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Effects of ciprofloxacin on drug P450 metabolic pathways in pigs

Sherry Cox

To the Graduate Council:

I am submitting herewith a dissertation written by Sherry Cox entitled "Effects of ciprofloxacin on drug P450 metabolic pathways in pigs." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Joe Bartges, Major Professor

We have read this dissertation and recommend its acceptance:

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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EFFECTS OF CIPROFLOXACIN ON DRUG P450 METABOLIC PATHWAYS IN PIGS

A Dissertation
Presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Sherry Cox
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DEDICATION

This thesis is dedicated to all the people who have supported and encouraged me over the many years it has taken to achieve this goal. In loving memory of Miss Ann who was my constant companion through the entire process and was her mom's pride and joy.

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ABSTRACT

Drugs are potentially toxic substances that elicit dosage-dependent therapeutic effects for specific disease conditions. The efficacy of a drug regimen depends on the concentration of the drug at the site of needed activity and the duration of time it is maintained. The selection of dosing regimens for different species requires the establishment of pharmacokinetic equivalency between species; that is, achieving equivalent peak serum and tissue concentrations and duration of drug exposure. Drug metabolism is a direct reflection of the multiple enzyme systems that characterize different species and is often the most important single factor in regulation if drug concentrations in the body. The largest concentration of enzymes catalyzing these reactions is located in the liver; however, significant concentrations also exist in other tissues such as the intestine. The cytochrome P450 system metabolizes the majority of drugs. Although cytochrome P450 enzymes exist in all species examined to date, minor changes in the structure or tissue distribution of the enzymes may lead to great differences in the metabolism and elimination of specific drugs. A lack of consideration of the rates of biotransformation and elimination of drugs in animals particularly those intended for food may result in drug residues, such as quinolones, in consumed meats. Alternatively, ineffective drug concentrations may prevent killing of bacteria, leading to contamination of meats. More important, low level antibiotic concentrations may favor development of resistant bacterial strains. In an effort to determine pharmacokinetic differences that can indirectly affect development of quinolone resistance in bacteria, differences in quinolone disposition will be identified by allometric analysis of pharmacokinetic data from different species will be analyzed. Activities of specific cytochrome P450 enzymes will be examined in swine.

TABLE OF CONTENTS

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

FIGURE PAGE

Chapter 1: Introduction

Designing Dosage Regimens

Drugs are potentially toxic substances that elicit dosage-dependent therapeutic effects for specific diseases. A drug is usually described by its most prominent effect or by the action thought to be the basis of the effect. However, such descriptions should not obscure the fact that most drugs produce multiple effects. A drug is characterized adequately only when its full spectrum of effects has been determined. The relationship between the dosages of a drug associated with undesirable as well as desirable effects is termed its therapeutic index, margin of safety, or selectivity. For therapeutic applications, selective action of a drug is clearly one of its more important characteristics (Nies and Spielberg, 1996). Furthermore, a drug may have more than a single therapeutic index, if different indications require varied dosages.

It is typical to administer multiple doses of a therapeutic agent at intervals appropriate for the drug preparation selected. The objective of the dosage regimen for most drugs is to maintain plasma concentrations of the drug or its active metabolites within the therapeutic range for the duration of treatment. Elucidation of the therapeutic (safe and effective) range of plasma concentrations for a drug relies on determination of concentration-response relationships for each therapeutic indication. Clinical evaluation is required, with precise physiologic and biochemical characterization of the response in a sufficient number of appropriately selected individuals. The effective concentrations and width of the therapeutic range are considered to reflect inherent activity and relative safety of a drug.

For some drugs, the pharmacologic effects are difficult to measure, toxicity and lack of efficacy are potential dangers, and/or the therapeutic index is narrow. In these circumstances, dosage regimens must be titrated carefully and a target-level strategy

applied. The desired plasma concentration of the drug is chosen that is expected to achieve effective tissue levels while remaining within the therapeutic range, and a dosage is computed that is expected to achieve this value. Monitoring of drug concentrations in blood is indicated when possible, for drugs that have narrow therapeutic indices. Although individual variation in response is usually present, the goal of treatment is to achieve the established therapeutic range for a drug in order to avoid ineffective treatment or toxicity.

Because patients differ in their response to drugs, treatment must be considered unique to that individual. Individualization of therapy for a particular patient requires a basic understanding of pharmacokinetics and pharmacodynamics. The success of a drug regimen depends on the achievement of effective concentration of the drug at the site of needed activity. How long the drug effect can be maintained influences the frequency of dosing, which impacts patient compliance. The pharmacokinetic disposition of a drug depends on the anatomy and physiology of the patient as well as specific properties of the drug itself. Factors that determine the relationship between prescribed drug usage and drug effect can be divided into three categories: those that affect the administered dose, those that affect the concentration at the site of action, and those that intensify the effect (Poklis, 1996).

Factors that affect the administered dose are: rate and extent of absorption, body size and composition, distribution in body fluids, binding to plasma proteins and tissues, and rate of elimination. Those that affect the concentration at the site of action are interaction with other drugs, development of tolerance, genetic factors, pathological factors, and physiological variables such as pH and pKa of drug. Factors that can intensify the effect are drug-receptor interaction, physiological state of the patient, and placebo effects. In order to design an appropriate therapeutic dosage regimen, all

sources of variation in response to the drug must be considered. Without consideration of these factors, therapy may be ineffective or produce deleterious effects. For example, inappropriate dosages of antibiotics such as quinolones used in animals intended for food may indirectly harm the consumer by favoring development of antibiotic-resistant bacteria, or through inadvertent consumption of drug residues.

Extrapolating Dosages Between Species using Allometry

The extrapolation of dosing regimens between species requires the establishment of pharmacokinetic equivalency; that is, achieving equivalent peak serum and tissue concentrations and duration of drug exposure (Frazier et al., 1995). The ability to achieve equivalency relates to the physiologic and morphologic characteristics of the tissues and organs responsible for drug absorption, distribution, metabolism and excretion. The appropriateness of drug dosage extrapolation between species depends on the similarity of the pharmacokinetic behavior of the drug for the species.

The allometric approach is a basic mathematical tool for analyzing differences in anatomy, physiology, biochemistry, and pharmacokinetics in animals of different sizes. At least 750 allometric equations have been reported (Calder, 1984). The usual allometric approach relates one biologic function or structure (y) to another (x) through an empirical power function:

$$y = a(x)^b$$

The extrapolation of pharmacokinetic parameters from one animal to another is based on allometric expressions of relative body size. This means that a small animal has a specific basal metabolic rate (kcal/kg) that is greater than that of a large animal (when both are of a group having the same mean core body temperature range) (Sedgwick, 1993). A smaller animal, with its higher metabolic rate, has a more rapid mean circulation time, higher densities of capillaries per unit of any given tissue, more

respiratory surface area for gas exchange, a higher glomerular filtration rate, greater hepatic enzyme activity, and higher intracellular densities of mitochondria and cytochrome C (important to tissue metabolism) per unit of body size than a larger animal (Schmidt-Nielsen, 1984). In other words, small-size animals are expected to have more metabolic activity per unit of body mass that may affect drug disposition than that of large animals.

To illustrate the importance of considering differences in metabolic rate when extrapolating treatment regimens, consider that a mouse is only 1/2800 the size of the human, but its dose rate and treatment frequency of an antibiotic were both found to be more than six-fold that of the human. If a clinician were to treat a mouse patient with the human antibiotic regimen, the therapeutic serum level could never be reached, but if the human patient were treated with the mouse regimen, it would surely prove toxic (Sedgwick, 1993).

The perceptive clinician must consider the mechanisms of drug absorption, biotransformation, distribution, and excretion to determine the appropriateness of extrapolating dosages between species. Allometric equations reveal quantitative differences, but not necessarily qualitative differences, in structure or function between animals associated with differences in body weight. Qualitative differences (in structure or function) can change the relationship to body weight and yield a different value for either a or b. Use of an incorrect estimate for either a or b can grossly over-estimate or under-estimate clearance of a drug (Frazier et al., 1995). Due to the development of bacterial resistance to older more established antibiotics, newer drugs such as quinolones are being used in a variety of species; however, not much is known about the allometric relationships of these drugs.

Effect of Drug Metabolism on Dosage Regimens

Although laboratory animals are considered to be adequate predictors of the effects of drugs on other species, species-dependent differences in drug metabolism often hinders extrapolation of efficacious, non-toxic dosages. For drug safety and efficacy reasons, it is of paramount importance to determine metabolic pathways, and to identify the active cytochrome P450 enzymes involved.

1. Mechanisms of Drug Biotransformation

Drug metabolism is a direct reflection of the multiple enzyme systems that characterize different animal species, and it is often the most important single factor in the regulation of plasma drug concentrations. Reactions catalyzed by these biotransforming enzymes are generally divided into two groups called phase I and phase II reactions. Phase I reactions involve oxidation, reduction, and hydrolysis. These reactions introduce a functional group (-OH, -NH_2, -SH, or -COOH) that increases water solubility and excretion, or provides a "handle" which is used in the second phase reactions. Phase II biotransformation reactions include glucuronidation, sulfation, acetylation, methylation, conjugation with glutathione, and conjugation with amino acids. Most phase II reactions result in a large increase in hydrophilicity of the compound; hence, they greatly promote excretion.

In many instances, chemical modification by metabolism also alters the biological effects of the compound. The importance of this principle is that some drugs must undergo biotransformation in order to exert their pharmacodynamic effect (i.e., it is the metabolite of the drug, and not the drug itself, that exerts the pharmacological effect) (Parkinson, 1996). In most cases, however, biotransformation decreases or terminates the pharmacological effects of a drug and lessens its toxicity.

The largest concentration of enzymes catalyzing biotransformation reactions is located in the liver. Significant concentrations of specific enzymes are also located in the skin, lung, nasal mucosa, eye, gastrointestinal tract, kidneys, adrenal gland, pancreas, spleen, heart, brain, testis, ovary, placenta, plasma, erythrocytes, platelets, lymphocytes, and aorta (Parkinson, 1996). The enzymes are located primarily in the endoplasmic reticulum (microsomes), or the soluble fraction of the cytoplasm (cytosol), with lesser amounts in the mitochrondria, nuclei, and lysosomes. Hepatic extraction and biotransformation of compounds absorbed from the gastrointestinal tract limits the systemic bioavailability of orally ingested compounds, a process known as first pass elimination (Parkinson, 1996; Back and Rogers, 1987). In some cases, biotransformation in the intestine contributes significantly to first pass elimination of compounds (Parkinson, 1996).

2. Cytochrome P450 Enzymes

Among the phase I biotransforming enzymes, the cytochrome P450 system ranks first in terms of catalytic versatility and the sheer number of compounds it detoxifies or activates to reactive intermediates (Parkinson, 1996; Guengerich, 1992). The largest concentration of P450 enzymes is located in the liver endoplasmic reticulum (microsomes), but they are located in virtually all other tissues. These enzymes also perform important roles in the activation of xenobiotics to toxic and/or tumorigenic metabolites as well as being involved in the biosynthesis of steroid hormones, bile acids, fat-soluble vitamins, and fatty acids. P450 enzymes play a very important role in determining the intensity and duration of action of drugs, and they also play a key role in the detoxification of xenobiotics.

Cytochrome P450 enzymes are present in all species examined, including bacteria, fungi, insects, plants, fish, amphibia, and mammals (Doehmer et al., 1993). At

least 221 different cytochrome P450 enzymes have been described (Nelson et al., 1993). Protein and gene sequence alignments and comparisons have revealed interrelationships between the different cytochrome P450 enzymes (Gotoh, 1992). They have been classified into 36 gene families of which 12 families exist in all mammals. These 12 families comprise 22 mammalian subfamilies. About 70 different human cytochrome P450 enzymes are known, and a uniform nomenclature has been developed and recommended. In general, P450 enzymes with less than 40 percent amino acid sequence identity are assigned to different gene families (gene families 1,2,3,4 etc.) P450 enzymes that are 40 to 55 percent identical are assigned to different subfamilies (2A, 2B, 2C, etc.). The P450 enzymes that are more than 55 percent identical are classified as members of the same subfamily (2A1, 2A2, 2A3, etc.) (Parkinson, 1996). Hepatic microsomal P450 enzymes involved in xenobiotic biotransformation belong to three main P450 gene families: CYP1, CYP2, and CYP3. Hepatic microsomes also contain P450 enzymes encoded by the CYP4 gene family. The substrates for the latter enzymes include several fatty acids and eicosanoids, but relatively few xenobiotics (Parkinson, 1996). P450 enzymes in liver microsomes of these gene families generally belong to a single subfamily (i.e., CYP1A, CYP3A, and CYP4A). The exception to the single family rule is the CYP2 gene family which contains five subfamilies (i.e., CYP2A, 2B, 2C, 2D, and 2E) (Parkinson, 1996). The number of P450 enzymes in a subfamily differs between species (Parkinson, 1996).

Recent studies have been directed at molecular mechanisms underlying the broad but specific metabolic capacities of P450 enzymes. These enzymes comprise a relatively limited number of catalysts that have partial overlap but distinct substrate specificities. There have been various experimental and analytical investigations involved in the determination of recognition or binding of substrates and substrate

specificity. These include site-directed mutagenesis, chemical modifications with substrate analogues, and sequence alignment (Gotoh, 1992; Guengerich, 1994). Sequence alignment has become much more feasible in the last several years due to the availability of commercial software. A large number of mammalian sequences are now accessible from databases such as GenBank, SwissProt and GenEMBL. Sequences for CYP1A1 and 1A2 have been reported for the following species: rat, human, rabbit, dog, hamster, monkey, guinea pig, mouse, trout, and chicken (Nelson et al., 1993). Sequences for the 3A family have been reported for: sheep, human, rat, rabbit, monkey, hamster, and dog (Nelson et al.,1993). 2E1 sequences have been reported for: human, rat, rabbit, monkey and mouse, while the 2D family has been found in: human, dog, mouse, and cow, rat and pig (Nelson et al., 1993).

A number of highly conserved sequences throughout the P450 family have been discovered using this alignment process. The alignment process has also revealed areas with excessively high non-synonymous amino acid substitutions, which coincide with potential substrate binding sites (Gotoh, 1992). These are called substrate recognition sites (SRS's). There are six of these proposed SRS sites that make up roughly 16% of a total sequence (Gotoh, 1992). Many site directed mutations that affect metabolism fall within these SRS's (Gotoh, 1992; Ellis et al., 1995). Certain amino acids or sequences are known to be well conserved across species (Lewis and Moereels, 1992; Edwards et al., 1989; Johnson et al., 1992; Yamazaki et al., 1993), while others in the P450 families vary (Gotoh, 1992; Ellis et al., 1995; Johnson et al., 1992; Negishi et al., 1996; Krainev et al., 1992). The emphasis to date has been on determining sequence homologies of cytochrome P450's (Lewis and Moereels, 1992; Edwards et al., 1989; Sakuma et al., 1994; Komori et al., 1992), with very few comparisons of the relationship between variability in sequences and xenobiotic biotransformation.

3. Species Differences in Metabolism

Variations in rates of drug metabolism have been recognized in humans (Nies and Spielberg, 1996; Poklis, 1996; Guengerich, 1992) and other species (Nies and Spielberg, 1996; Poklis, 1996; Smith, 1991; Mellett, 1969). The effect of genetic polymorphism in cytochrome P450 metabolizing enzymes can lead to dramatic changes in therapeutic concentrations at the site of action. When different individuals are administered identical dosages of a drug at similar time intervals, it is not uncommon to observe large differences in plasma concentrations of the drug. The classic example is the polymorphism described for CYP2D6. Two distinct classes of fast (those that never attain therapeutic plasma concentrations) and slow (those that attain excessive plasma concentrations) metabolizers have been observed among the human population. They are characterized by their efficiency in the metabolism of either debrisoquine or sparteine (Coutts, 1994; Gaedigk et al., 1991). CYP2D6 is also responsible for the metabolism of antidepressants, cardiovascular agents, morphine derivatives, psychoactive drugs and anticonvulsants (Doehmer et al., 1993; Coutts, 1994; Gaedigk et al., 1991). Results of studies indicate that slow (or poor) metabolizers have a total absence of the hepatic CYP2D6 protein (Gaedigk et al., 1991, Brosen, 1990). It is prudent to recognize that genetic factors can lead to significant variations in a therapeutic response to, and side-effects of, a particular drug. Although not routinely done, phenotyping of the patient prior to administration of a drug may aid in determining the proper therapeutic dose.

One of the main differences between humans and lower animals is the rate of the reactions which transform drugs (Smith, 1991). There are important species differences in the function and regulation of the cytochrome P450 enzymes. Variations have been attributed in part to the presence of different P450 enzymes, and to their inducibility

(Guengerich, 1992). Differences also occur in the response of cytochrome P450 enzymes between species (Smith, 1991; Boobis et al., 1990). Possible reasons for species differences are: the relative concentrations of different biotransforming enzymes, the active site of the enzyme differs among species, activity is catalyzed by different enzymes or isozymes (the enzyme may be in one species but completely missing in another), differences in the specificity of the isozyme, and differences in the pathways competing for the substrate (Parkinson, 1996; Mellett, 1969; Boobis et al., 1990). Any one or combination of these reasons can have a tremendous impact on the way a compound is metabolized by a particular species. A compound that is effectively used in one species could be useless because it is not metabolized in another, while a therapeutic dose in one species could prove to be lethal in another due to differences in metabolism.

Although food safety is of increasing concern to the public, little has been done to characterize specific P450 isozymes in food animals (Broad et al., 1995). Quinolones represent a class of antibiotics about which limited information on P450 enzyme metabolism is available in domestic animals. Because the use of these drugs is likely to increase in food animals, further information about the rates of metabolism to active and inactive metabolites is needed.

4. Distinguishing Between Parent Compound and Metabolite

The structural diversity of the multitude of modern therapeutic drugs is well documented (Nies and Spielberg, 1996; Poklis, 1996). To date, most pharmacokinetic drug studies have focused only on identification of parent compounds. Because the efficacy and toxicity of the administered drug may depend on one or more metabolites as well as the parent compound, the isolation and structural characterization of the

resulting array of metabolites is vitally important to understanding the physical and biological effects of the parent drug.

Isolation of drug metabolites from biological material is often difficult due to low concentrations in urine, bile or blood, and high concentrations of interfering endogenous compounds and food constituents (Dieterle and Faigle, 1983). The physico-chemical properties of the more polar metabolites, notably conjugates, further complicates their isolation in pure form (Dieterle and Faigle, 1983). Some metabolites may require chemical modification or thermal degradation in order to affect a separation. Furthermore, phase I metabolism of a drug often results in only minor structural modification of the parent compound, and determination of suitable chromatographic conditions to produce a separation of mixtures of drug metabolites is often particularly difficult (Naylor et al., 1996).

Quinolones consist of a bicyclic ring structure in which there is a substitution at position N-1, with various moieties. All of the current agents have a carboxyl group at position 3, a keto group at position 4, a fluorine atom at position 6, and a piperazinyl group or a methylpiperazinyl group at C-7. Differences in the moiety present at the N-1 and C-7 positions markedly influence both microbiological and pharmacokinetic properties. The extent to which different quinolones undergo hepatic biotransformation varies. Although glucuronide conjugates have been identified as minor metabolites, most metabolic reactions involving quinolones occur through microsomal oxidative mechanisms at the cytochrome P450 site.

Ciprofloxacin belongs to the potent antimicrobial group termed fluoroquinolones, and chemically is defined as 1-cyclopropyl-6-fluoro-1,4-dihyrdo 4-oxo-7-(1-piperazinyl)-3-quinolone carboxylic acid. Ciprofloxacin undergoes biotransformation in the liver to four metabolites: desethylene-ciprofloxacin (M-1), sulfo-ciprofloxacin (M-2), oxo-

ciprofloxacin (M-3) and formyl-ciprofloxacin (M-4). These metabolites are formed by piperazine-ring cleavage, sulfo-conjugation, piperazine-ring oxidation and N-formyl-conjugation. Ultimately these metabolites are excreted in both bile and urine of humans. However, very limited information is available with regard to metabolites that are formed in food animals. The M1 and M3 metabolites are products of cytochrome P450 metabolism. Sulfo-ciprofloxacin (M2) is produced by a reaction that is catalyzed by sulfotransferases, soluble enzymes that are found primarily in the liver and intestinal tract (Parkinson, 1996). Sulfation can occur with or without prior biotransformation by phase I enzymes, and most of the sulfated conjugates are excreted in the urine. Swine are deficient in this class of enzymes while chickens are not. Little is known about the formyl metabolite; however, it accounts for less than 1% of the metabolites recovered after oral administration of ciprofloxacin in humans (Outman and Nightingale, 1989).

Designing Dosage Regimens to Prevent Development of Bacterial Resistance

Pharmacodynamics is concerned with the relationship between concentration and the pharmacologic and toxicologic effects of drugs. The primary focus for antimicrobial agents is the relationship between drug concentration and the antimicrobial effect. The efficacy of these agents is increasingly being compromised by the development of bacterial resistance. It is clear that resistance to any class of antimicrobial agent increases as the level of use increases due to selective pressure (Smith and Lewin, 1993). Furthermore, the arbitrary use of antimicrobial agents exacerbates the development of resistance when used at insufficiently high dosages, for inappropriate durations of therapy, or for use in clinically ill patients who do not warrant such treatment. Both the human and veterinary medical professions must be made aware of the need to prescribe and/or administer antimicrobial agents more conscientiously in order to minimize the development of resistance.

The four mechanisms of antibiotic resistance are: inactivation or destruction of the drug, altered permeability of the bacterial cell wall or membrane, active expulsion of the drug by the cell efflux pump and altered target site (i.e., the ribosome) resulting in decreased susceptibility to the antibacterial agent (Smith and Lewin, 1993; Rasmussen et al., 1997; Prescott, 2000). These drug resistance determinants are mediated via one of two distinct genetic mechanisms; a mutation in the bacterial chromosome or by transferable elements (Smith and Lewin, 1993). Transferable drug resistance can occur by transduction, transformation, conjugation, transposons, and integrons (Prescott, 2000). Significant differences exist between these two types of drug resistance mechanisms. Transferable antibacterial resistance permits intraspecies and interspecies transfer to occur, and can lead to the rapid development of antibacterial resistance. Transferable resistance has proven difficult, if not impossible, to eradicate. In contrast, chromosomal resistance usually is only passed on to progeny and is a gradual stepwise process.

The fluoroquinolones are a series of synthetic antibacterial agents used in both human and veterinary medicine. They are rapidly bactericidal with a spectrum of activity against gram-negative bacteria, mycoplasma, and some gram-positive bacteria. They work through the inhibition of DNA gyrase, interfering with the supercoiling of bacterial chromosomal material. Mechanisms of resistance for quinolones have been characterized for several bacterial species. Three principal mechanisms have been described: decreased permeability of the bacterial cell wall caused by alterations of the hydrophilic pores; an efflux pump, which actively transports the fluoroquinolone molecule out of the cell as it approaches or passes through the bacterial membrane; and mutation of the DNA gyrase or topoisomerase IV, thus altering the binding site of quinolones (Prescott, 2000). Plasmid-mediated resistance has been described but not confirmed

(Cambau and Gutmann, 1993). Development of resistance is the greatest source of debate for the use of quinolones in animals. Because quinolones are the drugs of choice for many refractory and/or nosocomial infections in humans, regulatory pressure has restricted their use in animals in order to minimize the development of resistance.

Federal law now restricts the usage of quinolones in food producing animals, even though the agricultural community insists that quinolone usage in food animals is necessary to prevent production losses and bacterial contamination of meat. These restrictions have resulted from concerns from the health care community and the general public regarding the increasing development of drug resistant bacterial strains, and because of the problems this could pose to human and animal health.

The hypothesis of this study was that species-dependent differences in pharmacokinetic disposition of quinolones are due to differences in distribution or activity of cytochrome P450 isozymes. Differences in pharmacokinetic parameters can indirectly affect development of quinolone resistance by bacteria. The specific aims of the study were to: 1) Identify differences in quinolone disposition by allometric analysis of pharmacokinetic data of different species, and 2) identification of species-dependent metabolism pathways. This involves identifying differences in P450 metabolism in hepatic tissue of swine using specific substrates and inhibitors. Part two of the study also required the development of specific techniques in order to identify P450 differences.

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PART II: ALLOMETRIC ANALYSIS OF CIPROFLOXACIN AND ENROFLOXACIN PHARMACOKINETICS ACROSS SPECIES

Chapter 1: Abstract

The purpose of this study was to examine the allometric analysis of ciprofloxacin and enrofloxacin using pharmacokinetic data from the literature. The pharmacokinetic parameters used were half-life, clearance and volumes of distribution. Relationships between body weight and the pharmacokinetic parameter were based on the empirical formula $Y=aW^b$ where Y is half-life, clearance or volume of distribution; W is body weight; and a is an allometric coefficient (intercept) that is constant for a given drug. The exponential term b is a proportionality constant that describes the relationship between the pharmacokinetic parameter of interest and body weight. A total of 20 different species of animals were studied. Results of the allometric analyses indicated similarity between clearance and volume of distribution as they related to body weight for both drugs. Absence of significant relationships for half-life may be due to differences in the rate or extent of drug biotransformation. Results indicate that it is possible to use allometry to predict some pharmacokinetic variables of enrofloxacin or ciprofloxacin based on body size of species.

Chapter 2: Introduction

Drug dosage extrapolation among species assumes that pharmacodynamic similarities exist when pharmacokinetic equivalency is achieved. In other words, achieving equivalent peak serum and tissue concentrations and duration of drug exposure will achieve similar physiologic effects among species. Species-dependent differences at the site of drug action, such as the number of receptors or affinity of receptors for the drug, can preclude achieving equivalent effects for many classes of drugs. But achieving equivalent antimicrobial drug exposure in different species should achieve similar killing of microorganisms. The ability to achieve pharmacokinetic equivalency depends on the physiology and morphology of the tissues and organs responsible for drug absorption, distribution, biotransformation, and excretion of drugs.

The allometric approach is a basic mathematical tool for analyzing differences in anatomy, physiology, biochemistry, and pharmacokinetics in animals of different sizes. At least 750 allometric equations have been reported (Calder, 1984). The usual allometric approach relates one biologic function or structure (y) to another (x) through an empirical power function:

$$y = a(x)^b$$

Drug effect is often related to pharmacokinetic parameters of a drug, including half-life (t½), clearance (Cl), volume of distribution (Vd), or the area under the drug concentration versus time curve (AUC) (Baggot 1977; Rowland 1986; Benet et al., 1996). Because most pharmacokinetic parameters are dependent on physiologic functions, it is possible to consider drug dosages among species on the basis of allometric relationships; i.e., where y is the value of the pharmacokinetic parameter and x is body weight (Boxenbaum 1982; Riond and Riviere 1990; Pashov et al., 1997; Riviere et al., 1997). Allometry may be performed on any pharmacokinetic parameter;

however, the half-life profile, with abundant data in the literature is most often studied. Half-life is a composite of Vd and Cl. Therefore, Cl can be studied and may provide less biased information.

Interspecies scaling assumes that biochemical and physiologic processes responsible for rate of drug elimination vary in accordance to basal metabolic rate. A number of physiologic factors other than basal metabolism can modify these biochemical/physiological relationships and cause variations in half-life, volume of distribution and clearance of drugs that are unrelated to body mass. These factors include change in protein binding, saturation of drug elimination processes, diet, genetic polymorphism, drug-induced alterations in physiologic processes, biotransformation, interspecies differences in enterohepatic circulation, and tubular reabsorption as influenced by urinary pH (Mellett 1969; Williams 1973; Sorgel 1989; Riviere et al., 1997; Pashov et al., 1997). Differences in pharmacokinetic parameters and biologic activity of drugs among species may be related to physicochemical interactions of drugs with food, or to biodegradation of compounds in the rumen, caecum, or colon (Baggot 1977, 1980, 1990; Pashov et al., 1997).

Fluoroquinolones, such as enrofloxacin and ciprofloxacin, have similar distribution characteristics; however, elimination pathways and rates differ considerably among species. Oral absorption of fluoroquinolones is generally fast and substantial in humans, monogastric species, and pre-ruminant age calves, with up to 80% of the ingested dose absorbed into the systemic circulation (Vancutsem et al., 1990). These drugs have volumes of distribution greater than 1 l/kg. Binding to plasma proteins is variable among species (Bregante et al., 1999) and for the different quinolones (Zlotos et al., 1998a; Zlotos et al., 1998b). Major elimination pathways are renal excretion and hepatic metabolism. In the kidneys, fluoroquinolones are affected by glomerular

filtration, tubular secretion, and tubular reabsorption. In the liver, they are metabolized primarily by oxidation but also demethylation and deethylation of the parent molecule (Lode et al., 1989). Conjugative pathways are predominant for some fluoroquinolones in most species (Sorgel 1989); however, the degree of metabolism varies considerably across species.

Pharmacokinetic characteristics of ciprofloxacin and enrofloxacin, fluoroquinolones that inhibit DNA gyrase in bacterial cells, have been determined in several different species. The objectives of this study were to determine relationships between body weight, half-life, clearance and volume of distribution for these two fluoroquinolones that may impact interspecies scaling of drug doses. In particular, whether allometric scaling based on body weight could be used to predict these pharmacokinetic variables.

Chapter 3: Materials and Methods

The relationships between body mass and elimination half-life (t½), volume of distribution at steady state (Vdss), or total body clearance (Cl) of ciprofloxacin and enrofloxacin were analyzed using data from studies in 20 species: 13 for ciprofloxacin (Table 2-1) and 14 for enrofloxacin (Table 2-2). Data for t½, Vdss, and Cl of both drugs was obtained from previously published studies. Half-life values were determined during the elimination phases after intravenous (i.v.) administration of the drugs. The matrices of interest were serum, plasma, or blood. Data for body weights were obtained from these same studies. Mean values were used when a range of body weights was given. When body weights were not indicated, average values for the species and breed used in this study were obtained from the literature. Records were deleted if subjects were diseased or other drugs were co-administered. Analyses did not consider the influence of age or sex. Regression analyses were preformed on data from studies where HPLC analyses were done for both enrofloxacin and ciprofloxacin. Biological methods (microbiologic assay) do not differentiate between parent compounds and active metabolites of enrofloxacin and ciprofloxacin and were not included.

Data were separated into 2 groups for both drugs. The groups were: 1) all species (including mammals, fish, reptiles, and birds), and 2) mammals only.

Regression analysis of values for body weight, t½, Cl or Vdss was performed using SAS software (SAS Institute, Cary, NC, USA). The analyses were performed using mean values from individual citations. The linear regression of log half-life (t½, hr), log volume of distribution (Vdss, l/kg) or log clearance (Cl, ml/min/kg) versus log body weight (W, kg) was analyzed so that estimates of the intercept c and slope b could be computed by the following equations:

$$\log t_{1/2} = c + b(\log W), \quad \log Vdss = c + b(\log W) \text{ or } \log Cl = c + b(\log W)$$

TABLE 2-1. Ciprofloxacin animal species database.

Species	t½ (hr)	Cl (ml/min/kg)	Vdss (l/kg)	Source
Bos domesticus Cow	2.4	12.1		Nouws et al., 1988a
Sus scrofa Pig	2.6	17.3		Nouws et al., 1988a
Ovis ovis Sheep	1.2	18	*	Munoz et al, 1996
Orictolagus uniculus Rabbit	1.6	27.2	*	Aramayona et al., 1996
Canis famillaris	3, 2.2, 2.6	19, 18, 14	*	Abadia et al., 1994
Dog	2.8	7.8	1.9	Cester & Toutain 1997
Rattus rattus	2.2	26.7	4.6	Siefert et al., 1989
Rat	1.7	33	9.2	Nouaille et al., 1998
Macaca mulatta Monkey	4.3	4.7	1.8	Siefert et al., 1989
Homo sapiens	4.3, 4.4	8.3, 8.2	2.4, 2.4	Lettieri et al., 1992
Human	2.7, 2.9, 2.8	9.6, 9.6, 8.2	9.6, 9.6, 8.2	Dudley et al., 1987a
	2.9	9.3	*	Bergan et al., 1987
	4.2	8.2	2	Dudley et al., 1987b
	2.3	9.8	*	Deppermann et al., 1989
	3.6, 3.7, 3.5	8.8, 7.6, 7.8	2.1, 1.9, 1.8	Nix et al., 1992
	3.7	9.6	2.0	Wingender et al., 1984
	3.6, 3.7, 3.5,	9.2, 8, 8.7,	* * *	Bergan et al., 1988
	3.4, 4, 3.6	8.7, 8.7, 8.3	* * *	
	4.2	8.7	2.3	Catchpole et al., 1994
	3.3, 3.7, 3.5	8.1, 7.9, 7	2.2, 2.3, 1.9	Ljungberg et al., 1988
	4.8, 3.3	10.6, 10.2	*, *	Lode et al., 1988
Capra hircus Goat	2.7	19.6	3.4	Garcia Ovando et al., 2000
Gallus gallus domesticus	2.3	12.5	1.8	Garcia Ovando et al.
Chicken	3.1	15.5	4.0	1997
				Garcia Ovando et al., 1999
Cyprinus carpio Carp	14.5	2.5	2.7	Nouws et al., 1988b
Salmo gairdneri Trout	11.2	4.8	2.7	Nouws et al., 1988b
Clarias gariepinus African catfish	14.2	4.5	5.6	Nouws et al., 1988b

* Missing data. When more than one value is listed, they represent multiple doses in the article.

TABLE 2-2. Enrofloxacin animal species database.

Species	t½ (hr)	Cl (ml/min/kg)	Vdss (l/kg)	Source
Bos domesticus	6.6, 4.9	3.2, 6.5	1.8, 2.3	Kaartinen et al., 1997a
Cow	16.3	7.5	*	Martinez-Larranaga et al.,1997
Sus scrofa	7.3	6.2	3.9	Nielsen & Gyrd-Hansen 1997
Pig	7.7	4.5	2.7	Richez et al., 1997b
	3.5	7	*	Zeng & Fung 1997
	9.6	1.7	1.3	Anadon et al., 1999
	21	2.7	5.5	Post et al., 2002
Ovis ovis	3.7	9.2	3.0	Mengozzi et al., 1996
Sheep	3.8	4	*	Pozzin et al., 1997
Orictolagus uniculus	2.2	22.8	3.4	Cabanes et al., 1992
Rabbit	1.9	23.9	*	Aramayona et al., 1996
Canis famillaris	2.4	27.1	7	Kung et al., 1993
Dog	4.4	10.9	3.7	Monlouis et al., 1997
	2.3	12.2	2.5	Cester & Toutain 1997
Lama glama	3.4	11.7	3.5	Christensen et al., 1996
Llama				
Felis domestica	6.7	9.5	4	Richez et al., 1997a
Cat				
Camelus dromedarius	11.9, 5.8,	1.0, 1.4,	*, *, *, *, *	Harron et al., 1997
Camel	4.9, 5.8,	1.2, 1.4,		
	3.8	1.4		
Equus caballus	17.1	1.7	2.5	Bermingham et al., 2000
Horse	4.4	8.5	2.3	Kaartinen et al., 1997b
Capra hircus	1.1	13.5	1.2	Rao et al., 2000
Goat	4.0	4	1.2	Elmas et al., 2000
Gallus gallus domesticus	10.3	4.8	2.8	Anadon et al., 1995
Chicken	7.5	3	1.8	Garcia Ovando et al., 1997
	6.5	*	*	Kietzmann et al., 1997
	7.0	3.3	2.0	Garcia Ovando et al., 1999
	5.6	10.3	3.9	Knoll et al., 1999
Dormaius novaehollandiae	3.3	6	1.6	Helmick et al., 1997
Emu				
Chlamydotis undulata macqueenii	5.6	5.7	3.0	Bailey et al., 1998
Houbara bustard				
Salmo salar	34.2	2.3	6.1	Martinsen & Horsberg 1995
Atlantic salmon				

* Missing data. When more than one value is listed, they represent multiple doses in the article.

The allometric equation was then applied ($t_{1/2} = a(W)^b$, $Vdss = a(W)^b$ or $Cl = a(W)^b$), where a is the antilogarithm of c. Coefficients of determination and P-values were computed for each regression analysis under study. Double logarithmic plots of body weight vs. half-life, clearance or volume of distribution were constructed to demonstrate significance found in the regression analyses.

Chapter 4: Results

Results of the regression analyses conducted on the logarithm of half-life, volume of distribution or clearance versus the logarithm of body weight for ciprofloxacin are listed in Table 2-3. There was a statistically significant relationship between clearance (p = 0.0001) and volume of distribution (p = 0.0001) compared to body weight when all species were analyzed, while there was an absence of relationship between half-life and body weight. Ciprofloxacin half-life (Figure 2-1), clearance (Figure 2-2) and volume of distribution (Figure 2-3) were related to body weight in mammals, with p-values of 0.002, 0.0001 and 0.0001, respectively.

TABLE 2-3. Ciprofloxacin half-life, clearance and volume of distribution values for allometric equations.

Group	n	A	b	r^2	P-value
		Half-life			
All Species	40	4.2	-0.073	0.088	0.060 NS
Mammals	35	2.1	0.102	0.249	0.002
		Clearance			
All Species	40	5.8	1.12	0.914	0.0001
Mammals	35	20.7	0.816	0.905	0.0001
		Volume of Distribution			
All Species	23*	2.0	1.09	0.948	0.0001
Mammals	18*	3.1	0.979	0.870	0.0001

n = sample size; a = allometric coefficient; b = allometric exponent; r^2 = coefficient of determination; NS = not significant; *reference lacked data resulting in different n values between clearance, half-life and volume of distribution data

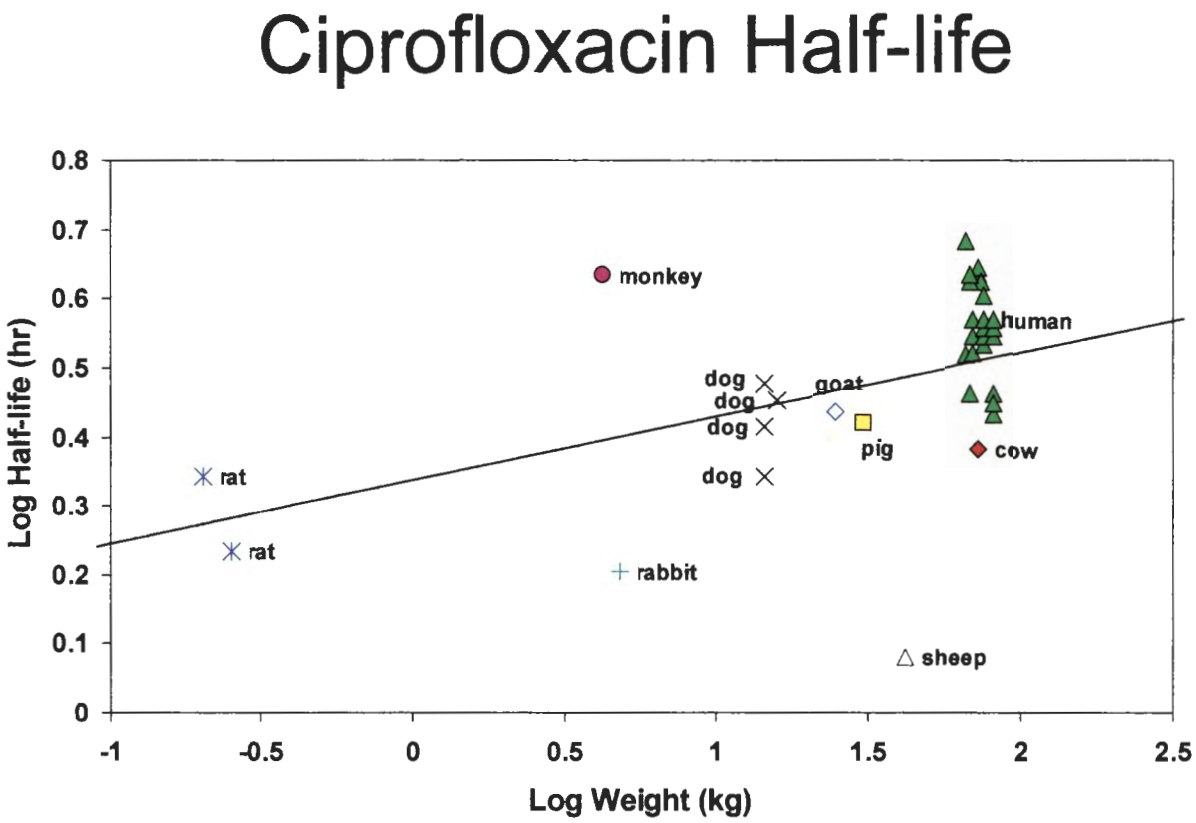

Figure 2-1. Allometric association for ciprofloxacin between half-life and body weight of mammals.

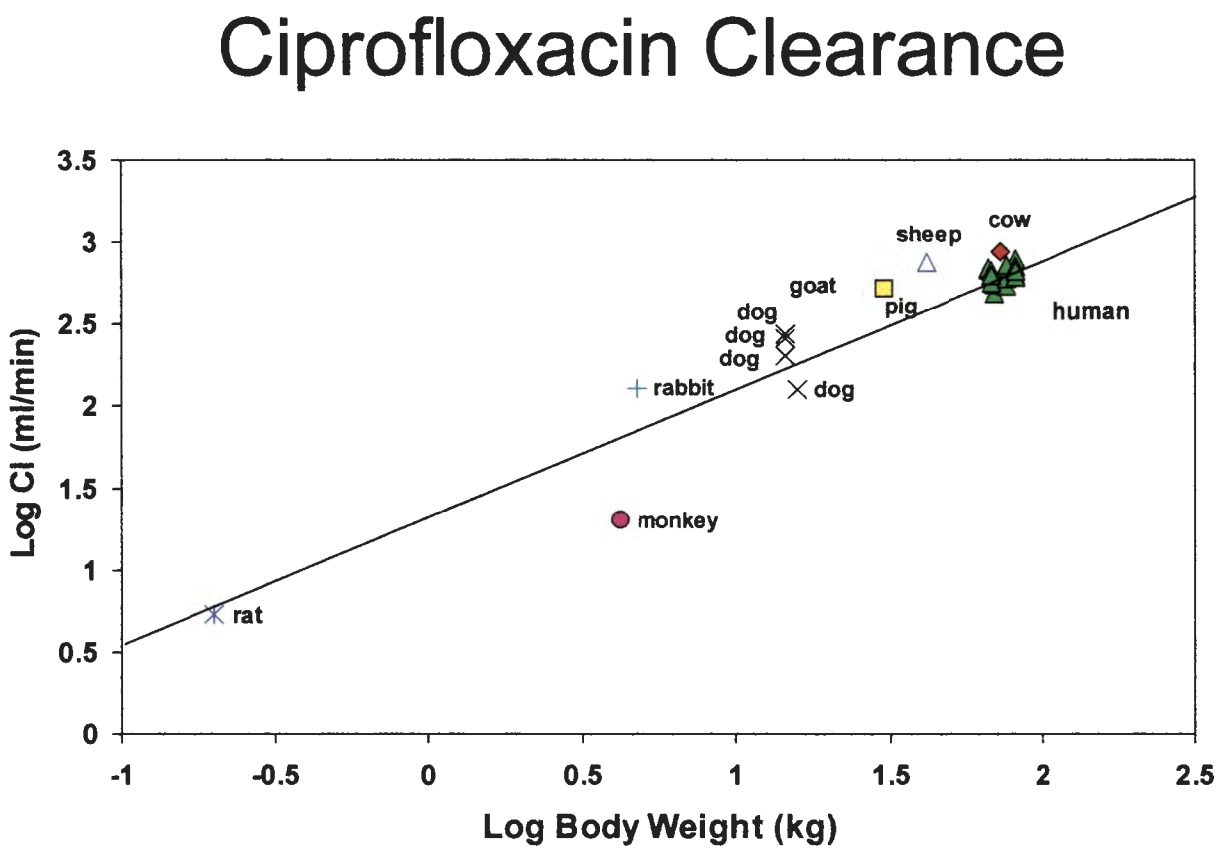

Figure 2-2. Allometric association for ciprofloxacin between clearance and body weight of mammals.

