THE EFFECT OF DILTIAZEM AND NIFEDIPINE ON THE RATE OF
METABOLISM OF MIDAZOLAM

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Midazolam is the preferred benzodiazepine in anesthesia during the perioperative period because of its amnestic and anxiolytic properties, and short duration of action. Several clinical trials have reported prolonged sedative effects when midazolam is administered with a calcium channel blocker such as diltiazem. The cause of the prolonged half-life of midazolam could be competitive inhibition of midazolam metabolism at the level of the P450 CYP3A enzymes when concomitantly administered with calcium channel blockers. In this experimental, quantitative in vitro study we examined the rate of inhibition of the metabolism of midazolam when coincubated with the calcium channel blockers diltiazem and nifedipine using three human liver microsomes. The results of our in vitro study demonstrated that formation of alpha-hydroxymidazolam was inhibited by both nifedipine and diltiazem. Nifedipine was the most potent inhibitor with a Ki of 5.73 + 0.31 uM. Diltiazem was one order-of-magnitude less potent with a Ki of 80.7 + 28.7 uM. Kinetic analysis showed a mixed type inhibition for both nifedipine and diltiazem.
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ABSTRACT

Midazolam is the preferred benzodiazepine in anesthesia during the perioperative period because of its amnestic and anxiolytic properties, and short duration of action. Several clinical trials have reported prolonged sedative effects when midazolam is administered with a calcium channel blocker such as diltiazem. The cause of the prolonged half-life of midazolam could be competitive inhibition of midazolam's metabolism at the level of the P450 CYP3A enzymes when concomitantly administered with calcium channel blockers. In this experimental, quantitative in vitro study we examined the rate of inhibition of the metabolism of midazolam when coincubated with the calcium channel blockers diltiazem and nifedipine using three human liver microsomes. The results of our in vitro study demonstrated that formation of alpha-hydroxymidazolam was inhibited by both nifedipine and diltiazem. Nifedipine was the most potent inhibitor with a Ki of 5.73±0.31 uM. Diltiazem was one order-of-magnitude less potent with a Ki of 80.7±or-28.7uM. Kinetic analysis showed a mixed type inhibition for both nifedipine and diltiazem.

Key words: anesthesia, benzodiazepine, calcium channel blocker, CYP3A, diltiazem, inhibition, midazolam, nifedipine, perioperative.
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THE EFFECT OF DILTIAZEM AND NIFEDIPINE ON THE RATE OF METABOLISM OF MIDAZOLAM

By

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THESIS
Presented to the Graduate School of Nursing Faculty of The Uniformed Services University of the Health Sciences In Partial Fulfillment Of the Requirements For the Degree of

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PREFACE

The knowledge of drug-drug interactions is imperative to the anesthesia provider.

This research study was conducted to provide information regarding the *in vitro* pharmacokinetic interaction between the frequently used benzodiazepine, midazolam, and the calcium channel blockers, diltiazem and nifedipine.
DEDICATION

To my daughters, Amber and Heather, I dedicate this paper. Thank you for being so understanding of the many long hours that were spent to achieve this goal.
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CHAPTER I: INTRODUCTION

Background

Certified Registered Nurse Anesthetists (CRNAs) administer many drugs in the practice of anesthesia. At times, CRNAs can administer up to five or more drugs of different classifications during an operative procedure. This does not take into account the drugs the patient may be required to take due to a preexisting disease. The occurrence of drug interactions and adverse drug reactions which may harm patients increases as the number of drugs patients take increases (Bovill, 1997). Nurse anesthetists must be aware of the possible complications that can occur when a drug interacts with a second drug and pharmacokinetically alters the second drug's absorption, distribution, metabolism, or elimination capabilities.

Benzodiazepines are frequently used in the practice of anesthesia as a preanesthetic medication, as well as during the induction and maintenance of anesthesia. Midazolam is preferred in anesthesia over other benzodiazepines such as diazepam because of the shorter onset of action, greater potency, and rapid metabolic elimination (Hardman, Limbard, Molinoff, Ruddon, & Gilman, 1996).

Calcium channel blockers (CCBs) are used in the management of hypertension, angina, myocardial infarction, and as antiarrhythmic agents (Hardman et al., 1996). Calcium channel blockers are classified by the World Health Organization (WHO) into two groups according to their mechanism of action. Group A (verapamil, diltiazem, nifedipine, nicardipine) are CCBs that are selective for slow calcium channels where as Group B (diphenylpiperazines, prenylamine derivatives and perhexiline) are non-selective for calcium channels (Durand, Lehot, & Foex, 1991). Calcium Channel Blockers are also divided into different classes. There are five different classes of compounds that have been investigated. Diltiazem belongs to the benzothiapine group and nifedipine is the prototype drug in the dihydropyridine group. Diltiazem interacts with the calcium
channel receptor in a different fashion than nifedipine of the dihydropyridine group. Diltiazem is more selective in blocking the calcium-dependent cells in the atrioventricular node and is therefore more beneficial for tachycardias and arrhythmias. On the other hand, the dihydropyridine prototypical drug nifedipine is more selective for blocking the calcium channels in smooth muscle and therefore has a less depressant effect on the heart. (Katzung, 1998).

Calcium is the major extracellular cation that plays a key role in excitation-contraction coupling in myocardial cells. When calcium enters in the cell, stored intracellular calcium is released from the sarcoplasmic reticulum causing the muscle to contract due to the actin-myosin complex. Two types of receptors, the receptor operated channels (ROC) and the voltage-operated channels (VOC) allow for the entrance of calcium into cells. The VOC can be divided into three different subtypes, (L, T and N). The L-type calcium voltage sensitive channel predominates in cardiac and smooth muscle (Katzung, 1998). CCBs work by blocking the entrance of calcium into the cell, thereby inhibiting the excitation-contraction coupling. Diltiazem, a CCBs approved by the Food and Drug Administration (FDA) and used in the United States, selectively blocks the L-type VOC. All CCBs have been approved clinically to decrease coronary vascular resistance and increase coronary blood flow, although the hemodynamic effects vary depending upon the CCB used (Hardman et al., 1996).

