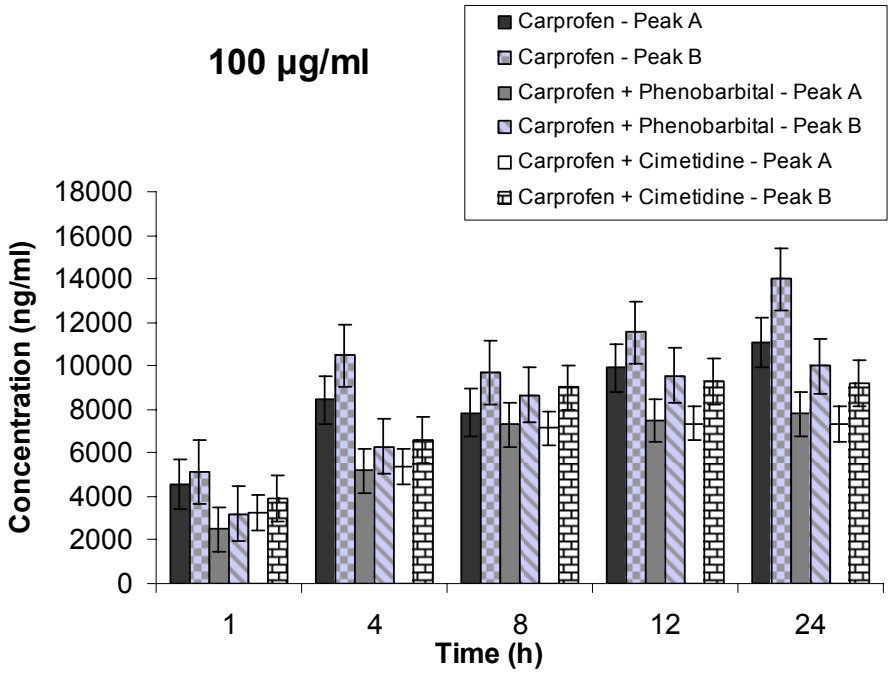

Fig. A-83. 100 µg/ml carprofen B hepatic slice supernatant concentrations alone and after phenobarbital or cimetidine incubation. Concentrations of carprofen enantiomers in supernatant alone and after incubation for 24 hours with phenobarbital or cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are an average of two samples per treatment per time point.

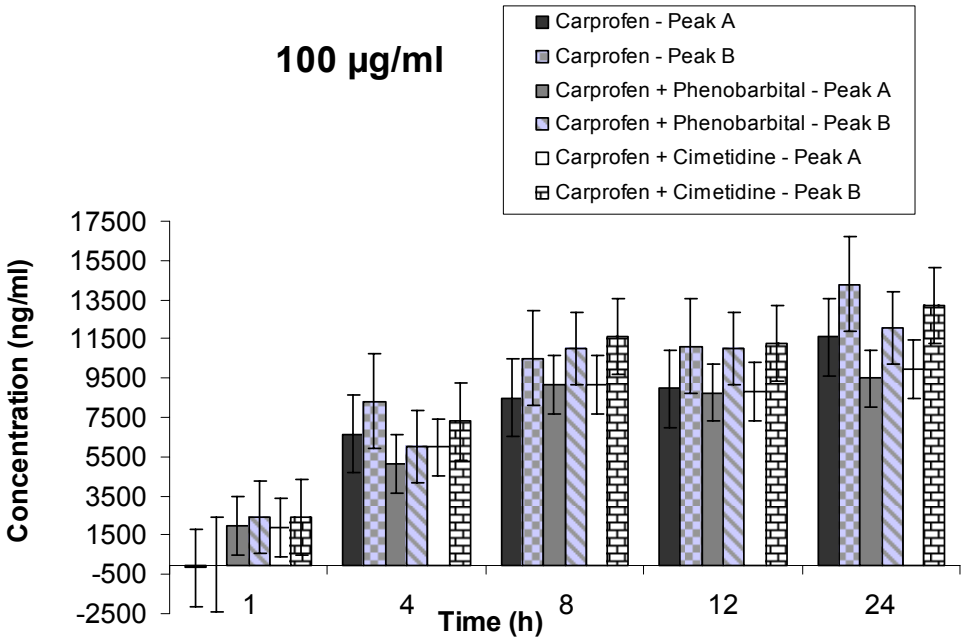

Fig. A-84. 100 µg/ml carprofen C hepatic slice supernatant concentrations alone and after phenobarbital or cimetidine incubation. Concentrations of carprofen enantiomers in supernatant alone and after incubation for 24 hours with phenobarbital or cimetidine. Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are an average of two samples per treatment per time point.

Charts and Statistics for Treatment Comparisons (Compiled Data)

Treatment Comparisons for 10 µg/ml Media and Supernatant

The mean media concentrations for slices incubated in 10 µg/ml carprofen alone or in conjunction with phenobarbital or cimetidine (Figure A-85) were compared using two-way ANOVA. For peak A, differences among treatments were noted (df=2, F-ratio=4.455, p-value=0.014). Media concentrations for slice treatments with carprofen and cimetidine were lower than those for slices treated with carprofen alone (Table A-87). The mean supernatant concentrations for slices incubated in 10 µg/ml carprofen media alone associated with phenobarbital or cimetidine were all below the lower limit of quantification.

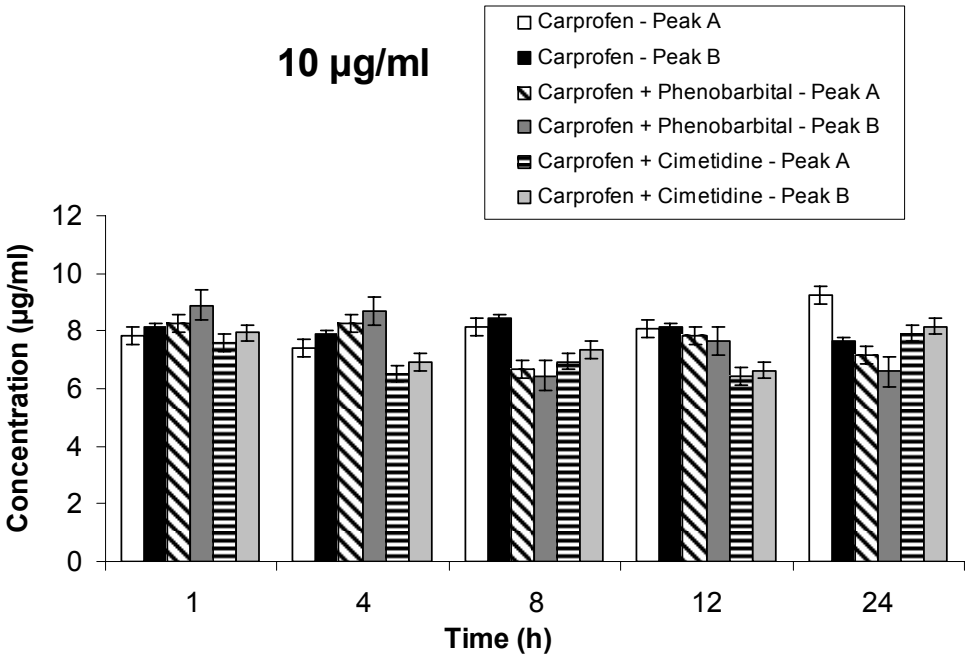

Fig. A-85. Mean media concentrations of carprofen for 10 µg/ml media alone and with phenobarbital or cimetidine incubation. Concentrations (µg/ml) of carprofen enantiomers (A or B) in media alone and after incubation for 24 hours with phenobarbital (75 µg/ml) or cimetidine (1000 µM). Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are a mean of nine samples per treatment per time point.