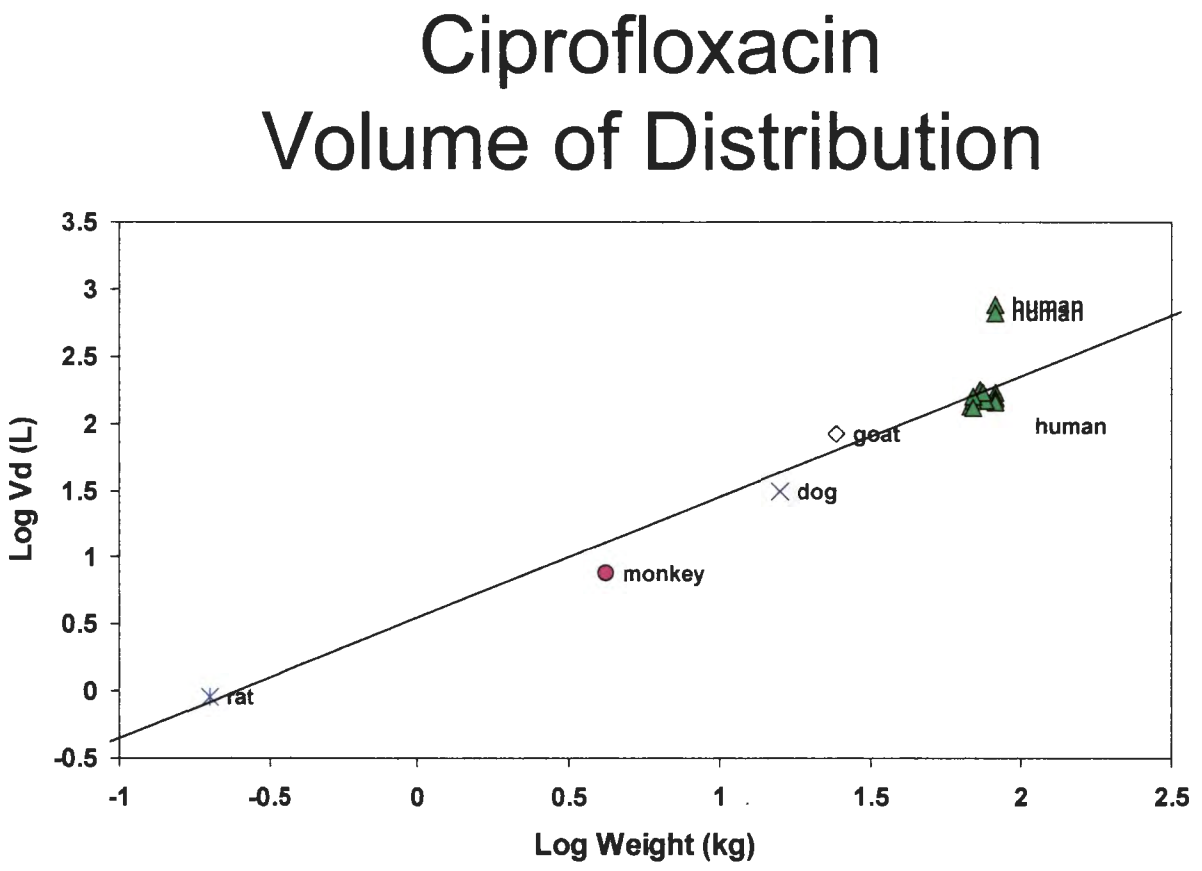

Figure 2-3. Allometric association for ciprofloxacin between volume of distribution and body weight of mammals.

The results of the regression analyses conducted for enrofloxacin half-life, volume of distribution and clearance are listed in Table 2-4. The enrofloxacin allometric analysis was similar to that of ciprofloxacin. There was a lack of association for half-lives and body weights among all species for enrofloxacin. Clearance ($p = 0.0001$) and volume of distribution ($p = 0.0001$) were significantly related to body weight in the analysis. Enrofloxacin half-life (Figure 2-4) was not significantly associated with body weight when mammals were analyzed. However, the analysis of volume of distribution (Figure 2-5) or clearance (Figure 2-6) and body weight in mammals produced a significant relationship ($p = 0.0001$).

TABLE 2-4. Enrofloxacin half-life, clearance and volume of distribution values for allometric equations.

Group	n	A	b	r^2	P-value
		Half-life			
All Species	34	6.6	-0.043	0.0167	0.468 NS
Mammals	26	3.4	0.120	0.0777	0.168 NS
		Clearance			
All Species	33*	8.8	0.818	0.782	0.0001
Mammals	26	28.7	0.541	0.545	0.0001
		Volume of Distribution			
All Species	24*	3.4	0.909	0.927	0.0001
Mammals	17*	4.0	0.886	0.839	0.0001

n = sample size; a = allometric coefficient; b = allometric exponent; r^2 = coefficient of determination; NS = not significant; *reference lacked data resulting in different n values between clearance, half-life and volume of distribution data

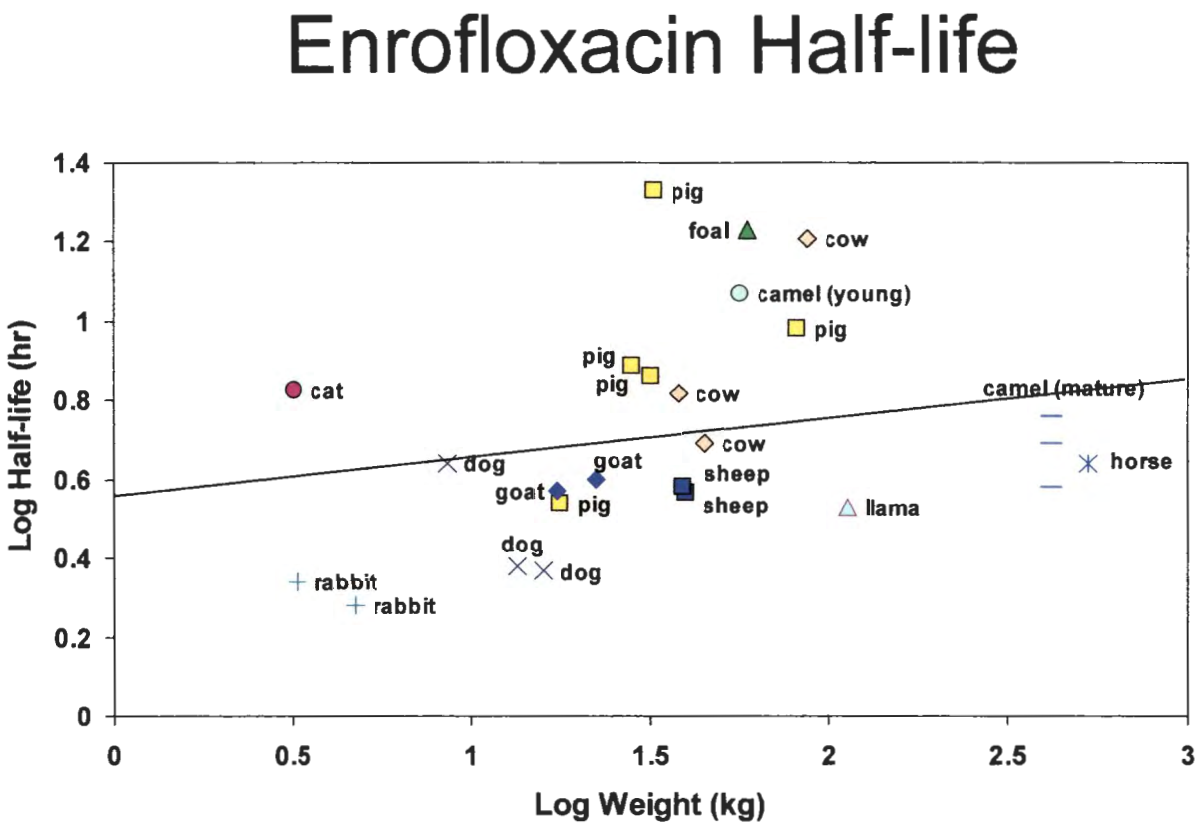

Figure 2-4. Allometric association for enrofloxacin between half-life and body weight of mammals.

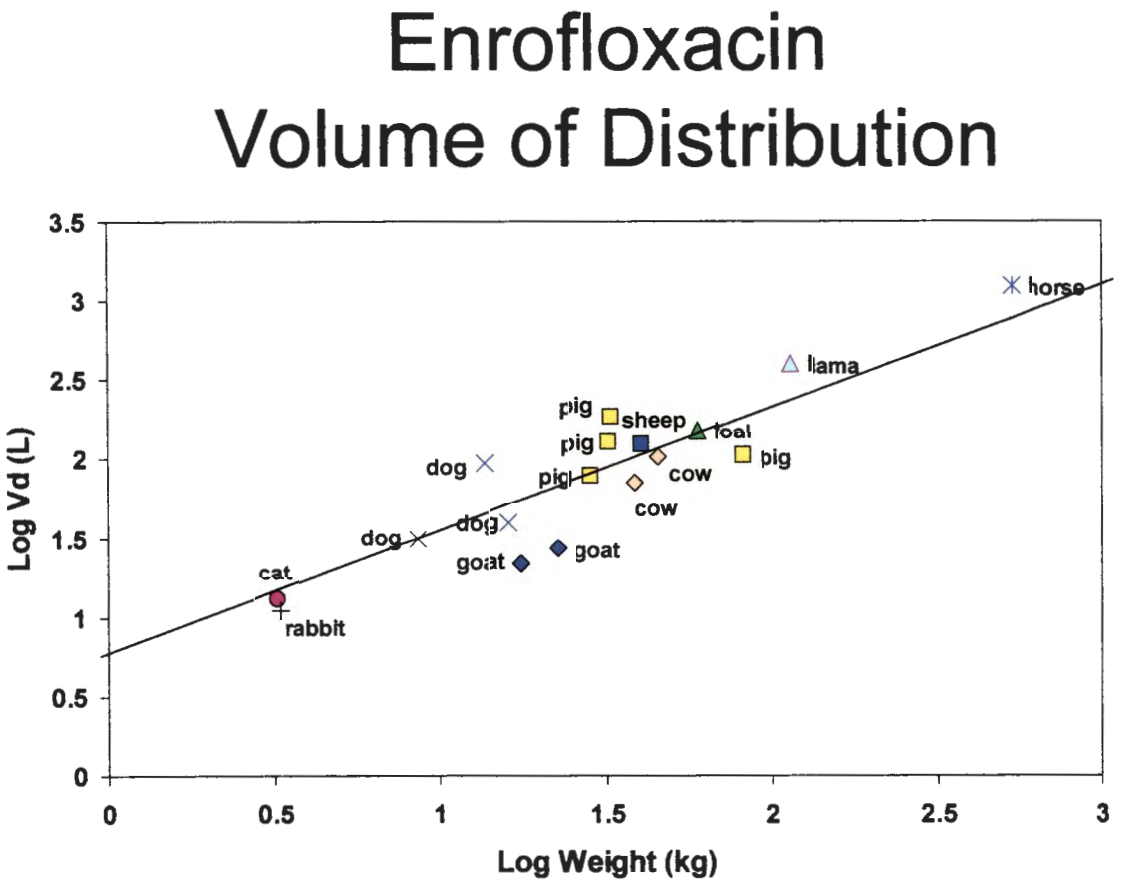

Figure 2-5. Allometric association for enrofloxacin between volume of distribution and body weight of mammals.

Enrofloxacin Clearance

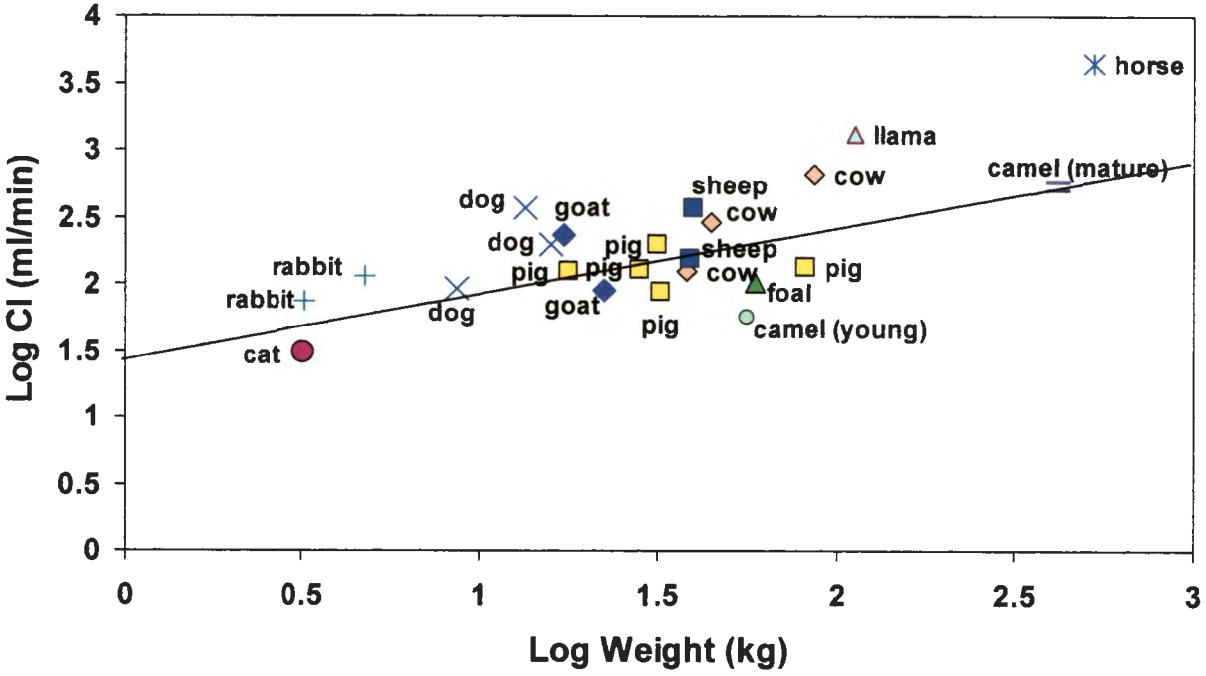

Figure 2-6. Allometric association for enrofloxacin between clearance and body weight of mammals.

Chapter 5: Discussion

In studies (Boxenbaum, 1982; Riviere et al., 1997; Bregante et al., 1999) where there has been an allometric relationship for half-life values for the allometric exponent (b in our study) have ranged from 0.2 to 0.3. We found that our allometric exponent is 0.1 or less for both ciprofloxacin and enrofloxacin.

The lack of statistically significant relationships between both ciprofloxacin and enrofloxacin half-lives may be due to considerable variation in biotransformation that exists among species. Ciprofloxacin is metabolized to desethylene-ciprofloxacin (M1), sulfo-ciprofloxacin (M2), oxo-ciprofloxacin (M3) and formyl-ciprofloxacin (M4) in humans as well as dogs, rats, mice, and monkeys (Outman and Nightingale 1989; Sorgel 1989). There have been trace amounts of ciprofloxacin metabolites detected in calves and pigs (Nouws et al., 1988a); however, the extent of metabolism varies. Enrofloxacin is N-deethylated to form ciprofloxacin; however, the rate and extent of this conversion varies among species. For instance, the extent of ciprofloxacin formation was 22% in dogs, (Monlouis et al., 1997) 10% in cats, (Richez et al., 1997a) 29% in goats, (Rao et al., 2000) 35% in sheep, (Mengozzi et al., 1996) 10% in chickens, (Garcia-Ovando 1999) 20-35% in horses, (Kaartinen et al., 1997b) and 35% in cows (Kaartinen et al., 1995). In pigs and foals, there were only trace amounts of ciprofloxacin metabolized from enrofloxacin (Zeng & Fung 1997; Bermingham et al., 2000; Nielson & Gyrd-Hansen 1997).

Species variation in drug half-life exists for many drugs that are eliminated mainly by hepatic metabolism. In mammalian species, the rates of phase I metabolic reactions are variable. There is less certainty associated with hepatic conjugation reactions because some are either defective or absent in certain species. As stated by Brodie "the differences in drug-metabolizing enzymes among species are so variable that,

similarities between animals and humans are often a matter of pure luck" (Brodie 1964). For example, cats are deficient in some glucuronidation reactions due to a lack of UDPglucuronyltransferase (Dutton et al., 1977; Dutton 1980). In this study, cats exhibited a longer half-life than dogs, goats, llamas, emus or camels.

In our study, total body clearance was associated with body weight with allometric exponents of 0.8 (all species) and 0.5 (mammals) for enrofloxacin, while ciprofloxacin exponents were 1.1 and 0.8 for all species and mammals. In other reports (Boxenbaum, 1982; Bregante et al., 1999), the exponents ranged from 0.60 to 0.82. The 0.8 allometric exponents in our study are consistent with previously reported values (Bregante et al., 1999). The larger value of 1.1 for ciprofloxacin (all species) and smaller value of 0.5 (mammals) for enrofloxacin may be due to the route of elimination or differences in plasma protein binding.

Enrofloxacin and ciprofloxacin are excreted by renal and hepatobiliary pathways in mammals (Lode et al., 1989). Renal excretion is variable; however, most quinolones, including enrofloxacin and ciprofloxacin, undergo glomerular filtration and active tubular secretion (Vance-Bryan et al., 1990; Vancutsem et al., 1990; Brown 1996). Elimination of enrofloxacin and ciprofloxacin given to trout, carp, African catfish, and rainbow trout occurs via a passive diffusion process in the glomerulus and the gills, which results in a longer half-life of elimination (Nouws et al., 1988b; Bowser et al., 1992).

Enrofloxacin has a low hepatic extraction ratio (Sorgel 1989). Therefore, hepatic clearance of enrofloxacin is influenced by plasma protein binding and intrinsic clearance, which is affected by the level of hepatic enzyme activity. No studies have shown that fluoroquinolones are so highly bound that this limits distribution to tissues. However, there does seem to be some variation between species. Ciprofloxacin plasma protein binding is 30% in humans (Lettieri et al., 1992); 14% in goats (Garcia Ovando et al.,

2000); 33% in rats (Siefert et al., 1986); 23% in monkeys (Siefert et al., 1986) 24% in pigs (Nouws et al., 1988a); 70% in calves (Nouws et al., 1988a); and 44% in dogs (Villa et al., 1997). Aramayona et al., (1996) found a two-fold difference in plasma protein binding between rabbits that were pregnant (30%) and those that were not (15%). Carp, catfish and trout have very similar plasma protein binding of 22%, 20% and 23% (Nouws et al., 1988b). Enrofloxacin seems to have the same type of variation between species for plasma protein binding as that of ciprofloxacin. For instance, plasma protein binding in cows is 35% (Kaartinen et al., 1995); in camels 17-24% (Gavrielli et al., 1995); in dogs 15-27% (Papich & Riviere 2001); in horses 22% (Villa et al., 1997); and in pigs 27% (Villa et al., 1997). Similar plasma protein binding occurs in a few species: in sheep, the value is 69% (Papich & Riviere 2001); in rats 50% (Papich & Riviere 2001); and in rabbits 35-50% (Papich & Riviere 2001).

Data was not corrected for plasma protein binding in our analyses since it was not available for all species that were studied. Thus, this may have influenced the half-life and clearance analyses. It is also difficult to compare results of studies in which protein binding was measured because of differences among laboratories and variations in methods used (Zlotos et al. 1998b). Bregante et al. (1999) did correct for plasma protein binding in their study by using the plasma free fraction, which may be why they had a better clearance allometric exponent for mammals. The data in the Bregante et al. (1999) study also originated from a single laboratory, which decreases the amount of variability compared to a study where data was derived from multiple sources.

Other possible reasons for variations in the exponents are the various conditions (lactating, pregnant, sex, breed, age, fasted or fed) of the animals used in the various studies. Nouws et al. (1988a) suggested that the age of the animal (maturity of renal function and metabolic capacity of liver) as well as breed difference may have affected

the ciprofloxacin plasma concentrations and thus its pharmacokinetic parameters. Aramayona et al. (1996) indicated that lactation and associated factors in rabbits could affect the binding of ciprofloxacin to plasma proteins. Lactation may be associated with different hormone levels that affect plasma protein binding. Siefert et al. (1986) noted that possible differences in ciprofloxacin metabolism existed between male and female rats. This may also be true in other species and could influence pharmacokinetic parameters of interest.

The allometric exponent for volume of distribution was 1.09 (all species) and 0.98 (mammals) for ciprofloxacin, while enrofloxacin exponents were 0.91 and 0.89 for both the all species and mammals groups, which is consistent with previously reported values (Bregante et al., 1999).

We concluded that volume of distribution is proportional to body weight for both drugs, while the elimination half-life for ciprofloxacin and enrofloxacin independent of body weight. The allometric exponent for clearance of both drugs suggests a differing rate of metabolism or extent of elimination in the species studied. Thus, our study supports the allometric modeling of enrofloxacin and ciprofloxacin across species using volume of distribution, but not half-life. The use of clearance is less conclusive, and further experimental allometric studies are indicated. Allometry can be an important tool for dose extrapolation of certain classes of drugs; however, this should be done only when strong correlations are found.

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PART III: DETERMINATION OF BUFURALOL AND 1'-HYDROXYBUFURALOL IN PORCINE MICROSOMAL PREPARATIONS

Chapter 1: Abstract

A simple, rapid and sensitive HPLC analysis of bufuralol and 1'-hydroxbufuralol in porcine microsome samples is described. Chromatography was performed on a Resolve C_{18} column using a mobile phase of 10 mM sodium perchlorate pH 2.45:acetonitrile (48:52). Fluorescence was measured at excitation 252 nm and emission 302 nm. The procedure produced a linear curve for the concentration range 10-1500 ng/ml. This assay produced rapid, accurate and repeatable results.

Chapter 2: Introduction

The cytochrome P450 monooxygenase enzyme system is important in terms of its catalytic versatility and sheer number of chemical agents it is capable of detoxifying, or activating to reactive intermediates (Guengerich, 1992;Parkinson, 1996). P450 enzymes are concentrated mainly in the liver endoplasmic reticulum (microsomes), but they are located in virtually all other tissues. These enzymes activate xenobiotics, including drugs that are toxic or that produce tumorigenic metabolites. The enzymes also detoxify xenobiotics, and aid in determining intensity and duration of action of drugs. Thus, it is essential to establish the activity and regulation of the P450 system for species selected for pharmacological and toxicological studies. In addition, impaired drug disposition in food-producing animals may lead to changes in residue levels of veterinary drugs and other xenobiotics in edible tissues, milk, or eggs. Much of the information known about P450 enzymes comes from studies conducted in rodents. However, the pig is becoming a popular alternative to traditional non-rodent species in pharmacological and toxicological testing (Skaanild and Friis, 1999). The information on the P450 enzyme system for this food animal species is limited.

P450 enzymes are mainly responsible for phase I metabolism, which adds or exposes polar functional groups on a lipophilic substrate. In the investigation of P450 enzyme-mediated xenobiotic metabolism, individual forms of P450 enzymes have been found to catalyze specific reactions with specific substrates. Thus, characterizing these specific enzyme activities allows their use as probes in drug metabolism studies. To date, at least one marker activity exists for the majority of human P450 enzymes (Sharer et al., 1995). The conversion of bufuralol to 1'-hydroxybufuralol is one of the methods used to characterize human cytochrome P450 enzyme CYP2D6 activity; but limited information exists in other mammalian species.

There are a few high performance liquid chromatography (HPLC) methods (Boobis et al., 1985; Kronbach et al., 1987; Lee and Moochhala, 1989; Guang and Lang-Li, 1996) used in the analysis of bufuralol and its metabolite. However, one method (Guang and Lang-Li, 1996) uses normal phase chromatography, while another method (Boobis et al., 1985) involves the use of a t-butyl ether extraction. A third method (Lee and Moochhala, 1989) does not report any validation for the procedure and uses tetrahydrofuran in the mobile phase, while another method (Kronbach et al., 1987) uses an expensive fluorometer and no internal standard. These methods (Boobis et al., 1985; Kronbach et al., 1987; Lee and Moochhala, 1989; Guang and Lang-Li, 1996) involve the analysis of human or rat microsome samples, but not porcine microsomal samples.

This article describes a rapid and efficient method for the in vitro analysis of 1'-hydroxybufuralol and bufuralol. This procedure provides a quick analysis that may facilitate characterization of P450 enzyme metabolism in the pig.

Chapter 3: Materials and Methods

Reagents and Standards

Acetonitrile was "HPLC" grade, while sodium perchlorate, and perchloric acid were reagent grade. All were purchased from Fisher Scientific (Pittsburgh, PA, USA). Bufuralol and 1'-hydroxbufuralol were purchased from Gentest (Woburn, MA, USA). Chloropropamide (internal standard) glucose 6-phosphate (G-6-P), glucose 6-phosphate dehydrogenase (G-6-PDH), and β-nicotinamide adenine dinucleotide phosphate (NADP) were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Stock standard solutions of bufuralol (100, 5 and 1 μg/ml) and 1'-hydroxybufuralol (100, 5, and 1 μg/ml) were prepared in water and stored at 4°C. The solutions were stable for six months. Working standards were prepared fresh daily by dilution of the stock standards. A stock standard solution of chlorpropamide (300 μg/ml) was prepared in methanol and stored at 4°C. This solution was also stable six months.

Apparatus

The analytical system consisted of a 626 solvent delivery system, a model 717 WISP autosampler, a 470 fluorescence detector and a computer equipped with Millennium software (Waters, Milford, MA, USA). The column was a Resolve C_{18} (5 μm 3.9 x 150 mm) equipped with a C_{18} Guard-Pak precolumn insert (Waters, Milford. MA, USA).

Chromatography

The mobile phase was an isocratic mixture of 10 mM sodium perchlorate: acetonitrile (48:52). The pH of the sodium perchlorate solution was adjusted to 2.45 with concentrated perchloric acid. It was prepared fresh daily using double-distilled deionized water, filtered (0.22 μM) and degassed before use. Flow rate was 1 ml/min for

the first four minutes, then ramped to 2.0 ml/min over 1.5 min, and maintained for 6 min then decreased back to 1 ml/min over 1 min. Column temperature was ambient and fluorescence was measured at an excitation 252 nm and emission 302 nm. All chromatograms were obtained with the following fluorometric conditions: gain 10X, attenuation 1, and filter time constant 0.5 s.

Sample Treatment

Spiked samples were prepared by addition of appropriate volumes of both bufuralol and 1'-hydroxybufuralol. The internal standard, chlorpropamide (100 μl of a 300 μg/ml) was added and appropriate amounts of the solutions used in microsomal preparation were added to produce a 0.5 ml final volume. Samples were vortex-mixed and a 125 μl sample was injected onto the liquid chromatograph.

Microsomal samples were prepared using Lake's (1984) ultracentrifugation method. Incubation mixtures contained 0.5 mg of microsomal protein, 100 mM phosphate buffer at pH 7.4 containing 6 mM magnesium chloride, 1 mM EDTA, and a NADPH-generating system (1 mM NADP, 10 mM G-6-P and 0.7 U of G-6-PDH) in a total volume of 0.5 ml. Incubation mixtures contained bufuralol and inhibitors and the reactions were initiated by addition of the NADPH-generating system after a 5 min pre-incubation step at 37°C. Reactions were quenched with 0.1 ml of ice cold acetonitrile after 10 min in a 37°C shaking water bath, and were then placed on ice for 1 hour. Samples were centrifuged at 16000 g for 15 min. The supernatants were removed and stored at -80°C until analysis could be preformed. Reaction rates were linear with incubation time under these conditions. Frozen samples were thawed on ice and vortex-mixed before use. Chlorpropamide (100 μl of a 300 μg/ml) was added to a 0.5 ml microsome sample and vortex-mixed. Those samples containing particulates were

centrifuged for 5 min at 16,000 g in an Eppendorf centrifuge (Brinkman Instruments, New York, NY, USA). A 125 μl aliquot of the supernatant was injected onto the liquid chromatograph.

Chapter 4: Results

A blank chromatogram for a microsomal sample with no drugs added is shown in Figure 3-1 with no interfering peaks. The chromatogram in Figure 3-2 is a porcine liver microsome sample after incubation with 10 μmole of bufuralol. Retention times for chlorpropamide, 1'-hydroxbufuralol, and bufuralol were 2.73, 5.00 and 9.94 min, respectively. The chromatogram in Figure 3-3 is a 250 ng/ml standard with retention times of 2.98, 4.80 and 9.80 min. There is a small peak that elutes immediately prior to chlorpropamide in all of the chromatograms. This is a contaminant of chlorpropamide, which does not interfere with elution of the other compounds.

This method produced a linear curve for the concentration range of 10-1500 ng/ml for bufuralol and its metabolite, with correlation coefficients ranging from 0.998 to 0.999 for both compounds. Replicate analyses performed on the same day for microsomal samples spiked with specific concentrations of bufuralol produced coefficients of variation (C.V.) of 9.6% for 40 ng/ml, 1.4% for 700 ng/ml and 2.2% for 1100 ng/ml. The metabolites' C.V. was 2.5%, 3.3%, and 2.5% at the same concentrations (Table 3-1).

Table 3-1. Intra-assay precision for bufuralol and 1'-hydroxybufuralol (n = 4)

Concentration Added (ng/ml)	Bufuralol Concentration measured (ng/ml) (mean±SD)	Coefficient of variation (%)	Hydroxybufuralol concentration measured (ng/ml) (mean±SD)	Coefficient of variation (%)
40	39±4	9.5	38±1	2.5
700	688±9	1.4	673±22	3.3
1100	1080±23	2.2	1095±28	2.6

SD = Standard deviation

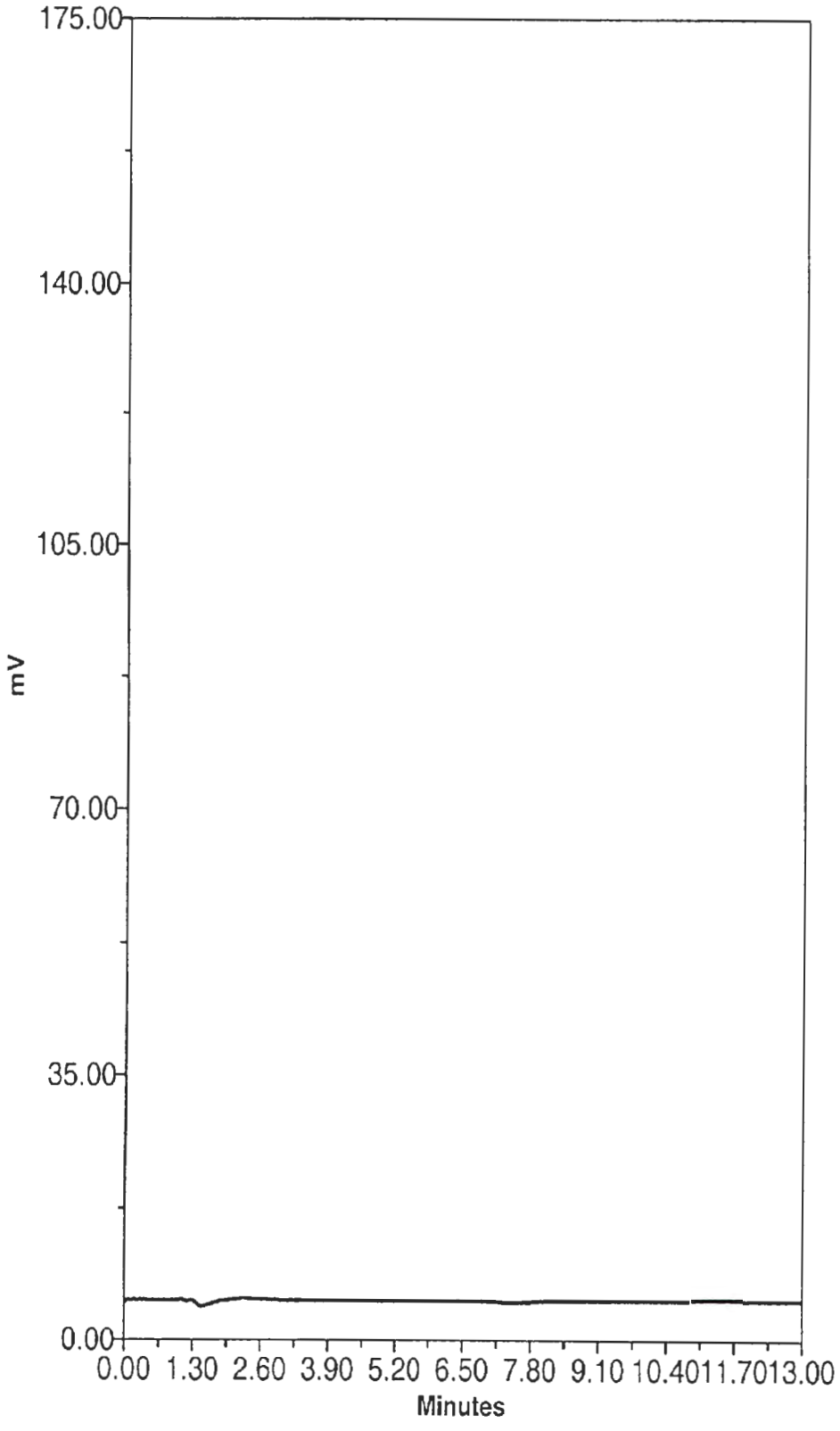

Figure 3-1. Chromatogram of microsomal sample with no drugs added.

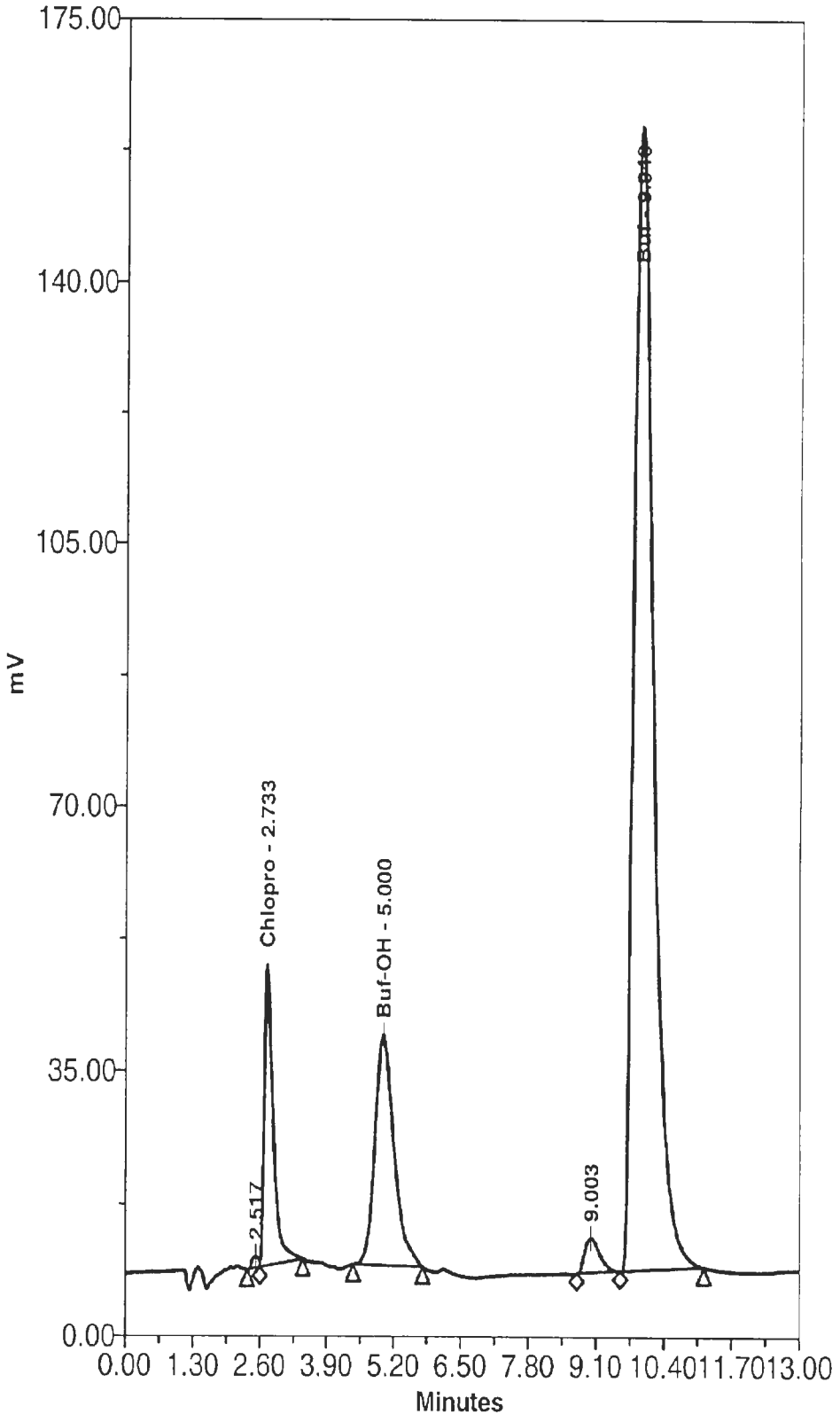

Figure 3-2. Chromatogram of a porcine microsomal sample after incubation with 10 μM of bufuralol for 10 min. Peaks: 2.52 = chlorpropamide contaminate; Chlorpro = chlorpropamide; Buf-OH = 1'-hydroxybufuralol; Buf = bufuralol.

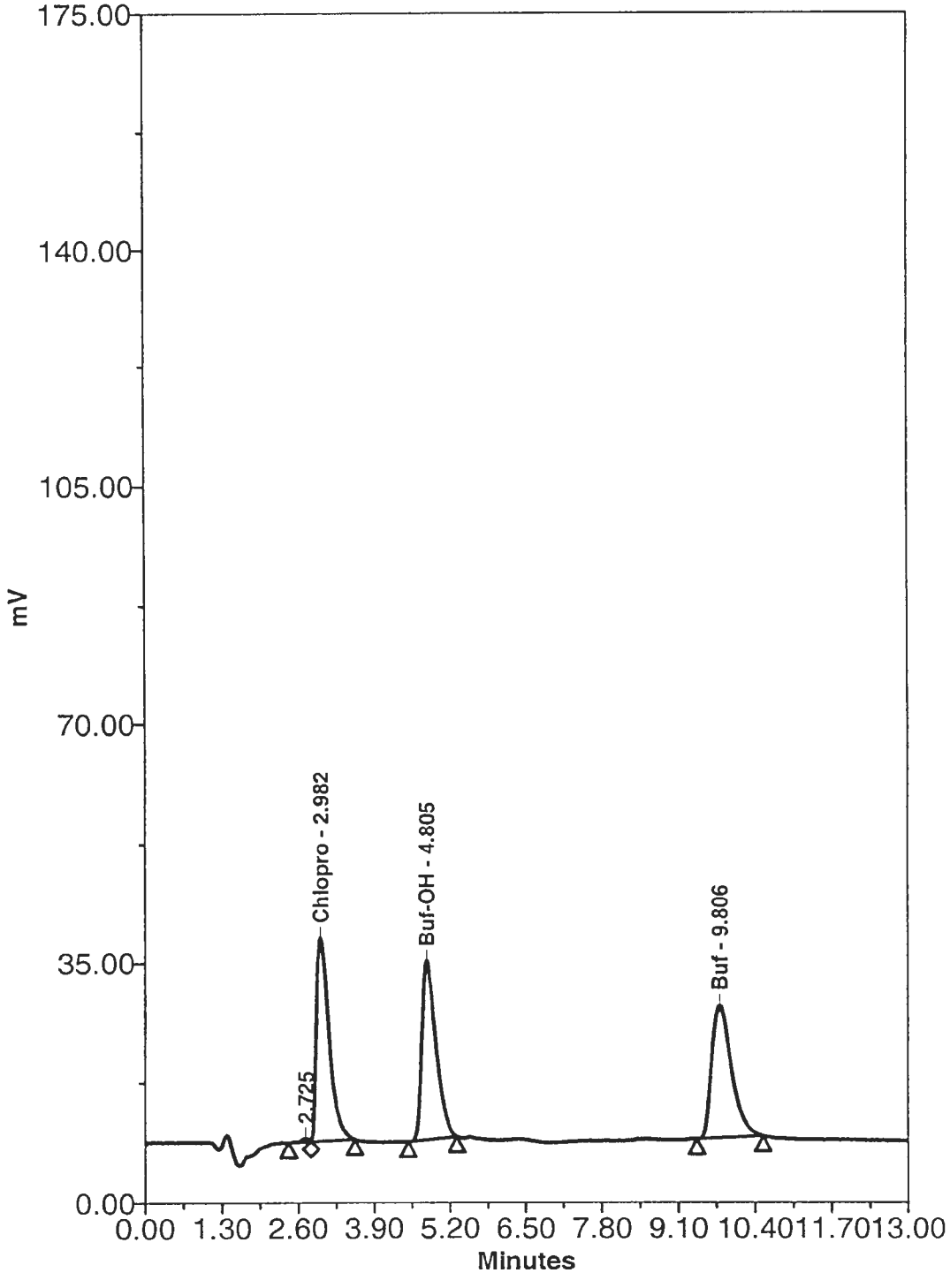

Figure 3-3. Chromatogram of a 250 ng/ml standard. Peaks: 2.73= chlorpropamide contaminate; Chlorpro = chlorpropamide; Buf-OH = 1'-hydroxybufuralol; Buf = bufuralol.