Serum concentration increases and/or clearance rate reductions have been documented for several drugs used in combination with CCBs, including benzodiazepines such as midazolam (Durand, Lehot, & Foex 1991). Bovill reports that this interaction may occur because diltiazem and probably verapamil are metabolized by cytochrome P-450 3A (CYP 34A) and both drugs are potent inhibitors of this enzyme (Boville, 1997, pg. 75S). Midazolam is also a drug metabolized by the enzyme CYP 34A. When midazolam is concurrently metabolized by the CYP 34A enzyme with diltiazem, a known potent inhibitors of this enzyme, the pharmacokinetics of midazolam may be
altered in such a way that the bioavailability of midazolam is increased. This increase in availability can prolong the half-life of midazolam and thus increase the sedative effects of the drug (Bovill, 1997). The effect of diltiazem and verapamil on the pharmacokinetics of midazolam was examined in a double-blind randomized cross-over clinical trial using nine healthy volunteers. This study demonstrated that oral doses of diltiazem or verapamil given in combination with an oral dose of midazolam caused prolonged sedative and hypnotic effects (Backman, Olkkola, Aranko, Himberg, & Neuvonen, 1994). Backman et al. suggested that the cause was an inhibition of CYP 34A by diltiazem and verapamil reducing the metabolism of midazolam. The authors concluded that the dose of midazolam needs to be reduced when given concurrently with diltiazem or verapamil.

**Conceptual/Theoretical Framework**

Drugs are metabolized primarily in the liver by two major types of physiologic reactions which are called phase I and phase II reactions. Phase I biotransformation reactions involve removal or addition of a functional group from the drug, thereby making the drug a more polar metabolite. A polar metabolite can be excreted from the body in the urine. Phase II reactions comprise the conjugation of a drug with an endogenous compound such as glucuronic acid, sulfate, glutathione, amino acids or acetate. The conjugated drug becomes highly polar and inactive and is rapidly excreted in the urine and feces (Hardman et al., 1996). Cytochrome P-450 (CYP) is a collection of enzymes located primarily in the hepatocyte on the membranes of the smooth endoplasmic reticulum. Extrahepatic tissues, which include these organelles in the cells of the gastrointestinal tract, the brain, and the lungs also have cytochrome P450 activity. The hemeprotein P-450 absorbs light maximally at 450 nanometers under spectral analysis, which is how it derives its name (Katzung 1998). The P-450 enzymes are the major catalysts of drug biotransformation reactions during phase I of drug metabolism. Twelve different cytochrome substrate families have been identified in
human beings, with the CYP3A substrate responsible for the majority of drug metabolism (Hardman et al., 1996).

Induction and inhibition of the CYP enzymes affect the biotransformation of drugs. Induction increases the amount of P-450 enzyme producing a higher rate of drug biotransformation, decreasing the amount of a drug availability (Hardman et al., 1996). There are many enzyme inducers that may lead to serious drug to drug interactions including barbiturates and anticonvulsants (D Arcy, McElnay & Welling, 1996).

Inhibition of the P-450 enzyme system causes an increased amount of available drug due to decreased biotransformation, prolonging the effects of the drug (Hardman et al., 1996). Inhibition of an enzyme has greater significance in anesthesia because inhibition of the hepatic CYP enzymes, which metabolize most anesthetic drugs, may result in an increased drug effect (Bovill, 1997). When two drugs compete for the same binding site on an enzyme, as has been documented with midazolam, the metabolism of the lower affinity drug is decreased causing a prolonged pharmacological effect of the drug (Hardman et al., 1996).

History of Midazolam

Midazolam is a short-acting imidazo benzodiazepine first synthesized by investigators at the Roche Laboratories in Basel, Switzerland in 1976 (White, 1997) (See Figure 8 in appendix) Chlordiazepoxide was the first benzodiazepine submitted for pharmacological testing in 1957 by Dr. Leo Sternbach. Brandt and colleagues were the first to report using chlordiazepoxide (Brandt et al., 1962) as a integral part of their preanesthetic plan secondary to the drugs reported sedative and muscle relaxant qualities demonstrated in animal studies. It was soon discovered that the benzodiazepine molecule exhibited other characteristics to include calming effects, anticonvulsant properties, and appetite stimulation. The search was on for an analog of chlordiazepoxide that was more potent than the original compound. Diazepam was successfully introduced into practice in 1964 for the relief of preoperative anxiety. It was three to ten times more potent than
chlordiazepine. Several other benzodiazepines were discovered in the search for greater potency to include lorazepam and the active metabolite of diazepam known as oxazepam. The synthesis of midazolam in 1976 was unique in that it was the first water-soluble benzodiazepine that required no irritating solvents as a carrier solution so it was less irritating to the veins as opposed to diazepam. Other characteristics such as greater amnestic qualities and a shorter half-life have given midazolam the distinct honor of being one of the most widely used intravenous drugs in the practice of anesthesia.

Today, benzodiazepines are known for their principal pharmacologic effects to include: anxiolysis, sedation, spinal cord-mediated skeletal muscle relaxation, anticonvulsant actions, and anterograde amnesia. They are currently widely used in clinical practice for their anxiolysis and sedating properties, and not the skeletal muscle relaxation property, which is inadequate for surgical procedures (Stoelting, 1998). Benzodiazepines such as midazolam, diazepam, lorazepam all share the same basic chemical structure composed of a benzene ring that is fused together with a seven-membered diazepine ring. Variations on the basic 5-aryl-1,4-benzodiazepine structure have yielded different compounds.

**Mechanism of Action of Benzodiazepines**

The Gamma-amino-butyric acid (GABA)-benzodiazepine receptor complex was first isolated and discovered in 1977 (White, 1997). Two GABA receptors have been identified, the GABA-alpha and GABA-beta receptors. The GABA-alpha receptor not only has binding sites for GABA and benzodiazepines, but also has separate binding sites for barbiturates and alcohol explaining the synergistic effects that can be produced by combining barbiturates, benzodiazepines and alcohol. Benzodiazepines produce their pharmacologic effects by directly binding to a specific site on the GABA-alpha receptor and allosterically modulating its activity (Hardman et al., 1996). Unlike GABA-alpha receptors, GABA-beta receptors are not altered by benzodiazepines.

Gamma-amino-butyric acid is the principal inhibitory neurotransmitter in the central
nervous system (CNS). Upon binding to the specific site on the GABA-alpha receptor, BZDs increase the affinity of this inhibitory neurotransmitter for the GABA receptor (Stoelting, 1998). This increased affinity of the receptor for GABA results in enhanced opening of gated chloride channels producing hyperpolarization of the postsynaptic membrane. Hyperpolarized neurons are more resistant to excitation, therefore explaining the proposed mechanism that benzodiazepines can produce their pharmacologic effects of anxiolysis, sedation, skeletal muscle relaxation and anticonvulsant activity. GABA-alpha receptors are primarily found in the central nervous system with the highest density if receptors found in the cerebral cortex.