Table A-87. Comparison among treatments of mean peak A media concentrations of carprofen in 10 µg/ml media for all times. Homogeneous groups for peak A two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Treatment	Homogeneous groups		Means (µg/ml)
carprofen alone	a		8.06005
carprofen with phenobarbital	a	b	7.64913
carprofen with cimetidine		b	7.07692

Treatment Comparisons for 50 µg/ml Media and Supernatant

The mean media concentrations of carprofen following slice incubation in 50 µg/ml carprofen media alone or in succession with phenobarbital or cimetidine (Figure A-86) were compared for differences. Two-way ANOVA by time and treatment revealed significant differences across time and treatment for

peak A (time: df=4, F-ratio=10.400, p-value=0.000; treatment: df=2, F-ratio=31.131, p-value=0.000) and peak B (time: df=4, F-ratio=8.330, p-value=0.000; treatment: df=2, F-ratio=10.409, p-value=0.000).

For peak A, media concentrations were higher one and four hours post-incubation than at 8, 12 and 24 hours post-incubation (Table A-88). Media concentrations for slices treated with carprofen alone were higher than those treated with phenobarbital and cimetidine (Table A-89). With peak B, concentrations one hour post-incubation were higher than those at 8, 12 and 24 hours, and four hour post-incubation concentrations were higher than 12 hours (Table A-90). Among treatments, carprofen concentrations for peak B were higher for carprofen alone than for slices treated with phenobarbital or cimetidine (Table A-91).

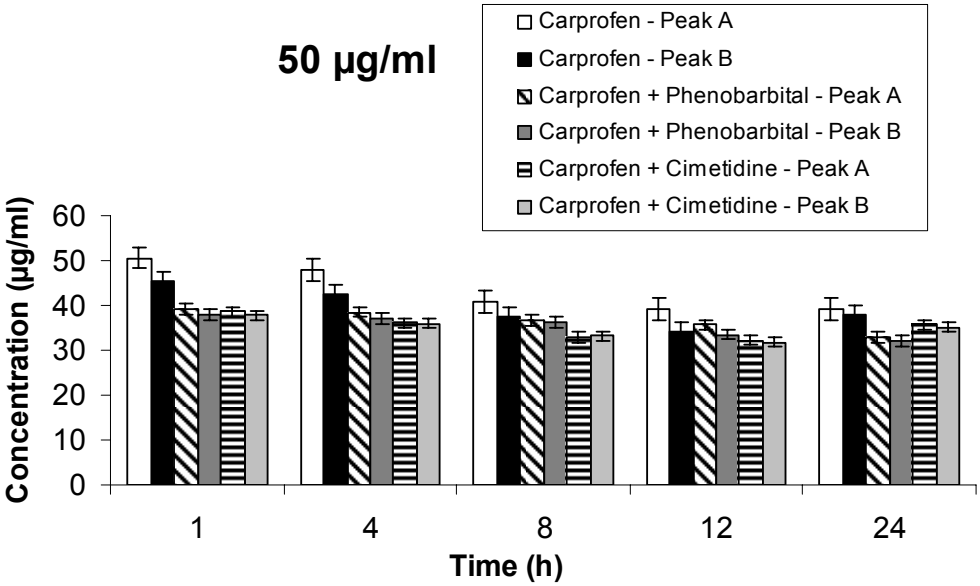

Fig. A-86. Average media concentrations of carprofen for 50 µg/ml media alone and with phenobarbital or cimetidine incubation. Concentrations (µg/ml) of carprofen enantiomers (A or B) in media alone and after incubation for 24 hours with phenobarbital (75 µg/ml) or cimetidine (1000 µM). Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are a mean of nine samples per treatment per time point.

Table A-88. Comparison across time of mean peak A media concentrations of carprofen for 50 µg/ml media for all treatments. Homogeneous groups for peak A two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (h)	Homogeneous groups	Means (µg/ml)
1	a	42.506
4	a	39.980
8	b	36.658
12	b	35.551
24	b	35.744

Table A-89. Comparison among treatments of mean peak A media concentrations of carprofen in 50 µg/ml media for all times. Homogeneous groups for peak A two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Treatment	Homogeneous groups	Means (µg/ml)
carprofen alone	a	43.270
carprofen with phenobarbital	b	36.599
carprofen with cimetidine	b	35.118

Table A-90. Comparison across time of mean peak B concentrations of carprofen for 50 µg/ml media for all treatments. Homogeneous groups for peak B two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (h)	Homogeneous groups	Means (µg/ml)
1	a	40.187
4	a b	37.977
8	b c	35.554
12	c	33.089
24	b c	34.932

Table A-91. Comparison among treatments of mean peak B media concentrations of carprofen in 50 µg/ml media for all times. Homogeneous groups for peak B two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Treatment	Homogeneous groups	Means (µg/ml)
carprofen alone	a	39.340
carprofen with phenobarbital	b	35.343
carprofen with cimetidine	b	34.754

The average hepatic slice supernatant concentrations for slices incubated in 50 µg/ml media alone or in conjunction with phenobarbital or cimetidine (Figure A-87) were determined and compared for differences. Two-way ANOVA revealed significant differences across time and among treatments for peak A (time: df=4, F-ratio=32.189, p-value=0.000; treatment: df=2, F-ratio=3.222, p-value=0.000) and peak B (time: df=4, F-ratio=37.509, p-value=0.000; treatment: df=2, F-ratio=3.465, p-value=0.036). For peaks A and B, supernatant concentrations at one hour post-incubation were lower than those at 4, 8, 12 and 24 hours (Table A-92). Four hour post-incubation concentrations were lower than 8, 12 and 24 hour concentrations. Twenty-four hour concentrations were higher than 8 and 12 hour concentrations. Among treatments, average supernatant concentrations for peak A and peak B were higher in slices incubated in carprofen with cimetidine than those incubated in carprofen with phenobarbital (Table A-93).

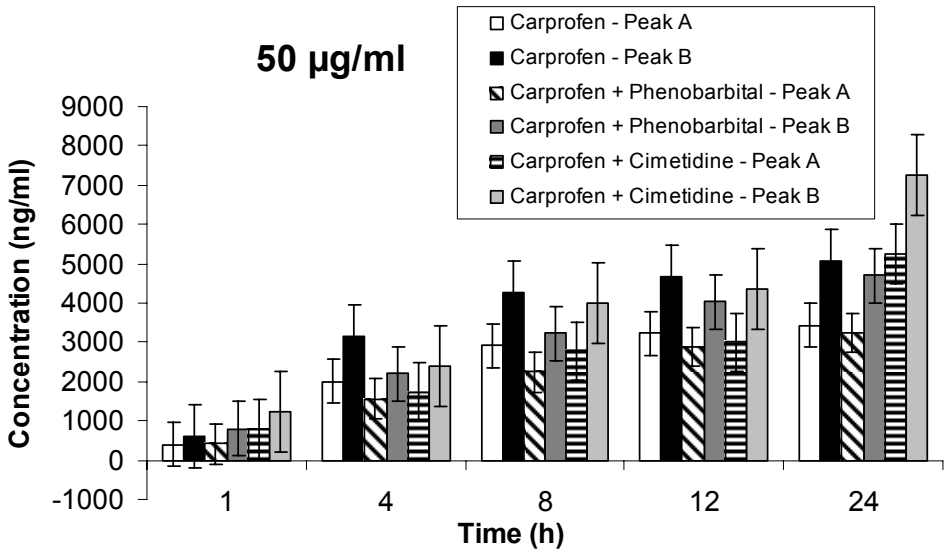

Fig. A-87. Average hepatic slice supernatant concentrations of carprofen for 50 µg/ml media alone and after phenobarbital or cimetidine incubation. Concentrations (µg/ml) of carprofen enantiomers (A or B) in supernatant alone and after incubation for 24 hours with phenobarbital (75 µg/ml) or cimetidine (µM). Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are a mean of six samples per treatment per time point.