Table 3-2. Inter-assay precision for bufuralol and 1'-hydroxybufuralol (n = 4)

Concentration added (ng/ml)	Bufuralol concentration measured (ng/ml)	Coefficient of variation (%)	Hydroxybufuralol concentration measured (ng/ml)	Coefficient of variation (%)
10	9	4.7	10	2.1
25	26	7.7	24	4.8
50	48	6.3	48	8.3
100	100	2.0	96	11.4
250	242	5.0	243	4.9
500	448	4.2	470	1.9
800	768	4.7	798	3.4
1000	952	5.7	991	1.9
1500	1354	3.8	1457	2.4

Day-to-day variability for microsomal replicates is shown in Table 3-2. The mean recoveries of bufuralol were 90%, 104%, 96%, 100%, 97%, 90%, 96%, 95%, and 90% for 10, 25, 50, 100, 250, 500, 800, 1000, and 1500 ng/ml, respectively. Mean recoveries of 1'-hydroxybufuralol were 95%, 97%, 95%, 96%, 97%, 94%, 100%, 99%, and 97% for 10, 25, 50, 100, 250, 500, 800, 1000, and 1500 ng/ml. The detection limit for both bufuralol and its hydroxymetabolite was 1 ng/ml. This represents a peak approximately three times baseline noise. No interference with the chromatographic procedure was observed from numerous drugs and chemicals used in inhibition studies.

Chapter 5: Discussion

To be useful for characterization of enzyme metabolism studies a method should be simple, rapid, sensitive and reproducible. Such an HPLC assay, utilizing fluorescence detection, has been developed to investigate the 1'-hydroxylation of bufuralol by microsomal fractions of porcine liver. The assay is sensitive, specific and reproducible, with a high recovery of the metabolite.

The detection limits and recoveries for 1'-hydroxybufuralol and bufuralol are equal to or better than existing methods for extraction and analysis of the compounds. However, Kronbach's (1987) method has a better limit of detection than our method, which is 0.1 ng/ml with a 100 μl injection volume and a 4 to 1 signal to noise ratio. This is due to the use of a more expensive dual monochromator fluorometer capable of higher sensitivities than are possible with a typical HPLC fluorescence detector. Use of chlorpropamide as the internal standard corrects for intra- and inter-assay variability in the method. It was discovered that due to the composition of the sample, injection filters could become clogged and thus affect the injection volume. Although the systems were cleaned at the end of each analysis in order to prevent this, an internal standard was added to prevent any changes in concentration from occurring due to this event. To prevent clogging of the column it was cleaned with dimethylsulfoxide (DMSO):water (1:1) injections after every 300 sample injections. With proper column cleaning this is a rugged procedure with the column still in use after 1500 injections.

This method was developed in order to determine the metabolism of bufuralol in porcine microsome samples. Our procedure eliminates the use of liquid-liquid extractions and normal phase chromatography. This method has been applied to metabolism studies in porcine microsomes involving bufuralol in this laboratory. In

conclusion, a simple, rapid, sensitive and useful HPLC procedure has been developed for analysis of 1'-hydroxybufuralol and bufuralol in microsome samples.

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PART IV: DETERMINATION OF 6-HYDROXYCHLORZOXAZONE AND CHLORZOXAZONE IN PORCINE MICROSOME SAMPLES

Chapter 1: Abstract

A simple, accurate and sensitive HPLC analysis of 6-hydroxychlorzoxazone and chlorzoxazone in porcine microsome samples is described. Chromatography was performed on a YMC-Pack ODS-AQ column using a mobile phase of 0.05% phosphoric acid pH 3:methanol (60:40). UV detection was carried out at 287 nm. The procedure produced a linear curve for the concentration range 25-2000 ng/ml. This assay produced quick, accurate, and repeatable results.

Chapter 2: Introduction

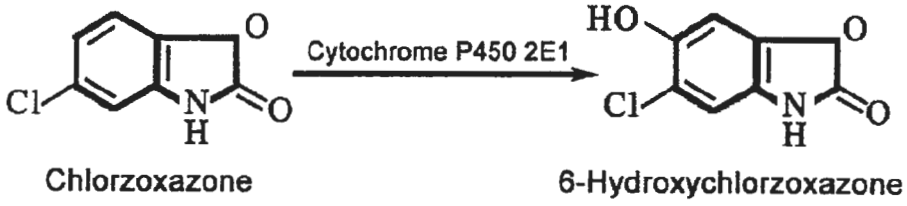
The cytochrome P450 monooxygenase enzyme system is important in terms of its catalytic versatility and the sheer number of compounds it detoxifies or activates to reactive intermediates (Guengerich, 1992; Parkinson, 1996). The largest concentration of P450 enzymes is located in the liver endoplasmic reticulum (microsomes), but they are located in virtually all other tissues in the body. These enzymes activate xenobiotics including drugs to toxic and/or tumorigenic metabolites, detoxify xenobiotics, and aid in determining intensity and duration of action of drugs. Therefore, it is essential to establish the activity and regulation of the P450 system for species selected for pharmacological and toxicological studies. In addition, impaired drug disposition in food-producing animals may lead to changes in residue levels of veterinary drugs and other xenobiotics in edible tissues, milk, or eggs. There is also concern about the increase in development of drug resistant bacterial strains in food animals. Much of the information known about P450 comes from studies conducted in rodents. However, the pig is becoming a popular alternative to traditional non-rodent species in pharmacological and toxicological testing (Skaanild and Friis, 1999). The information on the P450 system for this species is limited.

P450 enzymes are mainly responsible for phase I metabolism, which adds or exposes polar functional groups on a lipophilic substrate. In the investigation of P450 mediated xenobiotic metabolism, individual forms of P450 have been found to catalyze specific reactions with certain substrates, thus designating these activities as probes for these forms. To date, at least one marker activity exists for the majority of human P450 forms (Sharer et al., 1995). Cytochrome P4502E1 is a major mammalian hepatic enzyme, which catalyzes the biotransformation of many low molecular weight xenobiotics. However, one of the challenges to studying 2E1 has been to identify a

model substrate that is a sensitive and specific marker for the enzyme's activity. The conversion of chlorzoxazone (CZX) to 6-hydroxychlorzoxazone (OH-CZX) (Figure 4-1) is one of the methods used to characterize human cytochrome P450 enzyme CYP2E1 activity both in vivo and in vitro. But limited information exists in other mammalian species because of differences in biotransformation.

Several high performance liquid chromatography (HPLC) methods have been developed to measure CZX and OH-CZX in biological fluids and microsomes (Peter et al., 1990; Thummel et al., 1993; Chen & Yang, 1996; Haque & Stewart, 1997; Court et al., 1997; Chittur & Tracy, 1997; Frye et al., 1998; Leclercq et al., 1998; Lillibridge et al., 1998; Cummings et al., 2001; Taavitsainen et al., 2001). Some of the methods involve the use of liquid-liquid extractions (Peter et al., 1990; Thummel et al., 1993; Chen & Yang, 1996; Chittur & Tracy, 1997; Leclercq et al., 1998; Lillibridge et al., 1998; Cummings et al., 2001; Taavitsainen et al., 2001) while most use rat or human microsome samples.

We describe an easy and efficient method for the in vitro analysis of OH-CZX and CZX. This procedure provides a quick analysis that may facilitate the characterization of P450 metabolism in the pig. This characterization may allow the pig to be used in pharmacological and toxicological studies to determine detoxification of xenobiotics as well as aid in determining appropriate dosages and withdrawal times of drugs used in food animals.



Chlorzoxazone 6-Hydroxychlorzoxazone

Figure 4-1. Structures of chlorzoxazone and 6-hydroxychlorzoxazone.

Chapter 3: Materials and Methods

Reagents and Standards

The methanol used was "HPLC" grade; phosphoric acid was reagent grade while all other chemicals were enzyme grade. All of these chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA). Chlorzoxazone, glucose 6-phosphate (G-6-P), glucose 6-phosphate dehydrogenase (G-6-PDH), β-nicotinamide adenine dinucleotide phosphate (NADP) and phenacetin, the internal standard, were purchased from Sigma Chemical Company (St. Louis, MO, USA). Hydroxychlorzoxazone was purchased from RBI (St. Louis, MO, USA). Stock standard solutions of CZX (100, 5 and 1 μg/ml) and OH-CZX (100, 5, and 1 μg/ml) were prepared in methanol and stored at 4°C. Solutions were stable for six months. Working standards were prepared fresh daily by dilution of the stock standards. A stock standard solution of phenacetin (100 μg/ml) was prepared in methanol and stored at 4°C. This solution was also stable for six months. Phenacetin is light sensitive and all solution containers were wrapped in aluminum foil.

Apparatus

The analytical system consisted of a 626 solvent delivery system, a model 717 WISP autosampler, a 996 scanning UV detector and a computer equipped with Millennium software (Waters, Milford, MA, USA). The column was a YMC-Pack ODS-AQ (5 μm, 6 x 150 mm) equipped with a C_{18} Guard-Pak precolumn insert (Waters, Milford. MA, USA).

Chromatography

The mobile phase consisted of a mixture of (A) 0.05% phosphoric acid, pH 3.0 and (B) methanol. The mixture was pumped as a gradient starting at 60% A and 40% B and was maintained for 12 min. Over a 2 min period the mixture changed to 58% A and

42% B which was maintained for 5 min and then returned to initial conditions over the final 3 min period. The system was ready for the next injection without further equilibration. The mobile phase was prepared fresh daily using double-distilled deionized water, filtered (0.22 μM) and degassed before use. The flow rate was 1.7 ml/min. Column temperature was ambient and UV detection was measured at 287 nm.

Sample Treatment

Spiked samples were prepared by addition of appropriate volumes of both CZX and OH-CZX. The internal standard, phenacetin (30 μl of 100 μg/ml) was added. Appropriate amounts of the solutions used in microsomal preparations were added to produce a 0.5 ml final volume. Samples were vortex-mixed and a 190 μl sample injected onto the liquid chromatograph.

Microsomal samples were prepared using Lake's (1987) ultracentrifugation method. Incubation mixtures contained 0.5 mg of microsomal protein, 100 mM phosphate buffer at pH 7.4 containing 6 mM magnesium chloride, 1 mM EDTA, and a NADPH-generating system (1 mM NADP, 10 mM G-6-P and 0.7 U of G-6-PDH) in a total volume of 0.5 ml. Incubation mixtures contained CZX and inhibitors and the reactions were initiated by addition of the NADPH-generating system after a 5 min pre-incubation step at 37°C. Reactions were quenched with 0.1 ml of ice cold acetonitrile after 20 min in a 37°C shaking water bath and then placed on ice for 1 hour. Samples were centrifuged at 16000 g for 15 min. The supernatants were removed and stored at -80°C until analysis could be performed. Reaction rates were linear with incubation time under these conditions. Frozen samples were thawed on ice and vortex-mixed before use. Phenacetin (30 μl of 100 μg/ml) was added to a 0.5 ml microsome sample and vortex-mixed. Samples, which contained particulates, were centrifuged for 5 min at 16,000 g in

an Eppendorf centrifuge (Brinkman Instruments, New York, NY, USA). A 190 μl aliquot of the supernatant was injected onto the liquid chromatograph.

Chapter 4: Results

A blank chromatogram for a microsomal sample is shown in Figure 4-2 with a large peak at 2.32 min and a small peak at 2.95 min. These peaks are the result of NADPH generating solution used in the preparation of the microsome sample; however, they do not interfere with the peaks of interest. The x-axis on chromatograms 4-3 and 4-4 start roughly at 5 min in order to eliminate the large NADPH peak and provide a better image. The chromatogram in Figure 4-3 represents a 500 ng/ml standard. The retention times were 6.79, 11.78 and 20.28 min for OH-CZX, phenacetin and CZX, respectively. The chromatogram in Figure 4-4 represents a porcine liver microsome sample after incubation with 10 μM of CZX. Retention times for OH-CZX, phenacetin and CZX were 6.84, 11.92 and 20.67 min.

This method produced a linear curve for the concentration range of 25 - 2000 ng/ml for CZX and its metabolite, with correlation coefficients ranging from 0.998 to 0.999 for both compounds. Replicate analyses performed on the same day for microsomal samples spiked with specific concentrations of CZX produced coefficients of variation (C.V.) of 4.6% for 60 ng/ml, 2.9% for 800 ng/ml and 1.8% for 1700 ng/ml. The metabolites' C.V. was 3.2%, 0.3%, and 0.9% for the same concentrations (Table 4-1).

Table 4-1. Intra-assay precision for chlorzoxazone and 6-hydroxychlorzoxazone (n = 4)

Concentration added (ng/ml)	CZX Concentration measured (ng/ml) (mean±SD)	Coefficient of variation (%)	OH-CZX concentration measured (ng/ml) (mean±SD)	Coefficient of variation (%)
60	60±3	4.6	63±2	3.2
800	766±22	2.9	761±3	0.3
1700	1761±31	1.8	1749±16	0.9

SD = Standard deviation

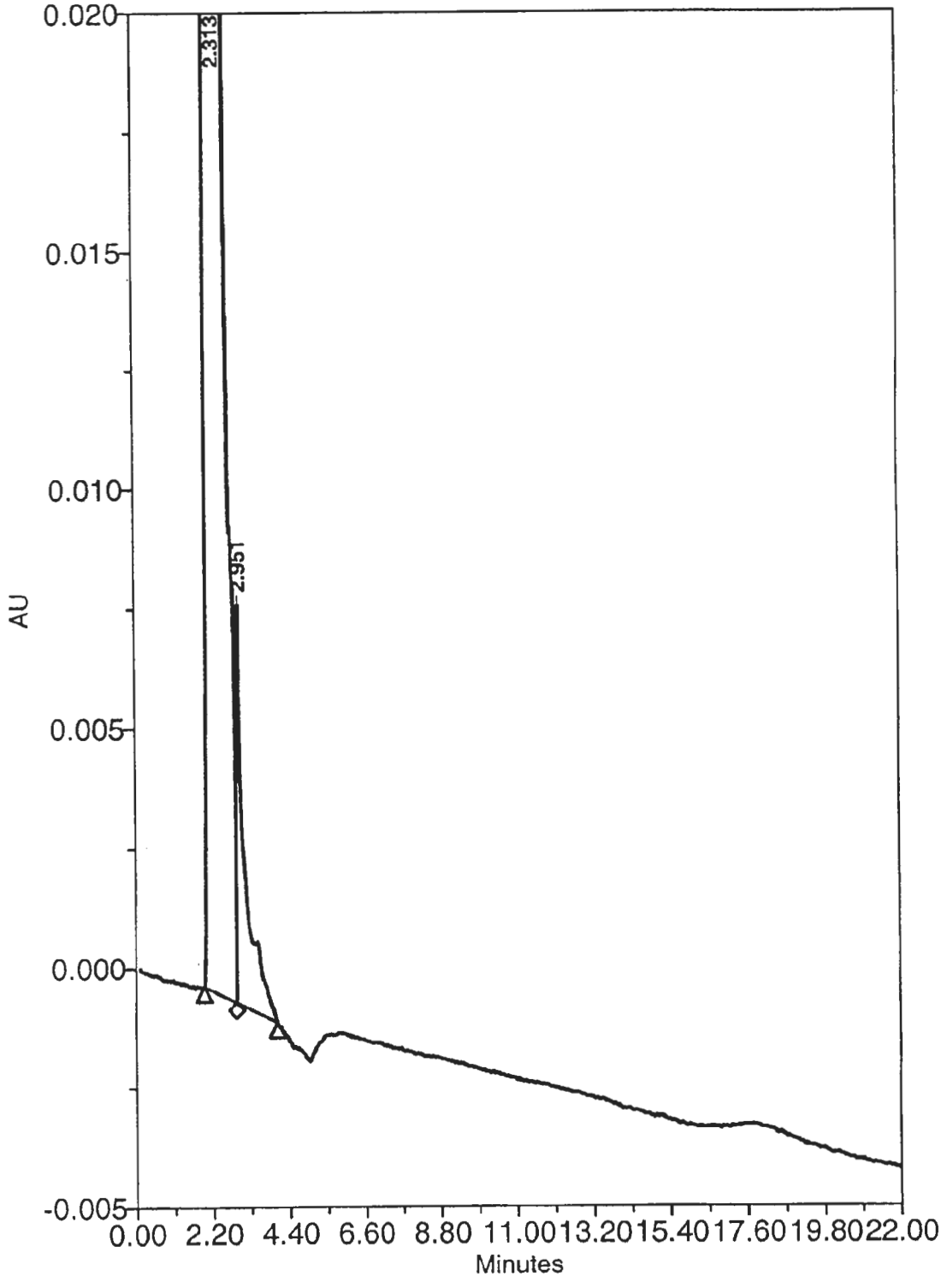


Figure 4-2. Blank microsome chromatogram with no drug added. Peaks 2.32 and 2.95 result from microsomal generating solution.

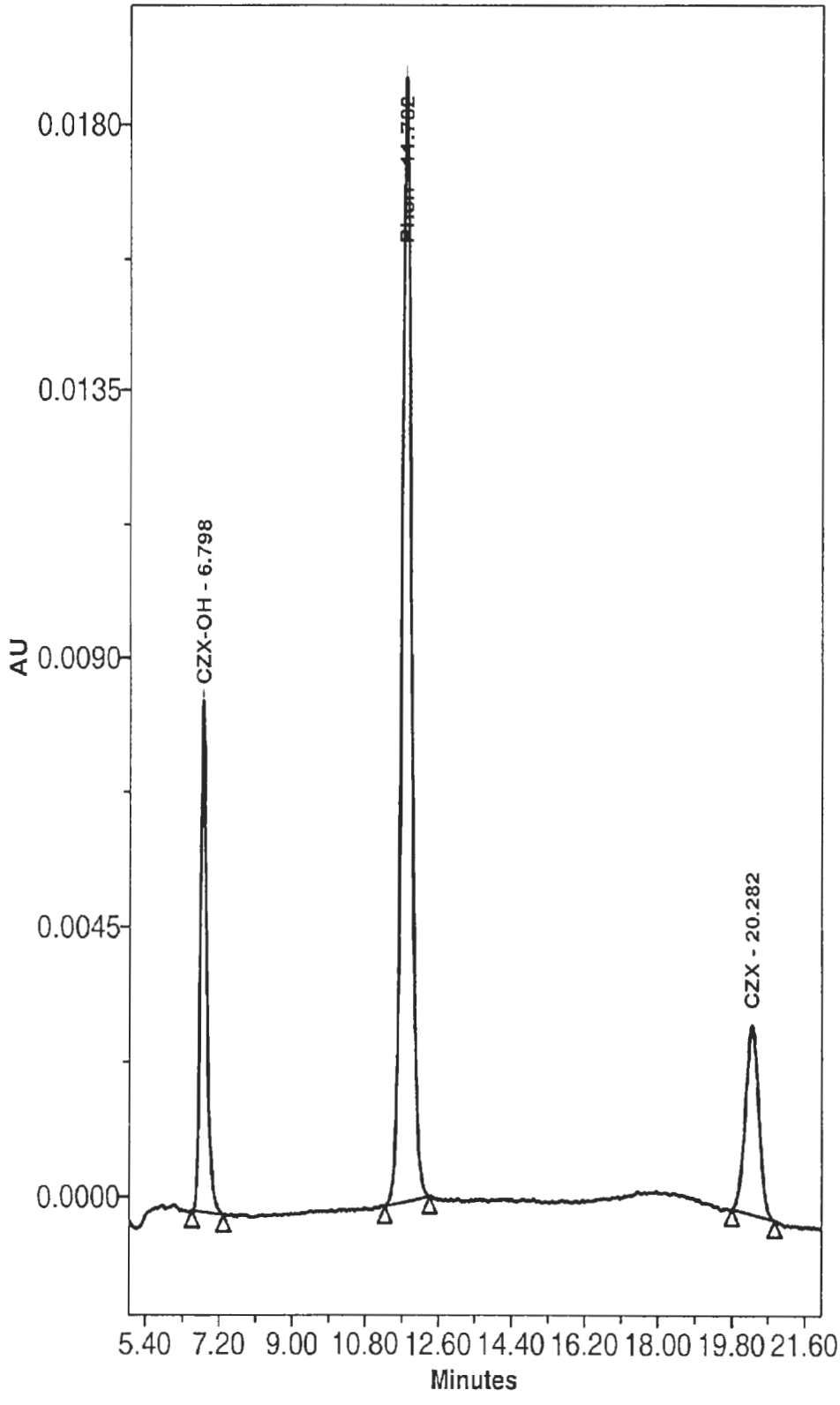


Figure 4-3. Chromatogram of a 500 ng/ml standard. Peaks: CZX-OH = OH-CZX; Phen = phenacetin; CZX = CZX.

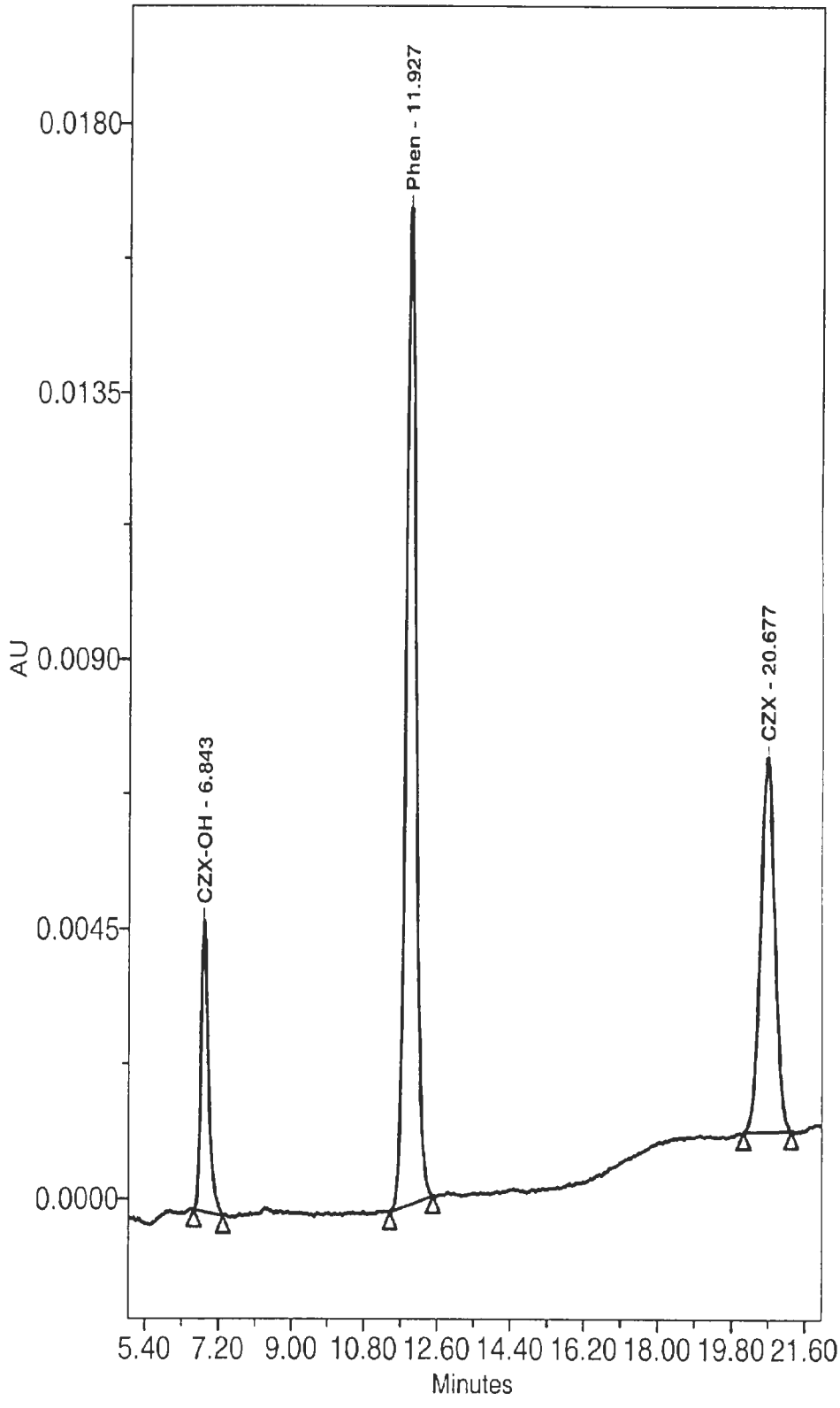


Figure 4-4. Chromatogram of a porcine microsomal sample after incubation with 10 μM of CZX. Peaks: CZX-OH = OH-CZX; Phen = phenacetin; CZX = CZX.

Table 4-2. Inter-assay precision for chlorzoxazone and 6-hydroxychlorzoxazone (n = 4)

Concentration added (ng/ml)	CZX concentration measured (ng/ml)	Coefficient of variation (%)	OH-CZX Concentration measured (ng/ml)	Coefficient of variation (%)
25	24	2.1	26	11
50	51	7.8	48	2.1
100	102	8.3	94	5.3
250	243	1.9	244	6.6
500	491	2.6	477	5.1
1000	989	1.5	975	6.0
2000	1897	6.0	1989	2.0

Day-to-day variability for microsomal replicates appears in Table 4-2. Mean recoveries of CZX were 96%, 101%, 102%, 97%, 98%, 99%, and 95 % for 25, 50, 100, 250, 500, 1000, and 2000 ng/ml. Mean recoveries of OH-CZX were 104%, 96%, 94%, 98%, 95%, 98%, and 99% for 25, 50, 100, 250, 500, 1000, and 2000 ng/ml. The detection limit for CZX was 20 ng/ml and its metabolite was 10 ng/ml. This represents a peak approximately three times that of baseline noise.

Numerous drugs and chemicals used in inhibition studies were tested for interference with the chromatographic procedure (Table 4-3). Initially, quinidine was found to co-elute with OH-CZX however, using the current chromatography and a change of pH to 3.2 eliminated this problem. Furafylline, which is used in microsomal assays, could interfere with the internal standard, phenacetin, but with the current gradient there is adequate separation.

Operating under linear conditions adapted from reported procedures, (Newton et al., 1995; Hickman et al., 1998) the formation of OH-CZX was easily detectable at 10 μM as seen in Figure 4-4. Enzyme activity for the sample was 216 nmole/min/mg protein. This was calculated by dividing the amount of product formed by incubation time and

microsomal protein content. In this particular sample of porcine liver microsomes, the estimated Km for the process was 43 μM.

Table 4-3. Chemicals tested for assay interference.

Chemicals	Retention Times
Quinidine	7.82
Furafylline	10.37
7.8-Benzoflavone	ND
Diethyldithiocarbamate	ND
Ketoconazole	ND
Ciprofloxacin	4.96
Bufuralol	ND
Itraconazole	ND
Phenacetin	11.92
NADP	2.32
Potassium phosphate	2.45
Magnesium chloride	ND
Glucose-6-phosphate	ND
Glucose-6-phosphate dehydrogenase	ND

ND = No peaks were detected.

Chapter 5: Discussion

Enzyme metabolism studies require a method that is simple, quick, sensitive and reproducible. An HPLC assay, utilizing UV detection, has been developed to investigate the 6-hydroxylation of CZX by microsomal fractions of porcine liver. The assay is sensitive, specific and reproducible, with a high recovery of the metabolite.

Because we did not use an extraction for our samples, the NADP generating solution produced a very large peak initially. In order to prevent any interference with OH-CZX, the mobile phase was adjusted to 60% A and 40% B. This percentage was maintained until the elution of the internal standard in order to prevent any interference from furafylline when it was used in microsomal preparations. We did try to optimize the chromatography after the elution of the internal standard; however, an increase in the percent methanol in the mobile phase higher than what we have listed caused a large shift in the baseline, which was not acceptable. If the quantitation of CZX is not necessary or important for the experiment of interest, the assay time can be shortened to eliminate CZX.

Most published procedures do not list validation parameters such as limit of detection or recoveries for OH-CZX and CZX which makes it difficult to compare our method with others. We do feel that our limit of detection and recovery for both compounds is more than adequate for microsomal studies. In cases where validation parameters are listed, ours are equal to or better than existing methods. Use of phenacetin as the internal standard corrects for intra- and inter-assay variability in the method.

HPLC procedures involving extraction of OH-CZX and CZX described by Thummel et al. (1993) used 5 ml of methylene chloride for extraction and have a quantitation range of 250-5000 ng/ml. While Peter et al. (1990) and Taavitsainen et al.

(2001) require microsome samples to be extracted 2 times with methylene chloride before evaporation and injection, with a limit of detection of 90 ng/ml. Lillibridge et al. (1998) also used methylene chloride and indicates that standard curves were linear over their respective ranges and interday and intraday coefficients of variation were less than 10%. Chittur and Tracey (1997) used an ether extraction, could quantify samples at amounts of 10 ng or greater and had a recovery between 40 and 45%. Cummings et al. (2001) used ethyl acetate and flash freezing in a dry-ice acetone bath. Leclercq et al. (1998) used a zinc sulfate homogenization procedure followed by filtering of the sample. Our procedure eliminates the use of time consuming liquid-liquid extractions involving toxic and expensive organic solvents and does not require the use of dry ice, homogenizers, nitrogen evaporation or sample filters. It is a rugged procedure with the column still in use after over 2000 injections and the guard column replaced roughly every 300 injections. We chose this particular column because of its longevity and ruggedness as well as its ability to tolerate the sample type used in the assay without effecting the resolution of the compounds of interest. Even after 2000, injections the resolution and peak shape are exceptional with very little increase in pressure.

The present study was conducted in order to develop a method to determine metabolism of CZX in porcine microsome samples from liver. The method has been applied to xenobiotic effects on porcine microsomes involving CZX in this laboratory. In conclusion, a simple, quick and sensitive HPLC procedure has been developed for analysis of OH-CZX and CZX in porcine hepatic microsome samples.

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Chapter 1: Abstract

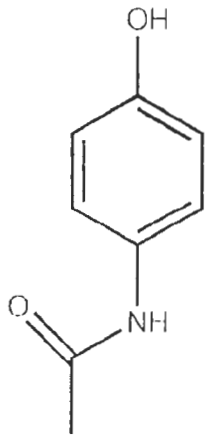
A simple, quick, and sensitive HPLC analysis of phenacetin and acetaminophen in porcine hepatic microsome samples is described. Chromatography was performed on a YMC-Pack ODS-AQ column using a gradient mobile phase consisting of 0.05% phosphoric acid (pH 3):methanol. UV detection was measured at 254 nm. The procedure produced a linear curve for the concentration range 10 -1500 ng/ml, and had a limit of detection of 5 ng/ml for both compounds. This assay produced accurate and repeatable quantification of acetaminophen and phenacetin.

Chapter 2: Introduction

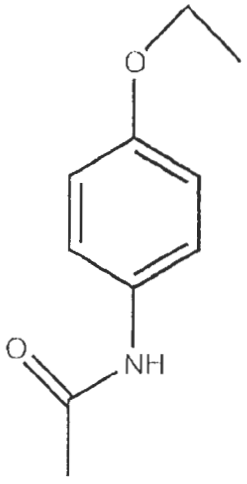
The cytochrome P450 monooxygenase enzyme system is important in terms of its catalytic versatility and for the sheer number of compounds it detoxifies or activates to reactive intermediates (Guengerich, 1992; Parkinson, 1996). P450 enzymes are located in virtually all tissues; however, the largest concentration of these enzymes is found in the liver endoplasmic reticulum (microsomes). These enzymes play a pivotal role in drug metabolism. They activate xenobiotics including drugs to toxic and/or tumorigenic metabolites, aid in determining intensity and duration of action of drugs and detoxify xenobiotics. Therefore, it is essential to establish the activity and regulation of the P450 system for species selected for pharmacological and toxicological studies. In addition, impaired drug disposition in food-producing animals may lead to changes in residue levels of veterinary drugs and other xenobiotics in edible tissues, milk, or eggs. There is also concern about the increase in development of drug resistant bacterial strains in food animals. Much of the information known about P450 enzymes comes from studies that were conducted in rodents. While very little is known about P450 enzymes in animals used for food, the pig is becoming a viable alternative to traditional non-rodent species in pharmacological and toxicological testing (Skaanild and Friis, 1999). However, information on the P450 system for this species is limited.

The hepatic P450 enzymes in microsomes are mainly responsible for phase I metabolism, which adds or exposes polar functional groups on a lipophilic substrate. In the investigation of P450 mediated xenobiotic metabolism, individual forms of P450 have been found to catalyze specific reactions with certain substrates. These activities are designated as probes for specific P450 enzyme action. To date, at least one marker activity exists for the majority of human P450 enzymes (Sharer et al., 1995).

Phenacetin-O-deethylation to acetaminophen (Figure 5-1) is one method used to



Acetaminophen



Phenacetin

Figure 5-1. Structures of phenacetin and acetaminophen.

characterize cytochrome P450 enzyme CYP1A2 activity. Several high performance liquid chromatography (HPLC) methods have been developed to measure acetaminophen and phenacetin in biological fluids and microsomes (Borm et al., 1983; Bartoli et al., 1996; McKillop et al., 1998; Lillibridge et al., 1998; Eagling et al., 1998; Dong et al., 1999; Nakajima et al., 1999; Kobayashi et al., 1999; Kudo et al., 2000; Zhang et al., 2001). Many microsome methods involve the use of liquid-liquid extractions and evaporation (Borm et al., 1983; McKillop et al., 1998; Lillibridge et al., 1998; Eagling et al., 1998; Nakajima et al., 1999; Kobayashi et al., 1999; Zhang et al., 2001) while one method (Kudo et al., 2000) uses extraction cartridges. The majority of the methods also involve the use of human or rat microsomes, but not porcine microsomes.

We describe an easy and efficient method for analysis of acetaminophen and phenacetin, which may facilitate characterization of P450 metabolism in the pig.

Chapter 3: Materials and Methods

Reagents and Standards

Methanol (HPLC grade), phosphoric acid (reagent grade), magnesium chloride (enzyme grade), potassium phosphate (enzyme grade), and ethylenediaminetetraacetic acid (EDTA) (enzyme grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Phenacetin, acetaminophen, glucose 6-phosphate (G-6-P), glucose 6-phosphate dehydrogenase (G-6-PDH), β-nicotinamide adenine dinucleotide phosphate (NADP) and trimethoprim (TMP), the internal standard, were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Stock standard solutions of phenacetin (100, 5 and 1 μg/ml) and acetaminophen (100, 5, and 1 μg/ml) were prepared in methanol and stored at 4°C. These solutions were stable for six months. Working standards were prepared fresh daily by dilution of the stock standards. Stock standard solutions of TMP (50, and 25 μg/ml) were prepared in methanol and stored at 4°C. These solutions were also stable six months. Phenacetin is light sensitive and all solution containers were wrapped in aluminum foil.

Apparatus and Chromatographic Conditions

The analytical system consisted of a 626 solvent delivery system, a model 717 WISP autosampler, a 996 scanning UV detector, and a computer equipped with Millennium software (Waters, Milford, MA, USA). The column was a YMC-Pack ODS-AQ (5 μm, 6 x 150 mm) equipped with a C_{18} Guard-Pak precolumn insert (Waters, Milford. MA, USA).

The mobile phase consisted of a mixture of (A) 0.05% phosphoric acid pH 3.0 and (B) methanol. The mixture was pumped as a gradient starting at 74% A and 26% B and maintained for 4 min. Then over a 5 min period, the mixture was changed to 66% A

and 34% B and maintained for 9 min. Over the next 4 min period, the system was returned to initial conditions and ready for the next injection without further equilibration. The mobile phase was prepared fresh daily using double-distilled, deionized water, filtered (0.22 μM) and degassed before use. Flow rate was 1.5 ml/min, column temperature was ambient, and detection was measured at 254 nm.

Sample Treatment

Spiked samples were prepared by the addition of standards (phenacetin, acetaminophen and TMP) to appropriate amounts of the solutions used in microsomal preparation to produce a 0.5 ml final volume. Samples were vortex-mixed and a 190 μl sample was injected onto the liquid chromatograph.

Microsomal samples were prepared using Lake's (1987) ultracentrifugation method. Incubation mixtures contained 0.5 mg of microsomal protein, 100 mM phosphate buffer at pH 7.4 containing 6 mM magnesium chloride, 1 mM EDTA, and an NADPH-generating system (1 mM NADP, 10 mM G-6-P and 0.7 U of G-6-PDH) in a total volume of 0.5 ml. Incubation mixtures contained phenacetin and inhibitors, and the reactions were initiated by addition of the NADPH-generating system after a 5 min pre-incubation step at 37°C. Reactions were quenched with 0.1 ml of ice cold acetonitrile after 20 min in a 37°C shaking water bath, and then placed on ice for 1 hour. Samples were centrifuged at 16,000 g for 15 min. The supernatants were removed and stored at -80°C until analysis could be performed. Reaction rates were linear with incubation time under these conditions. Frozen samples were thawed on ice and vortex-mixed before use. TMP (50 μl of 25 μg/ml) was added to a 0.5 ml microsome sample and vortex-mixed. Those samples containing particulates were centrifuged for 5 min at 16,000 g in

an Eppendorf centrifuge (Brinkman Instruments, New York, NY, USA). A 190 μl aliquot of the supernatant was injected onto the liquid chromatograph.

Chapter 4: Results

A blank chromatogram for a microsomal sample with no drug added is shown in Figure 5-2 with a large peak at 2.84 min and smaller peaks at 3.62 and 3.88 min. These peaks are the result of NADPH generating solution used in the microsome sample; however, they do not interfere with peaks of interest. The x-axis on chromatograms 5-3 and 5-4 start at 4.5 min in order to eliminate the large NADPH peak, and to provide a better image of the peaks of interest. The chromatogram in Figure 5-3 represents a porcine liver microsome sample after incubation with 10 μM of phenacetin for 20 min. Retention times for acetaminophen, TMP and phenacetin were 5.25, 8.08 and 19.48 min. The chromatogram in Figure 5-4 represents a 500 ng/ml standard with retention times of 5.28, 8.05 and 19.50 min for acetaminophen, TMP and phenacetin, respectively.

The method used produced a linear curve for the concentration range of 10 – 1500 ng/ml for phenacetin and acetaminophen, with correlation coefficients ranging from 0.998 to 0.999 for both compounds. Replicate analyses performed on the same day for microsomal samples spiked with specific concentrations of phenacetin, produced coefficients of variation (C.V.) of 1.5% for 60 ng/ml, 1.3% for 750 ng/ml and 0.1% for 1200 ng/ml. The metabolites' C.V. was 6.9%, 2.3%, and 1.0%, respectively, for the same concentrations (Table 5-1).

Table 5-1. Intra-assay precision for phenacetin and acetaminophen (n = 4)

Concentration Added (ng/ml)	Phenacetin Concentration measured (ng/ml) (mean±SD)	Coefficient of variation (%)	Acetaminophen concentration measured (ng/ml) (mean±SD)	Coefficient of variation (%)
60	62±1	1.5	58±4	6.9
750	720±9	1.3	722±17	2.3
1200	1206±1	0.1	1186±12	1.0

SD = Standard deviation

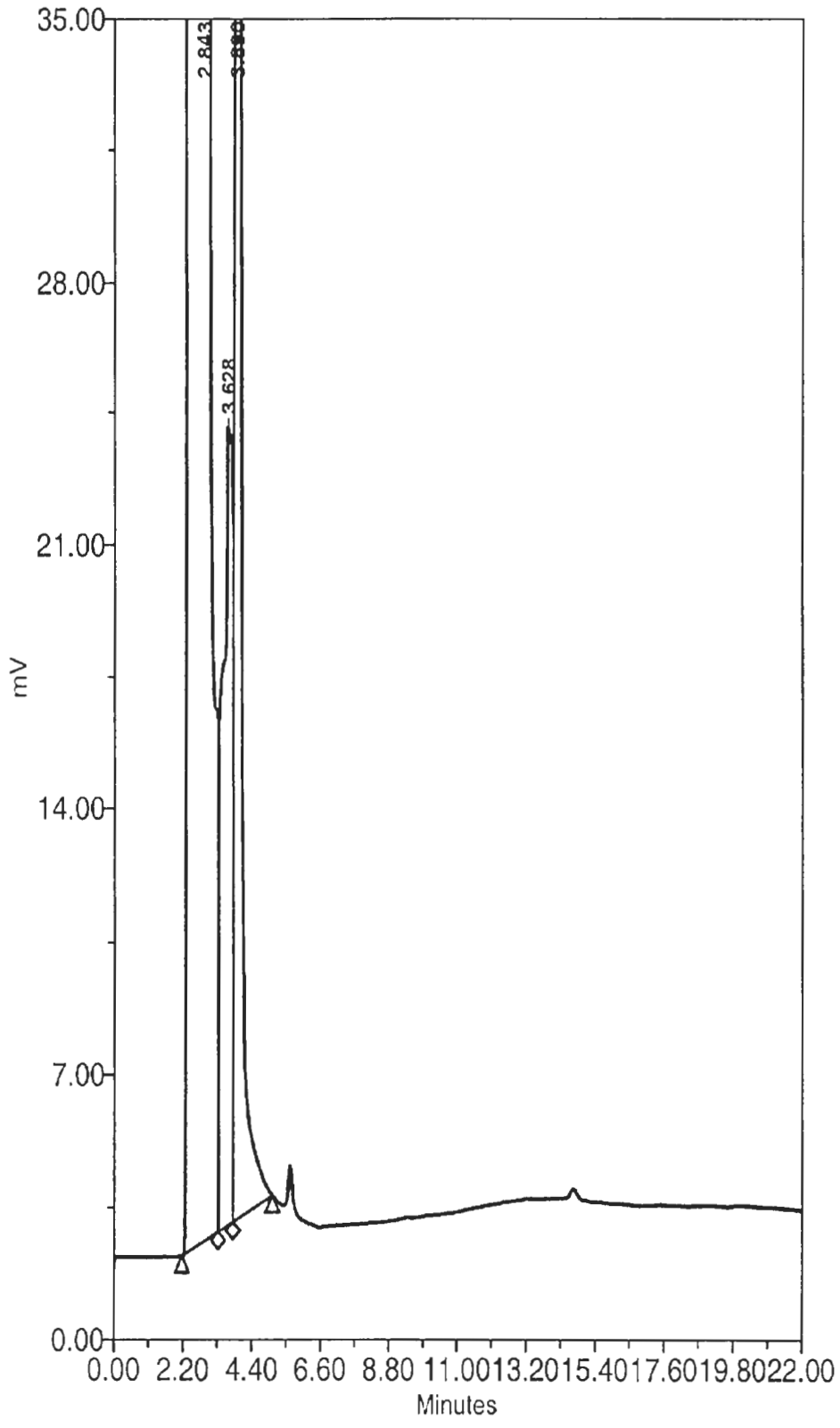


Figure 5-2. Blank microsome chromatogram with no drug added. Peaks 2.84, 3.62, and 3.88 result from microsomal generating solution.