**Chemical and Physical Properties of Midazolam**

Midazolam is unique in its chemical structure as a benzodiazepine in that it contains an imidazole ring in its structure. The imidazole ring gives midazolam its ability to be rapidly metabolized and remain stable in aqueous solutions (Stoelting, 1998). The midazolam structure also undergoes a pH dependent ring-opening phenomenon. Incorrectly stated in some literature, it is not the imidazole ring that opens and closes; conversely, it is the benzene ring in the midazolam structure that undergoes this pH dependent phenomenon. The benzene ring remains open in a pH environment less than 4 and therefore remains water soluble. In physiologic pH or a pH greater than 4 the benzene ring closes and midazolam becomes a highly lipid soluble drug. Midazolam has a pK of 6.5 and is prepared for clinical use by buffering the parental solution to an acidic pH of 3.5, thereby maintaining the drug in its water soluble state. Unlike diazepam, the water-soluble preparation of midazolam negates the need for a preparation such as propylene glycol to make it soluble and therefore, less burning occurs with midazolam upon intravenous administration.

Upon therapeutic intravenous administration of midazolam, minimal cardiovascular changes are observed in man, except for a minimal increase in heart rate. Respiratory changes include a decrease in tidal volume that does not affect minute volume due to
compensatory increase in respiratory rate. Midazolam is administered with caution because central depression of respiration can occur with repeated doses. Midazolam causes minimal changes in cerebral and coronary blood flow, however, a decrease in renal blood flow and function is noted (Dundee, Halliday, Harper, & Brogden 1984).

When administered orally, midazolam peak plasma concentrations occur in approximately 30 minutes. Administered intravenously, the peak concentration of midazolam occurs in about 15 minutes (Dundee et al., 1984). Midazolam is 96% bound to plasma proteins which prevents it from dispersing into lipid tissues, thus only about 4 percent is pharmaceutically active (Stoelting, 1998). Extensive in vitro studies demonstrate that midazolam is metabolized exclusively by P450 3A3, 3A4, and 3A5 enzymes (Bocker, Bohrer, Browne, Rugheimer, & Martin, 1994). The two metabolites, 1-hydroxymidazolam and 4-hydroxymidazolam, are converted in a rapid fashion to glucuronide conjugates and excreted in the urine (Paine et al., 1996).

**In Vitro Methods for Determining Human Hepatic Drug Metabolism**

In Vitro studies are a useful method of studying drug metabolism when long-term toxicology human studies are not possible because of the high risk for mortality and morbidity. They can identify drug metabolites and measure drug to drug interactions and environmental influences of drug metabolism directly on human tissue in a controlled environment (Wrighton, Vandenbranden, Stevens, Shipley & Ring, 1993).

The four in vitro methods used in human liver drug metabolism studies are experiments that use hepatic subcellular fractions, individual forms of cytochrome P450 phase II enzymes, isolated hepatocytes and liver slices. This study will be using the human liver slice method which has been shown to replicate the liver's metabolic capabilities (Wrighton et al., 1993).
Statement of the Problem

Adverse drug interactions can result in patient morbidity and mortality. Knowledge of adverse drug interactions is imperative in the practice of anesthesia. In 1994, it was reported that oral administration of CCB diltiazem or verapamil with midazolam prolonged the sedative and hypnotic effects of midazolam in nine healthy volunteers (Backman et al., 1994). Investigation into the effect of CCB on the metabolism of midazolam was warranted because these drugs are frequently administered in the practice of anesthesia.

Purpose of Study

The purpose of this study was to measure the rate of metabolism of midazolam in the presence of the calcium channel blockers diltiazem and nifedipine in a human liver microsome in vitro model. The research hypothesis was: Significant inhibition of the biotransformation of midazolam will occur in the presence of diltiazem and nifedipine.

Limitations of Study

1. This research was being performed in vitro, so it cannot be fully determined if the same results would be observed in vivo.
2. The sample size was limited by the amount of liver microsomes available for the experiment.
3. The microsome sample contained only microsomes from three human cadaver livers.
Assumptions of Study

1. The liver microsomes contained the same protein composition, thus yielding uniform results.
2. The stability of the drug metabolizing enzymes was not altered during culture and storage.
3. The sample of microsomes represented the microsome of the general population.
4. The liver was the major route of metabolism.

Definition of Terms

2. Bioavailability. The fraction of unchanged drug the systemic circulation following administration by any route (Katzung, 1998).
3. Biotransformation. The conversion of xenobiotics from one form to another within an organism associated with a change in pharmacologic activity (Katzung, 1998).
4. Calcium channel blocker (CCB). A group of drugs that block L-type voltage sensitive channels in arterial smooth muscle relaxation and vasodilation (Hardman et al., 1996).
5. Centrifugation. The process of separating the lighter portion of a solution, mixture or suspension from the heavier portions by centrifugal force (Katzung, 1998).
6. Half-Life. The time (in hours) required for the drug concentration in the plasma to decrease by 50% (Katzung, 1998).
7. **Metabolism.** The sum of the chemical changes occurring in living tissue, including anabolism and catabolism, and the biotransformation of xenobiotics (Katzung, 1998).

8. **Microsomes.** Particles derived from the endoplasmic reticulum of cell nuclei obtained when cells are broken up by centrifuging with a force 100,000 times that of gravity (Thomas, 1989).

9. **Pharmacodynamics.** The study of the intrinsic sensitivity or responsiveness of receptors to a drug and the mechanism by which these effects occur (Katzung, 1998).

10. **Pharmacokinetics.** The quantitative study of the absorption, distribution, metabolism and excretion of injected drugs and their metabolites (Katzung, 1998).

11. **Volume of Distribution.** The amount of drug present in the body divided by the concentration of the drug in the plasma expressed in liters (Katzung, 1998).

12. **Xenobiotic.** An antibiotic chemical substance not produced by the body, and thus foreign to it (Thomas, 1989).
CHAPTER II: REVIEW OF THE LITERATURE

Introduction

Benzodiazepines (BZD) are frequently used in the practice of anesthesia because of their many favorable pharmacologic characteristics. These characteristics include production of anterograde amnesia, minimal depression of ventilation or the cardiovascular system, specific site of action as anticonvulsants, relative safety if taken in overdose, and rarity of abuse or development of significant physical dependence (Stoelting, 1991). Midazolam is the preferred BZD in anesthesia because of the shorter onset of action, greater potency, and rapid metabolic elimination compared to other BZDs such as diazepam (Hardman et al., 1996).