Table A-92. Comparison across time of mean peak A and B supernatant concentrations of carprofen for slices incubated in 50 µg/ml media for all treatments. Homogeneous groups for peaks A and B two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (h)	Homogeneous groups	Peak A Means (ng/ml)	Peak B Means (ng/ml)
1	a	535.999	881.883
4	b	1732.818	2475.503
8	c	2647.300	3835.209
12	c	3037.234	4358.535
24	d	3968.487	5682.051

Table A-93. Comparison among treatments of mean peak A and B supernatant concentrations of carprofen for slices incubated in 50 µg/ml media for all times. Homogeneous groups for peaks A and B two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Treatment	Homogeneous groups	Peak A Means (ng/ml)	Peak B Means (ng/ml)
carprofen alone	a b	2373.041	3501.911
carprofen with phenobarbital	a	2069.555	2989.116
carprofen with cimetidine	b	2710.508	3848.882

Treatment Comparisons for 100 µg/ml Media and Supernatant

The average carprofen media concentrations for slices incubated in 100 µg/ml carprofen media alone or in combination with phenobarbital or cimetidine (Figure A-88) were compared using two-way ANOVA. For peak A, two-way ANOVA indicated significant differences for time (df=4, F-ratio=4.248, p-value=0.003) and treatment (df=2, F-ratio=22.240, p-value=0.000). Media concentrations one hour post-incubation were higher than 12 and 24 hour concentrations (Table A-94). Among treatments, media concentrations for carprofen alone were higher than those treated with phenobarbital or cimetidine (Table

A-95). Additionally, average media concentrations for slices treated with cimetidine were lower than those treated with phenobarbital. Peak B two-way ANOVA revealed a time-treatment interaction (df=8, F-ratio=3.710, p-value=0.001). There were significant main effects, and the interaction appeared to be orderly. Peak B media concentrations were higher at one hour than at 8, 12 and 24 hours, and four hour media concentrations were higher than 12 hour concentrations (Table A-96). For treatments, peak B concentrations for carprofen alone were higher than those for carprofen with phenobarbital or with cimetidine (Table A-97).

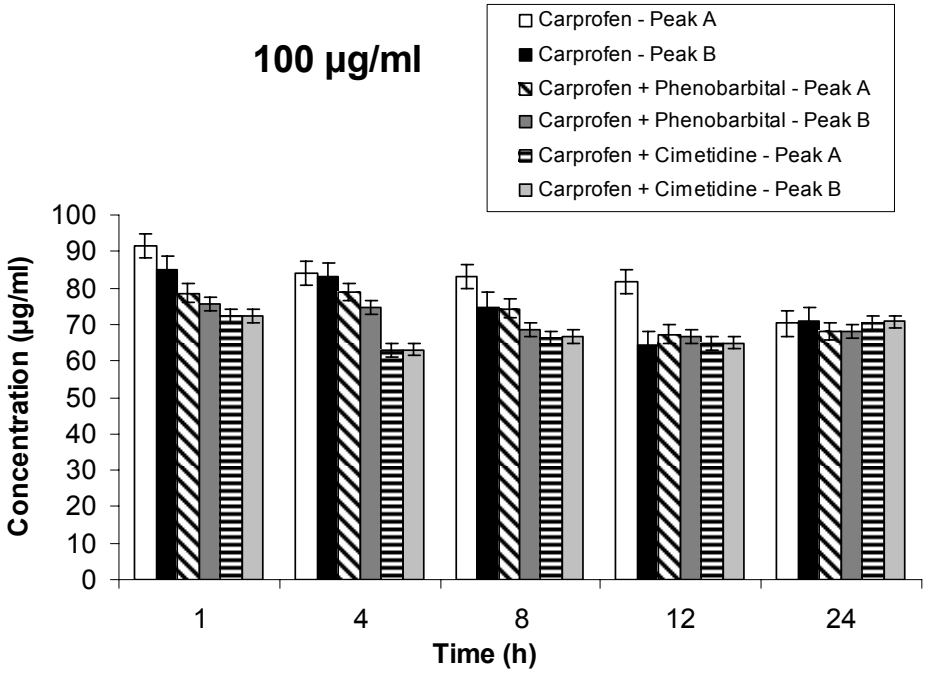

Fig. A-88. Average media concentrations of carprofen for100 µg/ml media alone and after phenobarbital or cimetidine incubation. Concentrations (µg/ml) of carprofen enantiomers (A or B) in media alone and after incubation for 24 hours with phenobarbital (75 µg/ml) or cimetidine (1000 µM). Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are a mean of nine samples per treatment per time point.

Table A-94. Comparison across time of mean peak A media concentrations of carprofen in 100 µg/ml media for all treatments. Homogeneous groups for peak A two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (h)	Homogeneous groups	Means (ug/ml)
1	a	80.384
4	a b	76.802
8	a b	74.225
12	b	70.906
24	b	69.620

Table A-95. Comparison among treatments of mean peak A media concentrations of carprofen in 100 µg/ml media for all times. Homogeneous groups for peak A two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Treatment	Homogeneous groups	Means (µg/ml)
carprofen alone	a	83.363
carprofen with phenobarbital	b	73.454
carprofen with cimetidine	c	67.342

Table A-96. Comparison across time of mean peak B media concentrations of carprofen in 100 µg/ml media for all treatments. Homogeneous groups for peak B two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (h)	Homogeneous groups	Means (µg/ml)
1	a	77.299
4	a b	75.080
8	b c	69.795
12	c	65.329
24	b c	69.872

Table A-97. Comparison among treatments of mean peak B media concentrations of carprofen in 100 µg/ml media for all times. Homogeneous groups for peak B two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Treatment	Homogeneous groups	Means (µg/ml)
carprofen alone	a	76.795
carprofen with phenobarbital	b	70.682
carprofen with cimetidine	b	67.539

The mean supernatant concentrations for slices incubated in carprofen 100 µg/ml media alone or in conjunction with phenobarbital or cimetidine (Figure A-89) were examined for differences. Two-way ANOVA revealed significant differences for peak A and peak B across time (peak A: df=4, F-ratio=73.088, p-value=0.000; peak B: df=4, F-ratio=49.960, p-value=0.000) and among treatments (peak A: df=2, F-ratio=11.504, p-value=0.000; peak B: df=2, F-ratio=7.441, p-value=0.001). For peaks A and B, supernatant concentrations at one hour post-incubation were lower than those at 4, 8, 12 and 24 hours (Table A-98). At 4 hours, peak A and B supernatant concentrations were lower than at 8, 12 and 24 hours. Among treatments, peak A and B supernatant concentrations for slices incubated in carprofen alone were higher than those for slices incubated with phenobarbital and cimetidine (Table A-99).