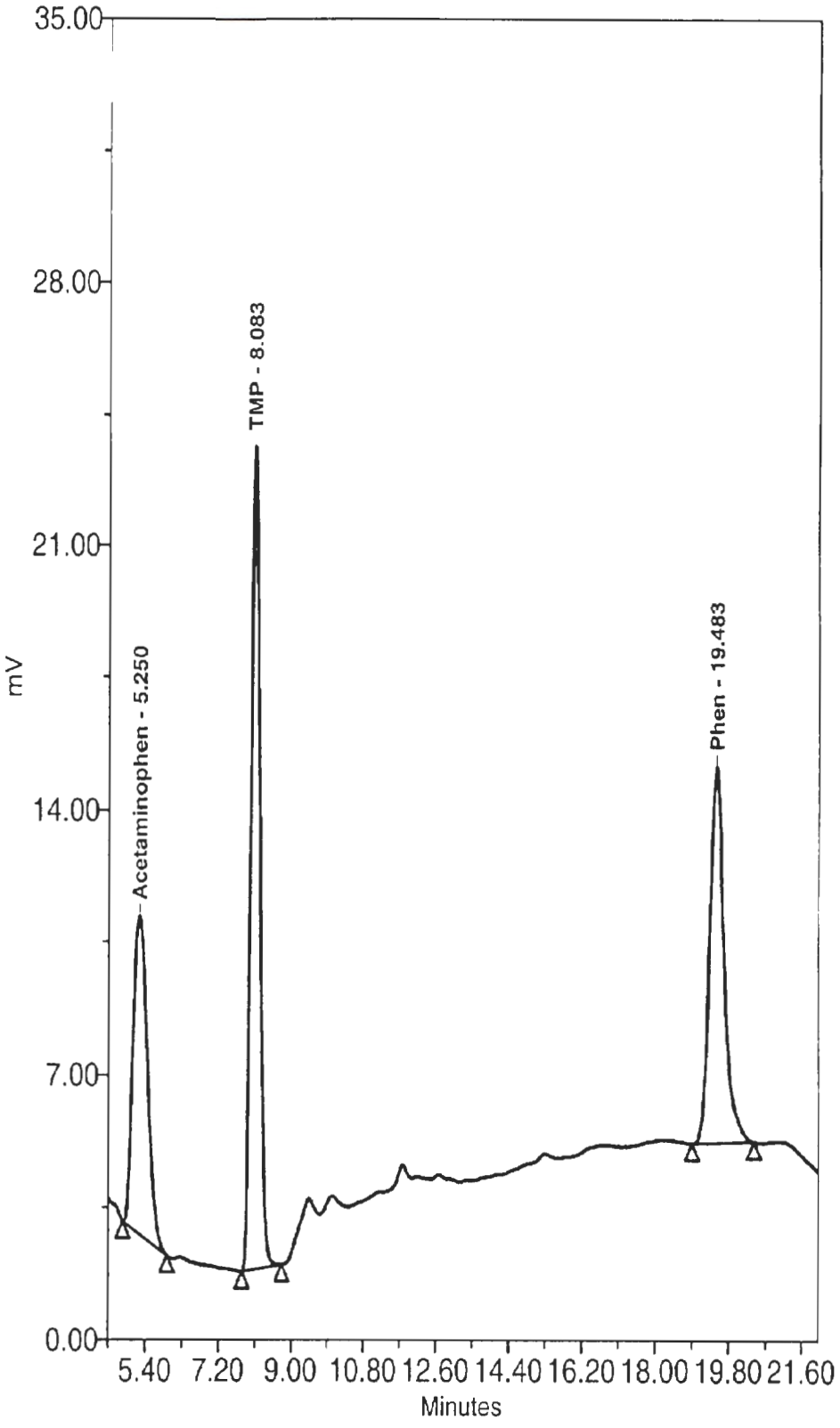


Figure 5-3. Chromatogram of a porcine microsomal sample after incubation with 10 μM of phenacetin. Peaks: acetaminophen; TMP (internal standard); Phen= phenacetin.

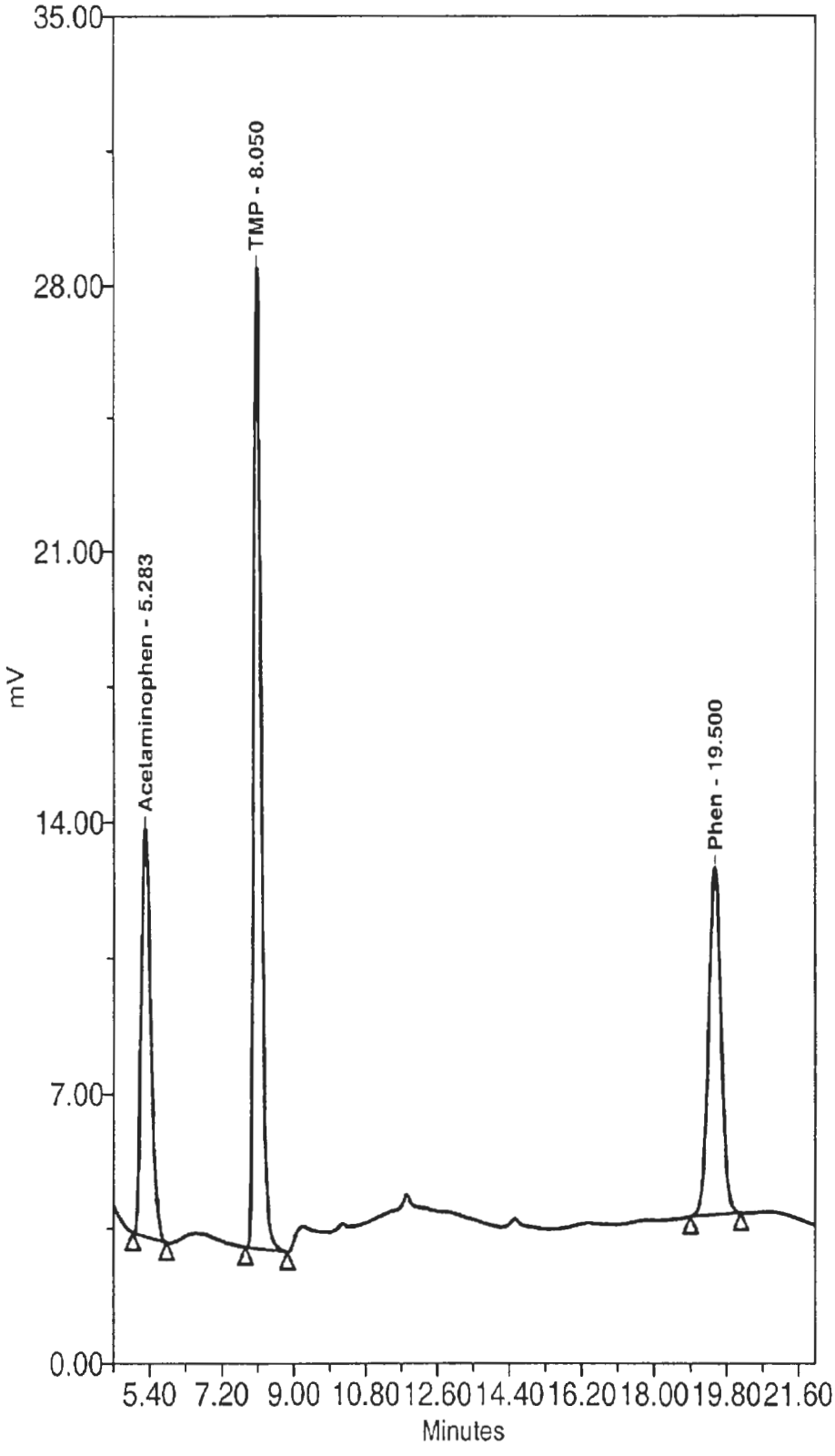


Figure 5-4. Chromatogram of a 500 ng/ml standard. Peaks: acetaminophen; TMP (internal standard); Phen= phenacetin.

Table 5-2. Inter-assay precision for phenacetin and acetaminophen (n = 4)

Concentration added (ng/ml)	Phenacetin Concentration measured (ng/ml)	Coefficient of variation (%)	Acetaminophen Concentration measured (ng/ml)	Coefficient of variation (%)
10	9.9	5.0	9.8	3.2
25	24	3.7	24	4.2
50	51	2.8	49	6.1
100	97	3.1	99	6.1
250	246	3.5	249	3.8
500	467	1.2	498	3.6
800	789	1.0	791	1.6
1000	978	1.0	987	3.3
1500	1481	1.2	1440	1.3

Results for day-to-day variability for microsomal replicates appear in Table 5-2. Mean recoveries of phenacetin were 99%, 96%, 103%, 97%, 98%, 94%, 98%, 98%, and 98 % for 10, 25, 50, 100, 250, 500, 800, 1000, and 1500 ng/ml. Mean recoveries of acetaminophen were 98%, 96%, 98%, 99%, 100%, 99%, 99%, 99%, and 96% for 10, 25, 50, 100, 250, 500, 800, 1000, and 1500 ng/ml. The detection limit for both compounds was 5 ng/ml. This represents a peak approximately three times baseline noise.

Numerous drugs and chemicals used in inhibition studies were tested for interference with the chromatographic procedure (Table 5-3). Furafylline was found to co-elute with phenacetin using the present gradient. This was corrected by changing the gradient. The first 10 min remains the same as the original gradient, however, the gradient changes from 66% A, 34% B to 76% A, 24% B from 10 min to 18 min. This is maintained for 8 min, then over 1 min the gradient is returned to the original conditions (74%A, 26%B).

Table 5-3 Chemicals tested for assay interference

Chemicals
Quinidine
Furafylline
7.8-Benzoflavone
Diethyldithiocarbamate
Ketoconazole
Ciprofloxacin
Bufuralol
Lidocaine
Itraconazole
Chlorzoxazone
NADP
Potassium phosphate
Magnesium chloride
Glucose-6-phosphate
Glucose-6-phosphate dehydrogenase

Chapter 5: Discussion

Enzyme metabolism studies require a method that is simple, quick, accurate, sensitive and reproducible. Such an HPLC assay, utilizing UV detection, has been developed to investigate the conversion of phenacetin to acetaminophen by microsomal fractions of porcine liver. The assay is sensitive, specific and reproducible, with a high recovery of the metabolite.

Because we did not use an extraction for our samples, the NADPH generating solution produced a very large peak initially. In order to prevent any interference with acetaminophen, the mobile phase was initially adjusted to 74% A and 26% B. We did try to optimize the chromatography in order to reduce the time between TMP and phenacetin; however, we found that quinidine, which is used in microsomal studies, would interfere with TMP when the percent methanol in the mobile phase was higher than 34%. A higher percentage of methanol also caused a large shift in the baseline, which was not acceptable. However, if the quantitation of phenacetin is not necessary or important for the experiment, the analysis time can be reduced.

Most of the procedures in the literature do not list validation parameters such as limit of detection or recoveries for acetaminophen and phenacetin. However, we feel that our limit of detection and recovery for both compounds are more than adequate for microsomal studies. In cases where validation parameters are listed, ours are equal to or better than existing methods. Use of TMP as the internal standard corrects for intra- and inter-assay variability in the method.

HPLC procedures involved in the determination of acetaminophen and phenacetin by McKillop et al. (1998) used a propanol:ethyl acetate extraction, flash freezing of the aqueous phase and evaporation, while Zhang et al. (2001) used an ethyl acetate extraction. Borm et al. (1983) used 10 ml of dichloromethane for 20 min,

followed by hydrochloric acid and then a back extraction into diethyl ether for 20 min, which must be evaporated. Eagling et al. (1998) used a similar procedure with 10 ml dichloromethane and 10ml ethyl acetate followed by evaporation, with inter- and intra-assay variabilities of 8.7% and 6.5%. Nakajima et al. (1999), Lillibridge et al. (1998) and Kobayashi et al. (1999) used 2ml of acetonitrile, vortexed for 10 min, then centrifugation and evaporation of the organic phase with a dri-block or vacuum evaporator. Kudo et al. (2000) used extraction cartridges in their procedure. Our procedure eliminates the use of time consuming liquid-liquid extractions involving toxic and expensive organic solvents, and does not require the use of dry ice, nitrogen, vacuum evaporation or extraction cartridges. It is a rugged procedure with the column still in use after over 2300 injections, and the guard column replaced roughly every 300 injections.

This method was developed in order to facilitate microsome studies involving phenacetin, and has been applied to metabolism studies conducted in porcine microsomes in this laboratory. In conclusion, a simple, quick and sensitive HPLC procedure has been developed for analysis of acetaminophen and phenacetin in hepatic microsome samples.

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PART VI: DETERMINATION OF MONOETHYLGLYCINEXYLIDIDE AND LIDOCAINE IN PORCINE MICROSOMAL PREPARATIONS

Chapter 1: Abstract

A simple, accurate, and sensitive HPLC analysis of monoethylglycinexylidide and lidocaine in porcine microsome samples is described. Chromatography was performed on a μBondapak C_{18} column using an isocratic mobile phase of 0.03M potassium dihydrogen phosphate:acetonitrile (87:13), pH 5.9. Detection was measured at 205 nm. The procedure produced linear curves for the concentration range 50-1000 ng/ml with a limit of detection of 10 ng/ml. Recoveries for both compounds were greater than 90%. This assay produced accurate and repeatable results.

Chapter 2: Introduction

Cytochrome P450 enzymes in the liver play a pivotal role in metabolism. They are important in terms of their catalytic versatility and sheer number of compounds detoxified or activated to reactive intermediates (Guengerich, 1992; Parkinson, 1996). P450 enzymes are present in all species examined to date. They are classified into families, which in turn are divided into subfamilies. The subfamilies in turn consist of highly related individual forms. The number of enzymes does differ between species (Parkinson, 1996). Enzyme levels can vary because of environmental as well as genetic factors. The largest concentration of P450 enzymes is located in the liver endoplasmic reticulum (microsomes), but they are located in virtually all tissues.

P450 enzymes activate xenobiotics including drugs to toxic and/or tumorigenic metabolites, detoxify xenobiotics, and aid in determining intensity and duration of action of drugs. Drug or xenobiotic metabolism is a direct reflection of the multiple enzyme systems that characterize different animal species and is often the most important single factor in the regulation of their concentrations. Therefore, it is essential to establish the activity and regulation of the P450 system for species selected for pharmacological and toxicological studies. In addition, impaired drug disposition in food-producing animals may lead to changes in residue levels of veterinary drugs and other xenobiotics in edible tissues, milk, or eggs. Much of the information known about P450 comes from studies conducted in rodents. However, the pig is becoming a popular alternative to traditional non-rodent species in pharmacological and toxicological testing (Skaanild and Friis, 1999). The information on the P450 enzyme system for this species is limited.

P450 enzymes are mainly responsible for phase I metabolism, which adds or exposes polar functional groups on a lipophilic substrate. In the investigation of P450 enzyme mediated xenobiotic metabolism, individual forms of P450 have been found to

catalyze specific reactions with certain substrates. Thus these specific activities or reactions can be utilized as probes for this particular kind of enzyme. To date, at least one marker activity exists for the majority of human P450 enzymes (Sharer et al., 1995). The conversion of lidocaine to monoethylglycinexylidide (MEGX) (Figure 6-1) by N-deethylation is one of the methods used to characterize cytochrome P450 enzyme CYP3A4 activity. Several high performance liquid chromatography (HPLC) methods have been developed to measure MEGX and lidocaine in biological fluids and microsomes (Kawai et al., 1985; Imaoka et al., 1990; Oshishi et al., 1993; Tanaka et al., 1994; Nakamoto et al., 1997; Leclercq et al., 1997; Kang et al., 1999; Wang et al., 1999; Wang et al., 2000). Many microsome methods involve the use of liquid-liquid extractions and evaporations (Kawai et al., 1985; Imaoka et al., 1990; Oshishi et al., 1993; Tanaka et al., 1994; Leclercq et al., 1997; Wang et al., 1999; Wang et al., 2000). The majority of methods also involve the use of human or rat microsomes, but not from swine.

This article describes a quick and efficient method for analysis of MEGX and lidocaine, which may facilitate characterization of P450 enzyme activity in the pig.

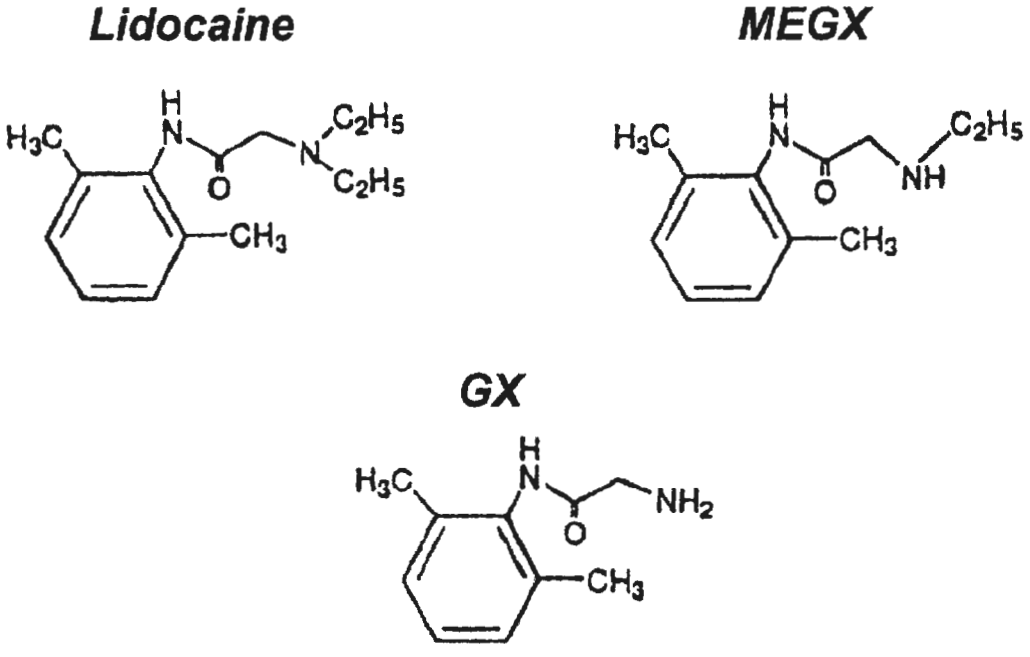

Figure 6-1. Structures of lidocaine, MEGX and GX.

Chapter 3: Materials and Methods

Reagents and Standards

Acetonitrile (HPLC grade), potassium dihydrogen phosphate (reagent grade), sodium hydroxide (reagent grade), magnesium chloride (enzyme grade), potassium phosphate (enzyme grade), and EDTA (enzyme grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Lidocaine was purchased from US Pharmacia (Rockville, MD, USA). MEGX and glycinexylidide (GX) were gifts from Astra laboratories (Westboro, MA, USA). Trimethoprim (TMP), glucose 6-phosphate (G-6-P), glucose 6-phosphate dehydrogenase (G-6-PDH), and β-nicotinamide adenine dinucleotide phosphate (NADP) were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Stock standard solutions of lidocaine (100, 5 and 1 μg/ml were prepared in methanol, while stock standard solutions of MEGX and GX (100, 5, and 1 μg/ml) were prepared in water. All solutions were stored at 4°C. Working standards were prepared fresh daily by dilution of the stock standards. Stock standard solutions of TMP (50 and 25 μg/ml) were prepared in methanol and stored at 4°C.

Apparatus and Chromatography

The analytical system consisted of a 626 solvent delivery system, a model 717 WISP autosampler, a 486 UV detector and a computer equipped with Millennium software (Waters, Milford, MA, USA). The column was a μBondapak C_{18} (10 μm, 3.9 x 300 mm) equipped with a μBondapak C_{18} Guard-Pak precolumn insert (Waters, Milford. MA, USA).

The mobile phase consisted of an isocratic mixture of 0.03M potassium dihydrogen phosphate pH 5.9 (87%) and acetonitrile (13%). It was prepared fresh daily

using double-distilled deionized water, filtered (0.22 μM) and degassed before use. The flow rate was 2.0 ml/min. Column temperature was ambient and detection was measured at 205 nm.

Sample Treatment

Spiked samples were prepared by the addition of appropriate volumes of GX, MEGX and lidocaine. The internal standard, TMP (25 μl of 25 μg/ml) was added and appropriate amounts of the solution used in microsomal preparations were added to produce a 0.5 ml final volume. Samples were vortex-mixed and a 190 μl sample injected onto the liquid chromatograph.

Previously frozen microsomal samples were prepared using Lake's (1987) ultracentrifugation method. Incubation mixtures contained 0.5 mg of microsomal protein, 100 mM phosphate buffer pH 7.4 containing 6 mM magnesium chloride, 1 mM EDTA, and a NADPH-generating system (1 mM NADP, 10 mM G-6-P and 0.7 U of G-6-PDH) in a total volume of 0.5 ml. Incubation mixtures contained lidocaine and inhibitors and the reactions were initiated by addition of the NADPH-generating system after a 5 min pre-incubation step at 37°C. Reactions were quenched with 0.05 ml of 1 M NaOH after 20 min in a 37°C shaking water bath and then placed on ice for 1 hour. Reaction rates were linear with incubation time under these conditions. Samples were centrifuged at 16,000 g for 15 min; the supernatant was removed and stored at -80°C until analysis could be preformed.

Frozen samples were thawed on ice and vortexed before use. TMP (25 μl of 25 μg/ml) was added to a 0.5 ml microsome sample and vortex-mixed. Those samples containing particulates were centrifuged for 5 min at 16,000 g in an Eppendorf centrifuge

(Brinkman Instruments, New York, NY, USA). A 190 μl aliquot of the supernatant was injected onto the liquid chromatograph.

Chapter 4: Results

A blank chromatogram for a microsomal sample with no drug added is shown in Figure 6-2 with large peaks at 1.12, 1.50 and 2.39 min, which are the result of the NADPH generating solution used in the microsome sample. However, none of these peaks interfere with the peaks of interest (MEGX, TMP, lidocaine). The x-axis on chromatogram 6-3 starts at 5 min in order to eliminate the large NADPH peaks and provide a better image of the peaks of interest. The chromatogram in Figure 6-3 represents a porcine liver microsome sample after incubation with 10 μM of lidocaine for 20 min. Retention times for MEGX, TMP and lidocaine were 7.20, 13.25 and 19.70 min. GX, which is another metabolite of lidocaine, was not detected in any of the sample chromatograms.

The method used in this study produced a linear curve for the concentration range of 50 – 1000 ng/ml for lidocaine and its metabolite, with correlation coefficients ranging from 0.998 to 0.999 for both compounds. Replicate analyses were performed on the same day for microsomal samples spiked with specific concentrations of 100 and 800 ng/ml MEGX. Coefficients of variation (C.V.) were 6.6% and 5.1%, respectively. The C.V. for lidocaine was 2.1%, and 7.9% for the same concentrations. Day-to-day variability for microsomal replicates appears in Table 6-1.

Table 6-1. Inter-assay precision for MEGX and lidocaine (n = 4)

Concentration added (ng/ml)	MEGX Concentration measured (ng/ml)	Coefficient of variation (%)	Lidocaine Concentration measured (ng/ml)	Coefficient of variation (%)
50	46	6.6	52	9.6
100	98	6.3	94	4.3
250	246	5.3	242	10.2
500	477	1.9	470	6.6
800	786	5.3	774	3.0
1000	990	5.0	897	1.0

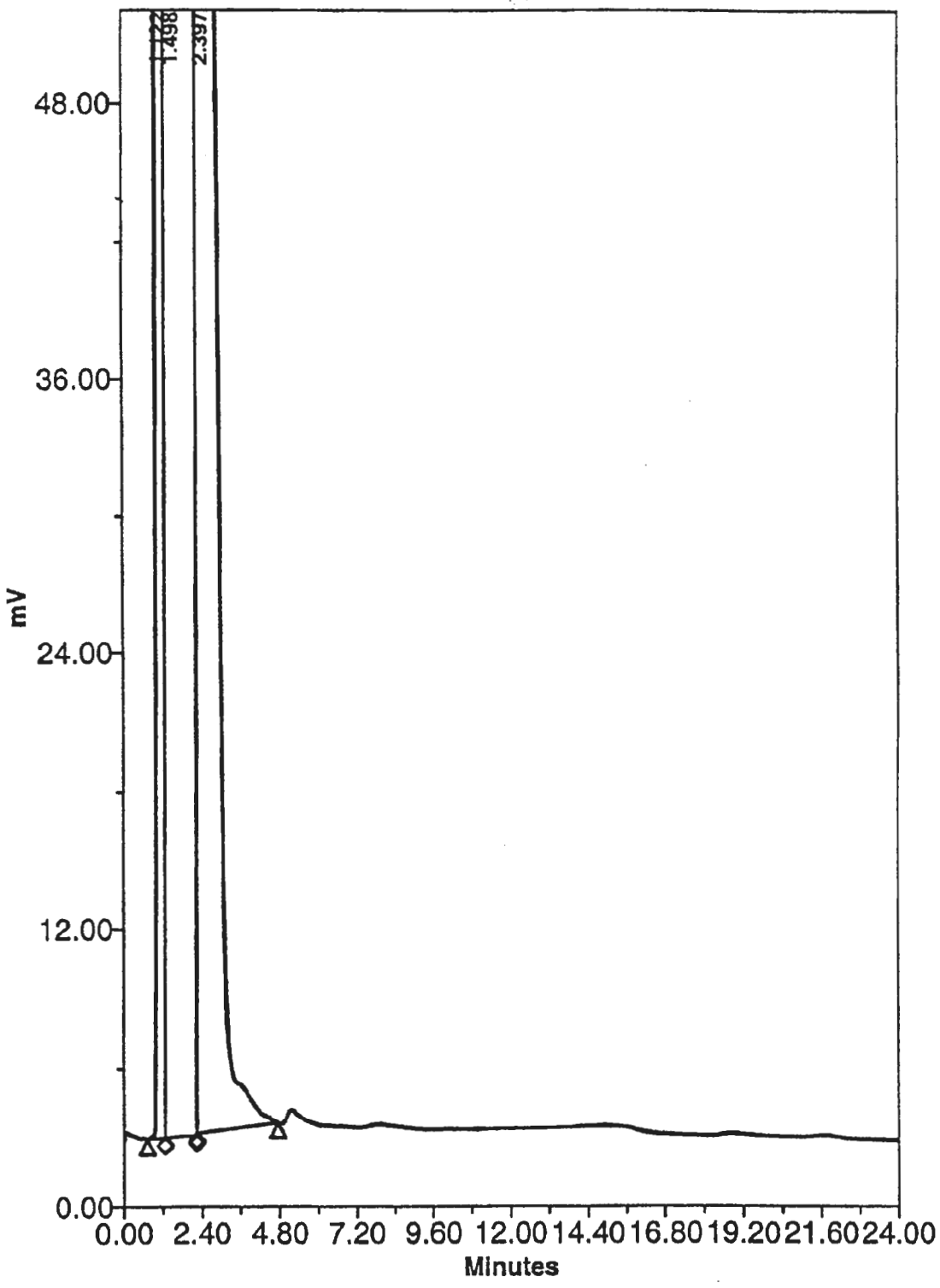

Figure 6-2. Blank microsome chromatogram with no drug added. Peaks 1.12, 1.50 and 2.40 result from microsomal generating solution.

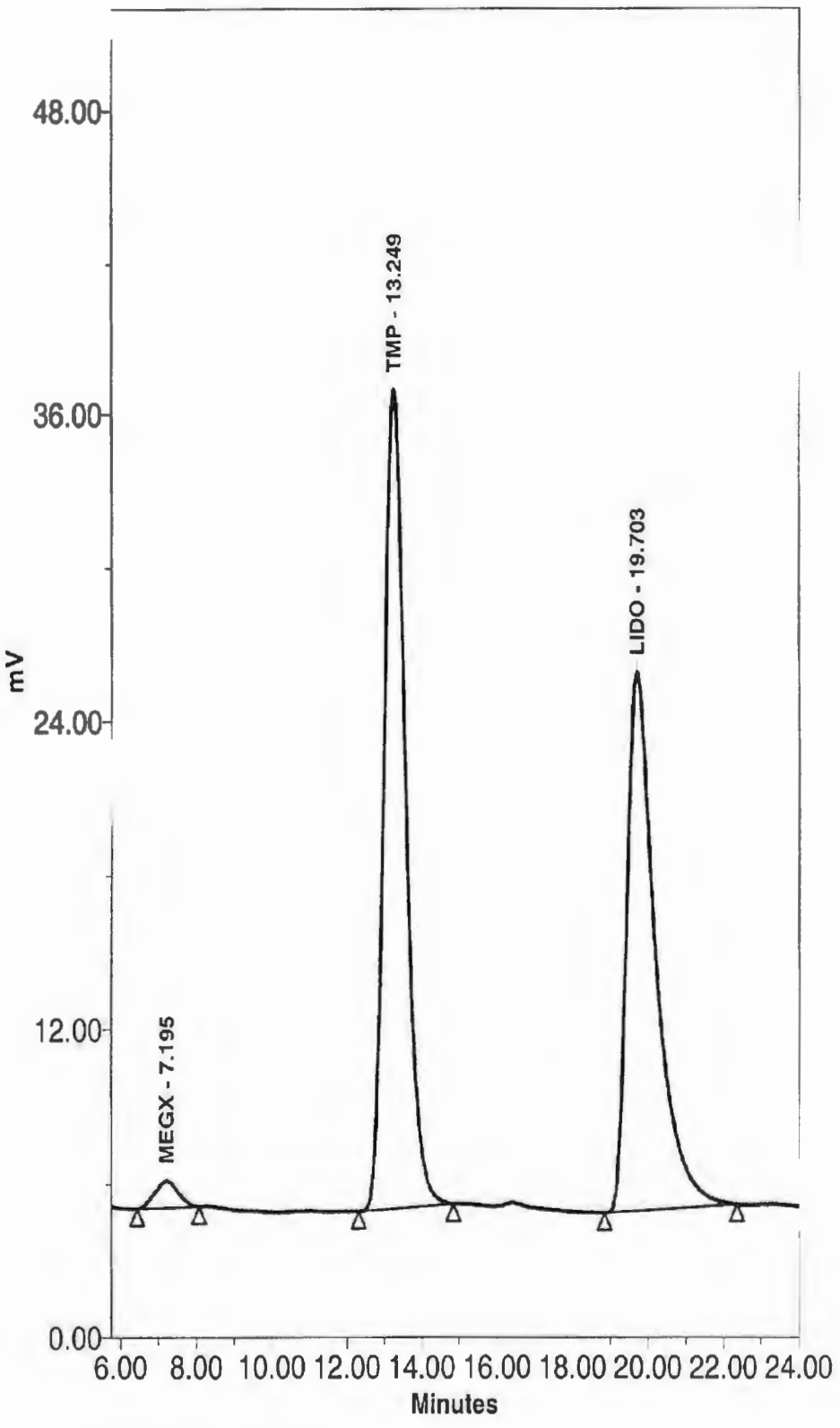

Figure 6-3. Chromatogram of a porcine microsomal sample after incubation with 10 μM of lidocaine. Peaks: MEGX; TMP (internal standard); LIDO= lidocaine.

Mean recoveries of MEGX were 93%, 98%, 99%, 95%, 98%, and 99 % for 50, 100, 250, 500, 800, and 1000 ng/ml. Mean recoveries of lidocaine were 103%, 94%, 98%, 94%, 96%, and 90% for 50, 100, 250, 500, 800, and 1000 ng/ml. The detection limit for both compounds was 10 ng/ml. This represents a peak approximately three times baseline noise. No interference from numerous drugs and chemicals used in inhibition studies was observed with the chromatographic procedure (Table 6-2).

Table 6-2. Chemicals tested for assay interference.

Chemicals
Quinidine
Furafylline
7.8-Benzoflavone
Diethyldithiocarbamate
Ketoconazole
Ciprofloxacin
Bufuralol
Chlorzoxazone
Itraconazole
Phenacetin
NADP
Potassium phosphate
Magnesium chloride
Glucose-6-phosphate
Glucose-6-phosphate dehydrogenase

Chapter 5: Discussion

The characterization of enzyme metabolism studies requires a method to be simple and accurate, sensitive and reproducible. Such an HPLC assay, utilizing UV detection, has been developed to investigate the N-deethylation conversion of lidocaine to MEGX by microsomal fractions of porcine liver. The assay is easy, sensitive, specific and reproducible, with a high recovery of MEGX.

We did not use an extraction for our samples, which resulted in very large peaks produced by the NADPH generating solution at the front of the chromatogram. We did optimize the conditions considering this problem and found that an isocratic mixture of 87% phosphate buffer (pH 5.9) and 13% acetonitrile premixed would prevent interference from the generating solution peaks. If the quantitation of lidocaine is not important for the experiment the analysis time can be reduced.

Most of the procedures in the literature do not list validation parameters such as limit of detection or recoveries for MEGX and lidocaine. However, we feel that our limit of detection and recovery for both compounds are more than adequate for microsomal studies. In cases where validation parameters are listed, ours are equal to or better than existing methods. The use of TMP as an internal standard corrects for intra- and inter-assay variability in the method.

In the determination of MEGX and lidocaine, Wang et al (1999; 2000) used a methyl tert-butyl ether extraction. Imaoka et al. (1990), Tanaka et al. (1994), Ohishi et al. (1993), Nakamoto et al. (1997) and Leclercq et al. (1997) used varying amounts of ethyl acetate which are then evaporated with vacuum or nitrogen. Kawai et al. (1985) used 10 ml of ethyl acetate followed by back extraction into sulfuric acid and neutralization with sodium hydroxide, which is evaporated under reduced pressure. Our procedure eliminates the use of time consuming liquid-liquid extractions involving toxic

and expensive organic solvents and does not require the use of nitrogen or vacuum evaporation. The method did include validation of GX and could be used for its analysis. However, GX was not detected in any of the porcine samples; therefore, its validation parameters were not included in this manuscript. It is a rugged procedure with the column still in use after over 1000 injections and the guard column replaced roughly every 300 injections.

The chromatographic method described, was developed in order to determine the metabolism of lidocaine in porcine microsome samples. The procedure has been applied by our group to metabolism studies of lidocaine in porcine microsomes. In conclusion, a simple, sensitive and useful HPLC procedure has been developed for analysis of MEGX and lidocaine in microsome samples.

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PART VII: THE EFFECTS OF CIPROFLOXACIN ON PORCINE HEPATIC CYTOCHROME P450 METABOLISM OF CHLORZOXAZONE

Chapter 1: Abstract

Fluoroquinolones are a relatively new class of antimicrobial drugs that are commonly used. They have the potential to interact with other drugs, and may alter the metabolism of both. For example fluoroquinolones administered together with methylxanthines, such as caffeine and theophylline, have resulted in inhibition of xanthine metabolism by P450 enzymes. Results of prior studies have indicated that quinolones inhibit P450 activity in companion animals and rodents. However, there is very little information available concerning the effects that quinolones have on P450 metabolism in food animals. This study was designed to determine what effect ciprofloxacin has on hepatic microsomal enzyme activity of swine with regard to the metabolism of chlorzoxazone (CZX), a drug that is used to characterize cytochrome P4502E1 enzyme activity. Part two of the study was designed to look at the effects of chemical inhibitors on CZX metabolism in swine in order to learn more about phase I biotransformation in this species.

Chapter 2: Introduction

Quinolone antibiotics are a recent addition to the antimicrobial class of drugs, and, therefore, limited information is available concerning comparative P450 enzyme activity. Specific P450 isozyme characterization is needed in food animals because of the food safety concerns of the public for their proper use (Broad et al., 1995). More information about the rate and extent of metabolism to both active and inactive metabolites is needed, in order for these drugs to continue to be used in animals. Inappropriate dosage regimens of antibiotics such as quinolones that are used in animals intended for food, may indirectly harm the consumer by favoring development of antibiotic-resistant bacteria. Improper dosing of these antibiotic agents may also result in inadvertent consumption of drug residues in edible tissues, milk or eggs.

A documented problem with fluoroquinolone use in human medicine is the interaction that occurs with these antimicrobial agents and other drugs that may alter the metabolism of both. Fluoroquinolones administered together with methylxanthines, such as caffeine and theophylline, have resulted in inhibition of xanthine metabolism by P450 enzymes (De Sarro and De Sarro, 2001, Fuhr et al., 1990, Sano et al., 1988). Among the phase I biotransforming enzymes, the cytochrome P450 system ranks first in terms of catalytic versatility and the sheer number of chemical agents it is capable of detoxifying, or activating to reactive intermediates (Parkinson, 1996; Guengerich, 1992). Results of prior studies have indicated that quinolones inhibit P450 activity in companion animals (Novotny and Shaw, 1991) and rodents (Vancutsem and Babish, 1996). However, there is very little information concerning the effects that quinolones have on P450 metabolism in food animals.

There are important species differences in the function and regulation of cytochrome P450 enzymes, as well as influence by environmental and genetic factors.

Some of these variations have been attributed, in part, to the presence of different P450 enzymes and their inducibility (Boobis et al., 1990; Smith, 1991; Guengerich, 1992,). Possible reasons for species differences are: the relative concentrations of different biotransforming enzymes; the active site of the enzyme differs amongst species; enzyme activity is catalyzed by different enzymes or isozymes (the enzyme may be in one species but completely missing in another); differences in the specificity of the isozyme; and differences in the biotransforming pathways competing for the substrate (Parkinson, 1996; Boobis et al., 1990 Mellett, 1969). Any one or combination of these reasons can have a tremendous impact on the way a chemical is metabolized by a particular species. A drug that is effectively used in one species could be useless in another because it is not metabolized efficiently. Likewise, a therapeutic dose in one species could prove to be lethal in another due to differences in metabolism.

The P450 enzyme system is mainly responsible for phase I metabolism, which adds or exposes polar functional groups on a lipophilic substrate. In the investigation of P450 mediated xenobiotic metabolism, individual forms of P450 enzymes have been found to catalyze specific reactions with specific substrates. Thus characterizing these specific enzyme activities with these substrates allows their use as probes for the P450 enzymes. To date, at least one marker activity exists for the majority of human P450 enzyme forms (Sharer et al., 1995). This study was designed to determine what effect ciprofloxacin has on hepatic microsomal enzyme activity of swine, with regard to the metabolism of chlorzoxazone (CZX). Chlorzoxazone is used to characterize human cytochrome P4502E1 enzyme activity. As part of this study, the effects of inhibitor probes on CZX metabolism in swine were examined to learn more about phase I biotransformation in this species.

Chapter 3: Materials and Methods

Chemicals and Reagents

Ketoconazole, 7, 8-benzoflavone, quinidine, phenacetin, diethyldithiocarbamic acid (DDC), chlorzoxazone, glucose-6-phosphate (G6P), β-nicotinamide adenine dinucleotide phosphate (NADP), and glucose-6-phosphate dehydrogenase (G6PDH) were purchased from Sigma Chemical (St. Louis, MO). Other chemicals were obtained from the following sources: furafylline from Gentest Corp. (Woburn, MA); ciprofloxacin from US Pharmacia (Rockville, MD); and hydroxychlorzoxazone from Research Biochemicals Incorporated (St. Louis, MO). All other high analytical grade reagents and solvents were purchased from Fisher Scientific (Fair Lawn, NJ).

Porcine Liver Microsomes

Four pigs (3 female and 1 castrated male) were used for the study. The pigs weighed 29 to 38 kg. and were fed standard pig chow. The animals had no known prior exposure to any drugs. Microsomes were prepared using an ultracentrifugation technique described by Lake (1987). The washed microsomes were resuspended in 10 mM Tris-acetate buffer (pH 7.4) containing 1 mM EDTA, and 20% sucrose, and were frozen at -80°C until used. Protein concentrations were determined using a Pierce reagent kit (Rockford, IL) and P450 content was determined according to the method of Omura and Sato (1964).

Enzyme Assays

Incubation mixtures contained 0.5 mg of microsomal protein, 100 mM phosphate buffer at pH 7.4 containing 6 mM magnesium chloride, 1 mM EDTA, and a NADPH-generating system (1 mM NADP, 10 mM G6P and 0.7 U of G6PDH) in a total volume of 0.5 ml. Incubation mixtures contained substrate and inhibitors and the reactions were initiated by addition of the NADPH-generating system after a 5 min pre-incubation step

at 37°C. Furafylline and DDC were pre-incubated for 15 min at 37°C with the generating solution and microsomes, followed by initiation of reactions by the addition of substrate. These inhibitors are mechanism-based and require NADPH-dependent complexation for activation. The substrate stock solutions were prepared in methanol, and the methanol content did not exceed 1% when added to the incubations. The substrate concentrations were 10, 100, 250, and 500 μM, while inhibitor concentrations were 1, 10, and 100 μM. All inhibitors were dissolved in methanol except DDC and ciprofloxacin, which were dissolved in water and 0.067M potassium dihydrogen phosphate (pH 3). Reactions were quenched with 0.1 ml of ice cold acetonitrile after 20 min in a 37°C shaking water bath, and then placed on ice for 1 hour. Microsome samples were centrifuged at 16,000 g for 15 min, after which the supernatants were removed and stored at -80°C until the analyses could be performed.

Enzyme inhibitors were also incubated without substrate in the assays under the same conditions, to ensure that their presence did not interfere with the quantitation of the respective metabolites in the assay.

HPLC System

The HPLC system consisted of a 626 pump, a 717 plus autosampler, a 996 scanning UV detector and a computer equipped with Millennium software (Waters, Milford, MA). The samples were analyzed using a method developed in our laboratory. Briefly, previously frozen samples were thawed on ice and vortexed. Thirty microliters of phenacetin (100 μg/ml) was added to a 0.5 ml sample and 190μl was injected onto the liquid chromatograph. Chromatography was conducted for the measurement of chlorzoxazone 6-hydroxylation on a YMC-Pack ODS-AQ column (5 μm, 6 x 150 mm) equipped with a C_{18} Guard-Pak precolumn insert (Waters, Milford, MA). The mobile

phase consisted of a mixture of (A) 0.05% phosphoric acid pH 3.0 and (B) methanol. The mixture was pumped as a gradient starting at 60% A and 40% B and maintained for 12 min; then over a 2 min period, the mixture was changed to 58% A and 42% B which was maintained for 5 min. Then over a 3 min period the system was returned to initial conditions. UV detection was measured at 287 nm. Calibration curves were constructed using the pure metabolite and phenacetin as the internal standard.

Data Analysis

Determination of apparent Ki values was carried out using Graphpad Prism (Graphpad Software, Inc., San Diego CA). Formation rates from the experiments with inhibitors were evaluated by using Dixon plots ([I] vs. 1/V).

Chapter 4: Results

The effect of the specific P450 inhibitors and ciprofloxacin on the metabolism of CZX can be seen in Figure 7-1 thru 7-8. The catalytic activities of controls (absence of inhibitor) were considered 100%.

7, 8-Benzoflavone, which is an inhibitor of P4501A2 activity, had no inhibitory effect on CZX 6-hydroxylase activity (Figure 7-1), even at the highest concentration (100μM). In contrast, the 10 and 100 μM 7,8-benzoflavone concentrations caused activation of the 2E1 mediated activity at lower substrate concentrations. Similar results were found for quinidine (P4502D6) (Figure 7-2). Both the 10 and 100 μM concentrations of quinidine increased CZX metabolism at several concentrations. At the highest concentration of quinidine, there was a small degree of inhibition (<25%) at 100 μM substrate concentration. Ketoconazole (P4503A4) had mixed results as illustrated in Figure 7-3. The 1 and 10 μM ketoconazole concentrations had very little effect on CZX metabolism except at the 10 μM concentration, where inhibition was 27% and 35%, respectively. The 100 μM ketoconazole concentration produced some inhibition at all CZX concentrations with its greatest effect seen at 10 μM (51%).