Despite midazolam’s favorable pharmacological characteristics, caution must be taken when administering this drug. In 1987 the Food and Drug Administration issued a bulletin warning about the use of midazolam with other drugs (FDA Drug Bulletin, 1987)). In 1988 the FDA again issued a bulletin stating that patients receiving midazolam must be carefully monitored (FDA Drug Bulletin, 1988). These warnings were issued due to several reports of apnea and other respiratory problems associated with the use of midazolam with other central nervous system depressants. Since midazolam’s approval by the FDA in 1985 and marketing for use in 1986, the literature has several documented cases of adverse effects related to the use of midazolam. The adverse outcomes were due to problems associated with clearance of the drug, accumulation of conjugated metabolites, and interactions with other drugs. Since the majority of drugs are metabolized by the CYP3A substrate, the potential for drug to drug interactions exists (Hardman et al., 1996).

Inhibition Studies of Midazolam

Erythromycin inhibits midazolam in vitro and several documented cases reported in the literature demonstrating this adverse affect in vivo. A case report in an article published by Hiller, Olkkola, Isohanni, and Saarnivaara in 1990 discussed the incidence
of an 8 year old boy suffering from asymptomatic ventricular septal defect who was given erythromycin as prophylaxis prior to surgery to have his adenoids removed. He received 0.5 mg/kg of midazolam orally as a premedication. After 200 mg of erythromycin had infused the boy lost consciousness. 45 minutes later the boy awoke spontaneously. Plasma concentration of midazolam 170 minutes after the premedication dose was given, was 134 ng/ml, a significantly greater plasma concentration than expected. In another case report discussing adverse effects of midazolam and erythromycin, a 61 year old man was admitted to the coronary care unit for atrial fibrillation. The patient was receiving erythromycin for suspected legionnaire's disease. After a total of 300 mg of midazolam was given over 14 hours for sedation for electrical cardioversion, the patient did not wake up for six days. The measured terminal half-life of midazolam in this patient was 24.8 hours. The normal expected half-life of midazolam is 1.5-2.5 hrs. The study concluded that competitive inhibition of midazolam and erythromycin by the same P-450 substrate caused the significant prolongation of midazolam's half-life (Gascon & Dayer, 1991).

**Conjugated Metabolites of Midazolam**

When midazolam is hydroxylated by the CYP3a enzyme during the first phase of metabolism two metabolites are formed. The major metabolite is alpha-hydroxymidazolam. This metabolite can be as sedative as the parent drug midazolam. The minor metabolite formed is 4-hydroxymidazolam. This metabolite is quantitatively unimportant. During the second phase of metabolism both metabolites are conjugated to glucuronides in the liver and eliminated in the kidneys. Conjugated glucuronides are commonly inactive, with the exception of morphine's metabolite. Accumulation of inactive metabolites should therefore not pose a threat of toxicity to the patient. However, in a study by Bauer and colleagues, the first to investigate the role of the conjugated metabolites in patients with unexplained prolonged sedation of midazolam, high concentrations of alpha-hydroxymidazolam glucuronide (5000-7000 ng/mL) were found
in patients who remained over sedated. These patients remained sedated despite a midazolam concentration (80 ng/mL) well below the normal sedative threshold. The study concluded that the conjugated alpha-hydroxymidazolam should be considered when the patient has a clinically questionable prolonged sedation with midazolam (Bauer et al., 1995).

Calcium Channel Blockers and Midazolam

In a study published in Anesthesia Analog, the calcium channel blockers, diltiazem and verapamil, were shown to be effective in attenuating the cardiovascular responses such as tachycardia and hypertension that can occur during tracheal intubation (Mikawa, Nishina, Maekawa, & Obara 1996). Since midazolam is often given as a premedication for sedation in anesthetic procedures, the probability that these two drugs will be given concurrently is high.

Calcium channel blockers were widely used in Japan and Europe before 1981 (Merin, 1987); however, it was not until 1981 when studies about apparent drug interactions with calcium channel blockers and anesthetics began to appear. At least 20 different studies have been published discussing the in vitro interactions of calcium channel blockers and the inhalation agents used in anesthesia; however, limited information has been published concerning the in vitro reactions of the calcium channel blocker diltiazem and the frequently used benzodiazepine midazolam.

Clinical Studies with Midazolam and Diltiazem

Clinical trials have been published in recent years that demonstrate the need for further investigation into the in vitro interactions of diltiazem and midazolam. A double-blind randomized cross-over study published in 1994 aimed to show the effects of diltiazem and verapamil on the pharmacodynamics and pharmacokinetics of midazolam. Nine healthy female volunteers were given oral doses of diltiazem and verapamil in the first two days and an oral dose of midazolam was also given on the second day. Plasma samples were then collected. The results of the study revealed the peak midazolam
concentration was doubled (P < 0.01) and the elimination half-life of midazolam prolonged (P < 0.05) by both verapamil and diltiazem treatments. Prolonged and profound sedative effects were also observed. The study concluded that if the administration of midazolam could not be avoided than the dose of midazolam should be reduced if given with diltiazem or verapamil (Backman et al., 1994). In another study published in 1996, 30 patients having coronary artery bypass grafting, were randomly assigned to receive either diltiazem two hours before induction of anesthesia with midazolam or a placebo in a double-blind study. The results of the study were that the mean half-life of midazolam was 43% (p < 0.05) longer in patients who received diltiazem. Tracheal extubation was also delayed on average 2.5 hours later (P=0.054) in the patients who received diltiazem than in those who received a placebo. The conclusion of the study stated that diltiazem slowed the elimination of midazolam and therefore delayed tracheal extubation (Ahonen, Olkkola, Salmenpera, Hynynen, & Neuvonen, 1996).