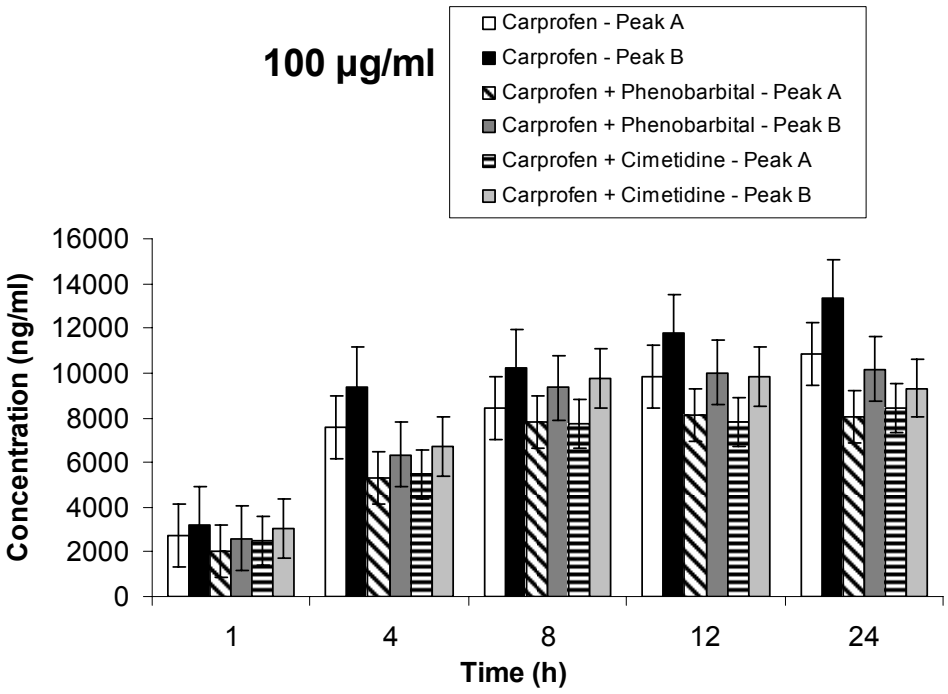

Fig. A-89. Average supernatant concentrations of carprofen for slices incubated in 100 µg/ml media alone and after phenobarbital or cimetidine incubation. Concentrations (ng/ml) of carprofen enantiomers in supernatant alone and after incubation for 24 hours with phenobarbital (75 µg/ml) or cimetidine (1000 µM). Time is the hour incubated in carprofen or post incubation with phenobarbital or cimetidine. The values are a mean of six samples per treatment per time point.

Table A-98. Comparison across time of mean peak A and B supernatant concentrations of carprofen in 100 µg/ml media for all treatments. Homogeneous groups for peaks A and B two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time (h)	Homogeneous groups	Peak A Means (ng/ml)	Peak B Means (ng/ml)
1	a	2428.702	2936.805
4	b	5916.870	7223.059
8	c	7979.768	9763.095
12	c	8564.749	10550.262
24	c	9086.225	10938.934

Table A-99. Comparison among treatments of mean peak A and B supernatant concentrations of carprofen in 100 µg/ml media for all times. Homogeneous groups for peaks A and B two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Treatment	Homogeneous groups	Peak A Means (ng/ml)	Peak B Means (ng/ml)
carprofen alone	a	7757.836	9428.244
carprofen with phenobarbital	b	6247.135	7690.740
carprofen with cimetidine	b	6380.817	7728.308

APPENDIX VII

ADDITIONAL CARPROFEN WITH PHENOBARBITAL INCUBATIONS

Single Study Charts

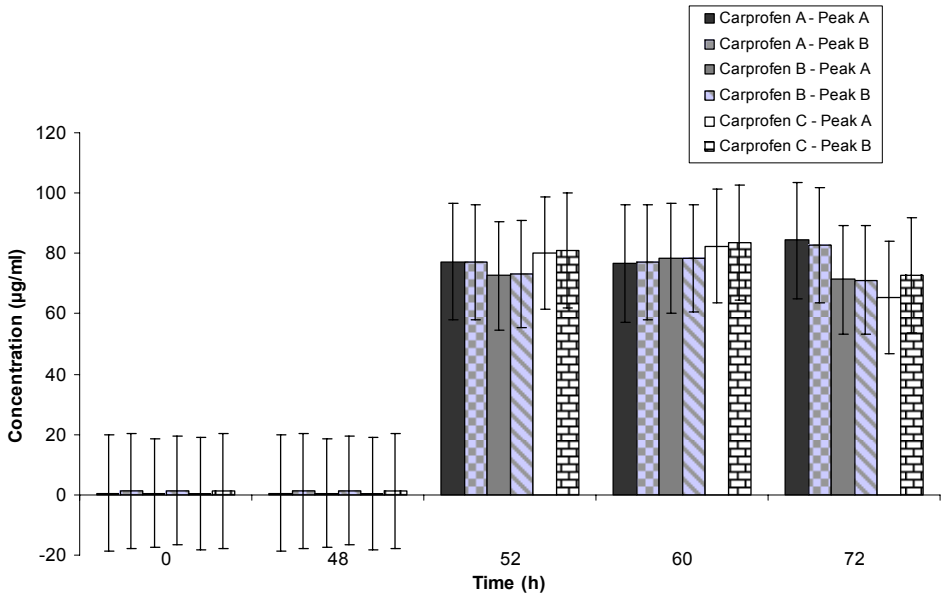

Fig. A-100. Carprofen A, B and C media concentrations following 48 hour phenobarbital (75 µg/ml) incubation. Concentrations of carprofen enantiomers in media after incubation for 48 hours with 75 µg/ml phenobarbital and incubated for an additional 4, 12 and 24 hours with carprofen A, B and C. The values are an average of three samples per carprofen type per time point.

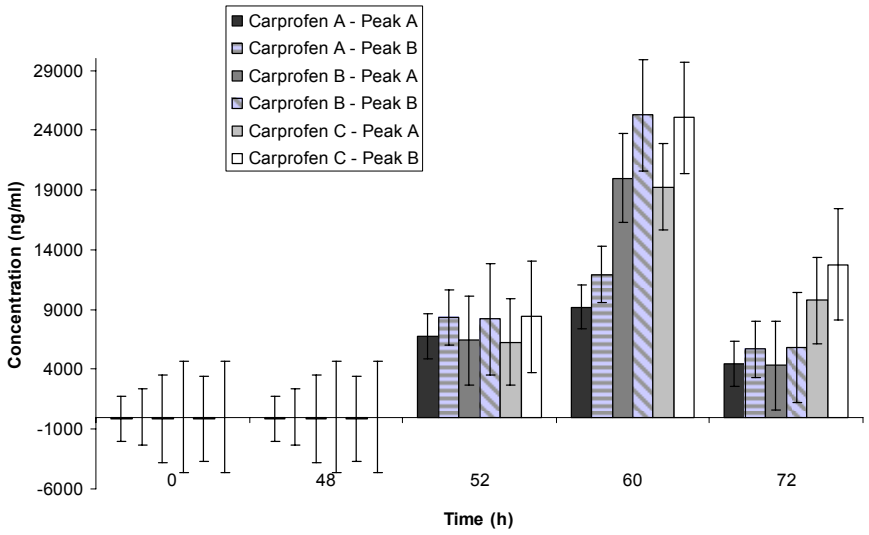

Fig. A-101. Carprofen A, B and C hepatic slice supernatant concentrations following 48 hour phenobarbital (75 µg/ml) incubation. Concentrations of carprofen enantiomers in hepatic slice supernatant after incubation for 48 hours with 75 µg/ml phenobarbital and incubated for an additional 4, 12 and 24 hours with carprofen A, B and C. The values are an average of two samples per carprofen type per time point.