Ciprofloxacin had partial inhibitory effects on CZX metabolism (Figure 7-4). The greatest inhibition occurred at the 1 and 10 μM concentrations (44% to 35%) on the 100 – 500 μM CZX concentrations. The 100 μM ciprofloxacin concentration produced inhibition from 25% to 36% for the 100 - 500 μM range. In contrast, this same ciprofloxacin concentration caused stimulation of CZX 6-hydroxylation at the 10 μM level.

The 100 μM concentration of DDC, which is a specific inhibitor of P4502E1, (Figure 7-5) caused 83%, 91%, 93%, and 87% inhibition in CZX 6-hydroxylase activity

for the concentrations 10 to 500 μM. The 1 μM DDC concentration produced over a 50% reduction in activity at all the substrate concentrations while the 10 μM concentration reduced activity by 60% or more. The type of intersection of the regression lines observed in the Dixon plot (Figure 7-6) is consistent with mixed non-competitive inhibition. The apparent Ki value was 2 μM.

Furafylline (Figure 7-7), a specific mechanism-based inhibitor of P4501A2, caused 56% to 68% inhibition of activity at its lowest concentration (1μM). Similar results were obtained with the 10 and 100 μM furafylline concentrations, with suppression of activity ranging from 53% to 70%. The regression lines in the Dixon plot (Figure 7-8) are consistent with non-competitive inhibition. The lines are parallel with no intersection, so Ki is irrelevant.

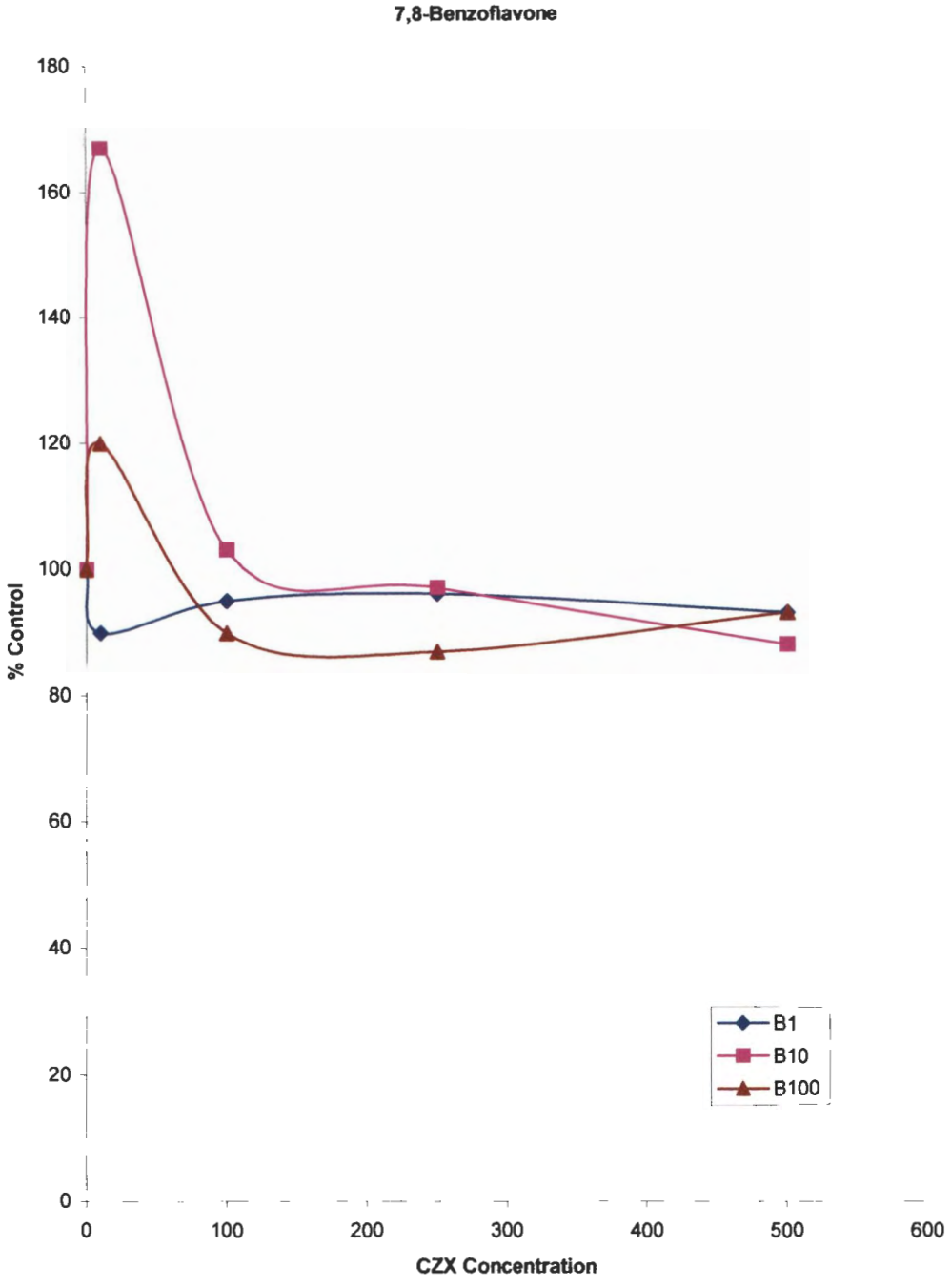

Figure 7-1. Effects of 7,8-benzoflavone on chlorzoxazone 6-hydroxylation.

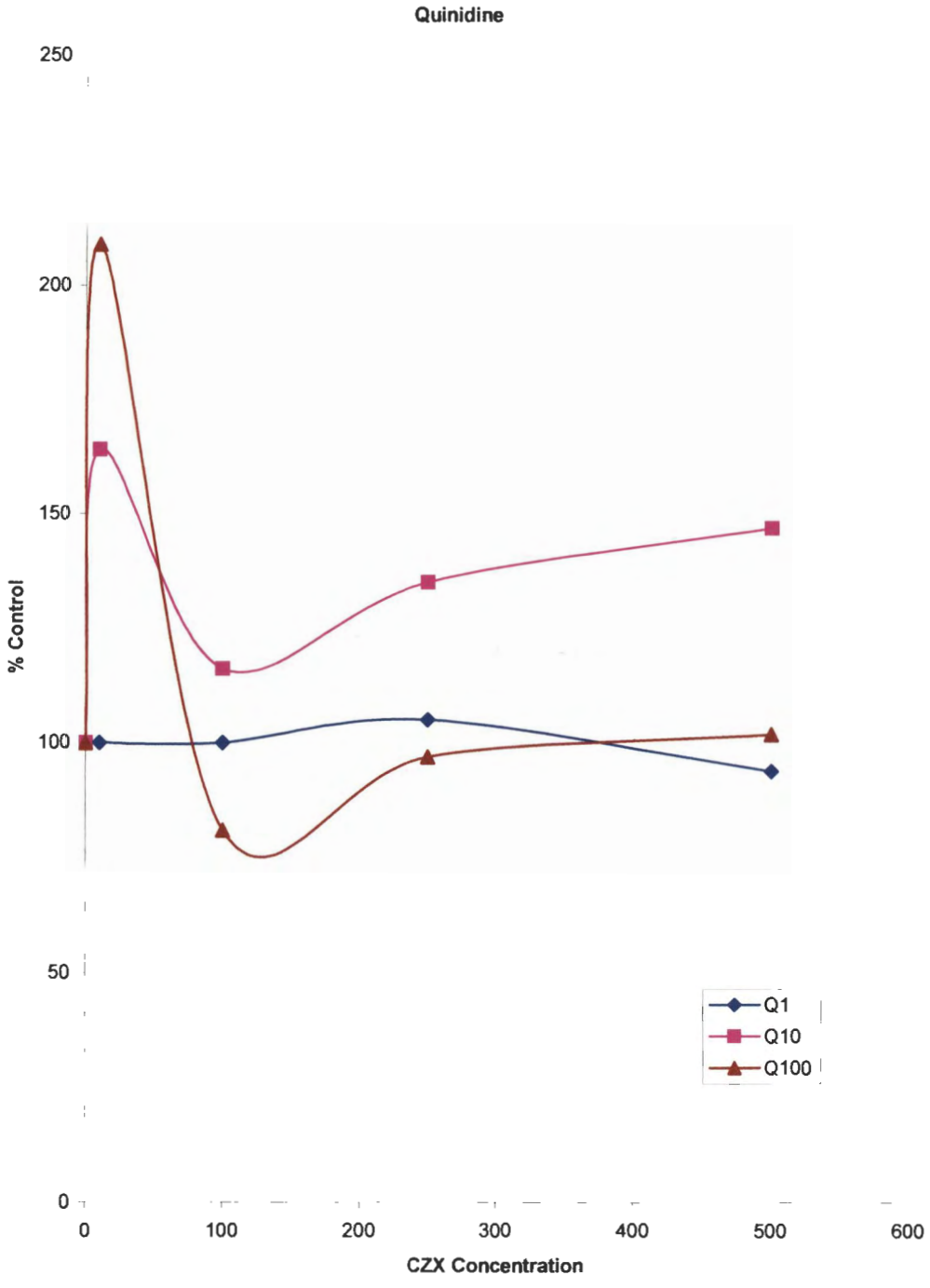

Figure 7-2. Effects of quinidine on chlorzoxazone 6-hydroxylation.

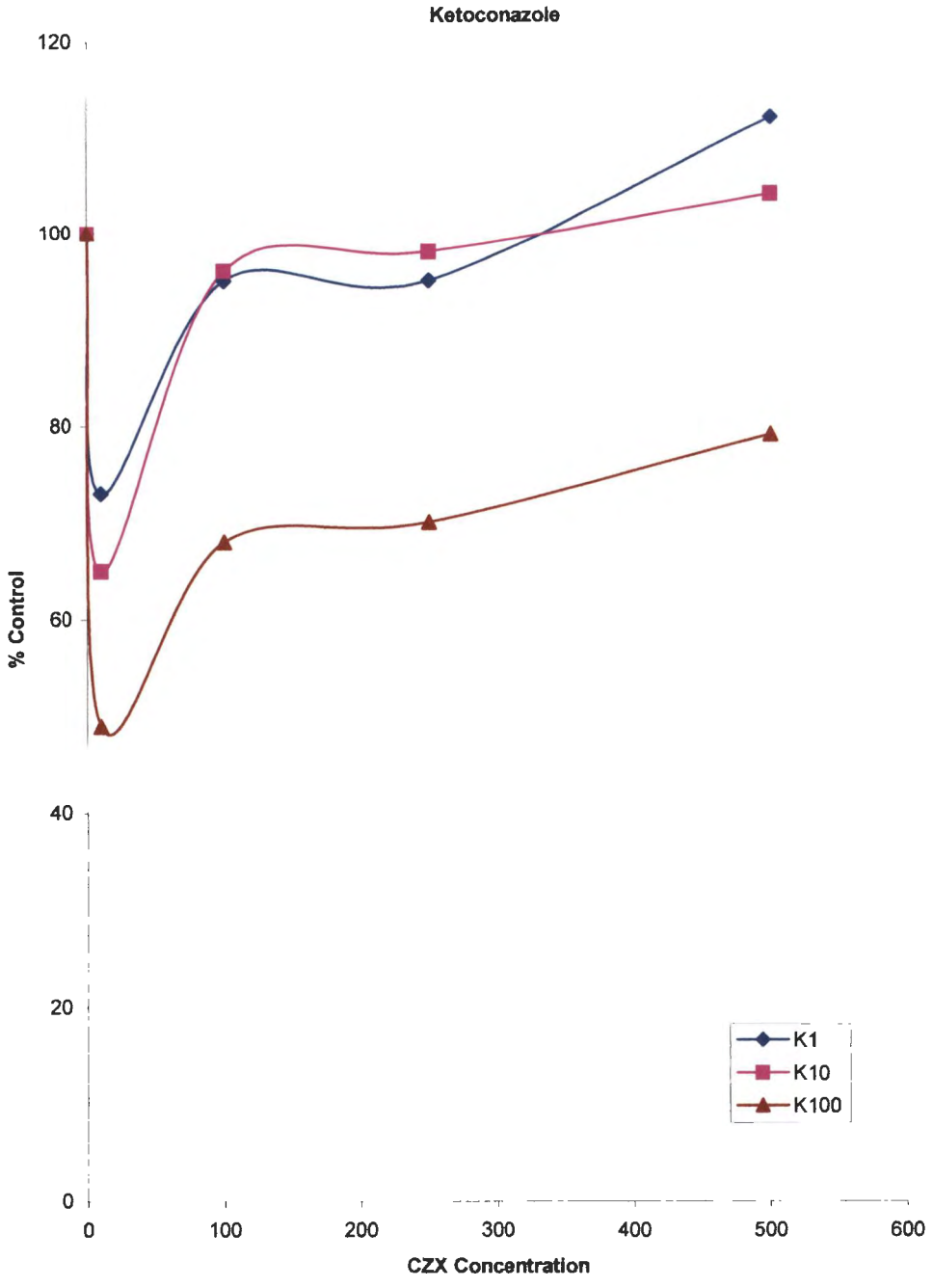

Figure 7-3. Effects of ketoconazole on chlorzoxazone 6-hydroxylation.

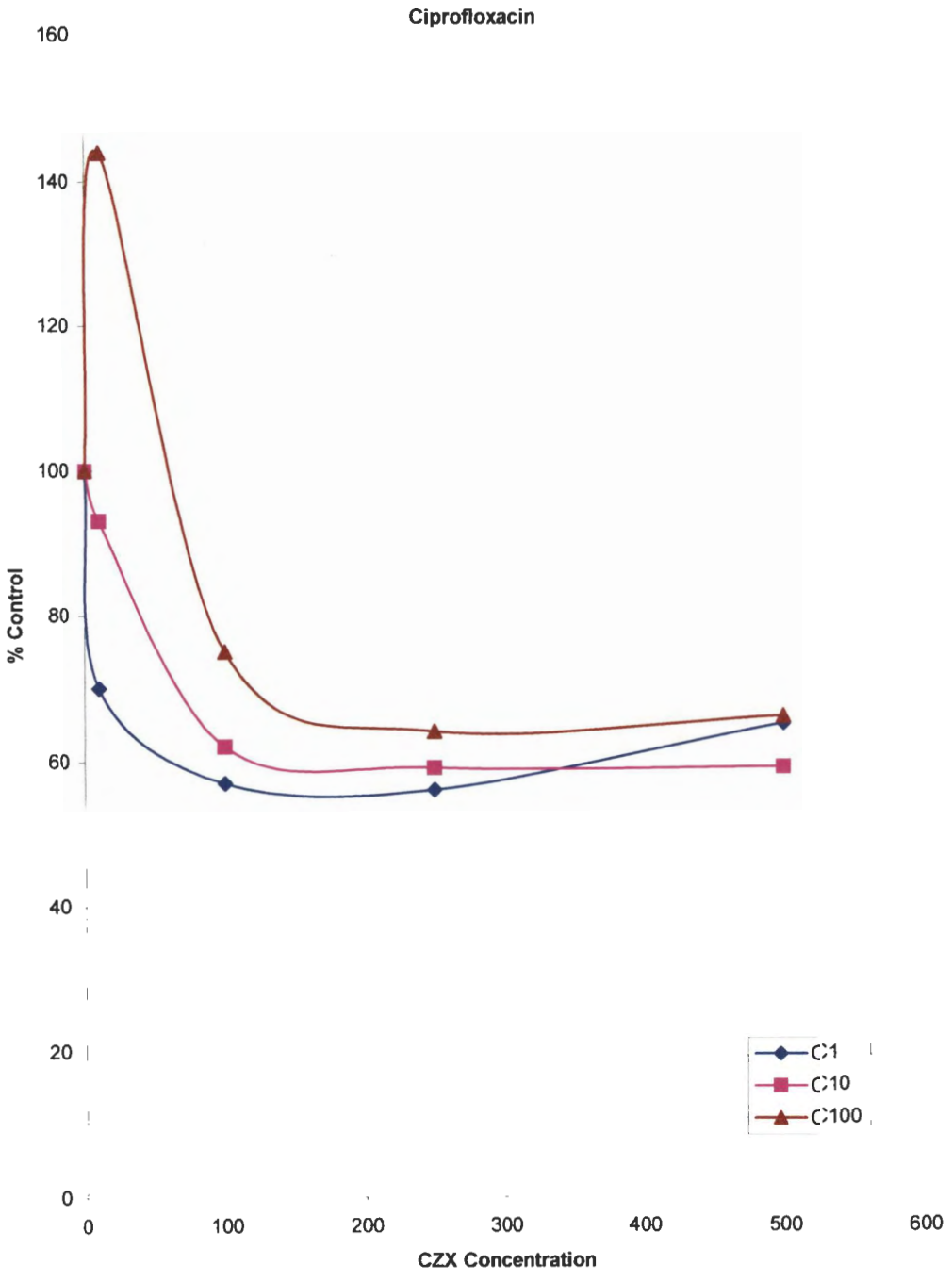

Figure 7-4. Effects of ciprofloxacin on chlorzoxazone 6-hydroxylation.

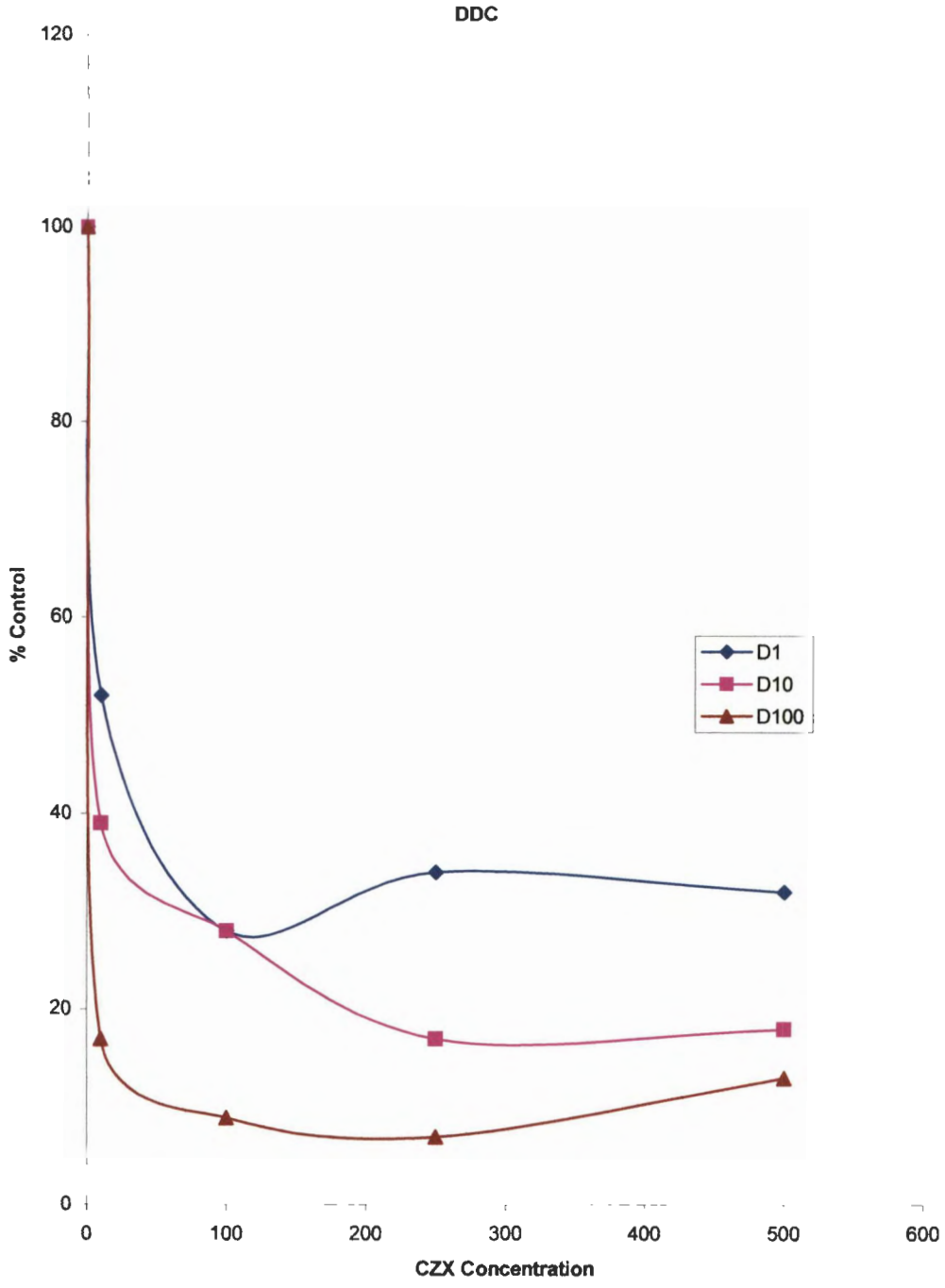

Figure 7-5. Effects of DDC on chlorzoxazone 6-hydroxylation.

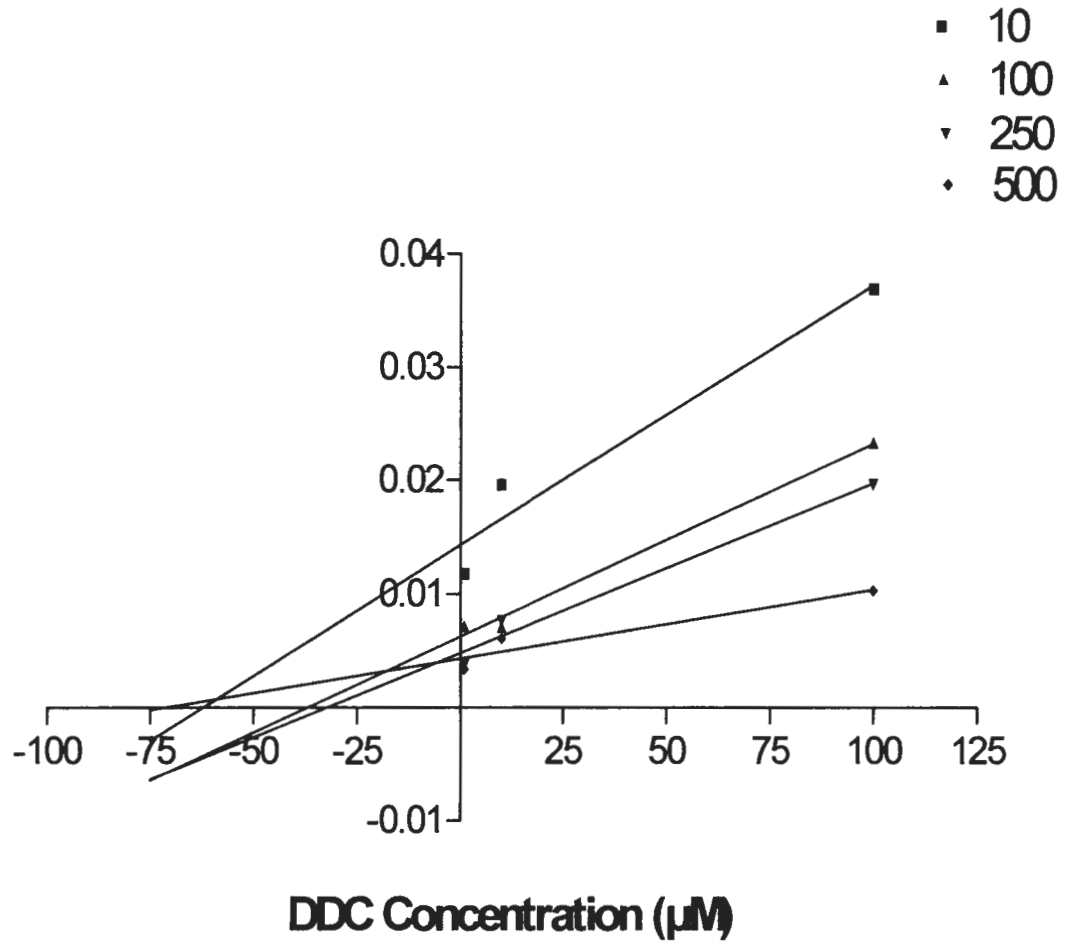

Figure 7-6. Dixon plot of the effects of DDC on chlorzoxazone 6-hydroxylation.

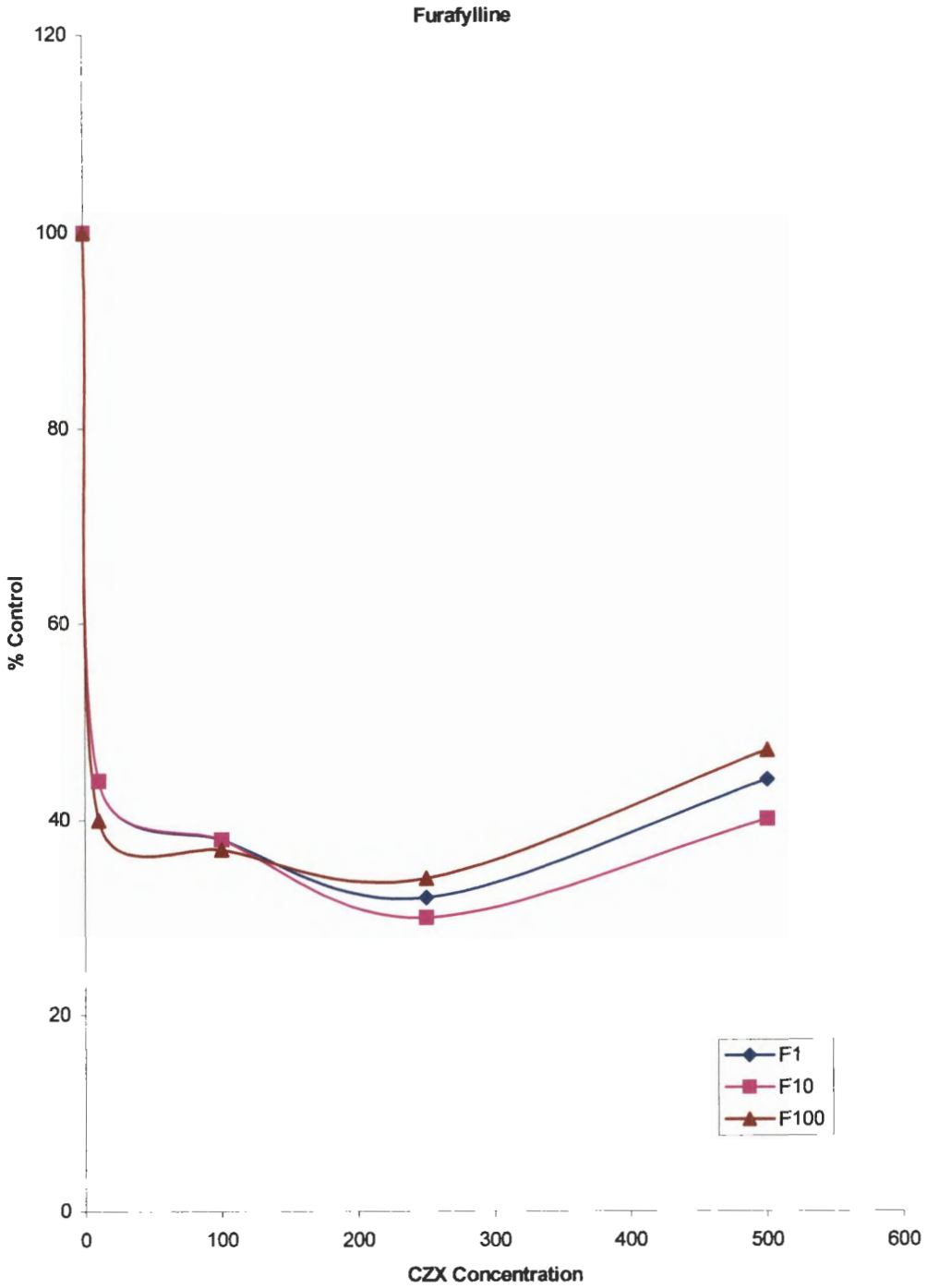

Figure 7-7. Effects of furafylline on chlorzoxazone 6-hydroxylation.

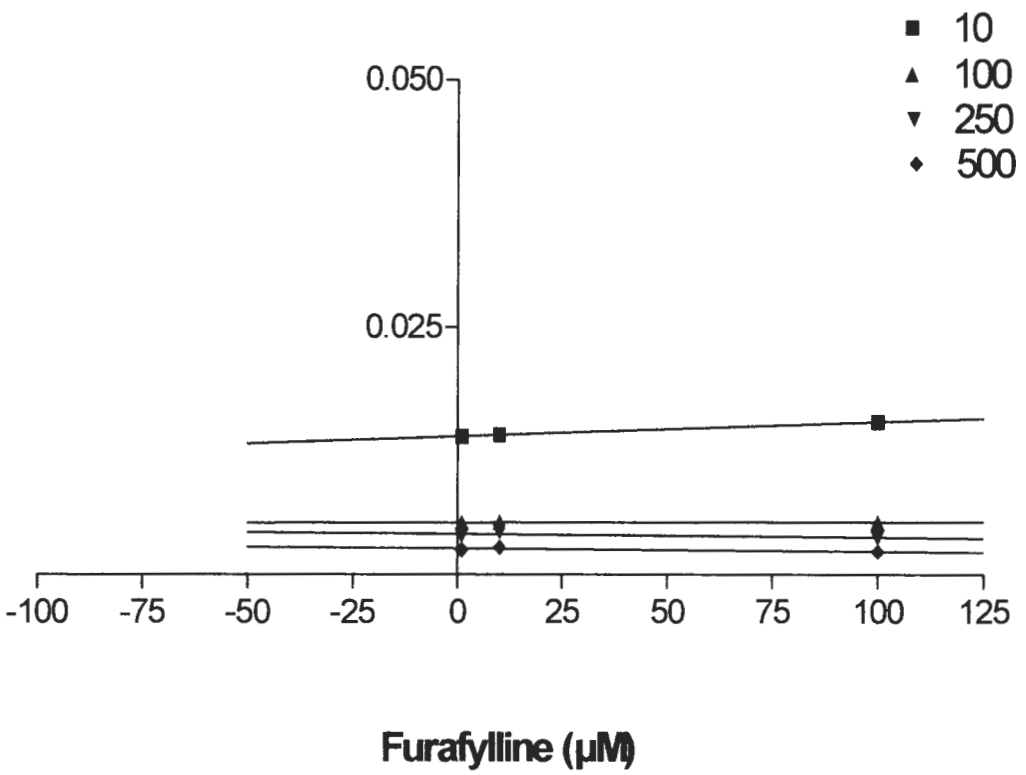

Figure 7-8. Dixon plot of the effects of furafylline on chlorzoxazone 6-hydroxylation.

Chapter 5: Discussion

The effects of ciprofloxacin on the metabolic activity of P4502E1 were evaluated in this study. As part of this process the effects that chemical inhibitors had on CZX metabolism in porcine liver microsomes were evaluated. Experiments were conducted using a wide range of concentrations for both substrate and inhibitor to give more definitive results. It should be pointed out that CZX and the inhibitors are specific for human P450 enzymes. Therefore, in animals, effective inhibition by the specific inhibitor probe will occur only if an enzyme homologous to the human enzyme is responsible for the activity.

7,8-Benzoflavone and quinidine had very little inhibitory effect on CZX 6-hydroxylase activity (<13%), and at some concentrations caused activation. These results are in agreement with studies conducted in human beings (Newton et al., 1995) and pigs (Diaz and Squires, 2000). Newton et al. (1995) reported that 7,8-benzoflavone produced a 2-fold increase in CZX metabolism in human microsomes. Diaz and Squires (2000) found an increase in activity when quinidine and α-naphthoflavone were used as inhibitors.

The selectivity of ketoconazole as an inhibitor of P4503A4 in human liver microsomes (Newton et al., 1995) and P4503A12 in canine liver microsomes (Kuroha et al., 2002) has been indicated. Newton et al. (1995) found that ketoconazole completely abolished testosterone β-hydroxylase activity (P4503A4 substrate) at concentrations greater than 5 μM. Kuroha et al. (2002) found that the administration of a therapeutic dose of ketoconazole may change the pharmacokinetics of other P4503A12 substrates in canines. Ketoconazole has also been implicated in the inhibition of the P4502C-mediated family (Back et al., 1988), and is thought to be an inhibitor of a broad spectrum of P450 isozymes (Ward and Back 1993). The results indicate that ketoconazole

produces only a slight inhibition at its highest concentration. This reduction in activity could be due to the fact that ketoconazole inhibits a broad spectrum of P450 enzymes or it could be due to non-specific inhibition observed at high concentrations of a specific inhibitor. The other two ketoconazole concentrations produced very little inhibition of activity, which suggests that the inhibition produced by the larger concentration is non-specific.

Ciprofloxacin produced some inhibition of P4502E1 enzyme activity in porcine microsomes. However, in both humans and rodents, quinolones are thought to exert their effects on the P4501A family (Vancutsem and Babish, 1996). It has been reported (Monshouwer et al. 1996) that porcine liver microsomes have P4501A1, 1A2 and 3A4 activities. Babol et al. (1998) found that CZX hydroxylation could be catalyzed by both P4501A1 and 3A in swine. In another study, the 6-hydroxylation of CZX in porcine liver microsomes required a two-enzyme model to describe the kinetic data (Court et al., 1997). Both high and low affinity activities were discerned indicating that two enzymes were involved in the metabolism of CZX. Court's et al (1997) study suggests that P4502E1 could be involved in CZX metabolism but does not identify the other enzyme involved. Similar results have also been documented for the oxidative metabolism of halothane by P4502E1 in humans (Spracklin et al., 1997). A high affinity/low capacity isoform and a low affinity/high capacity isoform were found to be involved. The fact that more than one enzyme seems to be involved in CZX metabolism and if that enzyme were to belong to P4501A family might explain why ciprofloxacin had some inhibitory effect on CZX hydroxylation. This may also provide an explanation of why furafylline, a mechanism-based inhibitor of P4501A2 produced an inhibitory effect on CZX hydroxylation. All three inhibitor concentrations produced a similar reduction of enzyme

activity. The type of inhibition furafylline exhibited was non-competitive, indicating that it binds to the enzyme substrate complex rather than to the free enzyme form.

The specific P4502E1 inhibitor DDC produced the greatest reduction of CZX hydroxylation activity of all the inhibitors used. The type of inhibition was non-competitive, which is similar to the results reported by Court et al. (1997). The suppression of activity by DDC observed in these experiments indicates that an enzyme similar to human P4502E1 could play a role in the CZX 6-hydroxylase activity in swine. However, other enzymes are probably involved as evidenced by the inhibition produced by furafylline and ciprofloxacin.

In conclusion, the results of this study suggest that a P450 enzyme in porcine liver microsomes belonging to the P4502E family is involved in CZX 6-hydroxylation. However, both ciprofloxacin and furafylline produced inhibitory effects; therefore, other enzymes are probably involved, most likely from the P450 1A family. It is clear that these inhibitors do not exhibit the same selectivity in porcine microsomal studies as human studies. This may be due to the differential selectivity of the inhibitors or due to the involvement of completely different P450 enzymes. Therefore, caution should be used when drugs metabolized by these enzymes are administered concomitantly with quinolones.

There is a limited amount of data available on hepatic cytochrome P450 enzymes in domestic animals, including substrate and inhibition specificities. With this in mind, these results should be used as guidelines for future studies. Since multiple enzymes appear to be involved in the biotransformation of CZX, and relatively minor differences exist between species in the amino acid sequence of P4502E1 substrate preference could be drastically effected. New drugs developed for domestic animals should be tested on the target species since the metabolism may be markedly different

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PART VIII: CIPROFLOXACIN'S EFFECTS ON PORCINE HEPATIC CYTOCHROME P450 BIOTRANSFORMATION OF BUFURALOL

Chapter 1: Abstract

Inappropriate dosages of antibiotics used in animals intended for food may indirectly harm the consumer by favoring development of antibiotic-resistant bacteria. This has caused public concern about food safety. Fluoroquinolones are a relatively newer class of antimicrobial drugs used in food animals and other species including human beings. Work has been done evaluating hepatic P450 enzyme metabolism of quinolones in several species. However, little has been done to characterize specific P450 isozymes in food animals. There are indications that quinolones inhibit P450 enzyme activity in companion animals and rats. However, there is very little information concerning the effects quinolones have on P450 metabolism in food animals. This study was designed to determine what effect ciprofloxacin has on the metabolism of bufuralol in porcine hepatic microsomes. Bufuralol is a drug that is used to characterize human cytochrome P4502D6 enzyme activity. The effects of chemical inhibitors on bufuralol metabolism in swine were also studied to learn more about phase I biotransformation in this species.

Chapter 2: Introduction

Fluoroquinolones were introduced into clinical medicine roughly 14 years ago. Because the fluoroquinolones are relatively new antimicrobial agents, limited information is available concerning P450 enzyme metabolic activity in domestic animals. Although use of quinolones may be limited due to food safety concerns of the public, specific P450 enzyme characterization is needed for their proper use in food animals (Broad et al., 1995). Inappropriate dosage regimens of antibiotics that are used in animals intended for food, may indirectly harm the consumer by favoring development of antibiotic-resistant bacteria. Improper dosing of these antibiotic agents may also result in inadvertent consumption of drug residues in edible tissues, milk or eggs.

A documented problem with fluoroquinolones used in human medicine is the interaction that occurs with these antimicrobials and other drugs that may alter the metabolism of both. Fluoroquinolones administered together with methylxanthines, such as caffeine and theophylline, have resulted in inhibition of xanthine biotransformation by P450 enzymes (De Sarro and De Sarro, 2001, Fuhr et al., 1990, Sano et al., 1988). Cytochrome P450 constitutes a superfamily of hemoproteins that play a pivotal role in the metabolism of a wide variety of xenobiotics and endogenous compounds. The system is responsible for converting a number of pro-drugs to their pharmacologically active metabolites. They are also responsible for a number of detrimental metabolic conversions such as rendering certain chemicals mutagenic or carcinogenic, and producing toxic metabolites (Parkinson, 1996; Guengerich, 1992). Previous studies have indicated that quinolones inhibit P450 activity in companion animals (Novotny and Shaw, 1991) and rodents (Vancutsem and Babish, 1996). However, there is very little information concerning the effects that quinolones have on P450 metabolism in food animals.

There are important species differences in the function and regulation of cytochrome P450 enzymes, as well as influence by environmental and genetic factors. Some of these species variations have been attributed in part, to the presence of different P450 enzymes and their inducibility (Guengerich, 1992; Smith, 1991; Boobis et al., 1990). Possible reasons for species differences in cytochrome P450 activity are: the relative concentrations of different biotransforming enzymes; the active site of the enzyme differs amongst species; the P450 activity is catalyzed by different enzymes or isozymes (the enzyme may be in one species but completely missing in another); differences in the specificity of the isozyme; and differences in the biotransformation pathways competing for the substrate (Parkinson, 1996; Boobis et al., 1990; Mellett, 1969). Any one or combination of these reasons can have a tremendous impact on the way a drug or xenobiotic is metabolized by a particular species. A drug that is effectively used in one species could be useless in another because it is not metabolized efficiently. Likewise, a therapeutic dose in one species could prove to be lethal in another due to differences in metabolism.

The P450 enzyme system is mainly responsible for phase I metabolism, which adds or exposes polar functional groups on a lipophilic substrate. In the investigation of P450 mediated xenobiotic metabolism, individual forms of P450 enzymes have been found to catalyze specific reactions with specific substrates. Characterizing these specific enzyme activities with these substrates allows their use as probes for P450 enzymes. To date, at least one marker activity exists for the majority of human P450 enzymes (Sharer et al., 1995). P4502D6 is an important human isoform and it is recognized to be involved in the metabolism of over 30 drugs (Jurima-Romet et al., 2000). P4502D6 is a genetically polymorphic enzyme, and many substrates predominantly metabolized by it are not well metabolized by other P450 isoforms. An

animal model for P4502D6 has been sought with little success. Neither rat nor mouse forms of P4502D are similar to the human enzyme in terms of enzyme characterization or at the primary sequence level (Jurima-Romet et al., 2000)

This study was designed to determine what effect ciprofloxacin has on the metabolism of bufuralol in porcine hepatic microsomes. Bufuralol is used to characterize human cytochrome P4502D6 enzyme activity, As part of this study, the effects of other inhibitor probes on bufuralol metabolism in swine were examined to learn more about phase I biotransformation in this species. Experiments were conducted using a wide range of concentrations for both substrate and inhibitors to give more conclusive results.

Chapter 3: Materials and Methods

Chemicals and Reagents

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Porcine Liver Microsomes

Four pigs (3 female and 1 castrated male) were used and weighed 29 to 38 kg. The animals were fed standard pig chow and had no known prior exposure to drugs. Hepatic microsomes were prepared using an ultracentrifugation technique described by Lake (1987). The washed microsomes were resuspended in 10 mM Tris-acetate buffer (pH 7.4) containing 1 mM EDTA, and 20% sucrose, and were then frozen at -80°C until used. Protein concentrations were determined using a Pierce reagent kit (Rockford, IL), and P450 content was determined according to the method of Omura and Sato (1964).

Enzyme Assays

Incubations contained 0.5 mg of microsomal protein, 100 mM phosphate buffer at pH 7.4 containing 6 mM magnesium chloride, 1 mM EDTA and a NADPH-generating system (1 mM NADP, 10 mM G6P and 0.7 U of G6PDH) in a total volume of 0.5 ml. Incubation mixtures contained substrate and inhibitors, and the reactions were initiated by the addition of the NADPH-generating system after a 5 min pre-incubation step at 37°C. Furafylline and DDC were preincubated for 15 min at 37°C with the generating

solution and microsomes, with reactions initiated by the addition of substrate. These inhibitors are mechanism-based and require NADPH-dependent complexation for activation. The substrate stock solutions were prepared in water and were 10, 50, and 100 μM, while inhibitor concentrations were 1, 10, and 100 μM. All inhibitors were dissolved in methanol except DDC and ciprofloxacin, which were dissolved in water and 0.067M potassium dihydrogen phosphate (pH 3). The methanol was evaporated before the start of the experiments. Enzyme reactions were quenched with 0.1 ml of ice cold acetonitrile after 10 min in a 37°C shaking water bath, and then placed on ice for 1 hour. Microsome samples were centrifuged at 16,000 g for 15 min after which the supernatants were removed and stored at -80°C until the analyses could be performed.

The specific enzyme inhibitors were also incubated without substrate in the assays under the same conditions, to ensure that their presence did not interfere with the quantitation of the respective metabolites.