Nifedipine in Anesthesia

Midazolam is an appropriate drug for induction of anesthesia for cardiac surgery because it does not cause detrimental hemodynamic changes when used alone (Yamaguchi, Kanmura, & Yoshimura, 1997). Calcium channel blockers that are structurally similar to nifedipine are used advantageously for the treatment of hypertension and cerebral and coronary vasospasm during anesthesia (Kazuo, 1993). The possibility of giving nifedipine and midazolam together preoperatively is extremely high. Limited research exists concerning the effect of nifedipine on the rate of midazolam’s metabolism. Does the dihydropyridine group of calcium channel blockers, such as nifedipine, have the same effect on midazolam as the reported effects of diltiazem of the benzothiapine group of calcium channel blockers? This question will be researched in this study. Clinical trials have only theorized that there may be interactions between diltiazem and midazolam at the level the CYP3A substrate.
Therefore, it is the purpose of this *in vitro* study investigate the effects of nifedipine on midazolam as well as to quantify the effects of diltiazem on the metabolism of midazolam. The results of this study will prove beneficial to clinicians since previous investigations concerning this matter are limited.
CHAPTER III: METHODOLOGY

Research Design

The methodology adopted for this research was developed by Dr. Louis R. Cantilena, Jr. in 1997 (Appendix A) and has been used successfully by other USUHS students for thesis work involving in vitro studies of midazolam. Hinkle (1997) was the first student to test the midazolam standard of procedure (SOP) for in vitro studies. It was necessary, for the purpose of this study, to make adjustments to the midazolam assay used by Hinkle. The design of this experiment consisted of four phases: (a) preparation of the microsomes, (b) incubation of midazolam, (c) extraction of midazolam's metabolites, and (d) high performance liquid chromatography (HPLC) analysis. The revisions made to the experiment design occurred in the HPLC phase. A shorter 75 milliliter (ml) absorbent column donated from the Phenomenex Company was used to allow for quicker HPLC run times of 20 minutes. The temperature of the column during HPLC analysis was maintained at 35 degrees Celsius. To provide an amicable environment for elution of midazolam, an 80:20 ratio of acetonitrile and HPLC grade water, called mobile phase, was used to facilitate the movement of midazolam through the column. A 0.5% solution of formic acid was added to the mobile phase to render an appropriate pH for midazolam. The mobile phase solution was pumped through the HPLC at a rate of 0.3ml/min. The flow rate gradients was adjusted for improved elution of midazolam and its metabolites. The flow rate gradients (time in min: flow rate in min/ml) for this experiments were 0:0.3, 10:0.35, 12:0.6, 14:0.6, and 15:0.3. The gradient conditions (time in min: % formic acid in acetonitrile) were 0:20, 10:55, 12:98, 14:98, and 15:20.

Preparation of Drugs

Preparing midazolam for the experiment first required dissolving it into 0.32 millimolar (mM) of ethanol. After properly dissolving the midazolam it was then diluted to the suitable concentrations of 0.5, 2.5, and 5.0 micromolar (uM). To promote validity of the experiment, lorazepam, a BZD similar to midazolam, was used as the internal
standard by which the peaks of midazolam were compared to during HPLC analysis. Lorazepam was dissolved in 3.11mM of ethanol and diluted to a concentration of 5.0 mM. The calcium channel blockers nifedipine and diltiazem were diluted to the concentrations 0.5, 1.0, 5.0, and 10.0 mM.

**Sampling and Setting**

This *in vitro* study used human liver microsomes. The sample size included three livers that were donated in 1997 for scientific research from the Washington Regional Transplant Consortium, Washington, D.C. When the livers were harvested from the donors they were sliced into cubes and their protein concentrations standardized to 0.2 mg/ml (Hinkle, 1997). The liver microsomes, packed in 50ml vials, were stored in liquid nitrogen in a freezer at USUSH at —80 degrees Celsius until it is necessary to thaw them for research. The identity of the donors is protected. Information to track the donors only included coded, descriptive data describing the gender and previous medication history. The USUHS Institutional Review Board (IRB) has approved the liver samples for research.

When it was time to perform the experiment, the stored microsomes were thawed. Samples from the three different microsomes were placed in separate test tubes and were run in triplicates for a total of 135 samples plus controls. Added to the 135 test tubes containing liver microsomes and buffer solution was a NADPH generating system that served as the catalyst for the reaction. The generating system was comprised of 423 mg of glucose 6 phosphate, 126 mg of NADP+, 1230 uL of buffer, and 270 uL of glucose 6 phosphate dehydrogenase. Finally the calcium channel blockers were added to the test tubes in four different concentrations. The test tubes were then placed in ice until it was time to start the reaction.
Incubation of Midazolam

To begin the incubation phase of the experiment, the test tubes were systematically removed from the ice and placed in a 37 degrees Celsius water shaker bath. The midazolam, in three different concentrations, was added to the test tubes and incubated for five minutes beginning at zero minutes. The test tubes were removed from the warmed water bath and placed in ice to stop the reaction. At this time 200 uL of the internal standard lorazepam was added to the test tubes.

Extraction of the Metabolites

Prior to HPLC analysis, it was necessary to extract the metabolites from the solution in the test tubes and remove any undesired protein. To achieve this, 5ml of acetonitrile was added to the test tubes and the test tubes were vortexed for 10 minutes. The test tubes were then centrifuged for 10 minutes, the contents of the test tubes transferred to clean tubes and then placed in a speed vacuum apparatus for the first evaporation phase. Once evaporated, twenty uL of acetonitrile and HPLC grade water (1:1) was added to the test tubes to reconstitute the contents and then vortexed for three minutes. An additional two mls of acetonitrile was added and the tubes vortexed for another 3 minutes. The tubes were centrifuged again for 10 minutes and then evaporated for the second time. After the evaporation process, 20uL of acetonitrile and water (1:1) was added to the tubes and vortexed for two minutes. 20 uL of HPLC grade water will then be added to the tubes and the tubes vortexed for an additional two minutes. Using a pipet, the contents of each test tubes were extracted and transferred to microvials and then placed into the HPLC machine. The HPLC system separated out the peak of midazolam from the peak of it s metabolite alpha-hydroxymidazolam. The peak of lorazepam was also visualized and used as the standard to compare midazolam s peaks.
Plan for Data Analysis

The height of the area under the peaks of midazolam and alpha hydroxymidazolam were entered into the Statistical Package for the Social Sciences (SPSS) database. The SPSS database calculated the data and the information provided was plotted on Line-Weaver Burke graphs to determine the percent of inhibition of midazolam by the calcium channel blockers, diltiazem and nifedipine.
CHAPTER IV: DATA ANALYSIS

Introduction

The design of this *in vitro* study was to investigate drug-drug interactions between midazolam and the calcium channel blockers (CCB) diltiazem and nifedipine. The benzodiazepine midazolam has two metabolites: alpha-hydroxy and 4-hydroxymidazolam. The effect of the CCBs diltiazem and nifedipine on the rate of the metabolism of midazolam to its major metabolite Alpha-hydroxymidazolam was measured in this study.