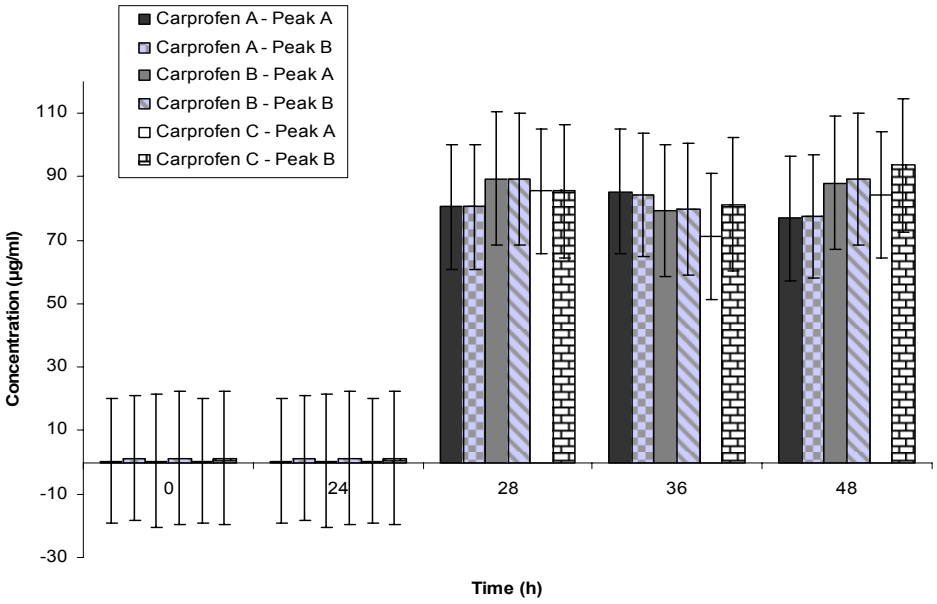

Fig. A-102. Carprofen A, B and C media concentrations following 24 hour phenobarbital (150 µg/ml) incubation. Concentrations of carprofen enantiomers in media after incubation for 24 hours with 150 µg/ml phenobarbital and incubated for an additional 4, 12 and 24 hours with carprofen A, B and C. The values are an average of three samples per carprofen type per time point.

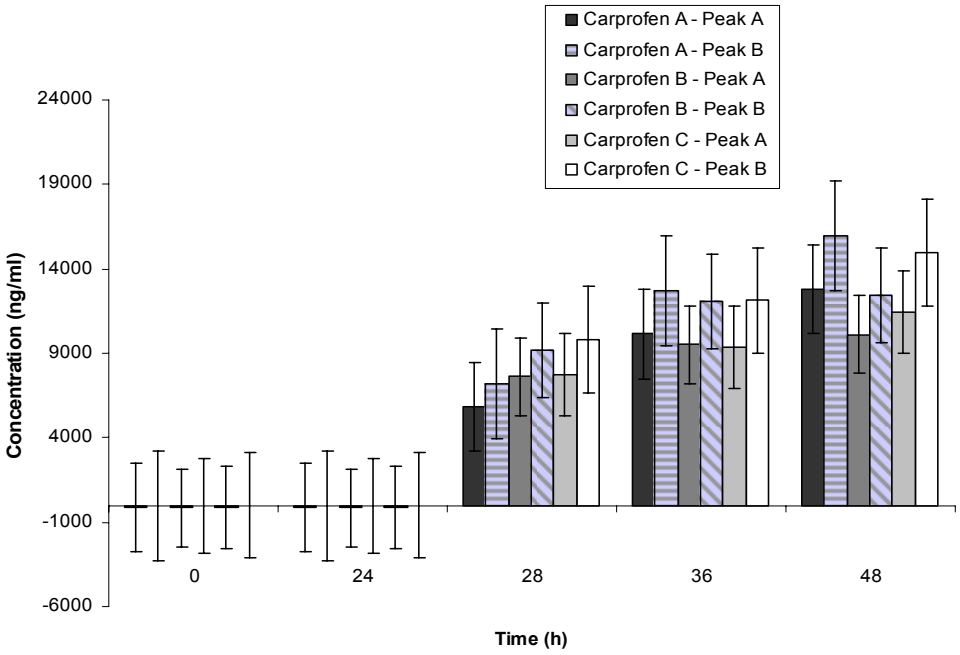

Fig. A-103. Carprofen A, B and C hepatic slice supernatant concentrations following 24 hour phenobarbital (150 µg/ml) incubation. Concentrations of carprofen enantiomers in hepatic slice supernatant after incubation for 24 hours with 150 µg/ml phenobarbital and incubated for an additional 4, 12 and 24 hours with carprofen A, B and C. The values are an average of two samples per carprofen type per time point.

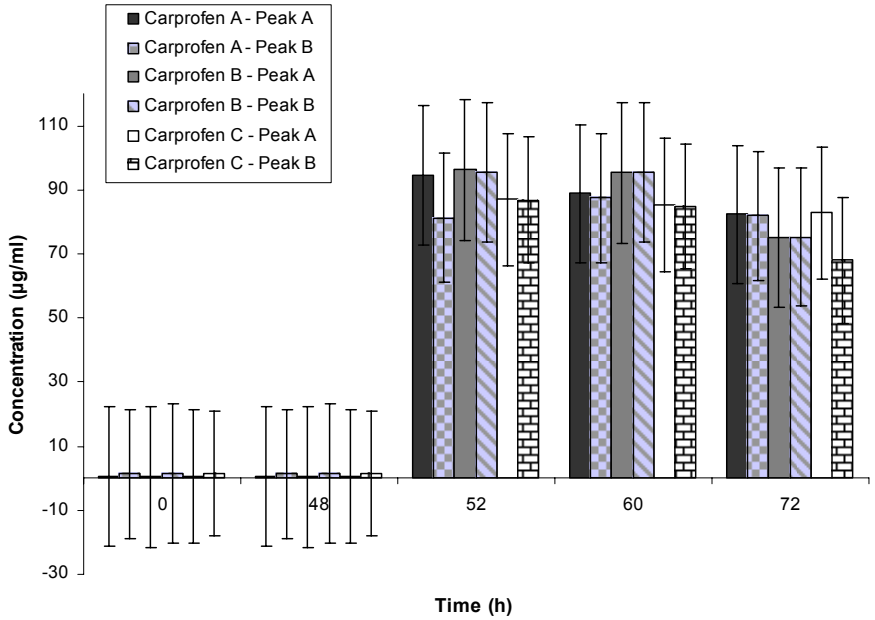

Fig. A-104. Carprofen A, B and C media concentrations following 48 hour phenobarbital (150 µg/ml) incubation. Concentrations of carprofen enantiomers in media after incubation for 48 hours with 150 µg/ml phenobarbital and incubated for an additional 4, 12 and 24 hours with carprofen A, B and C. The values are an average of three samples per carprofen type per time point.