HPLC System

The HPLC system consisted of a 626 pump, a 717 plus autosampler, a 470 fluorescence detector and a computer equipped with Millennium software (Waters, Milford, MA). The samples were analyzed by a method developed in our laboratory. Briefly, previously frozen samples were thawed on ice and vortexed. One hundred microliters of chloropropamide (300 μg/ml) was added to 0.5 ml sample and 125μl was injected onto the liquid chromatograph. Chromatography was conducted for the measurement of bufuralol 1'-hydroxylation on a Resolve C_{18} column (5 μm, 3.9 x 150 mm) equipped with a C_{18} Guard-Pak precolumn insert (Waters, Milford, MA). The mobile phase was sodium perchlorate:acetonitrile (48:52) pH 2.45 (adjusted with perchloric acid) at a flow rate of 1 ml/min. Fluorescence detection occurred at an

excitation 252 nm and emission 302 nm. All chromatograms were obtained with the following fluorometric conditions: gain 10X, attenuation 1, and filter time constant 0.5 s. Calibration curves were constructed using the pure metabolite 1'-hydroxybufuralol and chloropropamide as the internal standard.

Data Analysis

Determination of apparent Ki values was carried out using Graphpad Prism (Graphpad Software, Inc., San Diego CA). Formation rates from the experiments with inhibitors were evaluated by using Dixon plots ([I] vs. 1/V).

Chapter 4: Results

Specific chemical P450 inhibitors and ciprofloxacin were tested for their effects on bufuralol 1'-hydroxylation, which are illustrated in Figure 8-1 thru 8-9. The catalytic activities of controls (absence of inhibitor) were considered 100%.

Furafylline, which is a mechanism-based inhibitor of P4501A2, had very little inhibitory effect on bufuralol 1'-hydroxylation (Figure 8-1). In fact, furafylline and 7,8-benzoflavone, which is another probe for P4501A2 activity, (Figure 8-2) both produced a stimulatory effect. The greatest activation of hydroxylation activity occurred at the 10 and 50 μM bufuralol concentrations, and was produced by the 1 and 10 μM furafylline and the 1 and 100 μM 7,8-benzoflavone concentrations.

Quinidine, the specific P4502D6 inhibitor, had little effect on bufuralol metabolism (<12%) at any concentration (Figure 8-3). The inhibitor did however, produce an increase in activity at 10 and 50 μM bufuralol concentrations.

Ciprofloxacin produced mixed results as illustrated in Figure 8-4. The 1 μM inhibitor had very little effect on bufuralol 1'-hydroxylation except at the 100 μM substrate concentration (36%). The 10 μM ciprofloxacin concentration produced inhibition from 24% to 37%, while the highest concentration tested (100 μM) inhibited activity from 43% to 53%. The type of intersection of the regression lines observed in the Dixon plot (Figure 8-5) is characteristic of a mixed (predominantly competitive) inhibition. The apparent Ki was 161 μM.

As illustrated in Figure 8-6, DDC, a P4502E1 inhibitor, had some inhibitory effects at all 3 concentrations used (1, 10 and 100 μM). The greatest effect was seen with the 100 μM concentration, which produced a 22% to 53% reduction in bufuralol metabolism over the substrate concentrations used. The Dixon plot (Figure 8-7) reveals

that it is a non-competitive type of inhibition. Since the lines are parallel with no intersection the Ki is irrelevant to this type of inhibitor.

Ketoconazole, a P4503A4 inhibitor, caused some inhibition at all 3 concentrations used (Figure 8-8). The 10 μM concentration produced a range of 8% to 49% inhibition, while the 100 μM caused a 32% to 53% reduction in activity. The Dixon plot (Figure 8-9) is indicative of a mixed non-competitive type of inhibition with an apparent Ki of 265 μM.

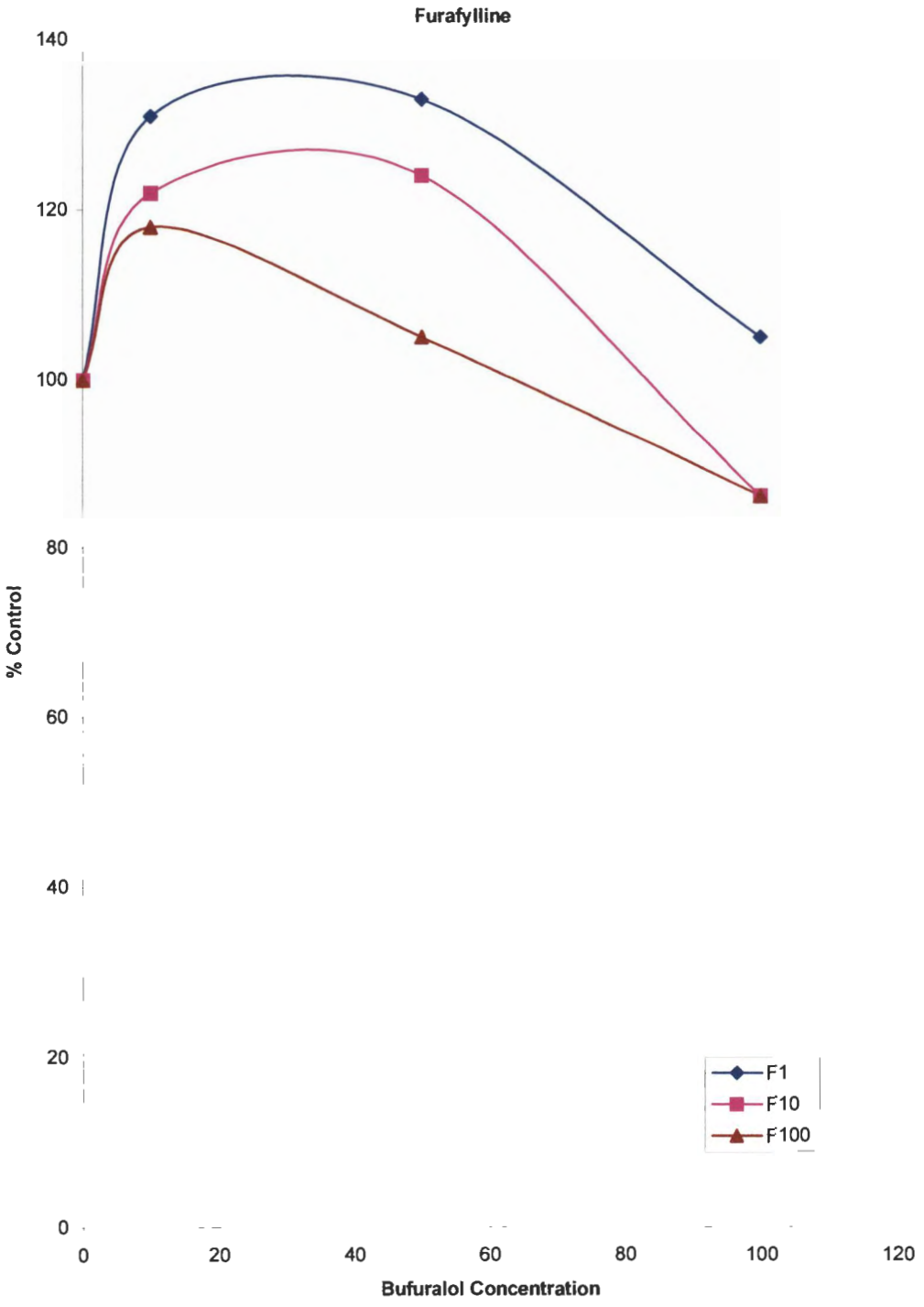

Figure 8-1. Effects of furafylline on bufuralol 1'-hydroxylation.

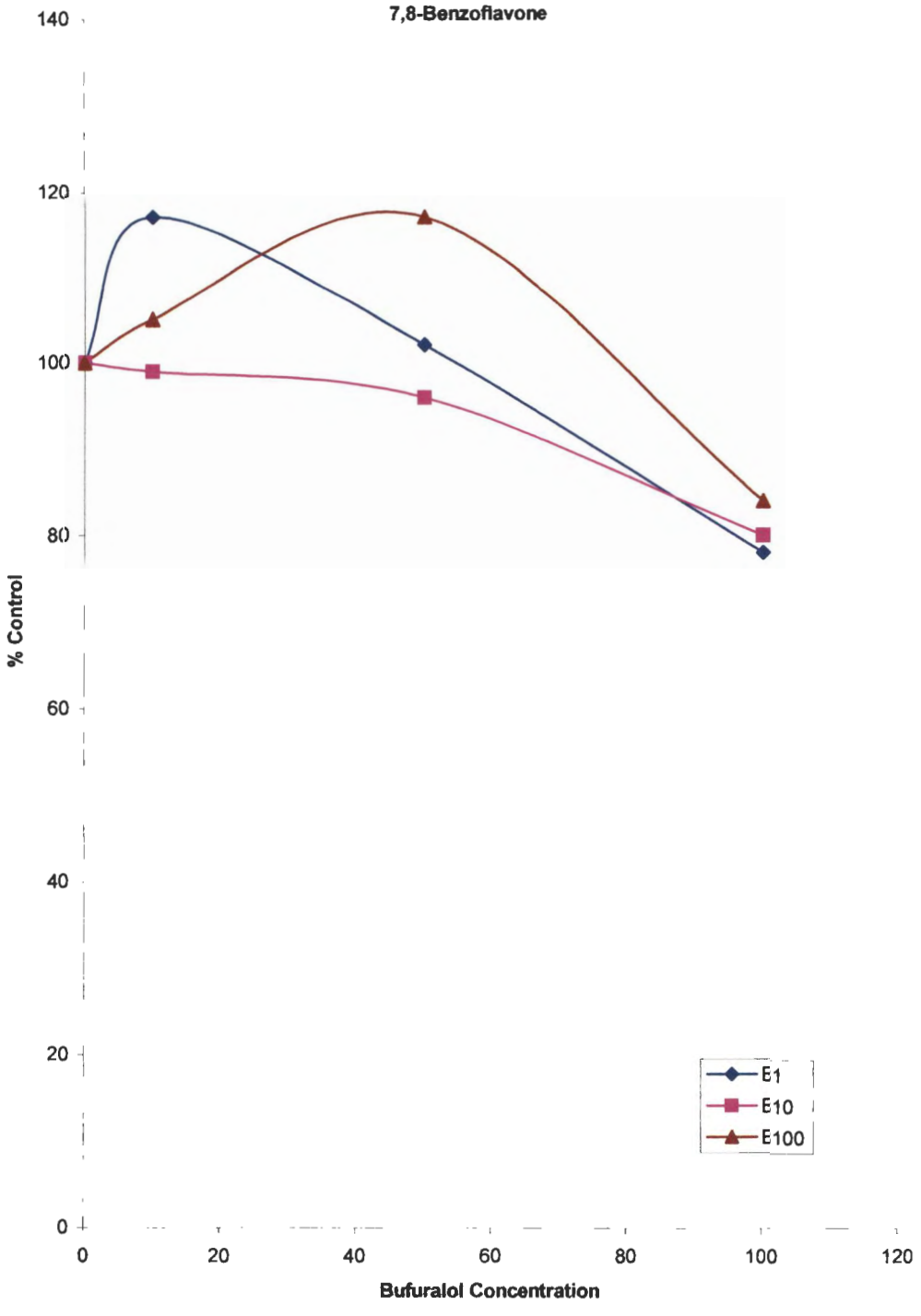

Figure 8-2. Effects of 7,8-benzoflavone on bufuralol 1'-hydroxylation.

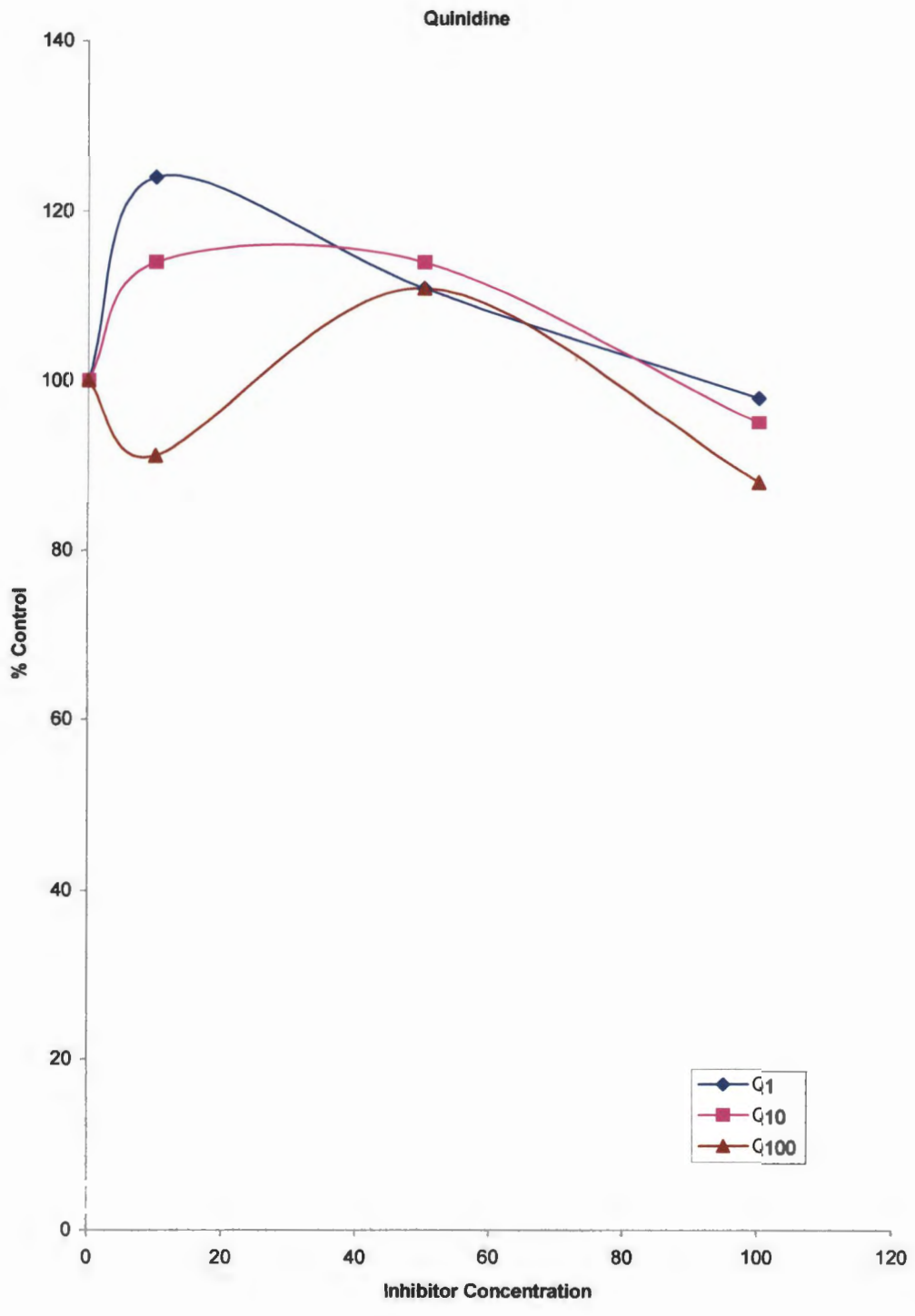

Figure 8-3. Effects of quinidine on bufuralol 1'-hydroxylation.

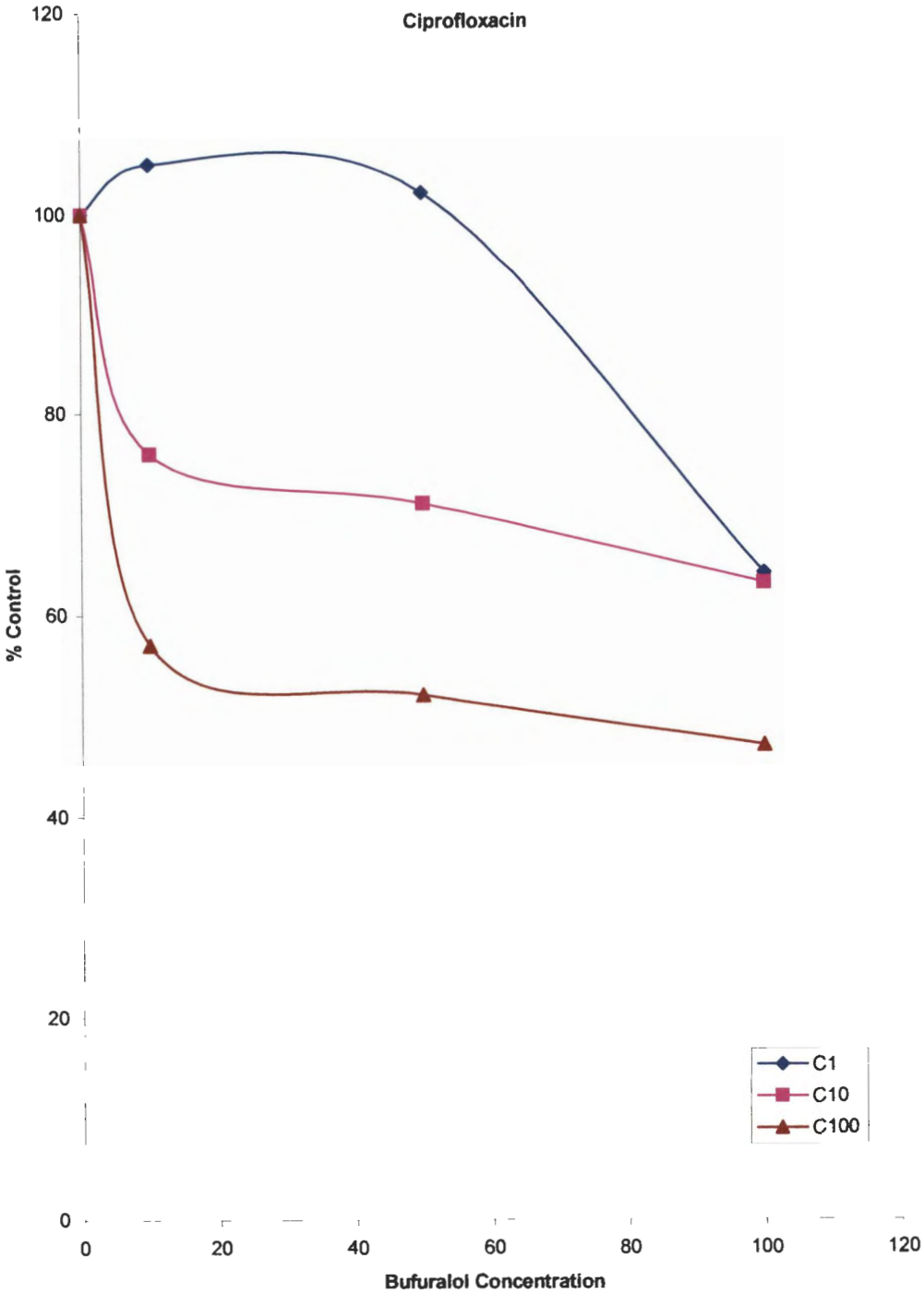

Figure 8-4. Effects of ciprofloxacin on bufuralol 1'-hydroxylation.

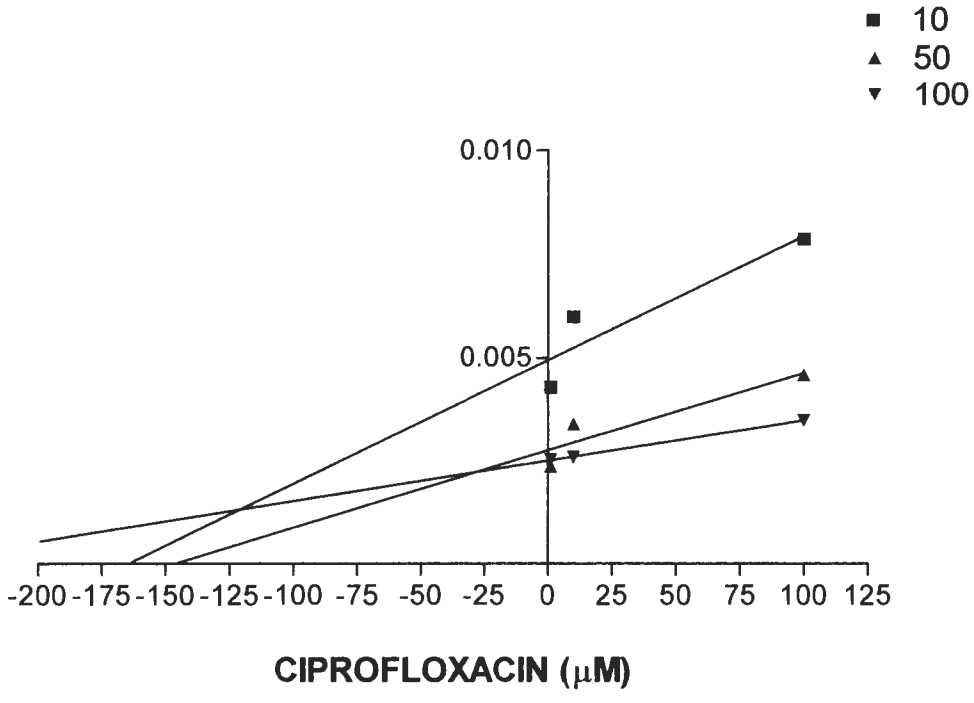

Figure 8-5. Dixon plot of the effects of ciprofloxacin on bufuralol 1'-hydroxylation.

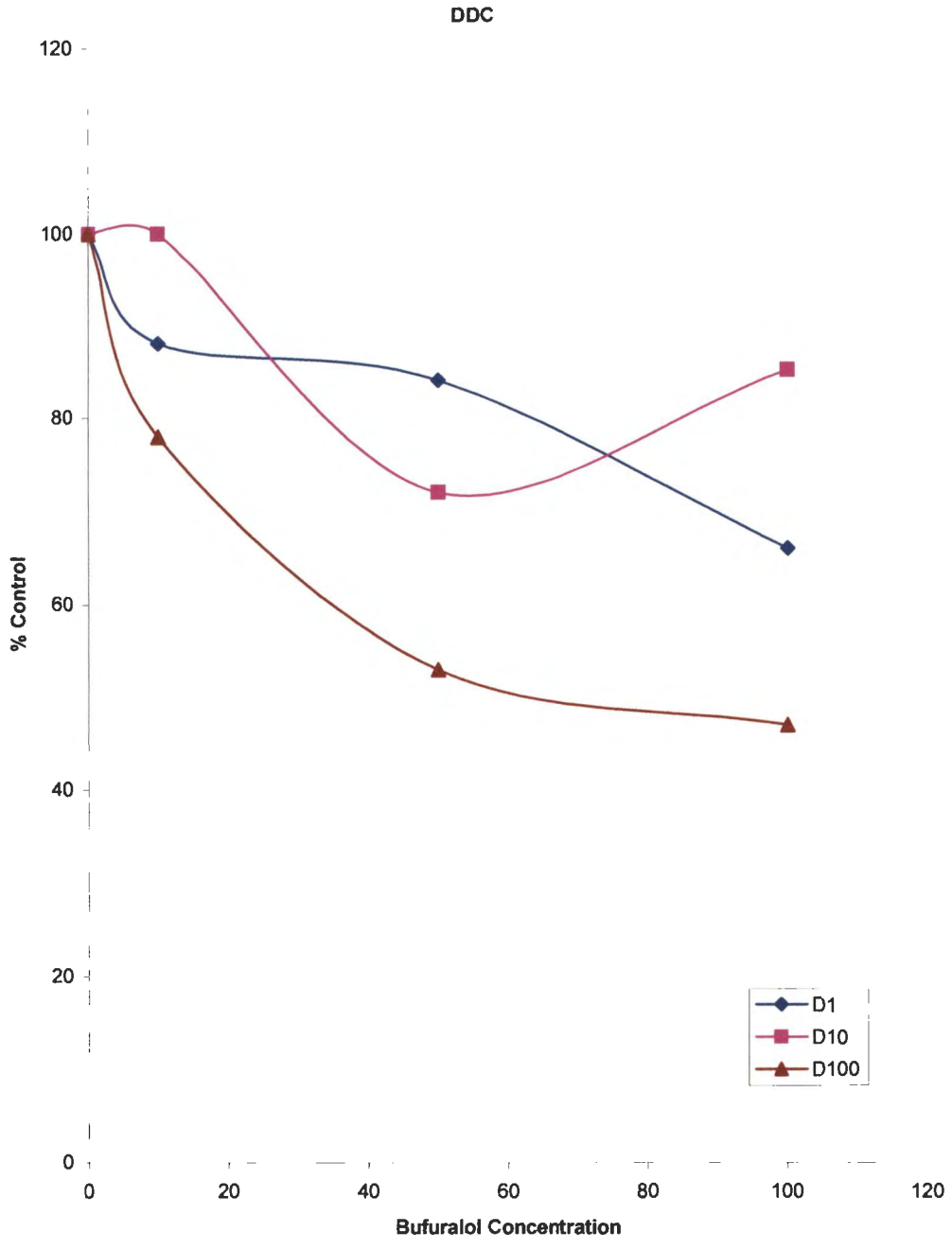

Figure 8-6. Effects of DDC on bufuralol 1'-hydroxylation.

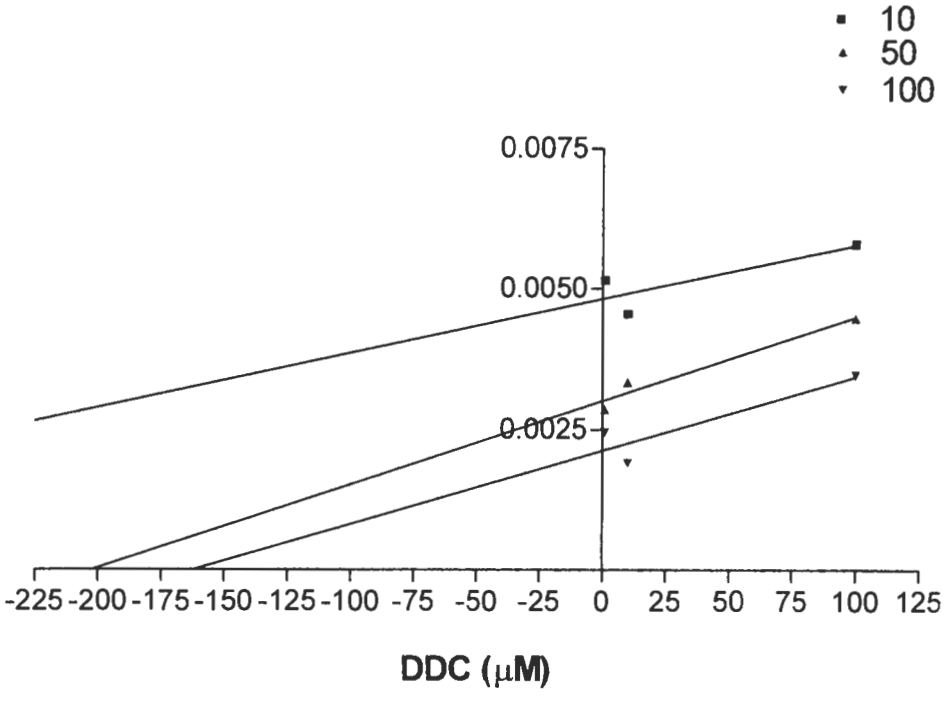

Figure 8-7. Dixon plot of the effects of DDC on bufuralol 1'-hydroxylation.

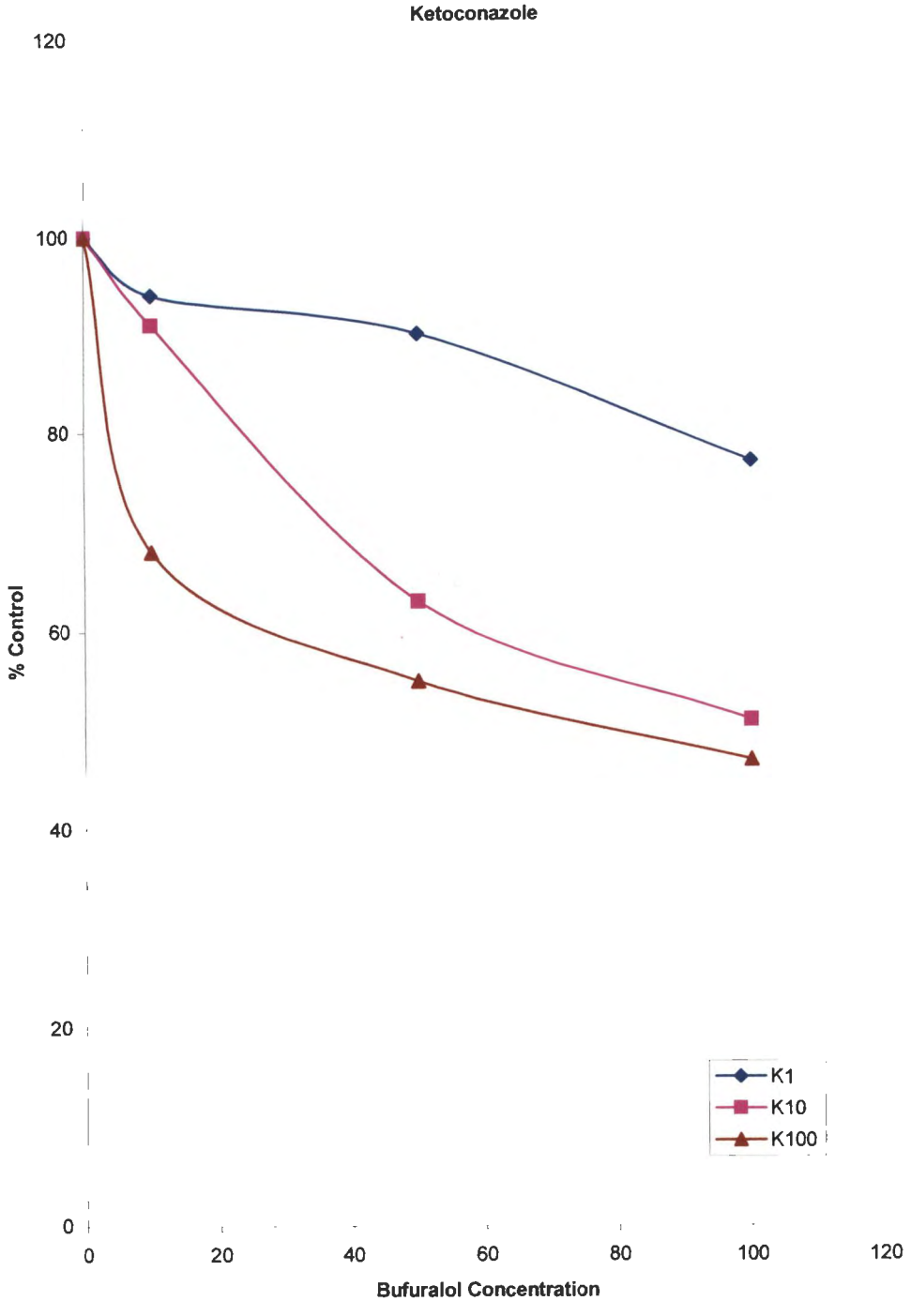

Figure 8-8. Effects of ketoconazole on bufuralol 1'-hydroxylation.

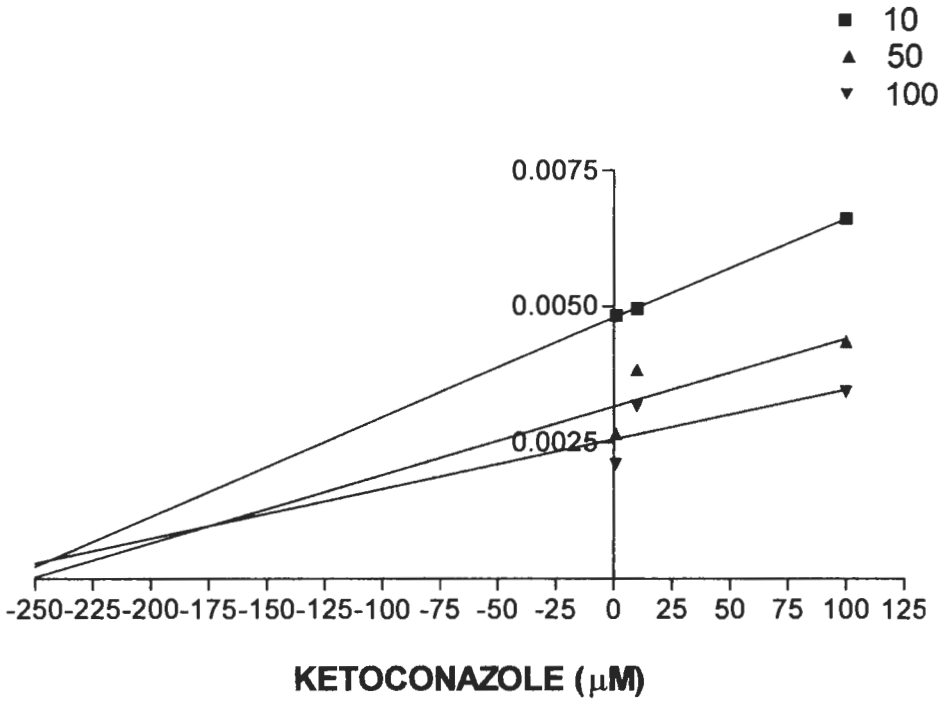

Figure 8-9. Dixon plot of the effects of ketoconazole on bufuralol 1'-hydroxylation.

Chapter 5: Discussion

In the present study, porcine liver microsomes were used to evaluate the possible effects that ciprofloxacin might have on the biotransformation of bufuralol, which in humans is mediated by P4502D6. As part of the study we also looked at the effects that specific chemical inhibitors (used in human P450 enzyme studies) had on bufuralol metabolism in porcine liver microsomes. In animals, effective inhibition by the specific inhibitor probe will occur only if an enzyme homologous to the human enzyme is responsible for the activity.

Inhibition by DDC at the two lower concentrations was very weak. Only at the highest concentration did notable inhibition occur. When enzyme inhibition is observed at only high inhibitor concentrations, this may represent non-specific inhibition. In a previous study (Chauret et al., 1997) found that DDC can cause up to 20% or greater inhibition of other P450 enzyme activities at high concentration ranges. This could explain why inhibition of activity occurred at the highest DDC concentration. Selectivity for DDC may also be less in porcine microsomes than seen in human microsomes.

Results indicate that ketoconazole does exhibit some inhibition at both the medium and high concentrations. Ketoconazole's selectivity as an inhibitor of P4503A4 has been indicated in earlier studies. But, Newton et al. (1995) reported that ketoconazole inhibited bufuralol 1'-hydroxylation by as much as 80% at 100 μM concentrations. Ketoconazole has also been implicated in the inhibition of P4502C reactions and is thought to be an inhibitor of a broad spectrum of P450 enzymes. This is due to the fact that P4503A4 has a particularly broad substrate specificity and therefore will play some role in the metabolism of the vast majority of drugs. Thus, even a relatively selective substrate for 2D6 may have its kinetics contaminated with P4503A4 interferences.

Ciprofloxacin, which is thought to exert its effects on P4501A2 in humans (Fuhr et al., 1990), did cause partial inhibition of enzyme activity in porcine microsomes. A study (Jurima-Romet et al., 2000) conducted in porcine microsomes using dextromethorphan as the P4502D6 substrate found that both a high affinity and low affinity component were involved in the metabolism of dextromethorphan. Similar biphasic kinetics for P4502D6 have been reported in human liver microsomes (Jurima-Romet et al., 2000). If the metabolism of bufuralol were via a two enzyme kinetic model this might explain why ciprofloxacin had some inhibitory effect in porcine microsomes. The other potent inhibitors of P4501A2, 7,8-benzoflavone and furafylline, had no effect on bufuralol metabolism, which would indicate that the P4501A family is not involved. However, difference in the levels of individual P450 enzymes, and in the expression of distinct isoforms may influence the selectivity of inhibitor probes (Eagling et al., 1998). Also discreet differences in the enzyme active site for substrate/inhibitor combinations might explain why ciprofloxacin had a greater inhibitory effect than the other two specific human 1A2 probes.

Quinidine, which is a potent human P4502D6 inhibitor, had no effect on bufuralol 1'-hydroxylation in porcine microsomes. In contrast Jurima-Romet et al. (2000) found both quinidine and quinine to be potent inhibitors of dextromethorphan, which is a human P4502D6 substrate, in porcine microsomes. In the same study Jurima-Romet et al. (2000) also used bufuralol as a P4502D6 substrate, but they used norfluoxetine as a specific inhibitor not quinidine. Norfluoxetine was a potent inhibitor of bufuralol metabolism in porcine microsomes in the study. Based on their results Jurima-Romet et al. (2000) concluded that a P4502D6-like enzyme did metabolize dextromethorphan and bufuralol. However, our substrate/inhibitor combination of bufuralol and quinidine do not support this conclusion. Skaanild and Friis (1997;1999) used debrisoquine as a probe

for P4502D6 activity in conventional pigs and minipigs and reported no activity. In another study (Monshower et al., 1998), investigators concluded that dextromethorphan O-demethylation may not be mediated by P4502D in the pig, and that the P4502D enzyme may be absent from this species. The reason for such discrepancies is unknown. Possible explanations for the results could be differences in experimental conditions, the role of various cofactors, the use of different buffers and discreet differences in the enzyme active site for certain substrate/inhibitor combinations. The activation and substrate inhibition may be highly dependent on a particular folding pattern of the protein. Slight changes in the flexible structure of the enzyme may alter the active site and influence the complex interactions between multiple molecules (Houston and Kenworthy, 2000).

The interpretation of the inhibition data in animal species, in which little data on P450 enzymes are available, can be difficult. This is particularly true of 2D6 where interspecies differences have been reported in terms of inhibition. For example it has been reported that the rat P4502D1 shares 70% homology with the human P4502D6 and that both species have similar substrate specifiicities. However, quinine is a potent inhibitor of rat P4502D1, while its stereoisomer quinidine is not. The converse is true for human P4502D6. In monkey liver microsomes, quinidine was 40-fold less potent as an inhibitor of dextromethorphan activity compared to human liver microsomes (Jurima-Romet et al., 2000).

In summary, the results of this study indicate that there is a P450 enzyme in porcine liver that is responsible for bufuralol 1'-hydroxylation. Both ciprofloxacin and ketoconazole produced inhibitory effects; therefore other enzymes are probably involved. However, since quinidine did not inhibit the activity we cannot say that the enzyme is P4502D6. It is clear that these inhibitors do not exhibit the same selectivity in

porcine microsomal studies as human studies. This may be due to the differential selectivity of the inhibitors or due to the involvement of completely different P450 enzymes. The need for further studies with different substrates and inhibitors at several concentrations and combinations are necessary to make this determination.

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PART IX: THE EVALUATION OF CIPROFLOXACIN ON PORCINE HEPATIC CYTOCHROME P450 BIOTRANSFORMATION OF PHENACETIN

Chapter 1: Abstract

Fluoroquinolones administered together with methylxanthines, such as caffeine and theophylline, have resulted in inhibition of the xanthine's metabolism by cytochrome P450 enzymes in humans. Previous studies have indicated that quinolones inhibit P450 activity in companion animals and rodents. However, there is very little information available concerning the effects that quinolones have on P450 metabolism in food animals. There are important species differences in the function and regulation of cytochrome P450 enzymes, as well as influence by environmental and genetic factors. Many factors can impact the way a drug is metabolized by a particular species.
This study was designed to determine what effect ciprofloxacin has on the metabolism of phenacetin in porcine hepatic microsomes. Phenacetin is used to characterize human cytochrome P4501A2 enzyme activity. As part of this assessment of cytochrome enzyme activity, we looked at the effects that other inhibitor probes had on phenacetin O-deethylation in porcine liver microsomes.

Chapter 2: Introduction

Fluoroquinolones are a series of synthetic antibacterial agents that are relatively new; therefore, limited information is available concerning P450 enzyme activity in animals. Quinolone use in animals may be limited due to consumer health-related concerns; therefore, to use them properly in food animals, specific P450 enzyme characterization is needed (Broad et al., 1995). Inappropriate dosage regimens of antibiotics such as quinolones, that are used in animals intended for food, may indirectly harm the consumer by favoring development of antibiotic-resistant bacteria. Improper dosing of these antibiotic agents may also result in inadvertent consumption of drug residues in edible tissues, milk or eggs.

A documented problem with fluoroquinolone use in human medicine is the interaction that occurs with these antimicrobials and other drugs that may alter the metabolism of both. Fluoroquinolones administered together with methylxanthines (such as caffeine and theophylline) have resulted in inhibition of the xanthine's metabolism by cytochrome P450 enzymes (De Sarro and De Sarro, 2001, Fuhr et al., 1990, Sano et al., 1988). Among the phase I biotransforming enzymes, the cytochrome P450 system plays a pivotal role in the metabolism of a wide variety of xenobiotics and endogenous compounds. They also are capable of converting drugs to active metabolites as well as rendering some chemicals mutagenic or carcinogenic, producing toxic metabolites (Parkinson, 1996; Guengerich, 1992). Results of previous studies have indicated that quinolones inhibit P450 activity in companion animals (Novotny and Shaw, 1991) and rodents (Vancutsem and Babish, 1996). However, there is very little information available concerning the effects that quinolones have on P450 metabolism in food animals.

There are important species differences in the function and regulation of cytochrome P450 enzymes, as well as influence by environmental and genetic factors. Some of these variations have been attributed in part, to the presence of different P450 enzymes and their inducibility (Guengerich, 1992; Smith, 1991; Boobis et al., 1990). Possible reasons for species differences in cytochrome P450 activity are: the relative concentrations of different biotransforming enzymes; the active site of the enzyme differs amongst species; the P450 activity is catalyzed by different enzymes or isozymes (the enzyme may be in one species but completely missing in another); differences in the specificity of the isozyme; and differences in the biochemical pathways competing for the substrate (Parkinson, 1996; Boobis et al., 1990; Mellett, 1969). Any one or combination of these reasons can have a tremendous impact on the way a drug is metabolized by a particular species. A drug that is effectively used in one species could be useless in another because it is not metabolized efficiently. Likewise, a therapeutic dose in one species could prove to be lethal in another due to differences in metabolism.

The P450 enzymes are mainly responsible for phase I metabolism, which converts drugs and other lipophilic chemicals, low in solubilities, to metabolites that are more hydrophilic and more readily eliminated. The human P4501A family contains 1A1 and 1A2. Human 1A1 metabolizes carcinogens but is essentially absent from the liver and is restricted to extrahepatic tissues. P4501A2 participates in the hepatic oxidation of drugs such as acetaminophen, clozapine and xanthines such as theophylline and caffeine. P4501A2 represents 10-15% of total P450 content in human liver, although there is substantial inter-subject variation in expression (Murray, 1999). In the investigation of P450 mediated xenobiotic metabolism, individual forms of human P450 enzymes have been found to catalyze specific reactions with specific substrates. Characterizing these specific enzyme activities with these substrates allows their use as

probes for the P450 enzymes. To date, at least one marker activity exists for the majority of human P450 enzymes (Sharer et al., 1995). This study was designed to determine what effect ciprofloxacin has on the metabolism of phenacetin in porcine hepatic microsomes. Phenacetin is used to characterize human cytochrome P4501A2 enzyme activity. As part of this process of examining P450 activity in hepatic microsomes we looked at the effects that other inhibitor probes had on phenacetin O-deethylation in porcine liver microsomes.