Demographics of the Liver Donors

The microsomes used for this study were obtained through the Washington Regional Transplant Consortium in Washington, D.C., prepared from cadaver livers found unsuitable for transplantation. The microsomes were stored at —80 C until use. See Table 1 for the demographic data of the three livers used for this study.

Table 1.

Liver Donor Data.

<table>
<thead>
<tr>
<th>Liver Code</th>
<th>Age</th>
<th>Race</th>
<th>Gender</th>
<th>Significant Medications</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>35</td>
<td>Caucasian</td>
<td>Male</td>
<td>Dopamine</td>
</tr>
<tr>
<td>18</td>
<td>28</td>
<td>Caucasian</td>
<td>Female</td>
<td>Cocaine, ETOH</td>
</tr>
<tr>
<td>16</td>
<td>30</td>
<td>Caucasian</td>
<td>Female</td>
<td>Cocaine, ETOH, Prozac</td>
</tr>
</tbody>
</table>
A total of 135 samples excluding controls were analyzed in this study using the High Performance Liquid Chromatography (HPLC) system to measure midazolam, its metabolite (alpha-hydroxymidazolam), and the internal standard lorazepam. Separation of Midazolam from its metabolite and the internal standard lorazepam gave retention times averaging 17.0, 19.6, and 28.4 respectively. The concentrations of alpha-hydroxymidazolam were calculated using standard curves of authentic standards prepared in microsomal medium with a protein concentration of 0.2 mg/ml with no NADPH. Figure 1 is a representation of a typical chromatogram of midazolam, its metabolite, and the internal standard.

Figure 1.

Typical Chromatogram of MDZ, MDZ Hydroxylated, and the Internal Standard.

Michaelis and Menton proposed a simple model that accounts for most of the features of enzyme-catalyzed reactions (Champe & Harvey, 1994). The Michaelis-Menton equation describes how velocity varies with substrate concentration:

\[ V_0 = \frac{V_{max} \cdot [S]}{K_m + [S]} \]

Where:
- \( V_0 \) = initial velocity reaction
- \( V_{max} \) = maximal velocity
- \( K_m \) = Michaelis constant
- \([S]\) = substrate concentration

Most enzymes show Michaelis-Menton kinetics. Figure 2 below shows the characteristic hyperbolic shape that occurs with the plot of reaction velocity (\( V_0 \)), against substrate concentration \([S]\).

**Figure 2.**

**Typical Michaelis-Menton plot.**

However, when the reaction velocity, \((V_o)\) is plotted against the substrate concentration, \(([S])\) as in the Michaelis-Menton equation, it is often difficult to determine when maximal velocity, \((V_{max})\) has occurred due to the gradual increase of the slope of the hyperbolic curve.

In contrast to the Michaelis-Menton equation, the Lineweaver-Burke plot (Champe & Harvey, 1994) takes the reciprocal ratio of velocity \(1/V_o\) plotted against the reciprocal ratio of substrate \(1/[S]\) and a straight line is produced. The equation describing the Lineweaver-Burke plot is as follows:

\[
1/V_o = \frac{K_m}{V_{max}} [S] + 1/V_{max}
\]

Figure 3 below is an example of a Lineweaver-Burke plot.

**Figure 3.**

*Example of a Lineweaver-Burke plot.*

Y axis = 1/velocity

X axis = 1/substrate concentration

\(K_m = \text{Michaelis constant}\)

\(V_{max} = \text{Maximal velocity}\)

Two advantages that the Lineweaver-Burke plot has over the Michaelis-Menton plot are a more accurate estimate of Vmax can be determined due to the straight line that is produced and information about inhibition of a reaction can be determined.

Any substance that can decrease the velocity (Vmax) of an enzyme-catalyzed reaction is called an inhibitor. There are three types of inhibition relevant in pharmacology: competitive inhibitors, non-competitive inhibitors, and suicide substrate inhibitors (Champe & Harvey, 1994). When the inhibitor binds to the enzyme an enzyme inhibitor complex is formed. Each inhibitor has a unique kinetic constant known as the Ki. Ki can be defined as the dissociation constant from the enzyme-inhibitor complex that is formed. A low Ki constant correlates with a strong inhibitor.

Figure 4 is an example of a lineweaver-Burke plot depicting competitive inhibition.

**Figure 4.**

**Example of Competitive Inhibition.**

\[ y \text{ axis} = 1/\text{velocity} \ , \ x \text{ axis} = 1/\text{substrate concentration} \ , \ 1/V_{\text{max}} = 1/\text{maximal velocity} \]

Increasing [I] = In the presence of a competitive inhibitor more substrate is needed to achieve half maximal velocity.

Kinetic Analysis Data

Kinetic constants were determined for alpha-hydroxymidazolam in the presence of the calcium channel blocker inhibitor nifedipine at a concentration range from 0.5 to 10 micromoles (UM) and the calcium channel blocker diltiazem at a concentration range from 10 to 100 micromoles (UM). In this study diltiazem was used at a higher than therapeutic concentration range because our pilot study using diltiazem determined its low potency in the inhibition of midazolam in vitro.

To construct a Lineweaver-Burke plot, the velocity of alpha-hydroxymidazolam was determined by taking the average of the three real concentrations of metabolite. The averaged data for the velocity of metabolite formation in the presence of diltiazem are presented in Table 2.
Table 2.

Averaged Data for Velocity of Metabolite Formation.