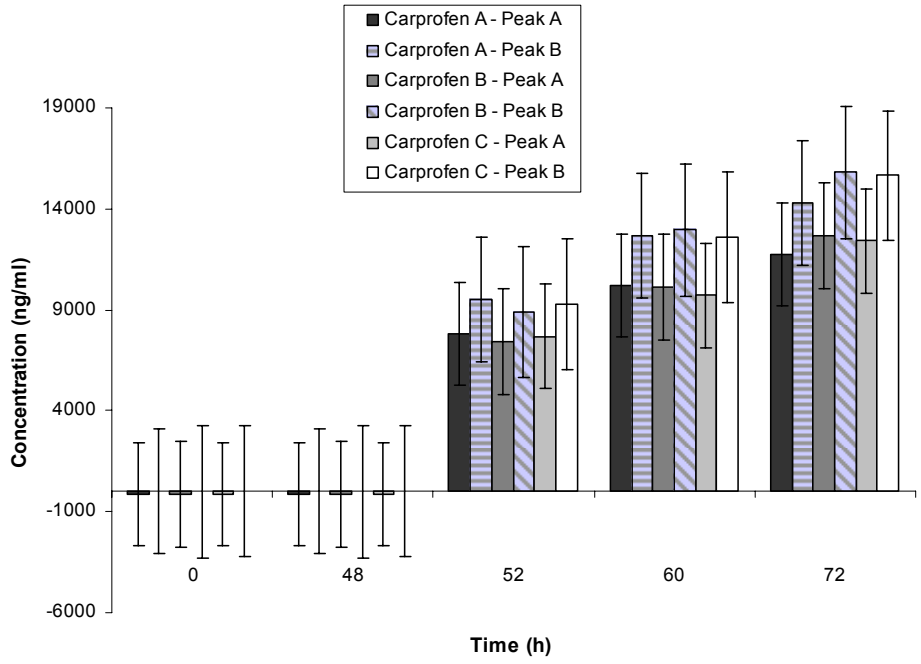

Fig. A-105. Carprofen A, B and C hepatic slice supernatant concentrations following 48 hour phenobarbital (150 µg/ml) incubation. Concentrations of carprofen enantiomers in hepatic slice supernatant after incubation for 48 hours with 150 µg/ml phenobarbital and incubated for an additional 4, 12 and 24 hours with carprofen A, B and C. The values are an average of two samples per carprofen type per time point.

Charts and Statistics for Mean Data

The following charts and tables describe the media and supernatant concentrations for slices incubated in 75 or 150 µg/ml phenobarbital for 24 or 48 hours and followed by an additional 24 hour incubation in media containing 0 or 100 µg/ml carprofen. Concentrations for media containing 0 µg/ml carprofen and associated supernatant samples were all below the lower limit of quantification.

Carprofen and Carprofen with 75 µg/ml Phenobarbital Media

The mean media concentrations of carprofen after slice incubation in carprofen for 4, 12 and 24 hours or in 75 µg/ml phenobarbital for 24 or 48 hours followed by incubation in 100 µg/ml carprofen media for an additional 4, 12 and 24 hours (Figure A-106) were analyzed for time and treatment differences.

Two-way ANOVA for peak A revealed a significant factor interaction (df=4, F-ratio=2.822, p-value=0.032) for time and treatment. Significant time (df=2, F-ratio=8.483, p-value=0.001) and treatment (df=2, F-ratio=5.618, p-value=0.005) effects were noted for mean peak A concentrations. Mean media concentrations at 4 and 12 hours were higher than those at 24 hours (Table A-100). For treatment, media concentrations of carprofen alone were higher than those of media following 24 hour incubation in phenobarbital (Table A-101).

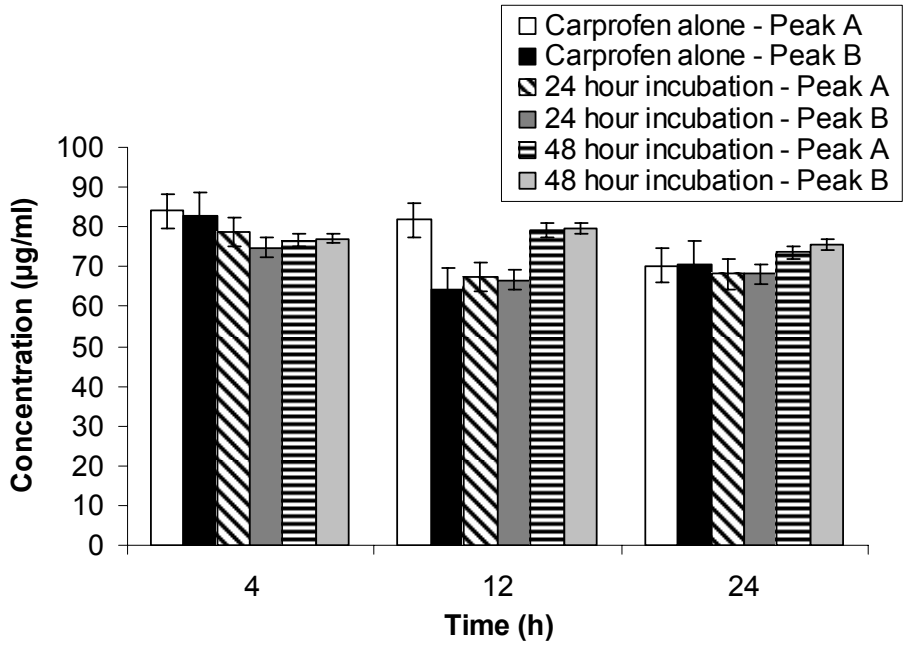

Fig. A-106. Mean media concentrations of carprofen alone and following 24 and 48 hour phenobarbital (75 µg/ml) incubation. Mean concentrations (µg/ml) of carprofen enantiomers (A or B) in media after incubation in only carprofen or incubated for 24 or 48 hours with 75 µg/ml phenobarbital and incubated for an additional 4, 12 and 24 hours with carprofen (100 µg/ml). The values are a mean of nine samples per treatment per time point.

Table A-100. Comparison across time of mean peak A media concentrations of carprofen in 100 µg/ml media for all treatments. Homogeneous groups for peak A two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time post-phenobarbital (h)	Homogeneous groups	Peak A Means (µg/ml)
4	a	79.332
12	a	75.861
24	b	70.712

Table A-101. Comparison among treatments of mean peak A media concentrations of carprofen in 100 µg/ml media incubated for 0, 24 or 48 hours with 75 µg/ml phenobarbital for all times. Homogeneous groups for peak A two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Length of incubation in phenobarbital	Homogeneous groups		Peak A Means (µg/ml)
0 hours	a		78.222
24 hours		b	71.436
48 hours	a	b	76.490

A significant factor interaction (df=4, F-ratio=3.083, p-value=0.022) was found for mean peak B carprofen media concentrations. Significant differences were noted for time (df=2, F-ratio=6.286, p-value=0.003) and for treatment (df=2, F-ratio=5.483, p-value=0.006) for peak B. Across time, mean carprofen concentrations at 4 hours were higher than those at 12 and 24 hours (Table A-102). Comparison of length of phenobarbital incubation revealed that mean carprofen media concentrations following 48 hour incubation were higher than those after 24 hour incubation (Table A-103).