Chapter 3: Materials and Methods

Chemicals and Reagents

Ketoconazole, 7, 8-benzoflavone, quinidine, phenacetin, trimethoprim (TMP), diethyldithiocarbamic acid (DDC), acetaminophen, glucose-6-phosphate (G6P), β-nicotinamide adenine dinucleotide phosphate (NADP), and glucose-6-phosphate dehydrogenase (G6PDH) were purchased from Sigma Chemical (St. Louis, MO). Other chemicals were obtained from the following sources: furafylline from Gentest Corp. (Woburn, MA) and ciprofloxacin from US Pharmacia (Rockville, MD). All other high analytical grade reagents and solvents were purchased from Fisher Scientific (Fair Lawn, NJ).

Porcine Liver Microsomes

Four pigs (3 female and 1 castrated male) were used. The pigs weighed 29 to 38 kg. The animals were fed standard pig chow and had no known prior exposure to any drugs. Microsomes were prepared using an ultracentrifugation technique described by Lake (1987). The washed microsomes were resuspended in 10 mM Tris-acetate buffer (pH 7.4) containing 1 mM EDTA, and 20% sucrose, and were then frozen at -80°C until used. Protein concentrations were determined using a Pierce reagent kit (Rockford, IL) and P450 content was determined according to the method of Omura and Sato (1964).

Enzyme Assays

Incubations contained 0.5 mg of microsomal protein, 100 mM phosphate buffer at pH 7.4 containing 6 mM magnesium chloride, 1 mM EDTA, and a NADPH-generating system (1 mM NADP, 10 mM G6P and 0.7 U of G6PDH) in a total volume of 0.5 ml. Incubation mixtures contained substrate and inhibitors, and the reactions were initiated by addition of the NADPH-generating system after a 5 min pre-incubation step at 37°C. Furafylline and DDC were preincubated for 15 min at 37°C with the generating solution

and microsomes. The reactions were initiated by addition of phenacetin. These inhibitors are mechanism-based and require NADPH-dependent complexation for activation. The phenacetin stock solutions were prepared in methanol, and the concentration did not exceed 1% when added to the incubations. The substrate concentrations were 10, 100, 250, and 500 μM, while inhibitor concentrations were 1, 10, and 100 μM. All inhibitors were dissolved in methanol except DDC and ciprofloxacin, which were dissolved in water and 0.067M potassium dihydrogen phosphate (pH 3). The methanol was evaporated before the start of the reaction. Enzyme reactions were quenched with 0.1 ml of ice cold acetonitrile after 20 min in a 37°C shaking water bath, and then placed on ice for 1 hour. The microsome samples were centrifuged at 16,000 g for 15 min after which the supernatants were removed and stored at -80°C until analyses could be performed.

Enzyme inhibitors were also incubated without substrate under the same conditions, to ensure that their presence did not interfere with the quantitation of the respective metabolites.

HPLC System

The HPLC system consisted of a 626 pump, a 717 plus autosampler, a 996 scanning UV detector and a computer equipped with Millennium software (Waters, Milford, MA). The samples were analyzed by a method developed in this laboratory. Briefly, previously frozen samples were thawed on ice and vortexed. Fifty microliters of TMP (25 μg/ml) was added to 0.5 ml sample and 190μl was injected onto the liquid chromatograph. Chromatography was conducted for the measurement of phenacetin O-deethylation on a YMC-Pack ODS-AQ column (5 μm, 6 x 150 mm) equipped with a C_{18} Guard-Pak precolumn insert (Waters, Milford, MA). The mobile phase consisted of a

mixture of (A) 0.05% phosphoric acid (pH 3.0) and (B) methanol. The gradient was started at 74% A and 26% B and was maintained for 4 min; then over a 5 min period, the mixture was changed to 66% A and 34% B and was maintained for 9 min; then over a 4 min period the system was returned to initial conditions. UV detection was measured at 254 nm. Calibration curves were constructed using the pure metabolite and TMP as the internal standard.

Data Analysis

Determination of apparent Ki values was carried out using Graphpad Prism (Graphpad Software, Inc., San Diego CA). Formation rates from the experiments with inhibitors were evaluated by using Dixon plots ([I] vs. 1/V).

Chapter 4: Results

The effects of specific P450 inhibitors and ciprofloxacin on the metabolism of phenacetin O-deethylation are illustrated in Figure 9-1 thru 9-8. The inhibitor probes used were 7,8-benzoflavone, furafylline, quinidine, DDC, and ketoconazole. The catalytic activities of controls (absence of inhibitor) were considered 100%.

Quinidine, the P4502D6 inhibitor, had very little effect on phenacetin O-deethylation (Figure 9-1). The 10 and 100 μM concentrations increased phenacetin metabolism at various substrate concentrations and 7,8-benzoflavone, a potent inhibitor of 1A2 activity, produced similar results (Figure 9-2). Both the 10 and 100 μM concentrations produced a stimulatory effect on phenacetin metabolism while the 1 μM concentration produced inhibition of 35% or less.

Ketoconazole, the P4503A4 inhibitor, produced mixed results as illustrated in Figure 9-3. The 1 and 10 μM concentrations caused very little inhibitory effect (<20%) with the exception of the 10 μM inhibitor concentration on the 10 μM phenacetin concentration (39%). The inhibition produced by the 100 μM ketoconazole ranged from 30% to 68% over the substrate concentrations used.

Furafylline, a mechanism-based inhibitor, is another probe for P4501A2 that was used in the study (Figure 9-4). The 1 μM concentration produced inhibition that ranged from 8% to 34%, while the 10 and 100 μM concentrations produced similar inhibitory results (32% to 41%).

Ciprofloxacin at the 100 μM concentration produced a 60% inhibition of phenacetin O-deethylation (Figure 9-5). The 1 and 10 μM concentrations produced very similar inhibitory results (<50%). The appearance of the Dixon plot (Figure 9-6) for

ciprofloxacin is characteristic of non-competitive or mixed inhibition. The apparent Ki was 178 μM.

As illustrated in Figure 9-7, the 100 μM concentration of DDC produced greater than 70% inhibition of phenacetin O-deethylation while the 1 and10 μM concentrations exhibited from 46% to 61% inhibition. The appearance of the Dixon plot (Figure 9-8) is characteristic of non-competitive or mixed inhibition. The apparent Ki was 131 μM.

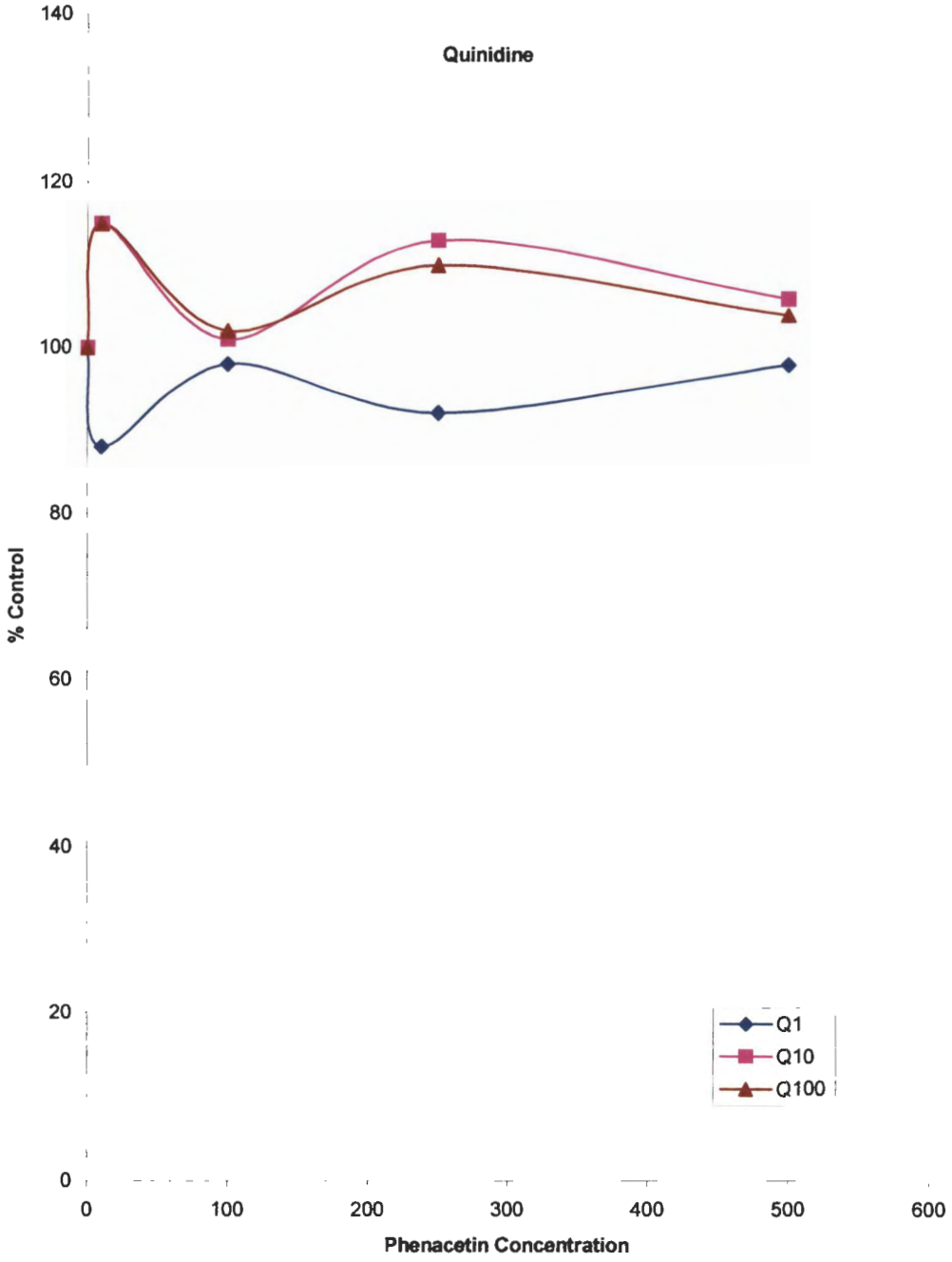

Figure 9-1. Effects of quinidine on phenacetin O-deethylation.

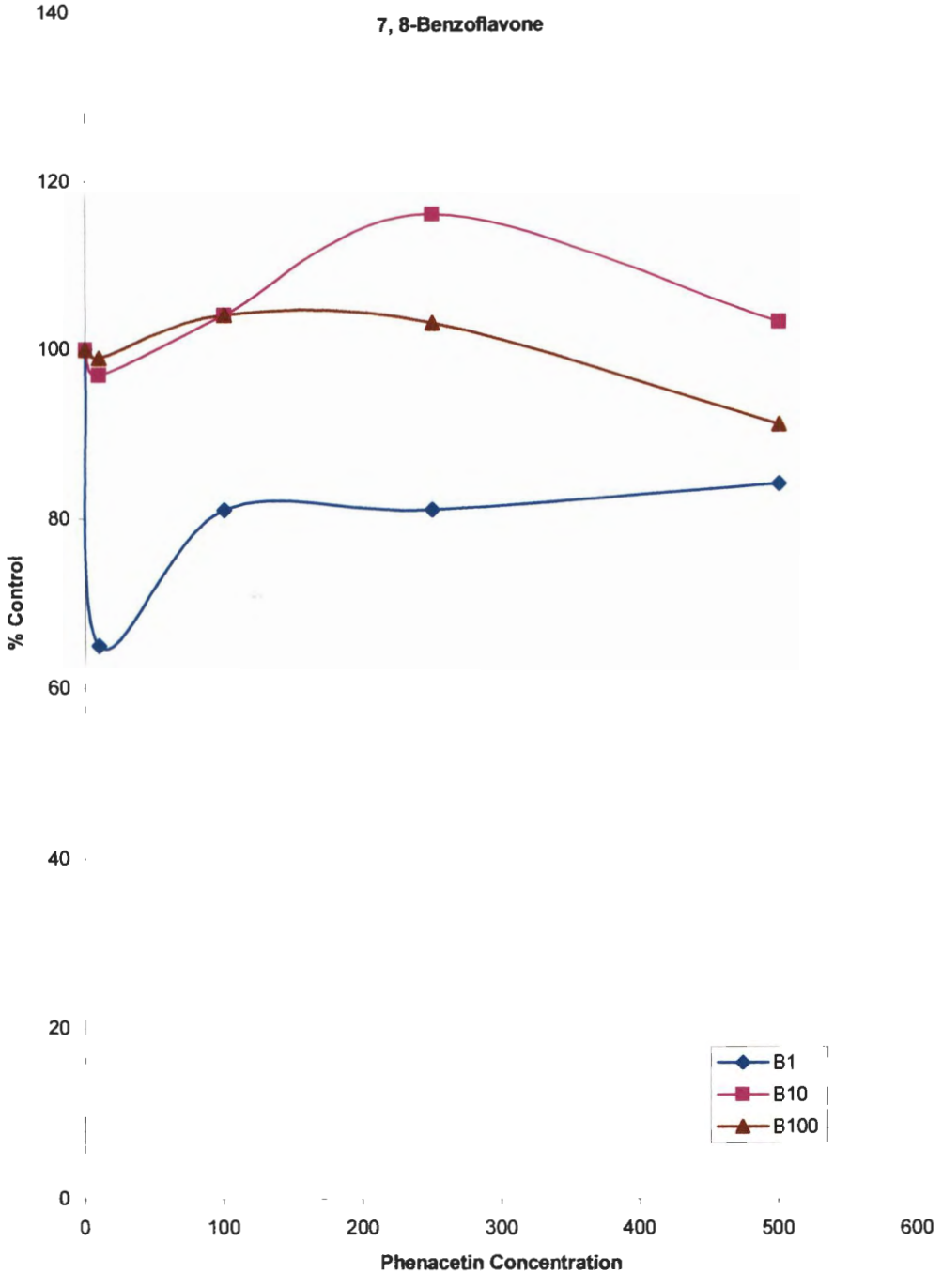

Figure 9-2. Effects of 7,8-benzoflavone on phenacetin O-deethylation.

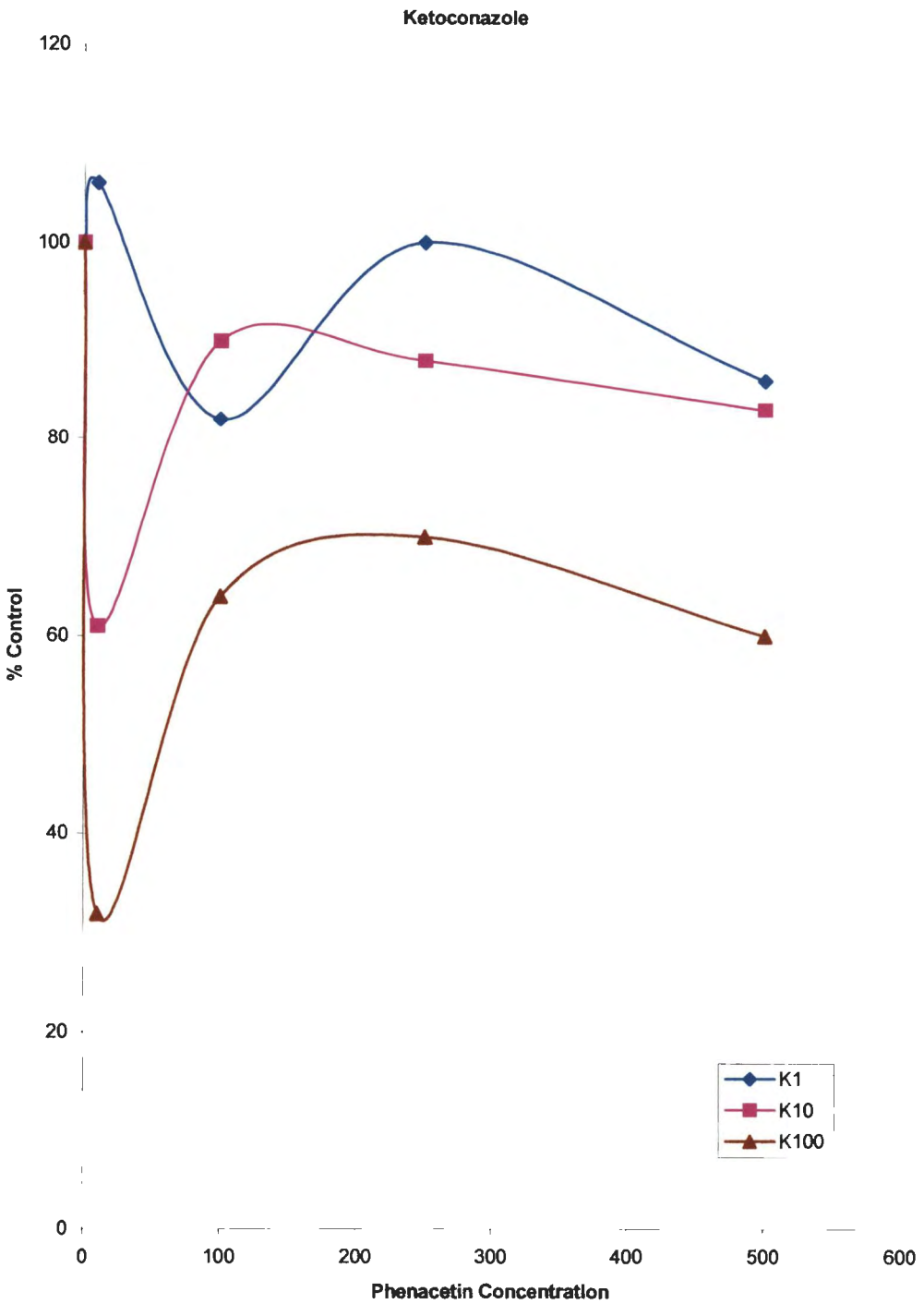

Figure 9-3. Effects of ketoconazole on phenacetin O-deethylation.

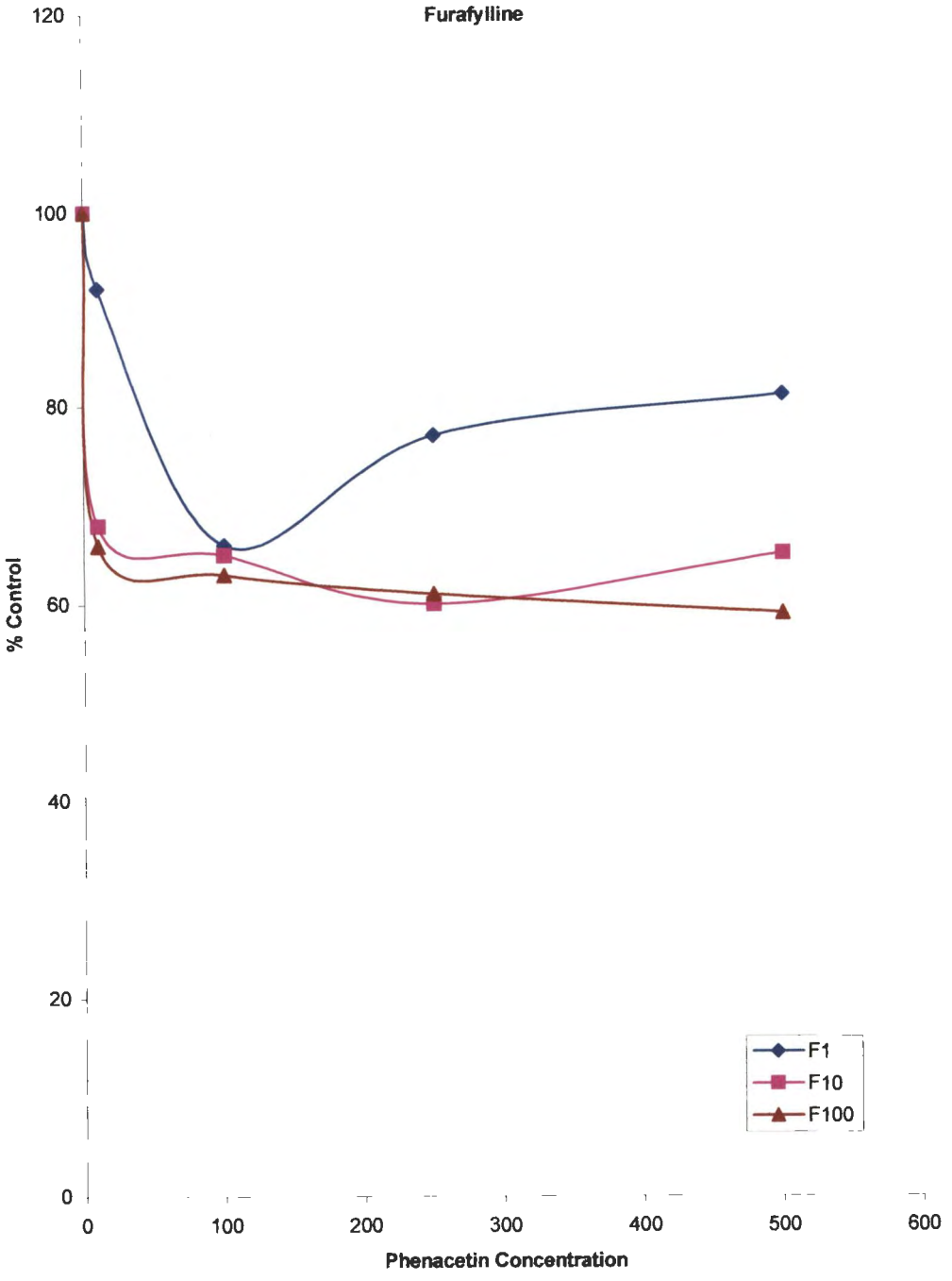

Figure 9-4. Effects of furafylline on phenacetin O-deethylation.

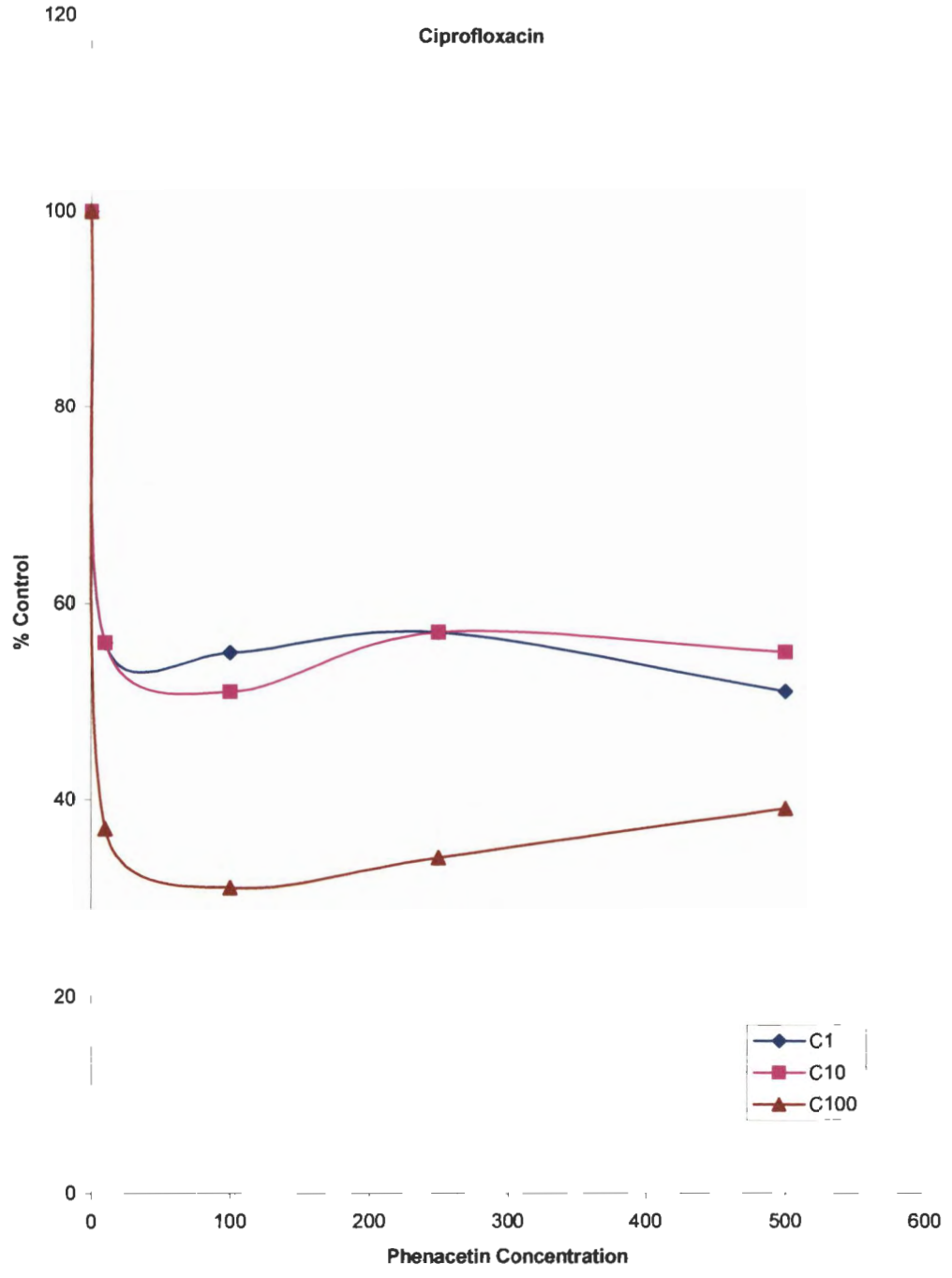

Figure 9-5. Effects of ciprofloxacin on phenacetin O-deethylation.

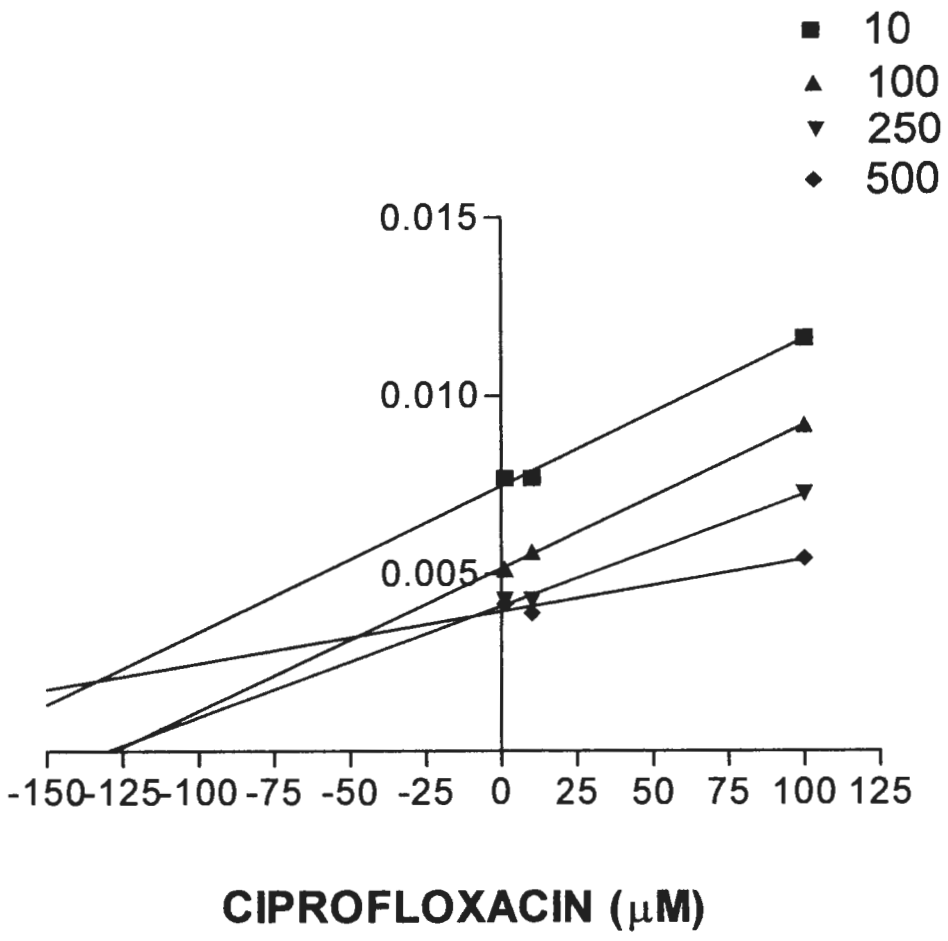

Figure 9-6. Dixon plot of the effects of ciprofloxacin on phenacetin O-deethylation.

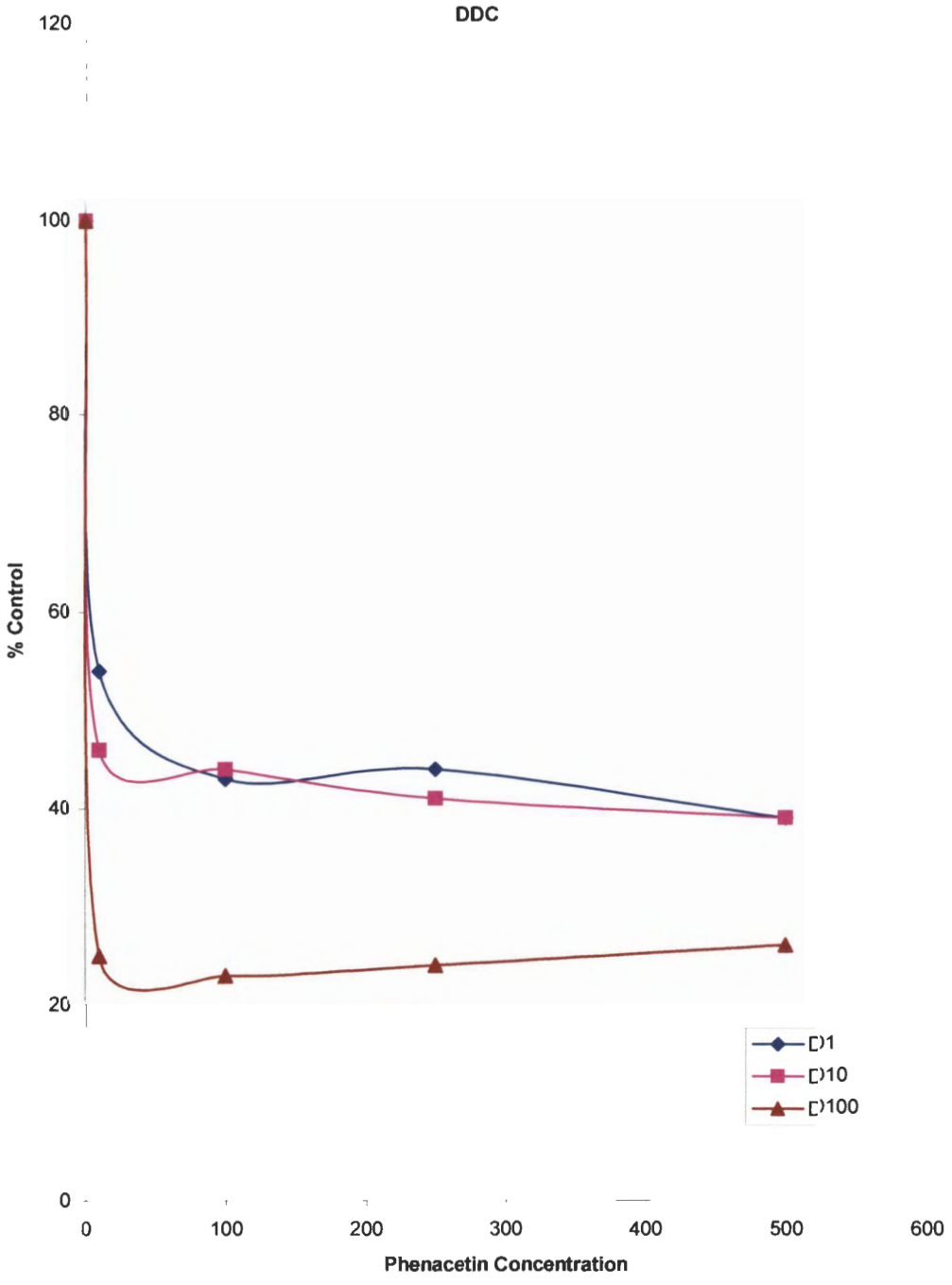

Figure 9-7. Effects of DDC on phenacetin O-deethylation.

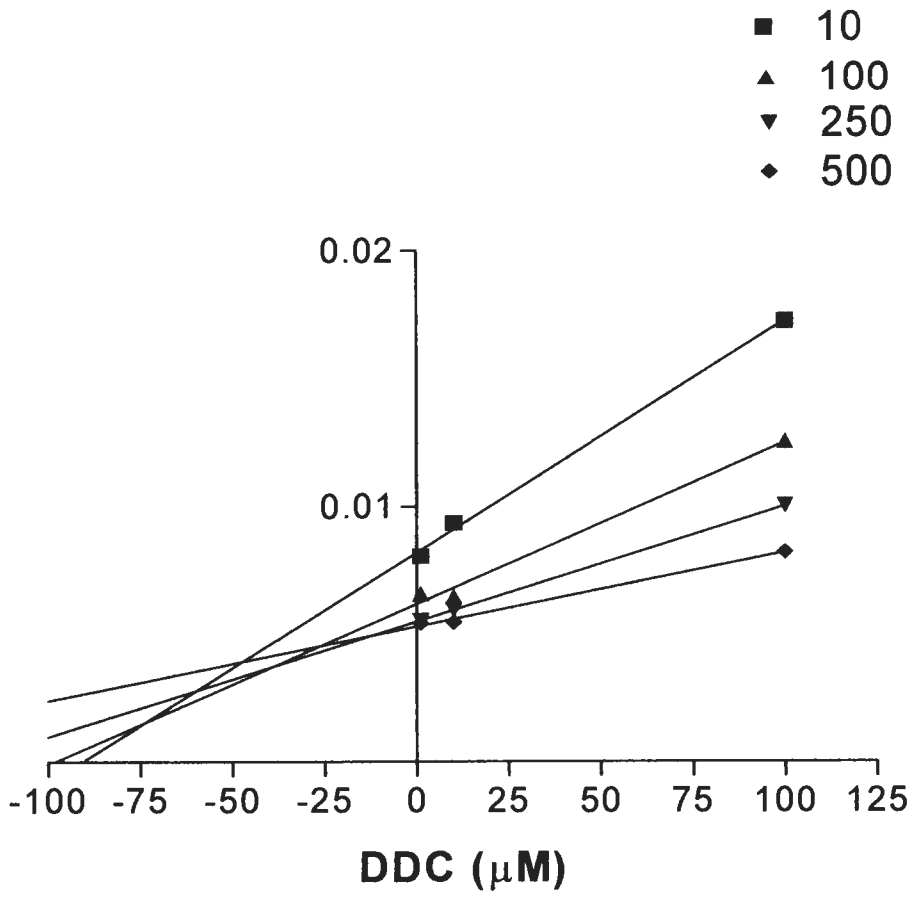

Figure 9-8. Dixon plot of the effects of DDC on phenacetin O-deethylation.

Chapter 5: Discussion

The specificity of an enzyme inhibitor can be evaluated by studying either the extent of inhibition (by varying the concentration of the inhibitor) or the kinetics of inhibition (by varying the concentration of the substrates at several fixed concentrations of inhibitor). We chose to do both to give more definitive results.

Several studies using 7-ethoxyresorufin as a probe (Skaanild and Friis, 1999; Skaanild and Friis, 1997; Monshouwer et al., 1996) have determined that pigs have P4501A2 and 1A1 activity. However, to our knowledge none have determined if this is a one or two enzyme Michaelis-Menten model. In humans and rats the metabolism of phenacetin involved both a high affinity and low affinity enzyme (Eagling et al., 1998). The data from our study indicates that more than one enzyme may be involved in phenacetin O-deethylation in porcine microsomes.

Quinidine, a potent 2D6 inhibitor, failed to inhibit the phenacetin O-deethylation. Therefore, cytochrome P4502D6 does not appear to be involved in phenacetin metabolism.

Both 7,8-benzoflavone and furafylline have been reported to be potent inhibitors of P4501A2 in human liver microsomes (Newton et al., 1995), although this activity was not evident in porcine microsomes. The flavone had very little effect on enzyme activity while furafylline had a slightly greater effect. In contrast, ciprofloxacin produced a much greater inhibition of enzyme activity than the two specific P4501A2 inhibitors. Fluoroquinolones are thought to exert their effects on the P4501A family of enzymes in humans, rodents and possibly in companion animals (Vancutsem and Babish, 1996). Differences in the levels of individual P450 isozymes, and in the expression of distinct isoforms, may influence the selectivity of inhibitor probes (Eagling et al., 1998). Also, relatively minor structural alterations in an inhibitor can have dramatic effects on P450

specificity, both of which might explain why ciprofloxacin had a greater inhibitory effect than the other two specific human 1A2 probes.

Ketoconazole produced an inhibitory effect on P450 activity, but only at its highest concentration. Enzyme inhibition that is observed at high concentrations may represent nonspecific inhibition (Chauret et al., 1997). Selectivity for ketoconazole may also be less in porcine microsomes than is seen in human microsomes. Ketoconazole was found to exhibit a much lesser degree of selectivity in rat microsomes when compared to those of humans (Eagling et al., 1998). Previous studies reported that ketoconazole was thought to be an inhibitor of a broad spectrum of P450 enzymes (Newton et al., 1995).

DDC produced the greatest inhibitory effect on phenacetin O-demethylation. It has been suggested that DDC is a selective inhibitor of P4502E1 in human liver microsomes (Newton et al., 1995). However, Chang et al. (1994) found that DDC was a potent inhibitor of P450 1A1, 1A2 and 2E1. Eagling et al. (1998) also found that DDC inhibited both P4501A2 and 2E1 activity in rat microsomes. If the metabolism of phenacetin were via a two enzyme kinetic model as found in humans and rats, this might explain why DDC had some inhibitory effect in porcine microsomes. It is also possible that since the two specific P4501A2 inhibitors had very little effect on phenacetin metabolism, that this reaction might be mediated by 2E1.

In conclusion, the results of this study indicate that a P450 enzyme in porcine liver is responsible for phenacetin O-deethylation. Both ciprofloxacin and DDC produced inhibitory effects; therefore, multiple enzymes are probably involved. However, since 7,8-benzoflavone and furafylline did not inhibit the activity, we cannot say that the enzyme is P4501A2. It is clear that these inhibitors do not exhibit the same selectivity in porcine microsomal studies as human studies. This may be due to the differential selectivity of

the inhibitors or due to the involvement of completely different P450 enzymes. The need for further studies with different substrates and inhibitors at several concentrations are necessary to make this determination. Caution must be exercised when extrapolating the effects of enzyme inhibitors between species and using this information to make predictions concerning the involvement of P450 enzymes.

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PART X: THE EFFECTS OF CIPROFLOXACIN ON PORCINE HEPATIC CYTOCHROME P450 METABOLISM OF LIDOCAINE

Chapter 1: Abstract

Fluoroquinolones are relatively new antimicrobial agents, with limited information available concerning P450 enzyme activity. Studies have indicated that quinolones inhibit P450 activity in companion animals and rats. However, there is very little information concerning the effects that quinolones have on P450 biotransformation in food animals. There are important species differences in the function and regulation of cytochrome P450 enzymes, as well as influence by environmental and genetic factors. This study was designed to determine what effect ciprofloxacin has on the metabolism of lidocaine in porcine hepatic microsomes. Furthermore, the inhibitory effects of 7,8-benzaflavone, furafylline, quinidine, DDC and ketoconazole on lidocaine N-deethylation were characterized in porcine liver microsomes.

Chapter 2: Introduction

Fluoroquinolones were introduced into clinical medicine roughly 15 years ago. These compounds were regarded as nearly ideal antimicrobial agents because of their broad spectrum of activity as well as their clinically advantageous pharmacokinetic properties. Although the use of fluoroquinolones in food animals may be limited due to consumer health-related concerns, P450 enzyme characterization is required to limit these concerns and to ensure their proper use (Broad et al., 1995). However, limited information is available concerning P450 enzyme activity of quinolones. Inappropriate dosage regimens of antibiotics that are used in animals intended for food, may indirectly harm the consumer by favoring development of antibiotic-resistant bacteria. Improper dosing of the antimicrobial agents may also result in inadvertent consumption of drug residues in edible tissues, milk or eggs.

A problem encountered with fluoroquinolones that are used in human medicine is the interaction occurring with these antimicrobials and other drugs that may alter the metabolism of both. Fluoroquinolones administered together with methylxanthines, such as caffeine and theophylline, has resulted in inhibition of xanthine metabolism by cytochrome P450 enzymes (De Sarro and De Sarro, 2001, Fuhr et al., 1990, Sano et al., 1988). Detoxification of many drugs occurs via P450 enzymes. The P450 system is also responsible for converting a number of prodrugs to active metabolites as well as rendering certain chemicals mutagenic or carcinogenic, producing toxic metabolites (Parkinson, 1996, Guengerich, 1992). Results of prior studies have indicated that quinolones inhibit P450 activity in companion animals (Novotny and Shaw, 1991) and rats (Vancutsem and Babish, 1996). However, there is very little information available concerning the effects that quinolones have on P450 biotransformation in food animals.

There are important species differences in the function and regulation of cytochrome P450 enzymes, as well as influence by environmental and genetic factors. Some of these variations have been attributed in part, to the presence of different P450 enzymes and their inducibility (Guengerich, 1992; Smith, 1991; Boobis et al., 1990). Possible reasons for species differences in cytochrome P450 activity are: the relative concentrations of different biotransforming enzymes; the active site of the enzyme differs among species; enzyme activity is catalyzed by different enzymes or isozymes (the enzyme may be in one species but completely missing in another); differences in the specificity of the isozyme; and differences in the biochemical pathways competing for the substrate (Parkinson, 1996; Boobis et al., 1990; Mellett, 1969). Any one or combination of these reasons can have a tremendous impact on the way a chemical is metabolized by a particular species. A drug that is effectively used in one species could be useless in another because it is not metabolized efficiently. Likewise, a therapeutic dose in one species could prove to be lethal in another due to differences in metabolism.

P450 enzymes are mainly responsible for phase I metabolism, which transforms drugs and lipophilic chemicals to metabolites that are more hydrophilic and readily eliminated. Cytochrome P4503A4 is the major form of P450 expressed in normal adult human liver microsomes. It plays a vital role in the metabolism of both endogenous substrates and numerous therapeutic agents. These enzymes are capable of oxidizing a wide variety of compounds ranging in size from monosubstituted benzenes to cyclosporin. In the investigation of P450-mediated xenobiotic metabolism, individual forms of P450 enzymes have been found to catalyze specific reactions with certain substrates. Characterizing these specific enzyme activities with these substrates allows their use as probes for the P450 enzyme. To date, at least one marker activity exists for the majority of human P450 enzyme forms (Sharer et al., 1995). This study was

designed to determine what effect ciprofloxacin has on lidocaine metabolism in porcine hepatic microsomes. Lidocaine is used to characterize human cytochrome P4503A4 enzyme activity. A second phase of the studied involved characterization of the inhibitory effects of 7,8-benzaflavone, furafylline, quinidine, DDC and ketoconazole on lidocaine N-deethylation in porcine liver microsomes.