<table>
<thead>
<tr>
<th>Diltiazem data</th>
<th>mcs#16</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>[Diltiazem]µM</td>
<td>0</td>
<td>10</td>
<td>25</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>[aOHMDZ]µM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1µM MDZ</td>
<td>0.75</td>
<td>0.6</td>
<td>0.59</td>
<td>0.51</td>
<td>0.4</td>
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<tr>
<td></td>
<td>2.04</td>
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<td>0.89</td>
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<td>0.67</td>
</tr>
<tr>
<td></td>
<td>2.2</td>
<td>1.81</td>
<td>1.73</td>
<td>1.48</td>
<td>1.28</td>
</tr>
<tr>
<td></td>
<td>3.16</td>
<td>2.83</td>
<td>3.01</td>
<td>2.9</td>
<td>2.8</td>
</tr>
<tr>
<td>mcs#18</td>
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<td>0.23</td>
<td>0.26</td>
<td>0.25</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
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<td>0.58</td>
<td>0.5</td>
<td>0.42</td>
<td>0.4</td>
</tr>
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<td></td>
<td>6</td>
<td>1.11</td>
<td>0.85</td>
<td>0.86</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.21</td>
<td>1.13</td>
<td>0.96</td>
<td>0.76</td>
</tr>
<tr>
<td>mcs#13</td>
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<td>0.29</td>
<td>0.22</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.96</td>
<td>0.49</td>
<td>0.41</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1</td>
<td>0.76</td>
<td>0.72</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.26</td>
<td>1.25</td>
<td>1.02</td>
<td>0.91</td>
</tr>
</tbody>
</table>

mcs = liver microsome
MDZ = midazolam
µM = micromolar
[aOHMDZ] = alpha- hydroxymidazolam concentration
[Diltiazem] = diltiazem concentration
The Lineweaver-Burke plot produced a series of slopes for each concentration of midazolam. See Figures 5 and 6.

**Figure 5.**

**Lineweaver-Burke Plot showing the Velocity of MDZ Hydroxylation for each Diltiazem Concentration to the Concentration of Midazolam.**

![](mcs16_Diltiazem-Midazolam.png)

- **y axis =** $1/\text{velocity (nmol/min/mg)}$
- **x axis =** $1/\text{concentration of midazolam [MDZ] in micromolar (uM)}$.
- **DIL =** diltiazem concentrations in uM.
- **mcs =** liver microsome
Figure 6.

Lineweaver-Burke Plot showing the Velocity of MDZ Hydroxylation for each Nifedipine Concentration to the Concentration of Midazolam.

y axis = 1/velocity (nmol/min/mg)

x axis = 1/concentration of midazolam [MDZ] in micromolars (uM).

NIF = nifedipine concentration in uM.

mcs = liver microsome
The Ki for diltiazem and nifedipine was then derived from plotting the slopes from the Lineweaver-Burke plots on a graph with the slope on the y axis and the substrate on the X axis. The Ki is determined where the line best intersects the x axis. (See Figures 7 and 8).

Figure 7.

**Plot of Slopes of Diltiazem Concentrations. The x Intercept Depicts the Ki.**
Figure 8.

Plot of Slopes of Nifedipine Concentrations. The x Intercept Depicts the Ki.
The apparent Ki values for inhibition of alpha-hydroxymidazolam are listed in Table 3 below. Ki results are shown as Mean + standard deviation (S.D.)

**Table 3.**

**Ki Values for Inhibition of MDZ Alpha-Hydroxylation.**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Nifedipine</th>
<th>Diltiazem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki (UM), MDZ alpha-hydroxy</td>
<td>5.73±0.31</td>
<td>80.7±28.7</td>
</tr>
</tbody>
</table>
CHAPTER V: RECOMMENDATIONS

Introduction

Midazolam is the preferred benzodiazepine in anesthesia secondary to its rapid onset of action and relatively short duration of action. Midazolam is metabolized exclusively by the cytochrome P-450 3A4 (CYP3A4) enzyme to two metabolites: alpha-hydroxymidazolam predominantly and 4-hydroxymidazolam to a lesser extent (Hardman et al., 1996). The CYP3A4 enzyme is responsible for the metabolism of the majority of drugs used today.

Calcium channel blockers are used for the treatment of several cardiovascular disorders to include angina, hypertension and arrhythmias. The two calcium channel blockers nifedipine and diltiazem are known inhibitors of the hepatic cytochrome P450 system. Since patients are required to continue taking their calcium channel blockers peri-operatively, the potential for drug-drug interactions when midazolam and calcium channel blockers are coadministered exists.

There have been numerous drug interactions reported in vitro and in vivo between midazolam and coadministered drugs utilizing the CYP3A enzyme. One in vivo study reported the prolongation of hypnotic effect of midazolam when coadministered with the calcium channel blocker diltiazem (Backman et al., 1994).

Conclusions

Finding from this study demonstrated that nifedipine and diltiazem inhibited MDZ hydroxylation in a concentration dependent manner in human liver microsomes. Nifedipine exhibited greater inhibition than diltiazem. Nifedipine was tested at therapeutic concentrations in human plasma at dosages of 0.5 to 10 uM and was one order of magnitude more potent than diltiazem in inhibiting MDZ alpha-hydroxylation. The mean maximum inhibition produced by nifedipine was 69.2-91.8% relative to control values. In this study, diltiazem was used at a higher than therapeutic range in plasma with concentration ranges from 10-100 uM because a performed pilot study
demonstrated low potency in the inhibition of MDZ alpha-hydroxylation \textit{in vitro}. The mean maximum inhibition produced by diltiazem was 71-80\% relative to control values. These results contradict the reported \textit{in vivo} interactions between orally administered midazolam and diltiazem producing a three-fold increase in the area under the time curve for plasma concentrations of midazolam. One possible explanation of this is the \textit{in vitro} model used in this experiment does not take into consideration factors such as enzyme induction and the binding of drugs to plasma proteins that occurs in the \textit{in vivo} model.

This study is the first investigation of the \textit{in vitro} interactions between midazolam and the calcium channel blockers nifedipine and diltiazem. In the presence of nifedipine and diltiazem concentrations Km values increased, whereas Vmax values decreased suggesting a mixed-typed inhibition mechanism. These findings support the hypothesis that nifedipine and midazolam will decrease the rate of metabolism of midazolam.

Considering the results of this \textit{in vitro} study, the coadministration of midazolam and nifedipine or diltiazem should be done cautiously. There is potential for significant drug interactions when midazolam is coadministered with certain calcium channel blockers at therapeutic plasma levels, therefore leading to increased sedative effects of midazolam. Clinical confirmation is suggested to determine the significance of this \textit{in vitro} finding.

Further research on the topic of \textit{in vivo} and \textit{in vitro} drug-drug interactions of midazolam and calcium channel blockers is warranted. Improvements on this study could include using a larger sample size of liver microsomes.


APPENDICES

Appendix A. Chemical Structure of Midazolam and its Major Metabolite.

Appendix B. *In Vitro* Midazolam Metabolism SOP.
APPENDIX A

Chemical Structure of Midazolam and its Major Metabolite.

midazolam  hydroxymethyl-metabolite
APPENDIX B

In Vitro Midazolam Metabolism SOP

The pooled microsomes were analyzed using the BioRad protein assay giving a concentration of 10.73 mg/ml. This required 18.6 uL to be used to attain a concentration of 0.2 mg/ml after dilution.