Table A-102. Comparison across time of mean peak B media concentrations of carprofen in 100 µg/ml media for all treatments. Homogeneous groups for peak B two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time post-phenobarbital (h)	Homogeneous groups	Peak B Means (µg/ml)
4	a	77.686
12	b	70.431
24	b	71.529

Table A-103. Comparison among treatments of mean peak B media concentrations of carprofen in 100 µg/ml media incubated for 0, 24 or 48 hours with 75 µg/ml phenobarbital for all times. Homogeneous groups for peak B two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Length of incubation in phenobarbital	Homogeneous groups		Peak B Means (µg/ml)
0 hours	a	b	71.787
24 hours	a		69.843
48 hours		b	77.420

Carprofen and Carprofen with 75 µg/ml Phenobarbital Supernatant

The mean supernatant concentrations for slices incubated in 100 µg/ml carprofen alone or in 75 µg/ml phenobarbital for 24 and 48 hours followed by incubation in 100 µg/ml carprofen media for an additional 4, 12 and 24 hours (Figure A-107) were analyzed for time and treatment differences. Two-way ANOVA for peak A revealed a significant factor interaction (df=4, F-ratio=10.319, p-value=0.000) for time and treatment. Significant time (df=2, F-ratio=18.679, p-value=0.000) and treatment (df=2, F-ratio=6.052, p-value=0.005) effects were noted for mean peak A concentrations. Across time, mean concentrations at 4 hours were lower than those at 12 and 24 hours; 12 hour concentrations were higher than 24 hour concentrations (Table A-104). Among treatments, mean supernatant concentrations for both carprofen alone and 48 hour phenobarbital incubation were higher than those for the 24 phenobarbital incubation (Table A-105).

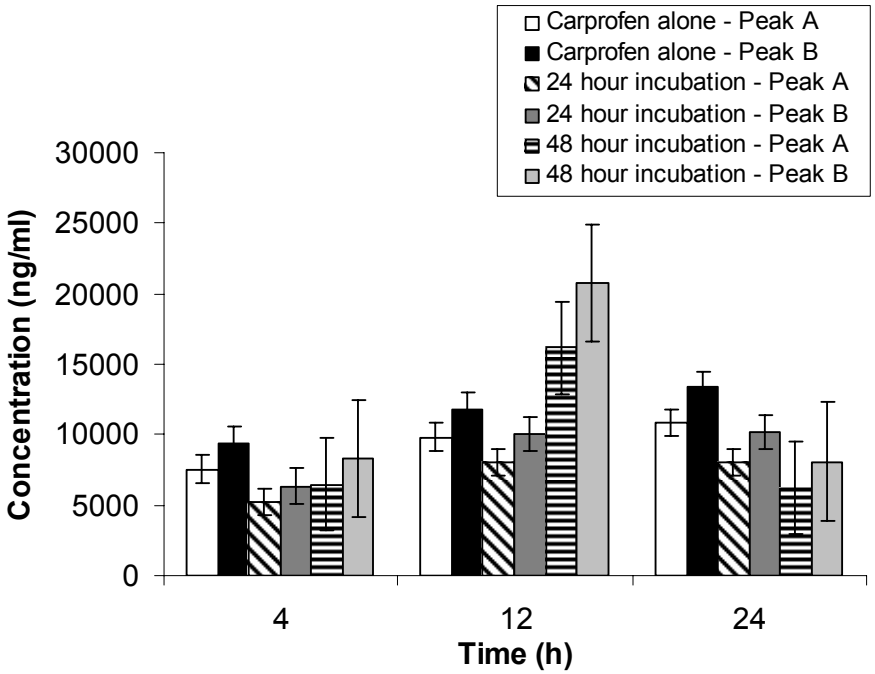

Fig. A-107. Mean hepatic slice supernatant concentrations of carprofen alone and following 24 and 48 hour phenobarbital (75 µg/ml) incubation. Mean concentrations (ng/ml) of carprofen enantiomers (A or B) in hepatic slice supernatant after incubation in only carprofen or incubated for 24 or 48 hours with 75 µg/ml phenobarbital and incubated for an additional 4, 12 and 24 hours with carprofen (100 µg/ml). The values are a mean of six samples per treatment per time point.

Table A-104. Comparison across time of mean peak A supernatant concentrations of carprofen in 100 µg/ml media for all treatments. Homogeneous groups for peak A two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time post-phenobarbital (h)	Homogeneous groups	Peak A Means (ng/ml)
4	a	6296.522
12	b	11354.041
24	c	8344.426

Table A-105. Comparison among treatments of mean peak A supernatant concentrations of carprofen in 100 µg/ml media incubated for 0, 24 or 48 hours with 75 µg/ml phenobarbital for all times. Homogeneous groups for peak A two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Length of incubation in phenobarbital	Homogeneous groups	Peak A Means (ng/ml)
0 hours	a	9635.661
24 hours	b	7123.391
48 hours	a	9606.952

Similar to peak A average supernatant concentrations, a significant factor interaction (df=4, F-ratio=10.914, p-value=0.000) for time and treatment was found for peak B mean concentrations. Additionally, significant time (df=2, F-ratio=18.264, p-value=0.000) and treatment (df=2, F-ratio=6.897, p-value=0.003) effects were noted for mean peak B concentrations. Twelve hour mean supernatant concentrations were higher than those at 4 and 24 hours (Table A-106). Among treatments, mean supernatant concentrations for the 24 hour phenobarbital incubation were lower than those for carprofen

alone or the 48 hour phenobarbital incubation (Table A-107). For both peak A and peak B, mean supernatant concentrations at 12 hours were higher than those at 4 and 24 hours. Mean supernatant concentrations for carprofen alone and following 48 hour phenobarbital incubation were higher than those for the 24 hour incubation.

Table A-106. Comparison across time of mean peak B supernatant concentrations of carprofen in 100 µg/ml media for all treatments. Homogeneous groups for peak B two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time post-phenobarbital (h)	Homogeneous groups	Peak B Means (ng/ml)
4	a	7856.020
12	b	14185.801
24	a	10534.050

Table A-107. Comparison among treatments of mean peak B supernatant concentrations of carprofen in 100 µg/ml media incubated for 0, 24 or 48 hours with 75 µg/ml phenobarbital for all times. Homogeneous groups for peak B two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Length of incubation in phenobarbital	Homogeneous groups	Peak B Means (ng/ml)
0 hours	a	11777.784
24 hours	b	8843.906
48 hours	a	12389.932

Carprofen and Carprofen with 150 µg/ml Phenobarbital Media

The mean media concentrations of carprofen alone or following incubation for 24 or 48 hours in 150 µg/ml phenobarbital (Figure A-108) were analyzed for time and treatment differences. When analyzed by two-way ANOVA significant differences were noted for time (df=2, F-ratio=4.647, p-value=0.013) and