Chapter 3: Materials and Methods

Chemicals and Reagents

Ketoconazole, 7, 8-benzoflavone, quinidine, trimethoprim (TMP) diethyldithiocarbamic acid (DDC), glucose-6-phosphate (G6P), β-nicotinamide adenine dinucleotide phosphate (NADP), and glucose-6-phosphate dehydrogenase (G6PDH) were purchased from Sigma Chemical (St. Louis, MO). Other chemicals were obtained from the following sources: furafylline from Gentest Corp. (Woburn, MA); lidocaine and ciprofloxacin from US Pharmacia (Rockville, MD) monoethylglycinexylidide (MEGX) and glycinexylidide (GX) were gifts from Astra laboratories (Westboro, MA). All other high analytical grade reagents and solvents were purchased from Fisher Scientific (Fair Lawn, NJ).

Porcine Liver Microsomes

Four pigs (3 female and 1 castrated male) were used, weighing 29 to 38 kg. The animals were fed standard pig chow and had no known prior exposure to any drugs. Microsomes were prepared using an ultracentrifugation technique described by Lake (1987). The washed microsomes were resuspended in 10 mM Tris-acetate buffer (pH 7.4) containing 1 mM EDTA, and 20% sucrose, and frozen at -80°C until used. Protein concentrations were determined using a Pierce reagent kit (Rockford, IL) and P450 content was determined according to the method of Omura and Sato (1964).

Enzyme Assays

Incubations contained 0.5 mg of microsomal protein, 100 mM phosphate buffer at pH 7.4 containing 6 mM magnesium chloride, 1 mM EDTA, and a NADPH-generating system (1 mM NADP, 10 mM G6P and 0.7 U of G6PDH) in a total volume of 0.5 ml. Incubation mixtures contained substrate and inhibitors, and the reactions were initiated by addition of the NADPH-generating system after a 5 min pre-incubation step at 37°C.

Furafylline and DDC were preincubated for 15 min at 37°C with the generating solution and microsomes. The reactions were initiated by the addition of substrate. These inhibitors are mechanism-based and require NADPH-dependent complexation for activation. The substrate stock solutions were prepared in methanol, which did not exceed 1% when added to the incubations. The substrate concentrations were 10, 100, 250 and 500 μM, while inhibitor concentrations were 1, 10, and 100 μM. All inhibitors were dissolved in methanol except DDC and ciprofloxacin, which were dissolved in water and 0.067M potassium dihydrogen phosphate (pH 3). Enzyme reactions were quenched with 0.05 ml of 1 M NaOH after 20 min in a 37°C shaking water bath, and then placed on ice for 1 hour. Microsome samples were centrifuged at 16,000 g for 15 min, after which the supernatants were removed and stored at -80°C until analyses could be performed.

The enzyme inhibitors were also incubated without substrate under the same conditions to ensure that their presence in the incubation did not interfere with the quantitation of the respective metabolites.

HPLC System

The HPLC system consisted of a 626 pump, a 717 plus autosampler, a 486 UV detector, and a computer equipped with Millennium software (Waters, Milford, MA). Samples were analyzed by a method developed in this laboratory. Briefly, previously frozen samples were thawed on ice and vortex-mixed. Twenty-five microliters of trimethoprim was added to 0.5 ml sample and 190μl was injected onto the liquid chromatograph. Chromatography was conducted for the measurement of lidocaine N-deethylation on a μBondapak C_{18} column (10 μm, 3.9 x 300 mm) equipped with a μBondapak C_{18} Guard-Pak precolumn insert (Waters, Milford, MA). The mobile phase

was potassium dihydrogen phosphte:acetonitrile (87:13) pH 5.9 at a flow rate of 2 ml/min. UV detection was measured at 205 nm. Calibration curves were constructed using the pure metabolites MEGX and GX and trimethoprim as the internal standard.

Data Analysis

Determination of apparent Ki values was carried out using Graphpad Prism (Graphpad Software, Inc., San Diego CA). Formation rates from the experiments with inhibitors were evaluated by using Dixon plots ([I] vs. 1/V).

Chapter 4: Results

The effects of the five specific P450 inhibitors and ciprofloxacin on the formation of MEGX are illustrated in Figure 10-1 thru Figure 10-7. Several of the curves in the figure are missing the 10 μM point due to the limit of detection for the assay. The catalytic activities of controls (absence of inhibitor) were considered 100%.

7,8-Benzoflavone is an inhibitor for P450 1A2 and had no inhibitory effect on lidocaine N-deethylation (Figure 10-1). In fact, it caused activation at all the concentrations evaluated.

The specific inhibitor of P450 2E1, DDC (Figure 10-2) had its greatest effect on lidocaine metabolism at the 100 and 250 μM substrate concentrations. Inhibition at these 2 concentrations ranged from 60% to 84%. There was some inhibition at the 500 μM concentration; however, it was less than the other 2 concentrations, with a reduction in activity ranging from 15% to 45%. The 100 μM DDC produced a stimulatory effect at the 10 μM lidocaine concentration.

The effect of quinidine, a potent P450 2D6 inhibitor is demonstrated in Figure 10-3. The largest decrease in activity occurred at the 100 and 250 μM substrate concentrations, and was produced by the 10 μM (72% & 66%) and 100 μM (75% & 50%) inhibitors. All three quinidine concentrations caused an increase in lidocaine metabolism at the 500 μM lidocaine level.

Furafylline, a mechanism-based inhibitor of P450 1A2, produced between 70% and 75% (1, 10 & 100 μM) inhibition of activity at the 100 μM lidocaine concentration (Figure 10-4). Inhibition for the 1, 10, and 100 μM concentrations was 49%, 54%, and 75% at the 250 μM lidocaine level. Very little inhibition occurred (<20%) at the 500 μM

level with the three concentrations used. The 100 μM furafylline concentration produced a stimulatory effect at the 10 μM lidocaine concentration.

Figure 10-5 represents the effect that ciprofloxacin had on lidocaine metabolism. The greatest reduction in activity occurred at the 100 μM and 250 μM substrate concentrations. The 100 μM ciprofloxacin concentration had the greatest effect at the lower lidocaine concentration and both the 10 μM and 100 μM ciprofloxacin concentrations produced an equal decrease in activity at 250 μM. Both the 1 μM and 10 μM ciprofloxacin concentrations caused a slight activation in activity at the 500 μM substrate concentration.

Ketoconazole (Figure 10-6), a P4503A4 inhibitor, produced 66% and 47% (1 μM), 86% and 83% (10μM) and 92% and 91% inhibition of activity at the 100 and 250 μM substrate concentrations. The 10 and 100 μM ketoconazole concentrations produced 46% and 83% inhibition of activity at the 500 μM lidocaine concentration. The intersection of the lines in the Dixon plot (Figure 10-7) is characteristic of a non-competitive type of inhibition. The apparent Ki value was 95 μM.

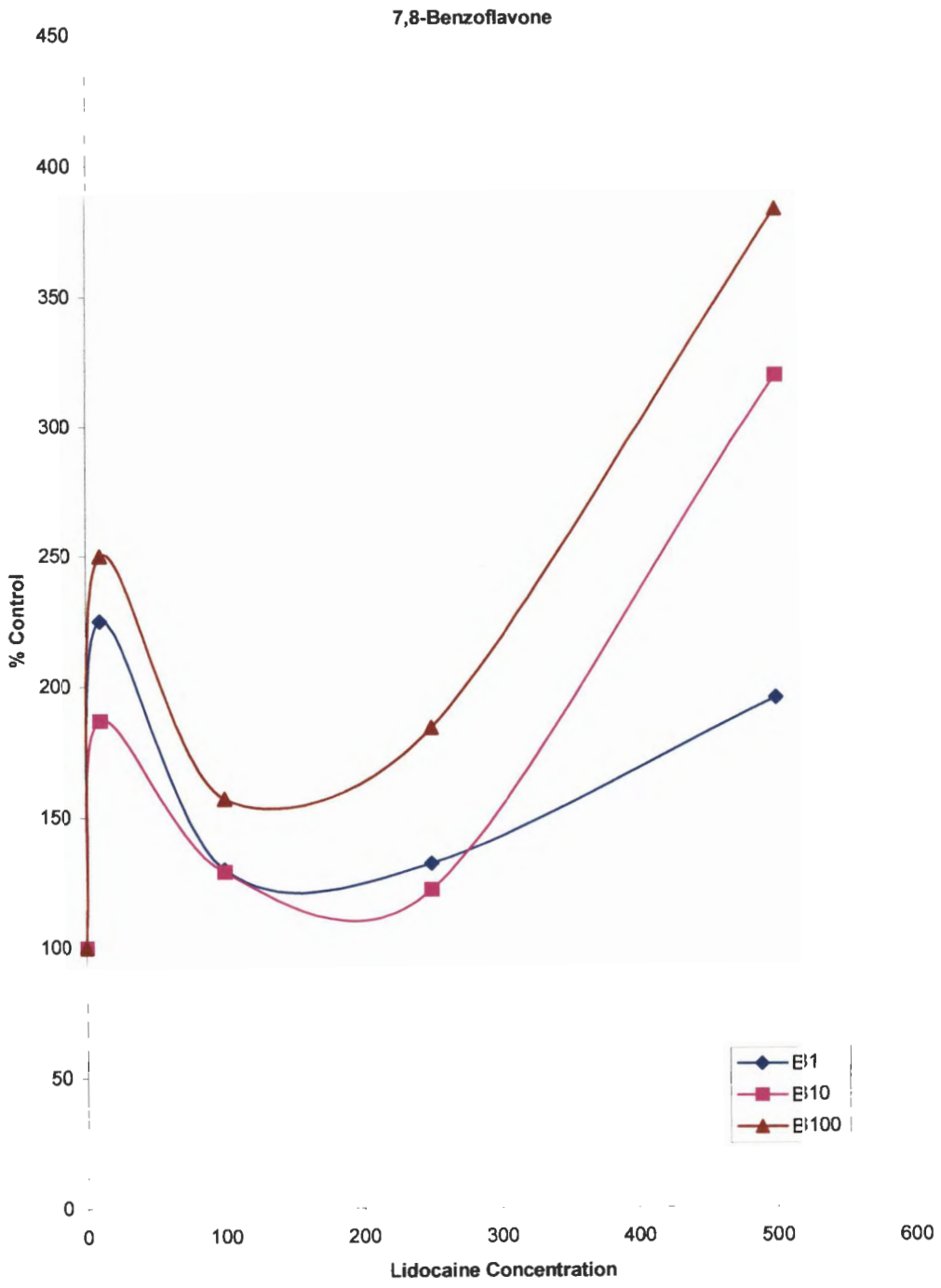

Figure 10-1. Effects of 7,8-benzoflavone on lidocaine N-deethylation.

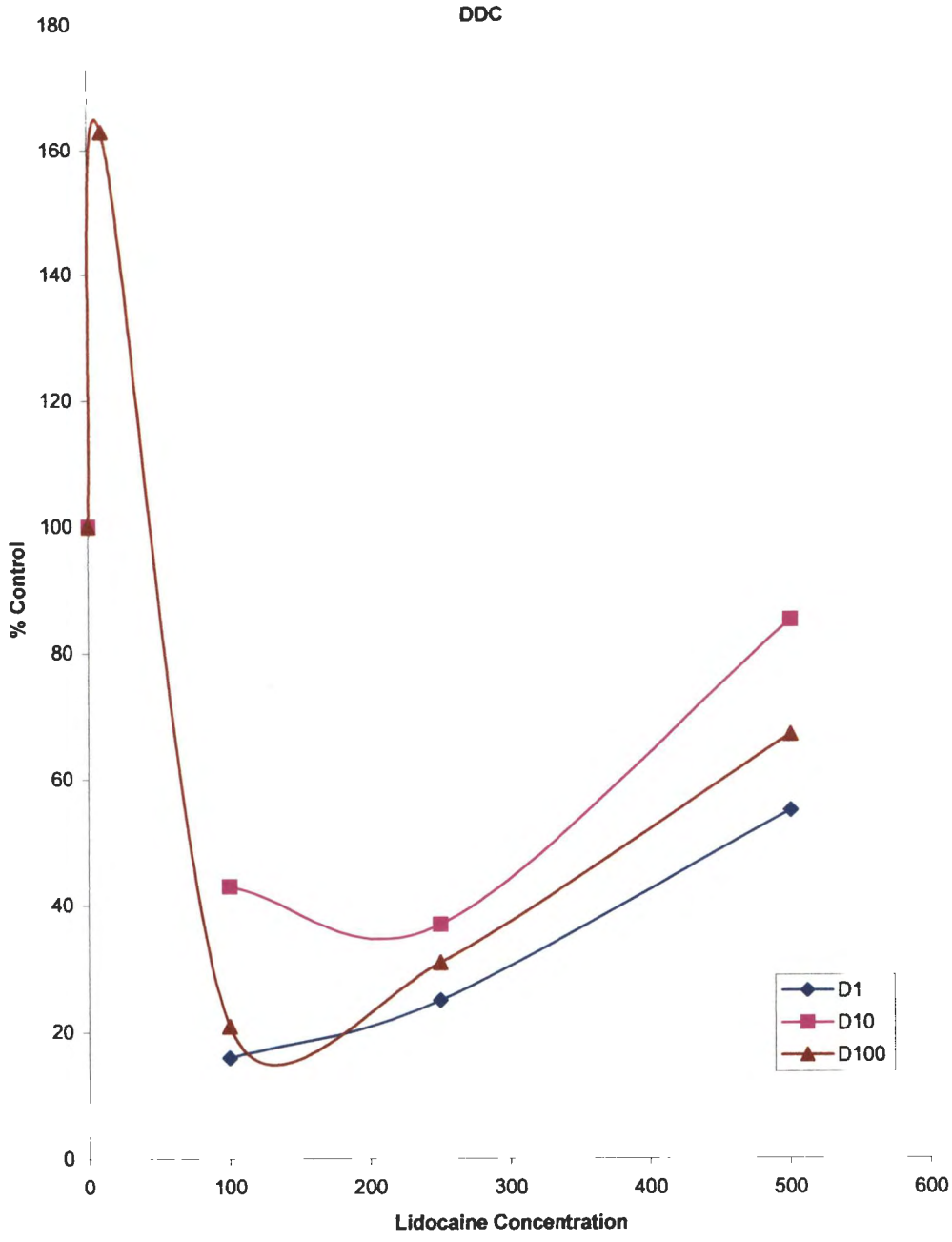

Figure 10-2. Effects of DDC on lidocaine N-deethylation.

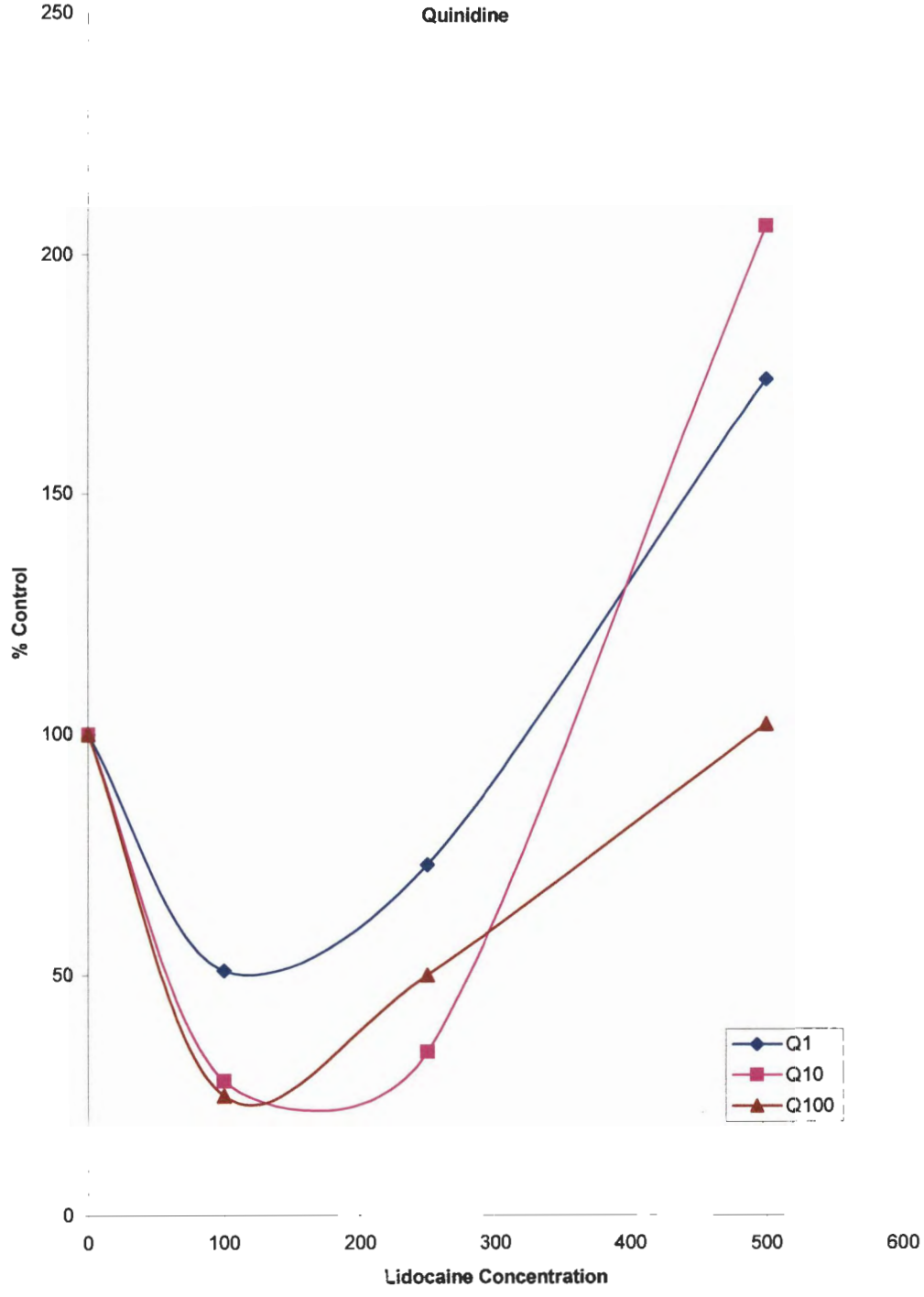

Figure 10-3. Effects of quinidine on lidocaine N-deethylation.

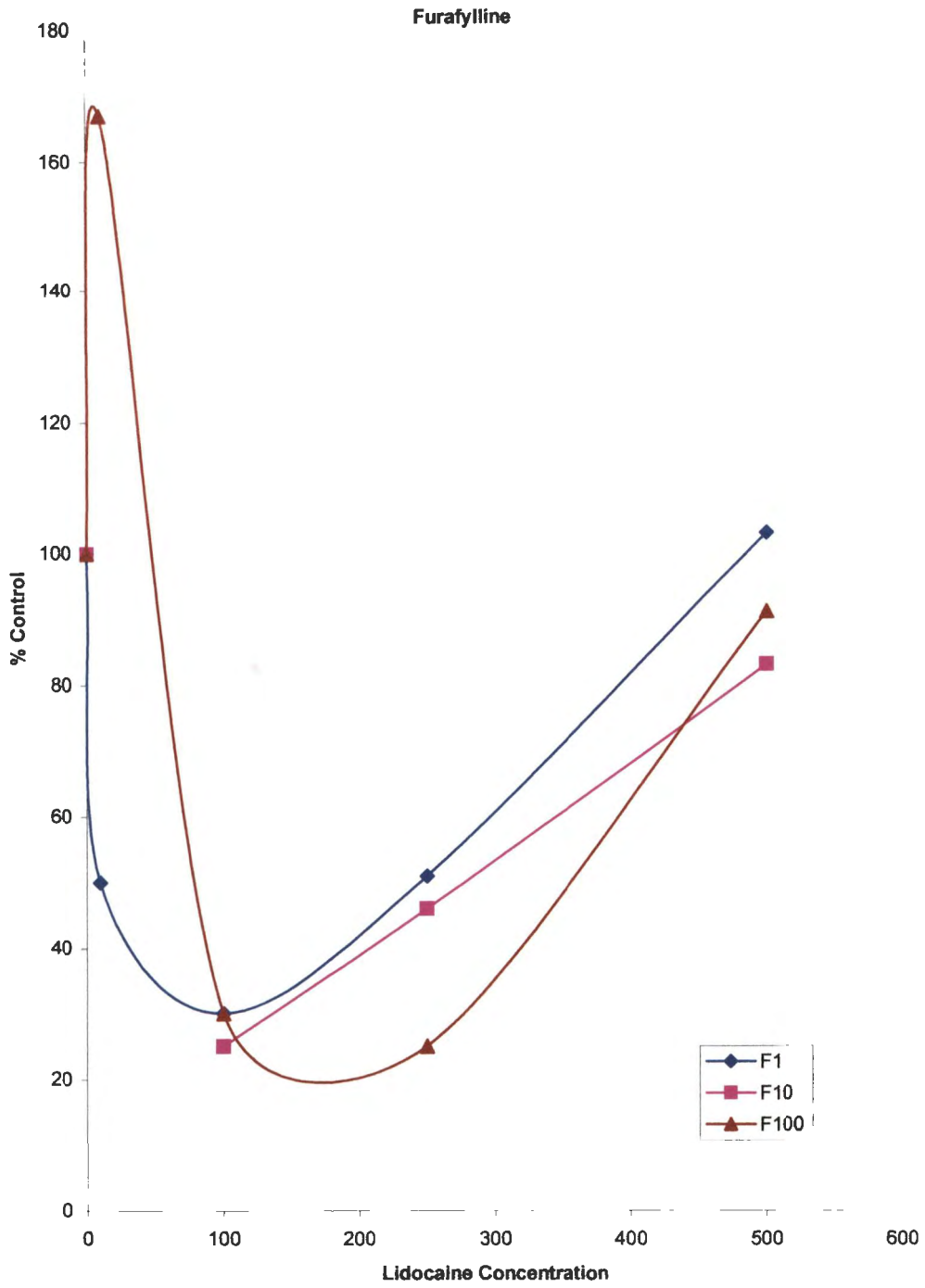

Figure 10-4. Effects of furafylline on lidocaine N-deethylation.

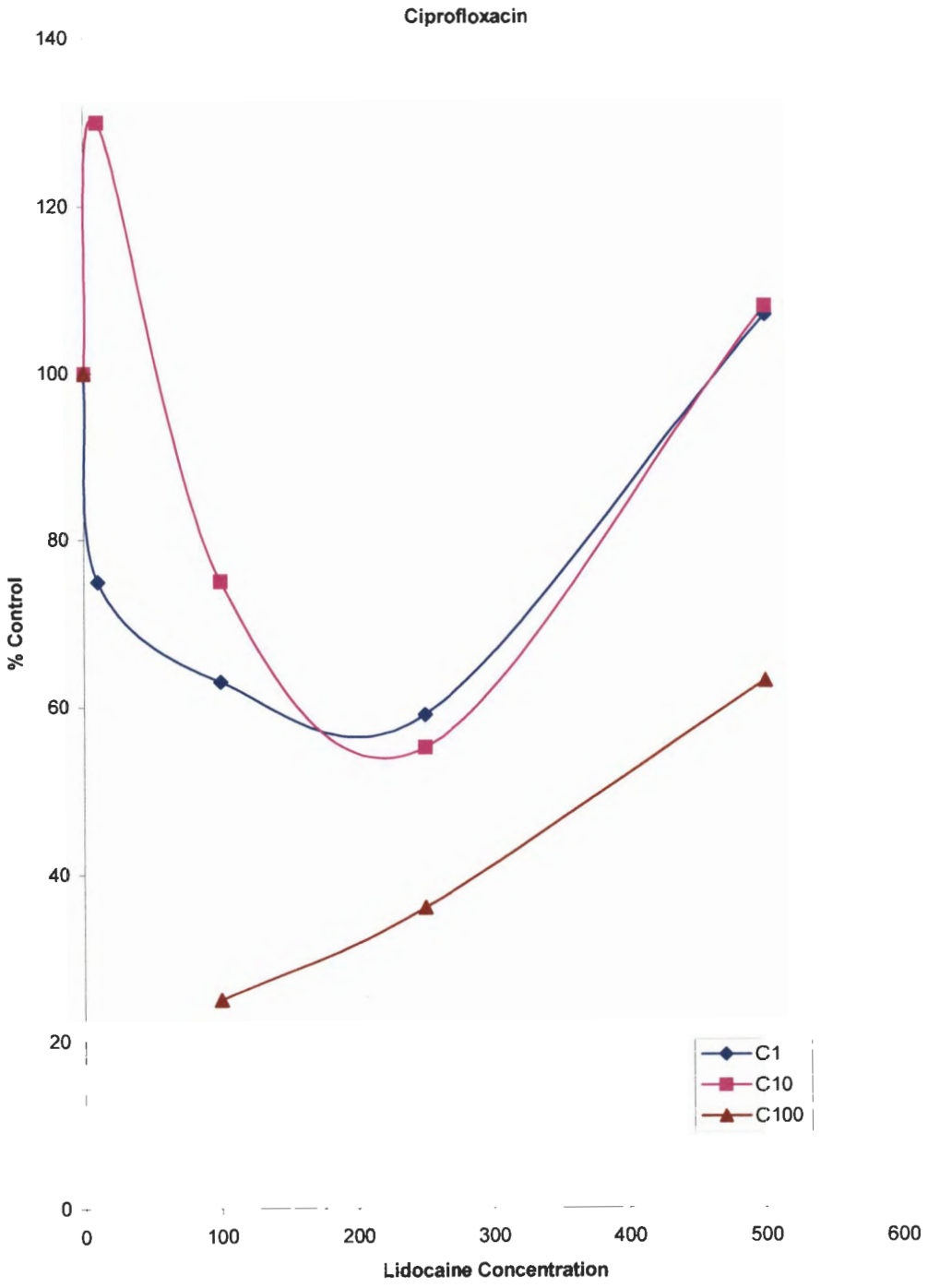

Figure 10-5. Effects of ciprofloxacin on lidocaine N-deethylation.

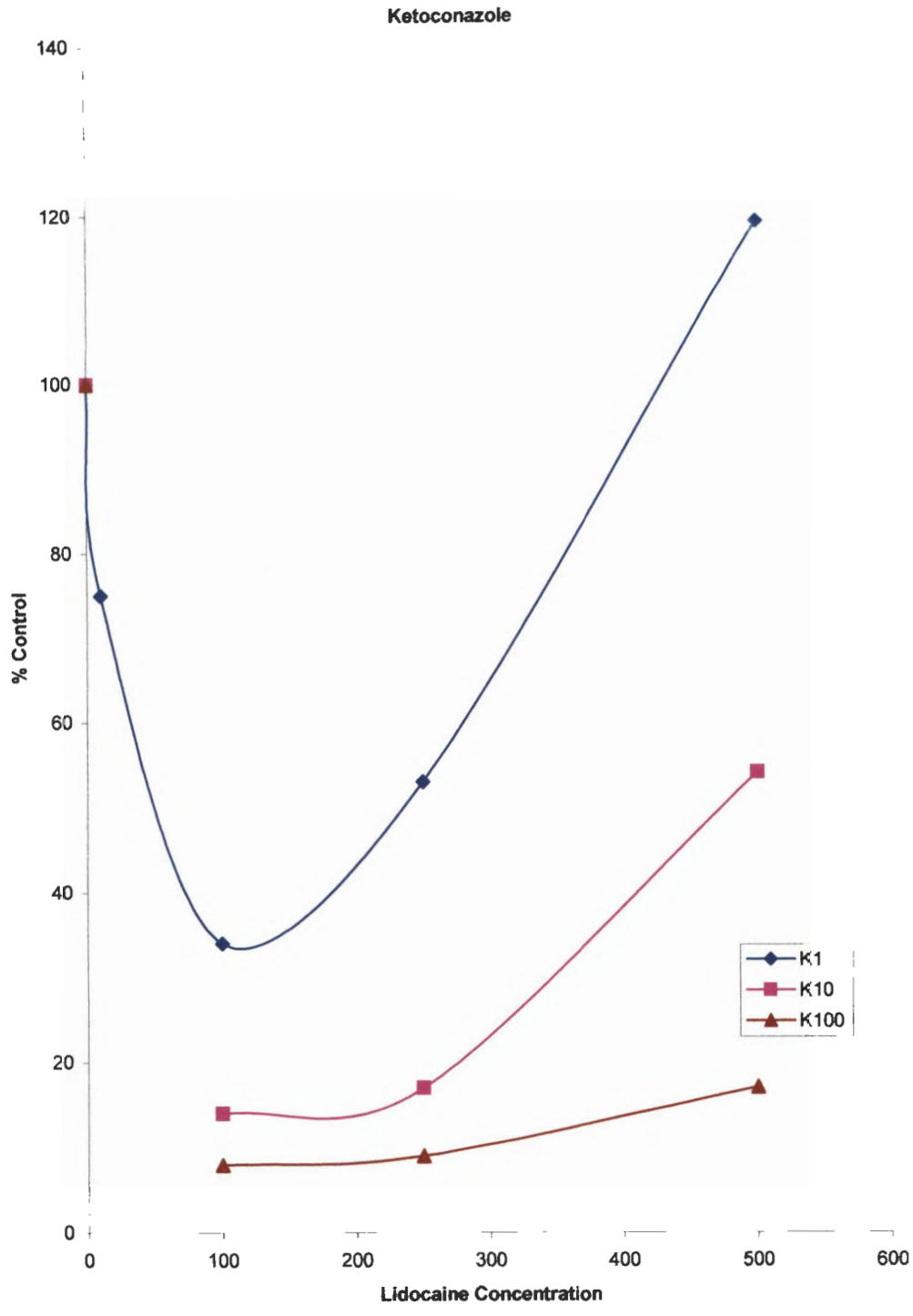

Figure 10-6. Effects of ketoconazole on lidocaine N-deethylation.

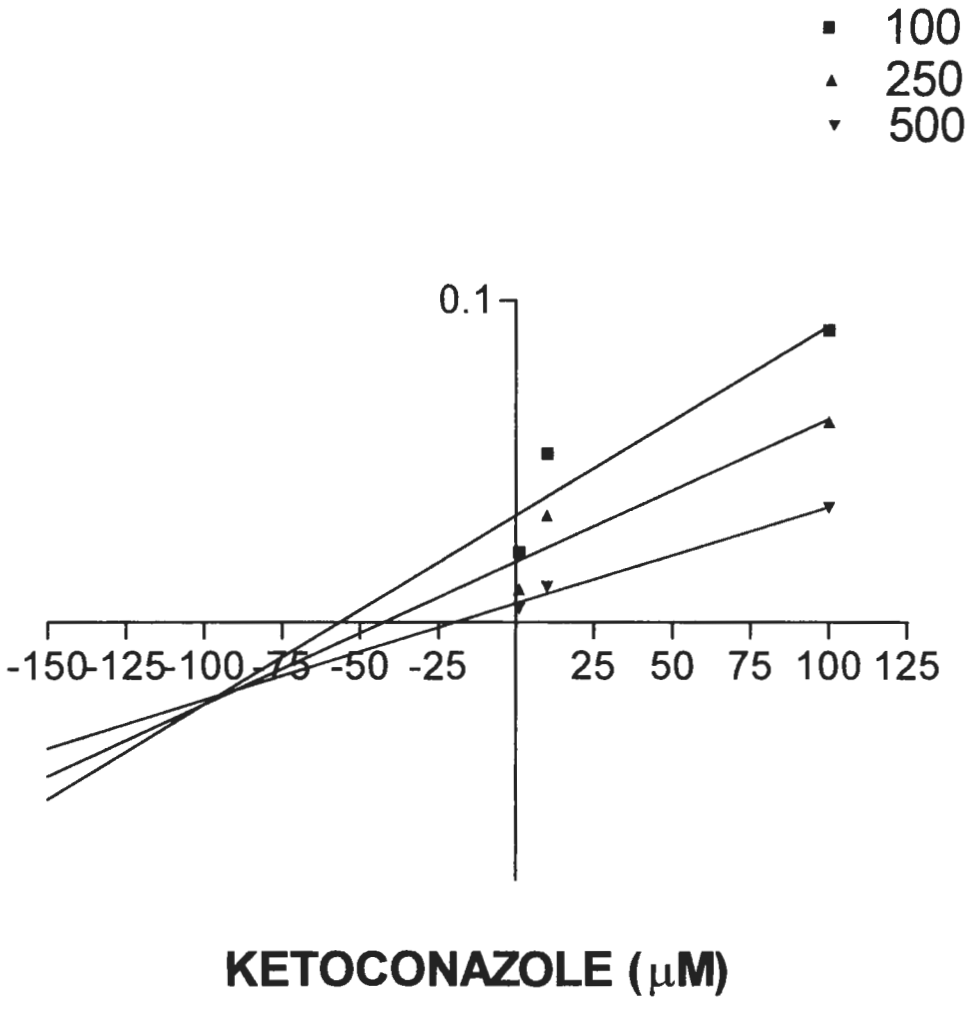

Figure 10-7. Dixon plot of the effects of ketoconazole on lidocaine N-deethylation.

Chapter 5: Discussion

In this study we evaluated the effects ciprofloxacin had on the metabolic activity of P450 3A4. As part of this process we also evaluated the effects that chemical inhibitors had on lidocaine N-deethylation in porcine liver microsomes. Experiments were conducted using a wide range of concentrations for both substrate and inhibitor to give more definitive results. Lidocaine and the five inhibitors are known to be specific for human P450 enzyme studies. In animals, effective inhibition can be expected if an enzyme is responsible for the activity and is homologous to the one reported for the activity of that specific probe.

Cytochrome P450 3A4 activity has been reported in pigs (Monshouwer et al., 1996; Skaanild and Friis, 1999) using testosterone as a probe. However to our knowledge it was not determined if this is a one or two enzyme Michaelis-Menten model. Studies (Nakajima et al., 2002; Wang et al., 2000) conducted in human liver microsomes indicated that the metabolism of lidocaine is biphasic, with a high affinity and low affinity component. The major enzymes involved in lidocaine biotransformation in humans were P4501A2 and 3A4.

Ketoconazole had the greatest inhibitory effect on lidocaine metabolism suggesting that 3A4 is involved in its metabolism. The biphasic kinetics might explain why furafylline, a mechanism-based inhibitor of P4501A2, produced an inhibitory effect on lidocaine deethylation. Ciprofloxacin also exerts its effects on P4501A2 and this may provide an explanation of why some reduction in activity occurred when it was used as an inhibitor.

Quinidine and DDC also produced some inhibition of lidocaine metabolism in porcine microsomes. Studies (Suzuki et al., 1993; Ohishi et al., 1993) conducted in rat liver microsomes indicated that lidocaine metabolism is mediated by an enzyme

belonging to the P4502D family. Therefore, it is possible that an enzyme from the 2D family could be involved in lidocaine metabolism in pigs. However, to our knowledge, no studies using lidocaine as a probe for P4503A4 enzyme activity or specific inhibitors have been conducted using porcine hepatic microsomes.

7,8- Benzoflavone produced stimulatory effect at all three concentrations used. This is in agreement with a study (Shou et al., 1994) conducted using human microsomes. Researchers found that 7,8-benzoflavone served as a substrate for P4503A4 and increased phenanthrene metabolism. Korzekwa et al. (1998) also found that 7,8-benzoflavone acts as good substrate for 3A4 in humans.

Most P450 oxidations follow standard Michaelis-Menten kinetic analyses (drug interactions can be predicted from inhibition studies). However, some P450 reactions have unusual enzyme kinetics, and most of these identified so far have been associated with P4503A4 (Korzekwa et al., 1998). The unusual kinetics fall into four categories: activation, autoactivation, partial inhibition and substrate inhibition. Atypical enzyme kinetics can also depend on the substrate and inhibitor employed in the study. Kinetic studies using hepatic microsomes contain a mixture of several P450 enzymes with overlapping specificity, and the observed rates of metabolism reflect the effect of several protein-drug interactions. In some cases, complications can arise due to the differing impact of several isoforms at different substrate concentrations (Houston and Kenworthy 2000).

In conclusion, the results of this study indicate that a P450 enzyme in porcine liver is responsible for lidocaine N-deethylation. Ketoconazole produced the greatest inhibition of enzyme activity indicating P4503A4 is probably involved. However, both ciprofloxacin and furafylline produced inhibitory effects, which indicates that the P4501A2 family is involved. Inhibition was also produced by quinidine and DDC,

indicating other enzymes are also involved. It is clear that these inhibitors do not exhibit the same selectivity in porcine microsomal studies as in human studies. This may be due to the differential selectivity of the inhibitors or it may be due to the involvement of completely different P450 enzymes. The need for further studies with different substrates and inhibitors at several concentrations are necessary to make this determination. There is a limited amount of information available on hepatic cytochrome P450 enzymes in domestic animals, including substrate and inhibition specificities. Keeping this in mind, these results should be used as guidelines for future studies.

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PART XI: CONCLUSIONS

The hypothesis of this study was that species-dependent differences in pharmacokinetic disposition of fluoroquinolone antibiotics are due to differences in distribution or activity of cytochrome P450 enzymes. Differences in pharmacokinetic parameters can indirectly affect development of bacterial resistance to quinolone antibiotics. The specific aims of the study were to: 1) identify differences in ciprofloxacin and enrofloxacin disposition by allometric analysis of pharmacokinetic data of different species, and 2) identify hepatic P450 metabolic pathways in pigs using specific substrates and enzyme inhibitors.

In order to determine if species differences existed in the disposition of the fluoroquinolones, allometric relationships were analyzed with regard to body weight, half-life, clearance, and volume of distribution. Previously published data from 20 different species of animals, including humans, were studied. Results of the allometric analyses indicated that there was a similarity between clearance and volume of distribution when related to body weights for both drugs. Absence of significant relationships for half-life of both drugs may be the result of variability in the rate or extent of drug biotransformation. Results of the current analyses indicate that it is possible to scale some pharmacokinetic variables of both enrofloxacin or ciprofloxacin based on body size of species. Further experimental allometric studies considering such factors as protein binding and specific conditions (sex, age, fasted or fed, and breed) of the animals should be conducted, which would better characterize the ability to scale across species.

The study of P450 metabolism in hepatic tissue of swine was a two-part process; 1) to see what effect ciprofloxacin had on the metabolism of four specific substrates that have been used to characterize human P450 enzyme activities; and 2) to look at the effects that other specific inhibitor probes had on the metabolism of these four substrates. In order to conduct these experiments, analytical methods had to be

developed to quantitate the different substrate biotransformation products that were produced by porcine microsomes. High performance liquid chromatographic methods were developed and validated for bufuralol, phenacetin, chlorzoxazone and lidocaine. This involved the selection of appropriate columns, mobile phases, and sample treatments. Each protocol was validated with regard to intra-assay and inter-assay variability, limit of detection and recovery.

Bufuralol, chlorozoxazone, phenacetin and lidocaine are specific substrate probes that have been used to characterize human cytochrome P450 enzymes (i.e., 2D6, 2E1, 1A2 and 3A4) while quinidine, DDC, furafylline, 7,8-benzoflavone and ketoconazole are specific inhibitors for these enzymatic reactions. These substrates and inhibitors were chosen to characterize porcine P450 enzymes as well as determine what effect ciprofloxacin had on the metabolism of these substrates.

The interpretation of enzyme inhibition data in animal species, in which little data on P450 enzymes are available, can be difficult. The results obtained indicate that a P450 enzyme in porcine liver is responsible for phenacetin O-deethylation, bufuralol 1'-hydroxylation, lidocaine N-deethylation and CZX 6-hydroxylation. The specific enzyme inhibitors (furafylline, 7,8-benzoflavone and quinidine) that were selected for the phenacetin and bufuralol studies failed to produce inhibitory effects. However, ketoconazole and DDC, which are specific inhibitor probes for lidocaine and CZX, did reduce the metabolism of their respective substrate. The results of the microsomal studies revealed that for each substrate more than one specific inhibitor probe could reduce the enzymatic activity, indicating that more than one enzyme is involved in their metabolism.

Ciprofloxacin exhibited slight inhibitory effects on bufuralol 1'-hydroxlation, CZX 6-hydroxylation and lidocaine N-deethylation. However, ciprofloxacin exerted its greatest

effect on phenacetin O-deethylation. Fluoroquinolones are thought to exert their effects on the P4501A enzyme family in humans, rodents and possibly companion animals. However, this may not be the case in swine since two specific inhibitors of the P4501A enzyme (furafylline and 7,8-benzoflavone) were without effect.

It is clear that the enzyme inhibitors that were chosen for these studies do not exhibit the same selectivity in porcine microsomes as humans, and that different microsomal enzymes may be responsible for metabolism of ciprofloxacin in swine. This may be due to the differential selectivity of the enzyme inhibitors, or due to the involvement of completely different P450 enzymes. Other explanations for the results could be differences in experimental conditions, the role of various cofactors, use of different buffers and discreet differences in the enzyme active site for certain substrate/inhibitor combinations. The activation and substrate inhibition may be highly dependent on a particular folding pattern of the protein. Slight changes in the flexible structure of the enzyme may alter the active site and influence the complex interactions between multiple molecules (Houston and Kenworthy, 2000).

Some of the results, especially those involving P4503A4, may be due to atypical enzyme kinetics. Most P450 oxidations follow standard Michaelis-Menten kinetic analyses (drug interactions can be predicted from inhibition studies) but, atypical enzyme kinetics can depend on the substrate and inhibitor employed in the study. Kinetic studies using hepatic microsomes contain a mixture of several P450 enzymes with overlapping specificity, and the observed rates of metabolism reflect the effect of several protein-drug interactions. In some cases, complications can arise due to the differing impact of several isoforms at different substrate concentrations (Houston and Kenworthy, 2000). Thus, there is a need for further studies with different substrates and inhibitors at several concentrations in order to determine which specific enzymes are

involved in the biotransformation process in porcine liver microsomes. Results of these studies provide a foundation for further definition of fluoroquinolone antibiotic disposition in swine. Further studies needed are: 1) use of the same substrates with different inhibitors; 2) use of different substrates with the same inhibitors; and 3) use of different concentrations of both substrates and inhibitors. In particular, studies should be conducted for comparative effect on fluoroquinolone disposition in food producing species with human health concerns.

The study results confirm that variability exists across species with regard to effect of P450 enzyme inhibitor and substrate utilization, and that caution must be exercised in extrapolating microsomal P450 enzyme kinetics on specific drug metabolism. The study results also have important implications for new drugs developed for domestic animals, which ideally would be tested on the target species since metabolism could be markedly different among species. Perhaps of greatest importance, these differences in kinetic handling of drugs like the quinolones have important implications for the development of resistant bacteria in addition to food residue concerns.

Houston J.B., Kenworthy K.E. 2000. In vitro-in vivo scaling of CYP kinetic data not consistent with the classical Michaelis-Menten model. Drug Metabolism and Disposition 28:246-254.

VITA

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