Materials:

1. Midazolam, alpha-hydroxymidazolam
2. Lorazepam
3. HPLC grade acetonitrile
4. Formic acid
5. NADPH generating system: NADP+, G6P, G6PDH
6. Human liver microsome samples that are pooled to a concentration of 10.73 mg/ml.
7. Protein assay: Bio-Rad Standard 11 dye, Bio-Rad, stock protein solutions ranging from 0.5-0.05 mg/ml, microliter plates.
8. 50 mM potassium phosphate buffer (pH 7.4)
9. Ethanol

Method:

1. Prepare stock solution of MDZ (3.2 mM):
   a. Formula weight MDZ = 325.8
   b. Make 5 ml of stock solution
   c. Weigh 5.213 mg MDZ in a volumetric flask
   d. Add up to 5 ml ethanol
   e. Store in refrigerator
2. Prepare stock solution of alpha-Hydroxymidazolam (3.2 mM):
   a. Formula weight = 341.8
   b. Make 50 ml of stock solution
   c. Weigh 5.469 mg alpha-Hydroxymidazolam in a volumetric flask
   d. Add up to 50 ml ethanol
   e. Store in refrigerator

3. Prepare 3.11 mM stock solution of Lorazepam (internal standard).

4. Make MDZ dilutions from stock (10 ml each) to use in incubations:
   a. 800uM------2500 uL stock, 7500 uL distilled water
   b. 600uM------1900 uL stock, 8100 uL distilled water
   c. 400uM------1250 uL stock, 8750 uL distilled water
   d. 200uM------625 uL stock, 9375 uL distilled water
   e. 100uM------313 uL stock, 9687 uL distilled water
   f. 50uM-------156 uL stock, 9844 uL distilled water

5. Make a mix of MDZ and its metabolites to use to construct calibration curves:
   a. 1000 uL MDZ stock (3.2 mM)
   b. 1000 uL 1-Hydroxymidazolam (3.2 mM)
   c. 1000 uL 4-Hydroxymidazolam (3.2 mM)
   d. 1000 uL distilled water
   e. The resting solution is 800 uM

6. Take 4ml of the MDZ mix (800 uM) and put 1 ml and 1 ml into two separate tubes. Add 1 ml water to one tube and add 3 ml water to the other tube. The result is 400 uM and 200 uM concentrations of the MDZ mix. Continue to make serial dilutions of the mix according to the following:
4ml of 800 uM solution

1 ml + 1 ml H2O = 400 uM (2ml)

1ml + 3 ml H2O = 200 uM (4 ml). Take 2 ml from this to make next dilution

1ml + 1ml H2O = 100 uM (2ml)

1ml + 3ml H2O = 50 uM (4ml). Take 2 ml for next dilution.

1ml + 1ml H2O = 25 uM (2ml)

1ml + 4ml H2O = 10 uM (5ml). Take 2 ml for next dilution.

1ml + 1ml H2O = 5 uM (2 ml)

1ml + 4ml H2O = 1 uM (5ml). Take 2 ml for next dilution.

1ml + 1ml H2O = 0.5uM (2ml)

1ml + 4ml H2O = 0.1 uM (5ml). Take 2 ml for next dilution.

1ml + 1ml H2O = 0.05uM (2ml)

1ml + 4ml H2O = 0.01 uM (5ml). Take 2 ml for next dilution.

1ml + 1ml H2O = 0.005 uM (2ml)

1 ml + 4ml H2O = 0.001 uM (5ml)

7. The pooled microsome samples will be subjected to 5 different concentrations of MDZ and all work will be done in triplicate.

8. Perform protein tests on all microsome samples. (See SOP for protein assay).

9. Prepare NADPH generating system:
   b. This will total 30 uL, enough for 300 samples.

10. Add sufficient microsome suspension to each test tube to obtain a final protein concentration of 0.2 uM/ml after dilution. Base this on the calculated protein content of each microsome sample. Add 10 uL of NADPH generating system and up to 0.980 ul of 50 mM potassium phosphate buffer.
11. Place test tubes in a shaking water bath for 3 minutes at 37 C. Tubes used for standards should be kept on ice.

12. Add 10 uL of MDZ solution at 0 minutes. (The tubes for standards will have solution added, then kept on ice without incubation.

13. Incubate tubes 5 minutes.

14. Plunge tubes into ice at 5 minutes. (see chart for time schedule).

15. Add 200 uL of the internal standard stock solution to each test tube. (samples and standards).

16. Add 5 ml acetonitrile to each incubated test tube.

17. Vortex each test tube for 10 minutes.

18. Centrifuge test tubes at 2000 g; 5 C; 10 minutes.

19. Transfer to clean test tubes and label.

20. Evaporate tubes to dryness with speed vac.


22. Vortex each tube for 3 minutes.

23. Add 2ml of acetonitrile to each tube.

24. Centrifuge tubes at 2000g; 5C;10 minutes.

25. Transfer to clean tubes and label.

26. Evaporate tubes to dryness with speed vac.

27. Add 20 uL acetonitrile:water (1:1 v/v) to each tube.

28. Vortex for 2 minutes at speed 5.

29. Add 20 uL water to each tube.

30. Vortex 2 minutes at speed 5.

31. Transfer each sample from test tube to microvial. Label and load onto HPLC system.
Chromatography:

1. Use of a Hewlett Packard 1050 HPLC system will be employed.
2. System filtered with micro bore tubing and Prodigy 5 u ODS (3) 1 00 A Column.
3. Flow rate = 0.2 ml/min.
4. Run Time = 20 minutes.
5. Temperature = 35 C.
6. Injection Volume = 10 uL.
7. Lamp wavelength = 220nm.
8. Solvent A = H2O.
9. Solvent B = .05% Formic acid in acetonitrile (pH 4.1).
10. Flow rate gradient:
    a. Time in minutes: Flow rate:
        0:0.2, 2:0.2, 3.5:0.25, 5:0.25, 30:0.25, 32:0.25, 33:0.4, 39.8:0.4, 40:0.25,
        45:0.25, 55:0.2.
11. Gradient conditions:
    a. Time in minutes:percent formic acid in acetonitrile.
        0: 15, 2:15, 3.5:15, 5:15, 30:45, 32:98, 33:98, 39.8:98, 40:98, 45:15, 55:15.