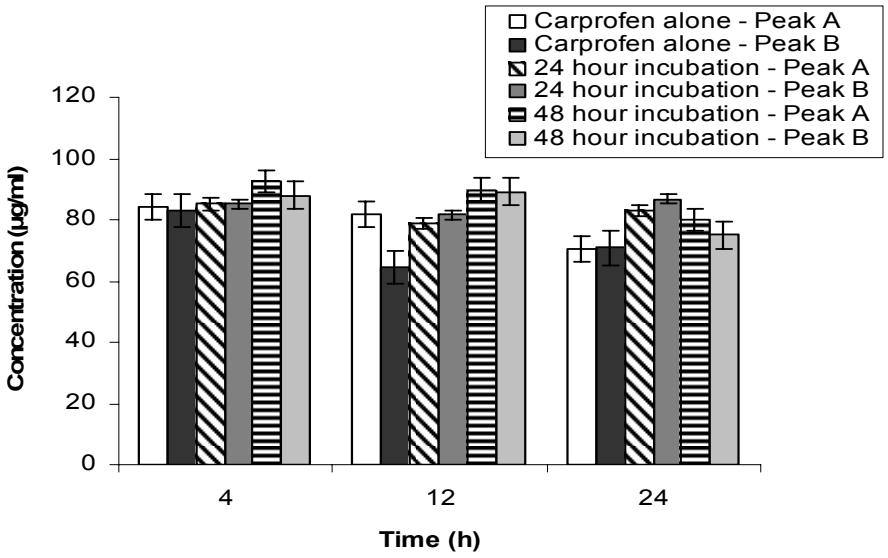

Fig. A-108. Mean media concentrations of carprofen alone and following 24 and 48 hour phenobarbital (150 µg/ml) incubation. Mean concentrations (µg/ml) of carprofen enantiomers (A or B) in media after incubation in only carprofen or incubated for 24 or 48 hours with 150 µg/ml phenobarbital and incubated for an additional 4, 12 and 24 hours with carprofen (100 µg/ml). The values are a mean of nine samples per treatment per time point.

treatment (df=2, F-ratio=3.964, p-value=0.024) for peak A. Across time, mean concentrations at 4 hours were higher than those at 24 hours (Table A-108). Among treatments, mean concentrations for the 48 hour phenobarbital incubation were higher than those for the carprofen only incubation (Table A-109).

Table A-108. Comparison across time of mean peak A media concentrations of carprofen in 100 µg/ml media for all treatments. Homogeneous groups for peak A two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time post-phenobarbital (h)	Homogeneous groups	Peak A Means (µg/ml)
4	a	87.672
12	a b	83.458
24	b	78.103

Table A-109. Comparison among treatments of mean peak A media concentrations of carprofen in 100 µg/ml media incubated for 0, 24 or 48 hours with 150 µg/ml phenobarbital for all times. Homogeneous groups for peak A two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Length of incubation in phenobarbital	Homogeneous groups	Peak A Means (µg/ml)
0 hours	a	78.222
24 hours	a b	82.290
48 hours	b	87.481

For peak B, a significant factor interaction was noted (df=4, F-ratio=3.145, p-value=0.020). In addition, a significant treatment (df=2, F-ratio=7.622. p-value=0.001) effect was found for mean peak B media concentrations of carprofen. The mean carprofen media concentrations were higher for the 24 hour and 48 hour phenobarbital incubations than for the carprofen only incubation (Table A-110).

Table A-110. Comparison among treatments of mean peak B media concentrations of carprofen in 100 µg/ml media incubated for 0, 24 or 48 hours with 150 µg/ml phenobarbital for all times. Homogeneous groups for peak B two-way ANOVA for treatment are represented as letters. Treatments with the same letter are not significantly different.

Length of incubation in phenobarbital	Homogeneous groups	Peak B Means (µg/ml)
0 hours	a	71.787
24 hours	b	84.558
48 hours	b	84.006

Carprofen and Carprofen with 150 µg/ml Phenobarbital Supernatant

The mean supernatant concentrations of carprofen following incubation of slices in carprofen alone or following incubation for 24 or 48 hours in 150 µg/ml phenobarbital (Figure A-109) were analyzed for time and treatment differences. For both peak A and peak B, a significant time effect was noted (A: df=2, F-ratio=58.075, p-value=0.000; B: df=2, F-ratio=62.938, p-value=0.000). For both peaks, mean supernatant concentrations of carprofen at 4 hours were lower than at 12 and 24 hours, and 24 hour concentrations were higher than 12 hour concentrations (Table A-111).

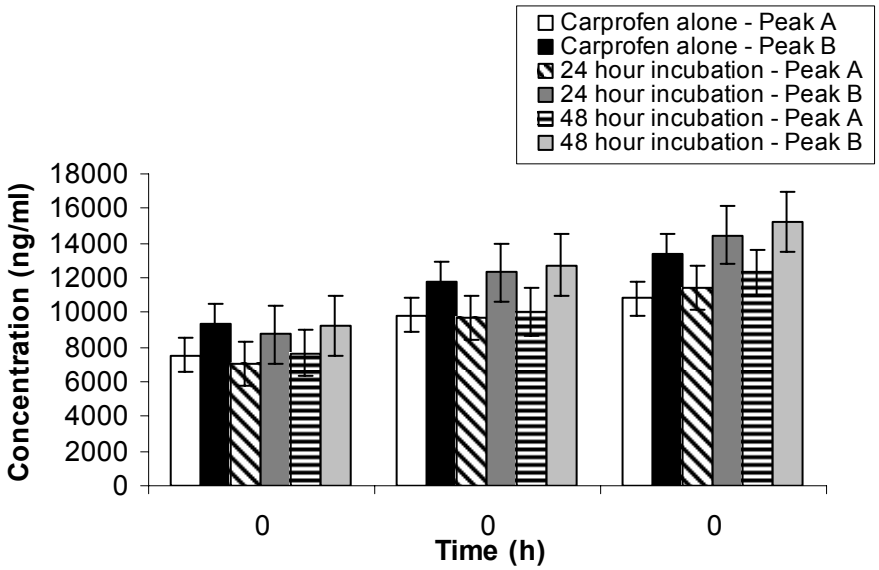

Fig. A-109. Mean hepatic slice supernatant concentrations of carprofen alone and following 24 and 48 hour phenobarbital (150 µg/ml) incubation. Mean concentrations (ng/ml) of carprofen enantiomers (A or B) in hepatic slice supernatant after incubation in only carprofen or incubated for 24 or 48 hours with 150 µg/ml phenobarbital and incubated for an additional 4, 12 and 24 hours with carprofen (100 µg/ml). The values are a mean of six samples per treatment per time point.

Table A-111. Comparison across time of mean peak A or B supernatant concentrations of carprofen in 100 µg/ml media for all treatments. Homogeneous groups for peaks A and B two-way ANOVA for time are represented as letters. Times with the same letter are not significantly different.

Time post-phenobarbital (h)	Homogeneous groups	Peak A Means (ng/ml)	Peak B Means (ng/ml)
4	a	7399.905	9081.241
12	b	9841.040	12273.708
24	c	11527.217	14354.917

VITA

Maya Millicent Scott

Permanent Address
761 Cedarwood Avenue
Fayetteville, AR 72701-1606
(479) 521-3677

Education

Doctor of Philosophy in Toxicology
Texas A&M University
College Station, Texas, 2005

Bachelor of Science in Animal Science
Summa Cum Laude
University of Arkansas
Fayetteville, Arkansas, 1996

Doctor of Veterinary Medicine
(top 10% of class)
Oklahoma State University
Stillwater, Oklahoma, 2000

Work Experience

Veterinary Clinical Associate — August 2000 – May 2005
Department of Veterinary Physiology & Pharmacology
Texas A&M University
College of Veterinary Medicine
College Station, Texas
Dawn M. Boothe

Veterinary Intern — November 1999 - December 1999
Pharmacia and Upjohn
Kalamazoo, Michigan
Scott A. Brown

Awards and Honors

Graduate School

United States Pharmacopeia Fellowship	2003-2004
Outstanding Ph.D. Graduate Student	2002
Graduate Student Council Representative	2002-2004
Regents Fellowship	2000-2001

Veterinary School

Merck Award for Academic Excellence	2000
Hill's Pet Nutrition Multicultural Scholarship	2000
John Montgomery Scholarship	1996-2000
Salsbury Scholarship	1999